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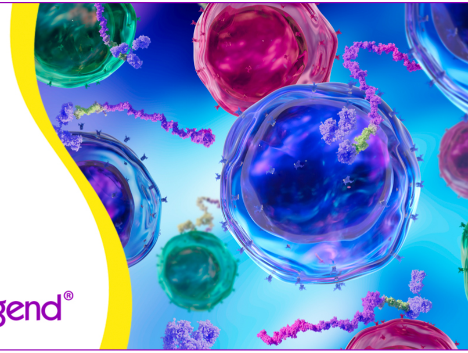

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THE ABILITY OF CULTURED LANGERHANS CELLS TO PROCESS AND PRESENT PROTEIN ANTIGENS IS MHC-DEPENDENT

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There is controversy regarding the ability of short term (2 to 3 days) cultured epidermal Langerhans cells (cLC) to process and present intact protein Ag to primed T cells. Some studies have shown that cLC are potent APC for both haptens and intact protein Ag, whereas in others cLC have been unable to process and present intact protein Ag. In an attempt to resolve this controversy, we tested the ability of Langerhans cells from several strains of mice to process and present intact protein Ag to T cell clones and T cell hybridomas. We found that both cLC and freshly prepared Langerhans cells from various Ia^k mice, including BALB.k mice, process and present intact protein antigens (i.e., hen egg lysozyme, cytochrome *c*, and OVA) to T cells. These functions are retained in cLC cultured for 7 days. In contrast, cLC from Ia^d mice do not process intact protein Ag, such as hen egg lysozyme and myoglobin, although they can present relevant peptides to specific T cells and are potent stimulators of allogeneic responses. Furthermore, cLC from (Ia^k × Ia^d)F$_1$ mice process and present intact protein Ag to Ia^k-restricted T cells, but not to Ia^d-restricted T cells. Although cLC that processed and presented intact protein Ag to T cells exhibited enhanced class II MHC expression, they were, on a per cell basis, somewhat less efficient than were fresh Langerhans cells. Finally, we found that if Ia^d Langerhans cells are pulsed with intact protein Ag and then cultured for 3 days, they are then fully capable of inducing Ag- and MHC-specific T cell proliferation.

It is well established that LC[2] are potent APC in the skin (1–8) and that LC, cultured for 2 to 3 days, as compared to fLC exhibit changes in surface phenotype and functional activity (7, 9). Indeed, cLC may be the in vitro equivalent of LC that have migrated from skin to draining lymph nodes to present Ag to lymphocytes (10, 11).

Although there is general agreement that cLC exhibit potent Ag-presenting functions for allogeneic and syngeneic T cell stimulation (6–9, 12), differing experimental results have been reported regarding the ability of cLC to process intact protein Ag for presentation to specifically sensitized T cells. In some studies, cLC effectively process intact protein antigens for presentation to naive T cells as well as to Ag-specific T cell hybridomas (7, 8). In contrast, other studies have demonstrated that cLC are unable to process intact protein Ag (13).

To address this controversy, we used a variety of T cell clones and hybridomas and different strains of mice and analyzed the characteristics of Ag processing functions of fLC and cLC. We found that, depending on the mouse strain and Ag tested, cLC may (Ia^k) or may not (Ia^d) process and present intact protein Ag. We also found that even though cLC from Ia^d mice cannot process intact protein Ag, when freshly isolated Ia^d LC were pulsed with these Ag and then cultured, they were very effective at presenting these soluble protein Ag. The latter finding suggests that when LC are exposed to Ag in the periphery (skin) they might be able to process the intact protein and present the relevant peptide to T cells after migration to the regional lymph nodes.

MATERIALS AND METHODS

Animals. C3H/HeN, AKR, B10.A, BALB/c, and DBA/2 mice were obtained from the Charles River Breeding Laboratory, Wilmington, MA. BALB.k mice were kindly provided by Dr. David Sachs at the National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD. We bred (C3H × BALB/c) F1 mice. Mice were used at 6 to 12 wk of age.

