Cultured Human Langerhans Cells Process and Present Intact Protein Antigens

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Epidermal Langerhans cells (LC) undergo profound phenotypic and functional alterations when cultured for 2 to 3 d. To determine whether the in vitro culture of human LC modulates their capacity to process and present intact protein antigens, we compared the ability of freshly isolated LC (fLC) and cultured LC (cLC) to stimulate in vitro T-cell proliferative responses to recall antigens. We found that human fLC and cLC were able to process and present recall antigens to primed T cells, inducing significant proliferative responses. For tetanus toxoid and *Candida albicans* extract, T-cell proliferative responses at 6 d to antigen-pulsed fLC were slightly greater than responses to antigen-pulsed cLC. For live influenza A virus, the T-cell responses induced by antigen-pulsed cLC were comparable or slightly greater compared with fLC. Allogeneic T-cell proliferation for both LC preparations were also comparable. The exogenous pathway of antigen processing was demonstrated by chloroquine inhibition. *J Invest Dermatol 99:331–336, 1992*

Epidermal Langerhans cells (LC) are very potent antigen-presenting cells (APC) of bone marrow origin [1] and normally comprise 2–5% of cells in mammalian epidermis [2]. These cells initiate allogeneic T-cell proliferation, antigen-specific T-cell activation, and cytotoxic T-cell proliferation [2–4]. LC play a critical role in both the afferent and efferent limbs of contact hypersensitivity reactions and skin-graft rejection and may contribute to immunosurveillance against cutaneous viral infections and neoplasia [5–7].

When murine LC are compared with other APC such as monocytes/macrophages, several differences emerge. LC express class II major histocompatibility complex (MHC) antigens constitutively, whereas macrophages express these antigens facultatively [8,9]. During short-term (2 to 3 d) culture, LC exhibit enhanced, and monocytes progressively lose, surface expression of class II MHC antigens and accessory cell function [9–11]. The effect of short-term culture on LC has been most extensively studied in the mouse. Murine cultured LC (cLC) become markedly dendritic and acquire increased surface expression of class II MHC antigens and ICAM-1 [10,12–14]. Concurrently, murine cLC lose or markedly reduce expression of ATPase [14], certain monocyte/macrophage markers, including nonspecific esterase, FcR, and F4/80 [14], and no longer exhibit their cytoplasmic (Birbeck) granules and endosomes [15]. Similar changes have been described in human cLC [16–18].

cLC are ten- to a hundredfold more potent than freshly prepared LC (fLC) in the induction of primary allogeneic or autologous T-cell proliferative responses, in the presentation of haptens to antigen-specific T-cell clones, and in the presentation of peptide antigens to T-cell hybridomas [10,14,19,20]. In addition, cLC are more potent than fLC as accessory cells for CD3-driven T-cell proliferation, despite an apparent reduction of surface Fc receptors on LC after culture [21]. Of particular interest is the finding that murine fLC and cLC can each present antigen to previously primed (memory) T cells [19], but only cLC are capable of initiating in vitro primary immune responses, i.e., the presentation of haptens or peptide antigens to naive resting T cells [22]. In the human system, LC have demonstrated greater immunostimulatory capacity than monocytes in allogeneic T-cell activation [3,23], and cLC are more potent than fLC in both autologous and allogeneic T-cell activation, but only when cultured in the presence of exogenous GM-CSF [24].

The antigen-processing ability of cLC has been primarily studied in the mouse. Contradictory findings that cLC lost [15,25,26] or retained [10,22] the ability to process soluble protein antigens were clarified when inbred strains were compared, and it was found that the antigen-processing capacity of cLC is dependent on MHC haplotype [27]. Human beings, however, represent an outbred population without a consistent MHC haplotype. To address the question of the relative capacity of human cLC to process and present protein antigens, we compared the ability of fLC and 3-d cLC from epidermis of normal volunteers to stimulate T-cell proliferative responses to common recall protein antigens (tetanus toxoid, live influenza A virus, and *Candida albicans* extract).

MATERIALS AND METHODS

Preparation of Epidermal Cells Single cell suspensions of epidermal cells (EC) were prepared from trypsinized suction blister roofs of normal volunteers, as previously described [28]. Briefly, suction blister roofs (epidermal sheets) were removed and floated in 0.5% trypsin in phosphate-buffered saline for 45 min at 37°C,

Manuscript received November 27, 1991; accepted for publication April 23, 1992.

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Abbreviations:
APC: antigen-presenting cells
CAND: *Candida albicans* extract
EC: epidermal cells
FACS: fluorescence-activated cell sorter
FcR: Fc receptor
FITC: fluorescein isothiocyanate
FLU: live influenza virus
GM-CSF: granulocyte/macrophage colony-stimulating factor
HLA: human leukocyte antigen
KLH: keyhole limpet hemocyanin
LC: Langerhans cells
cLC: cultured Langerhans cells
fLC: freshly prepared Langerhans cells
MHC: major histocompatibility complex
PBMC: peripheral blood mononuclear cells
TT: tetanus toxoid

0022-202X/92/$05.00

transferred to 0.05% DNAase in Hanks' balanced salt solution (HBSS), and disaggregated by repeated aspirations and expulsions of the epidermal sheets through a sterile syringe. Cells were filtered through a 100-μm nylon mesh, washed, suspended 1×10^6 cells/ml in keratinocyte growth medium (Clonetics, San Diego, CA) supplemented with 200 U/ml recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) (Genzyme, Boston, MA), and plated in 24-well plates (Flow Laboratories, McLean, VA). EC were then utilized as freshly isolated EC (fEC) or were cultured for 72 h at 37°C in a humidified 5% CO_2 atmosphere (cEC).

Antigen-Pulsing of Epidermal Cells fEC or cEC were cultured in the presence of a recall antigen for 18 h (antigen pulse). During this "pulsing" period, the fLC developed a dendritic morphology, which facilitated their quantification by hemocytometer. The cLC that were "pulsed" maintained their dendritic morphology during the 18-h pulse period. Live influenza A virus (FLU), tetanus toxoid (TT), and *C. albicans* extract (CAND) were used as recall antigens. FLU, as infectious allantoic fluid (gift of Dr. Gene Shearer), was used at a final concentration of 1:100. TT (Connaught Laboratories, Willowdale, Ontario, Canada) was used at a final concentration of 40 Lf/ml. CAND (Hollister-Stier, Spokane, WA) was used at a final concentration of 1:30.

Enrichment of Langerhans Cells Following the antigen pulse period, non-adherent EC were washed, resuspended in RPMI 1640 with 10% human AB serum, layered over a Ficoll-sodium metrizoate density gradient (density 1.077) (Lymphoprep, Nycomed, Oslo, Norway), and centrifuged at $400 \times g$ for 25 min. Typically, 5 to 20% of interface cells had dendritic morphology and stained positively with fluorescein isothiocyanate (FITC)-conjugated human leukocyte antigen (HLA)-DR (Becton-Dickinson, Mountainview, CA) under fluorescent microscopy (data not shown). Enriched LC were irradiated (2000 rad, ^{137}Cs) and utilized for functional studies.

Preparation of T Cells Autologous T cells were prepared from healthy epidermal cell donors. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of heparinized whole blood over Lymphoprep at $600 \times g$ for 15 min at 20°C. Interface cells were incubated with 5 mM L-leucine methyl ester (Sigma) in serum-free RPMI 1640 for 1 h at room temperature [29]. The resulting cells and cellular debris were centrifuged and reconstituted in complete medium, consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% human AB serum (Sigma, St. Louis, MO) heat-inactivated for 60 minutes at 56°C, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml fungizone (Gibco), 2 mM L-glutamine (Gibco), 10 mM HEPES buffer solution (Gibco), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma), and 1 μg/ml indomethacin (Sigma). This suspension was incubated for 1 h in a nylon wool column at 37°C, and non-adherent cells were eluted [30].