Serologic reagents. The following mAb directed against murine Ia Ag were used: 10.2-16, specific for I-A^k (14) (cells from American Type Culture Collection (ATCC), Rockville, MD), M5/114.5.2, specific for I-A (b,d,q haplotypes) and I-E (d,k haplotypes) (15) (cells from ATCC), and MK-D6, specific for I-A^d (16). Anti-I-A^k antibody was purified from the culture supernatant of 10.2-16 on a protein G affinity membrane disk after the manufacturer's recommendation (Genex, Gaithersburg, MD). M5/114.5.2 was grown in ascites fluid and the antibody was purified by ion exchange chromatography. For FITC conjugation of mAb, 2 mg/ml of purified mAb was adjusted to pH 8.4 with 1 M $NaHCO_3$, pH 9.5 and an appropriate amount of FITC at 5 mg/ml in ethanol was added, quickly mixed, and allowed to incubate at 37°C for 2 h. FITC-conjugated mAb were separated from free FITC on a PD-10 column packed with Sephadex G-25M (Pharmacia, Uppsala, Sweden) and suspended in PBS. FITC-conjugated MK-D6 was kindly provided by Dr. Ada Kruisbeek, NCI.

Culture medium. RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine (GIBCO Laboratories, Chagrin Falls, OH), penicillin, streptomycin, and fungizone antibiotic solution (GIBCO), non-essential amino acid solution (GIBCO), sodium pyruvate solution (GIBCO), 10 mM HEPES buffer solution (GIBCO), 5×10^{-5} M 2-ME, and 1 μg/ml indomethacin (Sigma Chemical Co., St. Louis, MO) (complete medium) was used for the culture of epidermal cells, hybridomas, the T cell clone A.E7, and allogeneic ELR. The T cell clone TK.G4, was cultured in a 1:1 mixture of RPMI 1640 and EHAA medium (GIBCO) supplemented with the same reagents used in the complete medium mentioned above. For culture of CTLL, complete medium without 2-ME was used.

Preparation of cell suspensions. Single cell suspensions of EC and spleen cells were prepared as previously described (17, 18). The epidermal cell suspensions were applied to Lympholyte M (Cedarlane Laboratories Limited, Canada) density gradients and centrifuged at 300 × *g* for 10 min at room temperature. The interface cells were

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[1] Address correspondence and reprint requests to Dr. Stephen I. Katz, Dermatology Branch, NCI/NIH, Bldg. 10/12N238, Bethesda, MD 20892.
[2] Abbreviations used in this paper: LC, Langerhans cells; cLC, cultured LC; EC, epidermal cells; fLC, fresh LC; HEL, hen egg lysozyme.

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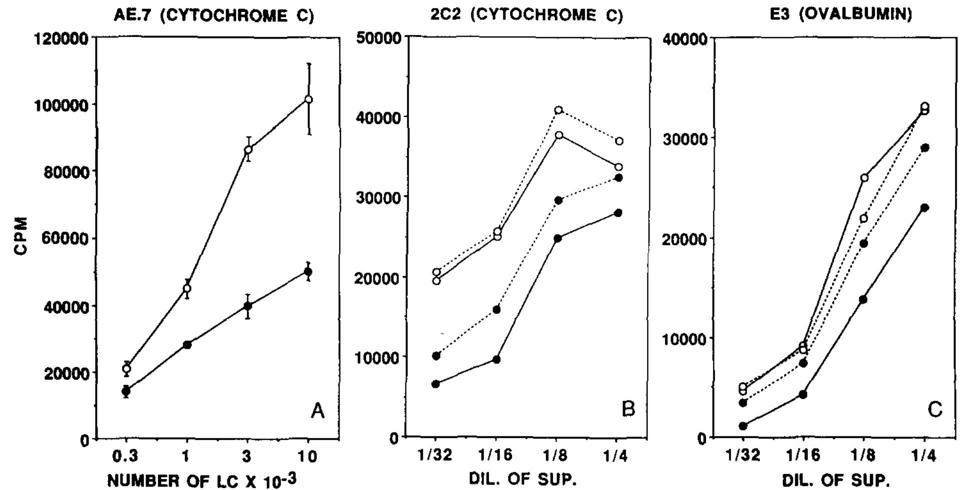