T-Cell Proliferation Assay T-cell proliferative responses were measured by [^{3}H]-TdR incorporation, using a slight modification of a previously described technique [28]. For antigen-driven lymphocyte proliferation, as well as autologous and allogeneic epidermal cell-lymphocyte reactions, cells were cultured for 6 d in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 37°C, under a humidified 5% CO_2 atmosphere. One microcurie of [^{3}H]-TdR (Amersham, Arlington Heights, IL) was added to each microtiter well during the last 15–18 h of culture. [^{3}H]-TdR-labeled cells were harvested using a semi-automated cell harvester (PhD Cell Harvester, Cambridge Technology, Cambridge, MA), and [^{3}H]-TdR incorporation was measured by a liquid scintillation counter (Beckman, Fullerton, CA). Responses were reported as mean cpm ± SEM from triplicate samples.

Phytohemagglutinin T-Cell Stimulation Assay T cells (10^5/well) were cultured for 3 d in the presence of 1 μg/ml phytohemagglutinin (Burroughs-Wellcome, Research Triangle, NC) in 96-well flat-bottom microtiter plates. [^{3}H]-TdR was added in the last 15–18 h of culture and [^{3}H]-TdR uptake was measured.

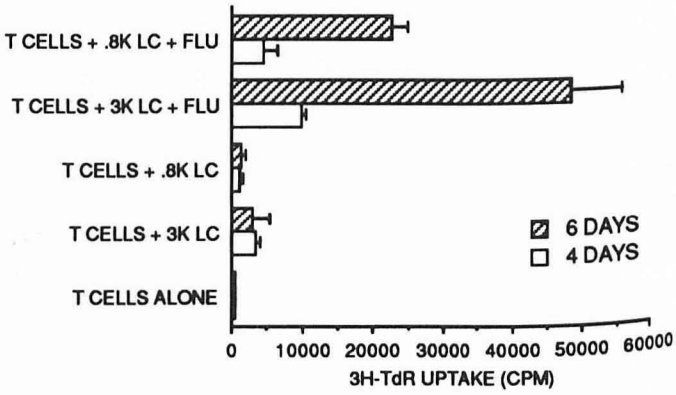

Figure 1. Time-dependent proliferative response of T cells in the presence of antigen-pulsed LC. fLC were incubated with FLU for 18 h and then co-cultured with autologous T cells (10^5/well). T-cell proliferation was determined by measuring [^{3}H]-TdR incorporation after 4 and 6 d of co-culture.

Staining and Flow-Cytometric Analysis Cells were washed and resuspended in Ca^{++}- and Mg^{++}-free HBSS with 1% bovine serum albumin (Sigma), 0.01% DNAase (Sigma), and 0.2% disodium EDTA (Sigma), designated as fluorescence-activated cell sorter (FACS) buffer. Epidermal cells were stained with FITC-conjugated anti-HLA-DR (class II MHC) or control FITC-conjugated IgG_{2a} specific for keyhole limpet hemocyanin (KLH) (Becton-Dickinson, Mountainview, CA). T cells were stained with FITC-conjugated monoclonal antibodies Leu-1 (CD5), HLA-DR (class II MHC), Leu-M3 (CD14), and Leu-12 (CD19) or control FITC-conjugated IgG_1 and IgG_{2a} specific for KLH (Becton-Dickinson). Stained cells were incubated for 30 min at 4°C, washed three times, resuspended in FACS buffer, transferred to polystyrene tubes, and analyzed by flow cytometry on a FACScan (Becton-Dickinson).