Figure 1. cLC from C3H mice can process and present protein Ag to Ag-specific T cell clone and hybridomas. The pigeon cytochrome c-specific, I-E^{k}-restricted T cell clone, A.E7 was stimulated with varying numbers of fLC (*open circles*) and cLC (*closed circles*) from C3H mice in the presence of 50 μg/ml of pigeon cytochrome c (*A*). [^{3}H]-TdR incorporation was used as an index of T cell proliferation. [^{3}H]-TdR incorporation of the clones cultured with fLC or cLC but without Ag was less than 10,000 cpm. The cytochrome c-specific, I-E^{k}-restricted T cell hybridoma, 2C2, and, the OVA-specific, Ia^{k}-restricted T cell hybridoma, E3 were stimulated with varying numbers (6,000—*continuous line* or 20,000—*interrupted line*) of fLC (*open circles*) or cLC (*closed circles*) from C3H mice in the presence of 2.5 mg/ml of cytochrome c and 1 mg/ml of OVA, respectively (*B* and *C*). The supernatants that were generated after 24 h were cultured in various dilutions with CTLL and [^{3}H]-TdR incorporation by CTLL was used as an index of IL-2 production by hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from non-Ag exposed cultures was less than 1000 cpm.

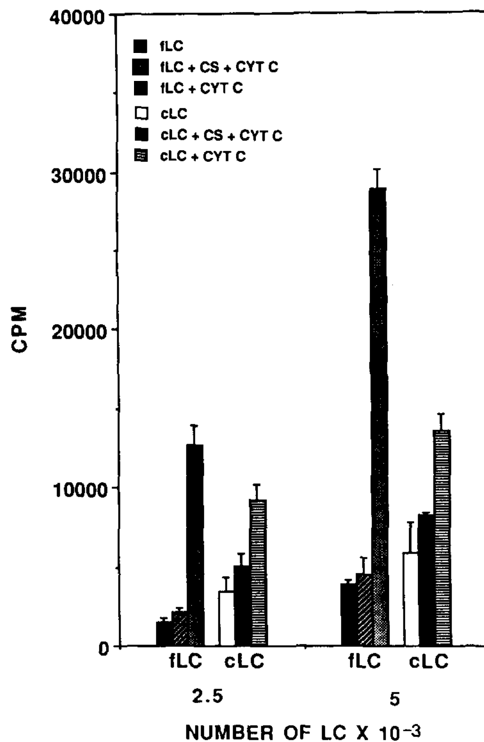

Figure 2. Pretreatment of LC with chloroquine sulfate (CS) can abrogate T cell stimulatory activity of LC. Both fLC and cLC were treated with or without 300 μM chloroquine sulfate (*CS*), and then incubated with 50 μg/ml cytochrome c (*CYT C*) for 2 h at 37°C, and washed. These LC were cultured with the cytochrome c-specific, I-E^{k}-restricted T cell clone, A.E7, and [^{3}H]-TdR incorporation was used as an index of T cell proliferation. fLC and cLC were also cultured with A.E7 without Ag.

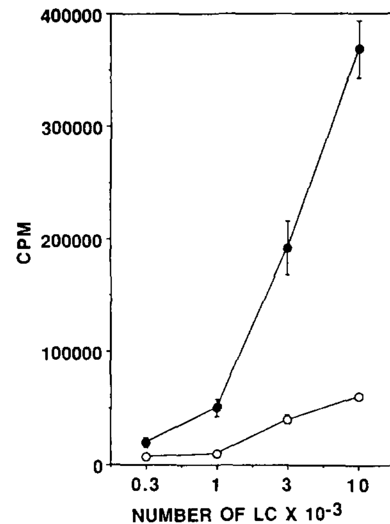

Figure 3. cLC can induce very vigorous allogeneic T cell response. Various numbers of fLC (*open circles*) and cLC (*closed circles*) from C3H mice were cultured with 200,000/well of nylon-wool passed splenic T cell from BALB/c mice for 4 days. [^{3}H]-TdR incorporation was used as an index of T cell proliferation.

recovered, washed three times, and suspended in complete medium. These preparations (fLC) contained from 8 to 18% LC, and were used to examine the functional activity of freshly prepared LC.

EC culture. Single cell suspension of EC (10^{7} cells) were cultured in complete medium at 37°C in 5% CO_2 using 25 ml T flasks (Falcon, Falcon Plastics, Oxnard, CA). After 72 h, the cells were harvested and applied to Lympholyte M density gradients, and centrifuged at 1000 × *g* for 10 min at room temperature. The interface cells (cLC) were washed three times and suspended in complete medium. cLC routinely contained from 30 to 55% Ia Ag-bearing cells. In some experiments, cLC were cultured for an additional 2 to 4 days, in complete medium supplemented with 10 U/ml human IL-1α (Genzyme Corporation, Boston, MA) and 250 U/ml mouse granulocyte macrophage-CSF (Genzyme) and assayed for functional activity.