Inhibition of Antigen Processing by Chloroquine fEC and 3-d cEC were pretreated with 300 μM chloroquine (Sigma) for 15 min to 1 h at 37°C, incubated with a recall antigen for 2–3 h at 37°C in the continued presence of chloroquine, and then washed free of chloroquine and antigen, as described [31]. These cells were cultured an additional 18 h, enriched for LC, and co-cultured with T cells, as described above. Control experiments were performed wherein chloroquine was added to the cultures for 2 h *after* the antigens were cultured with the LC.

Statistical Analysis p values were determined by the Student t test (two-tailed, paired) and considered statistically significant at $p \leq 0.01$.

RESULTS

Kinetics of the T-Cell Recall Response When fLC were cultured with T cells for varying periods, we found that significant proliferative responses were detected by 6 d of co-culture (Fig 1). When assayed on day 6, T-cell proliferation induced by both antigen-pulsed fLC and cLC was consistently in a growth phase for all antigens used (data not shown).

Human cLC Process and Present Protein Antigens to Primed T Cells To determine whether cLC retained the ability to process and present antigens, freshly prepared and 3-d cultured EC were incubated with recall antigens (FLU, TT, or CAND) for 18 h, enriched for LC by centrifugation over a density gradient, x-irradiated, and co-cultured with accessory cell-depleted autologous T cells for 6 d. A constant number of T cells (10^5 per well) was co-cul-

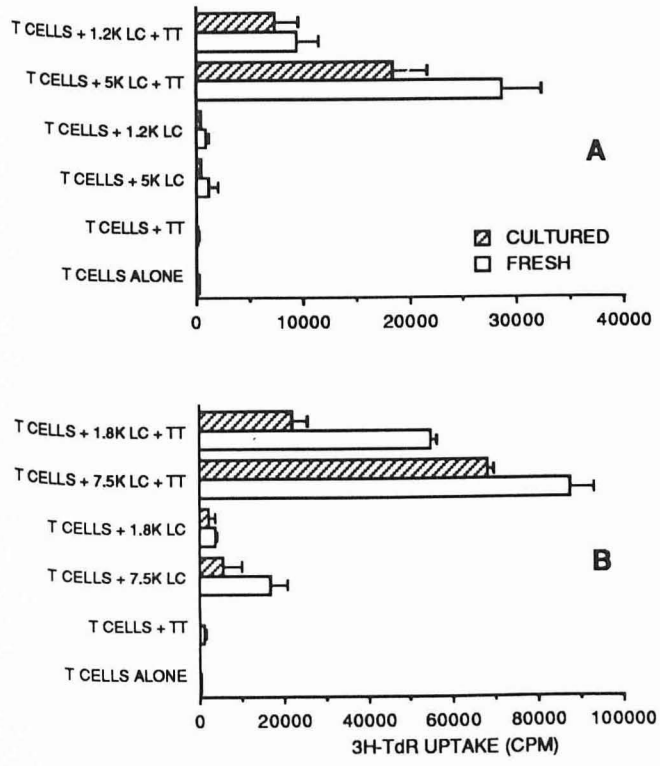

Figure 2. Processing and presentation of tetanus toxoid by freshly prepared and cultured human LC. Two representative experiments shown (*A* and *B*). TT-pulsed (40 Lf/ml) fLC or cLC were co-cultured for 6 d with 10^5 autologous monocyte-depleted T cells. T-cell proliferation was assessed by the incorporation of [^{3}H]-TdR, which was added in the last 15–18 h of co-culture.

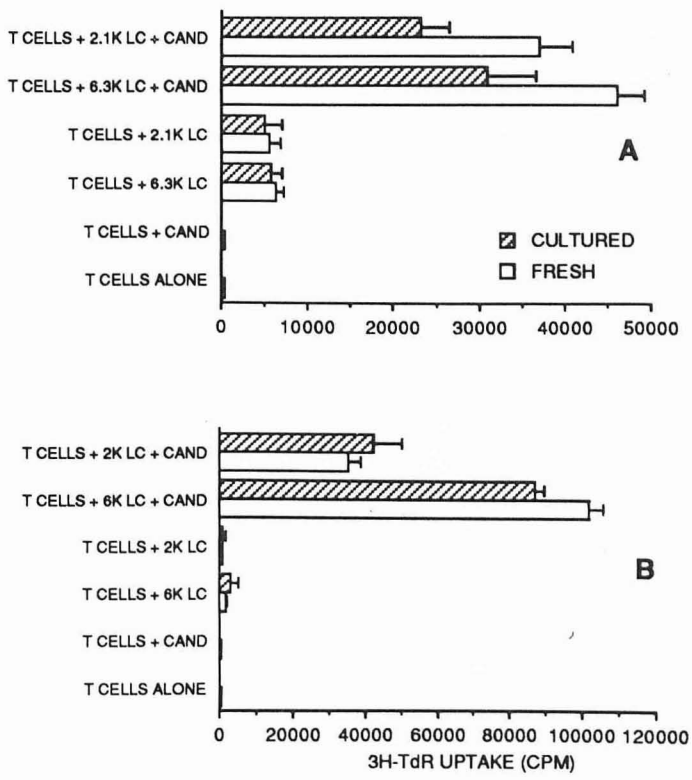

Figure 3. Processing and presentation of *C. albicans* extract by freshly prepared and cultured LC. Two representative experiments shown (*A* and *B*). LC were exposed to *C. albicans* extract (1:30) and assayed for antigen-processing and -presenting ability, as described in Fig 2.

tured with various numbers of LC, ranging from 800 per well to 7500 per well. T-cell proliferation was assessed by [^{3}H]-TdR incorporation, added in the last 15–18 h of culture. For TT, freshly isolated- and antigen-pulsed LC (fLC) and 3-d–cultured then pulsed LC (cLC) induced significant T-cell proliferation, in a dose- (number of LC) dependent manner (Fig 2). The control autologous reaction showed a low background level of proliferation. In two representative experiments, fLC induced T-cell proliferative responses somewhat greater (36% and 22%, not statistically significant) than cLC (Fig 2). CAND-pulsed LC also induced a significant proliferative response in a dose-dependent manner. In two representative experiments, CAND-pulsed fLC induced proliferative responses again somewhat greater (33% and 15%, not statistically significant) than CAND-pulsed cLC (Fig 3). In contrast to TT and CAND, in most experiments, FLU-pulsed cLC induced slightly greater T-cell proliferative responses than FLU-pulsed fLC. In two representative experiments, FLU-pulsed cLC induced T-cell proliferative responses slightly greater (34% and 20%, not statistically significant) than FLU-pulsed fLC (Fig 4). cLC processed and presented recall antigens in 20 of 23 experiments.

In our system, fLC were antigen pulsed for 18 h, so that at the time of co-culture with T cells these were effectively 1-d cLC. Similarly, 3-d cLC were antigen-pulsed for 18 h, so that at the time of co-culture these were effectively 4-d cLC. To address the possibility that the presentation ability of 1-d cLC may differ from that of 4-d cLC, we compared the allostimulatory potency of these LC preparations. We found that both 1-d cLC and 4-d cLC induced comparable T-cell proliferative responses (Fig 5). This is not surprising, as both of these cLC preparations exhibit markedly enhanced class II MHC expression. To confirm that T-cell proliferation was a consequence of antigen presentation by LC, we demonstrated that no other functional antigen-presenting cells were present in co-cultures. The purified T-cell preparations were uniformly unresponsive to exogenous antigens (Figs 2–4) and phytohemagglutinin (data not shown). The virtual absence of monocytic cells and B cells within the T-cell preparation was demonstrated by flow cytometry, with 95%, 1.6%, 0.3%, and 0.6% of cells positive for CD5 (pan-T cell), HLA-DR (class II MHC), CD14 (monocyte), and CD19 (B cell) (Becton-Dickinson), respectively (data not shown).