Flow cytometry. fLC or cLC from various strains of mice were first incubated with mAB 2.4G2 (culture supernatant) for 10 min to prevent Fc-binding of reagents (19), and then incubated with saturating amounts of FITC-conjugated anti-Ia mAb or nonreactive isotype-matched controls (Becton Dickinson Immunocytometry System, Mountain View, CA) (1 to 5 μg/10^{6} cells) for 30 min at 4°C. The cells were then washed three times with balanced salt solution without Ca and Mg HBSS containing 1% BSA, 0.02% EDTA, and 0.01% DNase (Sigma). Propidium iodide was added at a final concentration of 0.5 μg/ml to each sample just before analysis to identify dead cells. The cell suspensions were then analyzed by FACScan (Becton Dickinson). The number of LC was calculated as: number of LC = cell numbers of trypan blue non-staining EC × fraction of Ia^{+} cells in PI non-staining EC.

Ag. OVA, HEL, and cytochrome c (from pigeon breast muscle) were purchased from Sigma. Pigeon cytochrome c fragment 81-104 and HEL fragment 103-120 were kindly provided by Dr. J. Ashwell (NCI, Bethesda, MD) and Dr. P. E. Jensen (The Department of Pathology, Emory University School of Medicine, Atlanta, GA), respectively. Sperm whale myoglobin was prepared as described previously (20), and sperm whale myoglobin fragment 102-118, synthesized as described (21), were gifts of Dr. J. A. Berzofsky (NCI, NIH).

Cell lines. A panel of T cell clones and hybridomas reactive with protein Ag were used in this study. Ia^{k}-restricted T cell hybridomas

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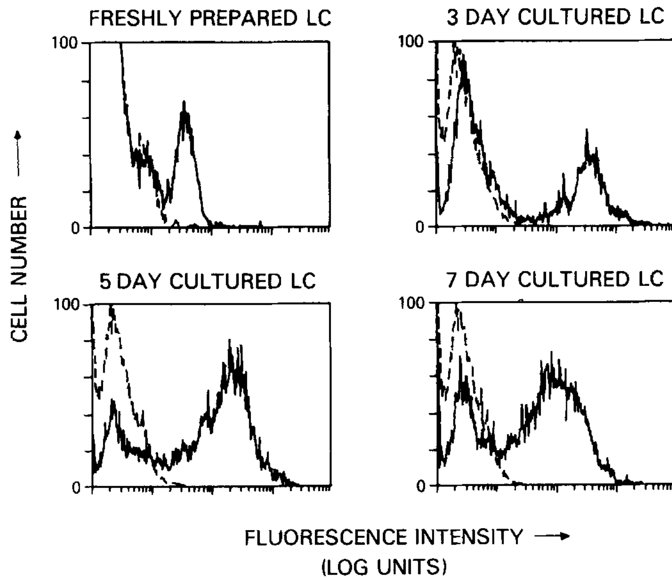

Figure 4. Class II MHC Ag expression by cLC decreases after several days of culture. fLC and LC cultured for 3, 5, and 7 days were stained with FITC-conjugated M5/114.5.2 (*solid lines*), or with an FITC-conjugated isotype (*dotted lines*), and analyzed by FACScan. The mean fluorescence intensity of the fLC was 53 compared to that of 3, 5, and 7 days which was 529, 274, and 186, respectively.

(E3 and E8) specific for OVA were established in our laboratory (22). The pigeon cytochrome *c* specific and I-E^k-restricted T cell hybridoma, 2C2, the HEL-specific, I-A^k-restricted T cell hybridoma C10.9, and the cytochrome *c*-specific, I-E^k-restricted T cell clone A.E7 (gift from Dr. J. Ashwell), (23–25) the HEL-specific, I-E^d-restricted T cell hybridoma HD1-A.C5 (26) (gift from Dr. P. E. Jensen), and the myoglobin-specific I-A^d-restricted T cell clone TK.G4 (gift from Dr. J. A. Berzofsky) were also used as described (27).