The Exogenous Pathway of Antigen Processing is Utilized by LC To verify that human LC processed antigens by the exogenous (acidic endosomal) pathway, we studied the antigen-processing ability in the presence of chloroquine, a selective inhibitor of the exogenous pathway [32,33]. We found that chloroquine regularly inhibited T-cell proliferation by antigen-pulsed fLC and cLC to an extent greater than the control allogeneic T-cell proliferation (Figs 6 and 7). Furthermore, when chloroquine was added to LC after antigen pulsing, there was no significant inhibition of T-cell proliferation (data not shown). Chloroquine inhibition of allogeneic proliferation ranged from 7 to 56%. To determine whether the inhibitory effects of chloroquine on antigen-driven or allogeneic T-cell proliferation were related to the surface expression of class II MHC or EC viability, we subjected fEC to a 4-h antigen pulse, a 4-h incubation with antigen plus chloroquine, or a 4-h sham treatment. Cells were then cultured for 18 h, and the entire non-adherent cell population was analyzed by flow cytometry for the expression of class II MHC (HLA-DR) antigens and for viability by propidium iodide staining. Each treatment yielded a comparable percentage of class II MHC bearing cells, comparable mean fluorescent intensity of class II MHC on LC, and comparable viability for both keratinocytes and LC (data not shown).

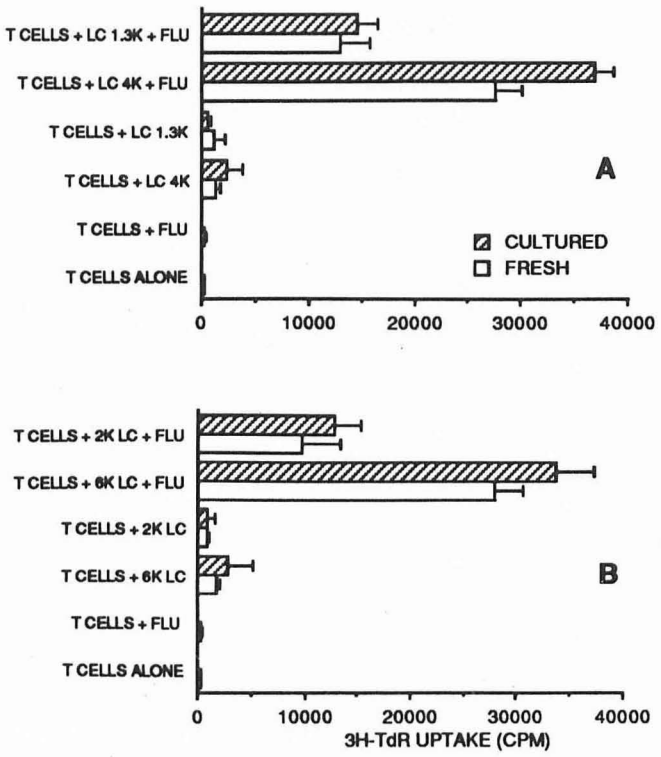

Figure 4. Processing and presentation of influenza A virus by freshly prepared and cultured LC. Two representative experiments shown (*A* and *B*). LC were exposed to live influenza A virus (1 : 100) and assayed for antigen-processing and -presenting ability, as described in Fig 2.

DISCUSSION

Murine and human LC undergo profound changes in phenotype and immunostimulatory function during short-term culture. Phenotypic changes include enhancement in the expression of the class II MHC antigens, with concomitant reduction or loss of Birbeck granules and acidic organelles (endosomes), as well as certain monocyte/macrophage markers, non-specific esterase, FcR, and the F4/80 antigen [10,12–16]. Although studies of the antigen-processing ability of murine cLC had yielded contradictory data [10,15,22,25,26], it appears that the retention or loss of antigen processing in murine cLC is MHC-dependent [27]. Despite the down-regulation of acidic organelles and Birbeck granules in cLC in both the mouse and human [15,16], data from the murine system and the data obtained in the current study of human LC demonstrate that antigen-processing ability is a property of both fLC and cLC.