Proliferation of T cell clones. After resting for more than 14 days in complete culture medium with 10% culture supernatant of rat T cells simulated with Con A (Collaborative Research Inc., Bedford, MA), the responder clones, A.E7 and TK.G4, were cultured for 64 to 70 h with varying numbers of APC with or without Ag. These responder cells were treated with a combination of anti-Ia (M5/114.5.2), anti-rat κ (MAR 18.5, ATCC) (28) and complement (Low-tox-M, Cedar Lane, Inc., Ontario, Canada) before addition to the assay in order to deplete them of residual APC. Culture was performed at 37°C in 5% CO_2 for 64 to 70 h in complete medium in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). [^{3}H]-TdR (1 μCi/well) (New England Nuclear, Boston, MA) was added for the final 16 to 18 h of culture. The cells were then harvested by using a semi-automated cell harvester (PHD Cell Harvester, Cambridge Technology Inc., Cambridge, MA) and incorporation of [^{3}H]-TdR was measured by a liquid scintillation counter. Data were expressed as the arithmetic mean cpm ± SEM. To decrease background proliferation, EC and spleen cells were exposed to 1500 and 3000 rad, respectively, from a γ cell 1000 irradiator.

IL-2 production by T cell hybridoma. The T cell hybridomas were cultured with varying numbers of APC and with intact protein Ag or peptides in complete culture medium in 96-well flat-bottom microtiter plates. After a 24-h culture period, the supernatants were assayed for IL-2 using the IL-2-sensitive cell line, CTLL (from ATCC). A total of 10^4 cells was incubated with various dilutions of supernatant for 34 to 38 h. During the final 10 to 12 h of culture, [^{3}H]-TdR (1 μCi/well) was added. Cells were harvested, and incorporation of radioactivity was assessed as described above.

Chloroquine pretreatment. In some experiments, LC were pretreated with 300 μM chloroquine at 37°C for 1 h, washed, incubated with 50 μg/ml pigeon cytochrome *c* or the relevant cytochrome *c* peptide 81-104 for 2 h at 37°C, and washed (29). The ability of these LC to stimulate A.E7 proliferation was determined as described above.

Epidermal lymphocyte reaction. For the primary allogeneic epidermal lymphocyte reaction, responder lymphocytes were obtained from spleens of various strains of mice. Purified T cells were obtained by passage of RBC-depleted spleen cells through nylon wool columns. These T cells were co-cultured (2 × 10^5/well) with varying numbers of allogeneic stimulator fLC, cLC, or RBC-depleted spleen cells in 96-well flat-bottom microtiter plates for 4 days at 37°C in a 5% CO_2 humidified atmosphere. During the last 16 h of culture, the cells were pulsed with 1 μCi/well of [^{3}H]-TdR. Cells were harvested, and incorporation of radioactivity was assessed as described above in "proliferation of T cell clones." To decrease background proliferation, EC and spleen cells were irradiated as described above.

Ag-dose response of T cell clones. To determine whether increasing the dosage of intact protein Ag affected the response of the Ag-specific T cells, we used varying amounts of intact protein Ag in culture.

Purification of cLC. In some experiments, freshly prepared epidermal cell suspensions were treated with anti-Thy-1.2 antibody (Becton Dickinson) and complement (Low-tox-M), and then applied onto Lympholite M, and centrifuged at 1000 × *g* for 10 min at room temperature. The interface cells were treated with anti-Ly-5.1 antibody (New England Nuclear, Boston, MA) for 30 min on ice, and then Ly-5.1$^+$ epidermal cells were separated by using Dynabeads M-450 coated with sheep anti-mouse IgG (Dynal, Oslo, Norway). These Ly-5.1$^+$ cells were cultured for 3 days in the presence of 10 U/ml human IL-1α (Genzyme Corp., Boston, MA) and 250 U/ml mouse granulocyte macrophage-CSF (Genzyme), and only Dynabead-free cells (separated using a magnet) were used as purified cLC for flow cytometry analysis and for functional assay.