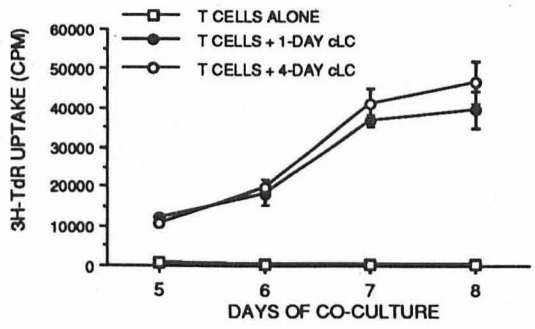

Figure 5. Time course of allogeneic T-cell response, using 1-d and 4-d cLC as stimulators. *Open circles,* T cells plus 4-d cLC. *Closed circles,* T cells plus 1-d cLC. *Open squares,* T cells alone.

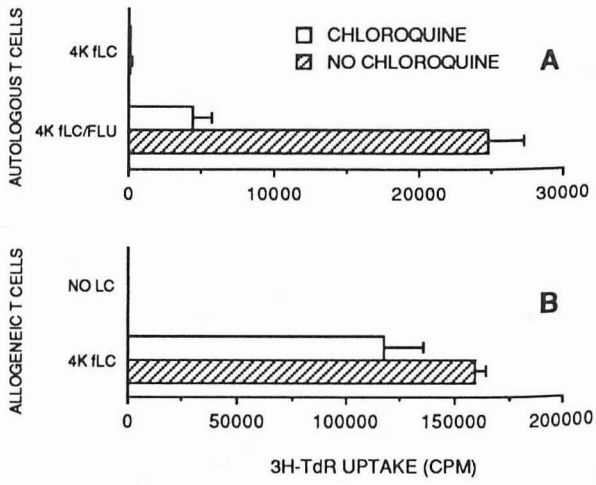

Figure 6. Effects of chloroquine on antigen processing of FLU by fLC on alloantigen presentation. In the presence of chloroquine, there was an 82% decrease in the ability of fLC to induce antigen-specific T-cell proliferation *(A)*, compared with a 26% decrease in alloantigen-induced T-cell proliferation *(B)*.

We found that the ability of human LC to process and present native antigens to primed T cells is retained after 3 d of culture. In general, differences between the antigen-processing/presenting ability of fLC and cLC were most apparent at higher numbers of stimulator cells. For both CAND and TT, we found that antigen-pulsed fLC consistently induced slightly greater T-cell proliferative responses than antigen-pulsed cLC. These findings contrast somewhat with those of Teunissen et al [18], who recently reported that cLC were "consistently better stimulator cells" than fLC. The reason for the varied findings may be accounted for by the method used for the preparation of their fLC. Teunissen et al positively selected fLC using a panning technique with OKT6 and antimouse immunoglobulin. Furthermore, GM-CSF was not added to any of their cultures [18]. Their T-cell responses when fLC were used were in the order of 3,000–8,000 cpm and 5,000–18,000 cpm to CAND and TT, respectively [18]. Our findings differed considerably in that

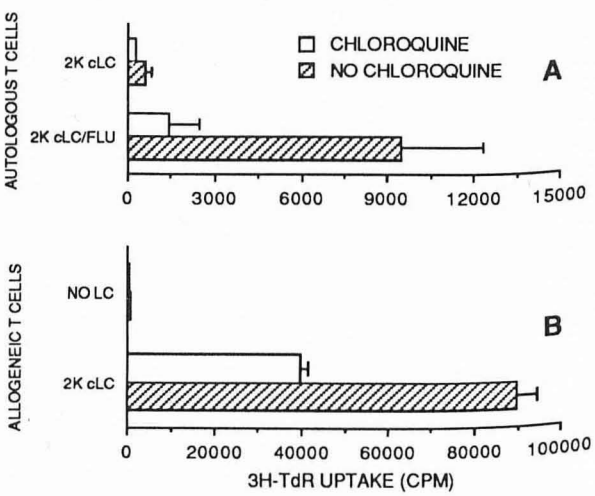

Figure 7. Effects of chloroquine on antigen processing of FLU by cLC on alloantigen presentation. In the presence of chloroquine, there was an 85% decrease in the ability of cLC to induce antigen-specific T-cell proliferation *(A)*, compared with a 56% decrease in alloantigen-induced T-cell proliferation *(B)*.

optimal numbers of fLC induced 47,000–100,000 cpm and 30,000–85,000 cpm to CAND and TT, respectively. Notwithstanding these differences, both studies demonstrate that both fLC and cLC can process and present exogenous protein antigens.

Viral antigens are usually presented by the endogenous pathway, which is chloroquine resistant [34]; however, there are exceptions [35], such as FLU, which is presented by the exogenous pathway [35]. Live FLU-pulsed cLC induced comparable or greater T-cell proliferative responses than did fLC. Because the end-point of our assay was T-cell proliferation, in which the direct measurement of antigen processing independent of presentation was not possible, more specific inferences about the antigen-processing machinery of fLC versus cLC cannot be made. The general scheme of the exogenous (MHC class II) pathway of antigen processing and presentation can be summarized: 1) endocytosis of the antigen; 2) proteolysis within acidic endosomes; 3) transport to the endosomes of class II MHC, either newly synthesized or recycled from the cell surface; 4) "rescue" of antigenic peptides from further degradation by binding to class II MHC within endosomes, with concomitant stabilization of class II-antigen complex; and 5) transport of the class II–antigen complex to the cell surface for presentation. Thus, measurement of the T-cell response gives no indication of the number of class II–antigen complexes necessary to produce such a response [36,37]. It is possible that fLC and cLC may have utilized different pathways of antigen processing, e.g., a different repertoire of endosomal proteases, yielding different or the same epitopes [38–40]. This latter point cannot be determined in our studies, as peripheral blood T cells rather than T-cell clones were used as responder cells.

Chloroquine is a specific inhibitor of the exogenous pathway of antigen processing [32,33]. We found that the processing of each recall antigen in both fLC and 3-d cLC was inhibited by chloroquine, indicating that the exogenous pathway of antigen processing was required. However, chloroquine consistently induced a lesser degree of inhibition (from 7 to 56%) of control allogeneic T-cell proliferation. This is in keeping with recent data that indicate that chloroquine specifically inhibits allorecognition when fLC are used as stimulators [41]. Chloroquine inhibits the dissociation of the invariant chain from the class II heterodimer in both the murine and human system [32,42] and has been shown to slightly decrease the biosynthesis of class II in mice [42]. Our flow-cytometric analysis, however, showed no change in the surface expression of HLA-DR for EC treated with chloroquine. In addition, chloroquine treatment did not alter the viability of EC or the HLA-DR–positive subset, suggesting that chloroquine did not exert its inhibitory effects on allogeneic T-cell proliferation by direct toxicity to EC.

The possibility of collaboration between LC and other accessory cells [43,44] made the elimination of accessory cells from the T-cell preparation particularly important. The abrogation of accessory cell activity in the T-cell preparation was consistently demonstrated in functional assays, and the depletion of monocytes and B cells was additionally confirmed by flow cytometry in several experiments.

In the murine system, dendritic cells have the unique property of initiating primary immune responses. Murine cLC have been used in vitro to induce primary sensitization of naive resting T cells to haptens and peptide antigens [22]. In the human system as well, the stimulation of resting T cells appears to be restricted to dendritic cells [45]. The goal of our future studies is to determine whether human cLC can induce primary in vitro sensitization and thus generate antigen-specific T cells that respond to viral or tumor antigens.

We thank Mr. Jay Linton and Mr. Lincoln Liburd for their excellent technical assistance.

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