RESULTS

cLC from C3H mice stimulate T cell clones and hybridomas in presence of intact protein Ag. fLC and cLC from C3H mice induce significant proliferation or IL-2

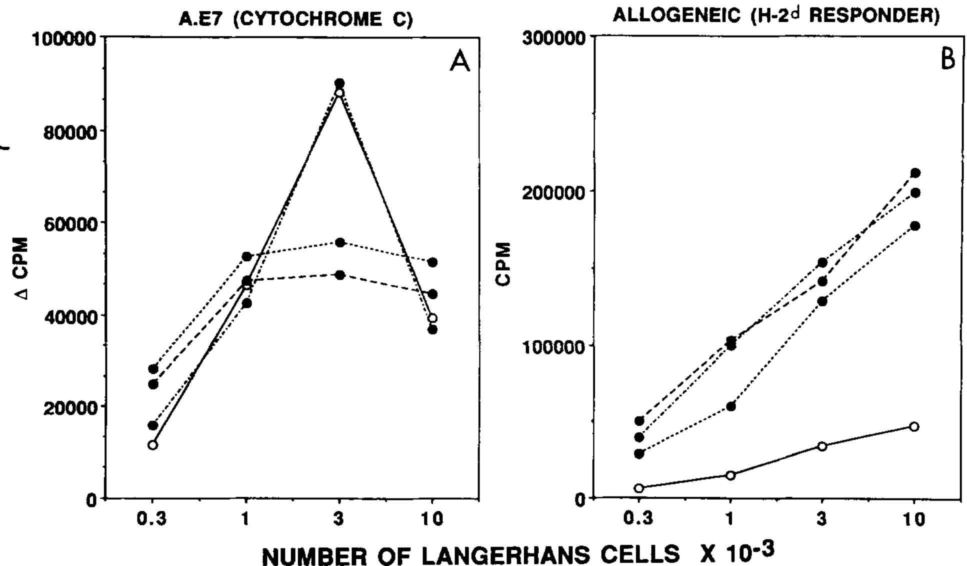

Figure 5. cLC (*closed circles*), even after more than 3 days culture, stimulate both the cytochrome *c*-specific T cell clone A.E7 (*A*) as well as allogeneic BALB/c T cells (*B*). [^{3}H]-TdR incorporation by responder T cells was used as an index of T cell proliferation. fLC (*open circles*), 3 day cLC (–·–·–), 5 day cLC (– – –), 7 day cLC (– – –).

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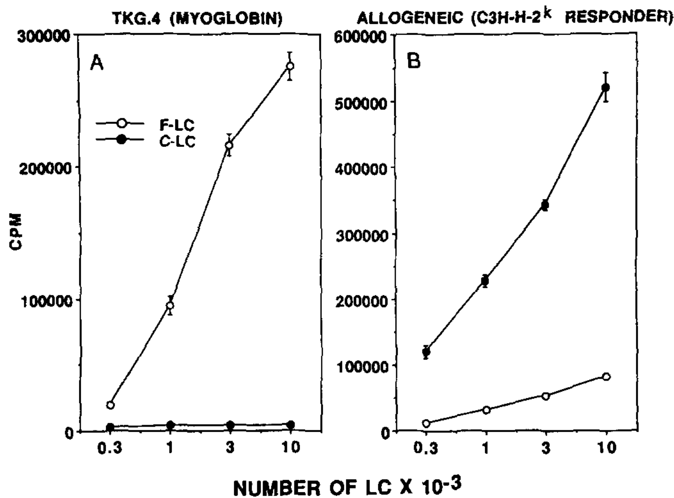

Figure 6. cLC from BALB/c mice cannot process and present protein Ag to a specific T cell clone, although they can stimulate allogeneic T cells vigorously. The myoglobin-specific, I-A^d-restricted T cell clone, TK.G4 (*A*), and nylon-wool passed allogeneic splenic T cells (*B*) were stimulated with varying numbers of fLC (*open circles*) and cLC (*closed circles*) from BALB/c mice. T cell clones were cultured with or without 2 μM myoglobin. [^{3}H]-TdR incorporation by the clone cultured with fLC or cLC without Ag was less than 1000 cpm. [^{3}H]-TdR incorporation of allogeneic T cells alone was less than 1000 cpm.

production by protein Ag-specific T cell clones (A.E7) and hybridomas (2C2 and E3) in the presence of pigeon cytochrome *c* (A.E7 and 2C2) or OVA (E3) (Fig. 1). In some experiments, on a per cell basis, stimulation of the clone or hybridoma was the same using either fLC or cLC. However, in most experiments (75%), fLC exhibited more potent Ag-presenting function using the intact protein Ag. Pretreatment of the fLC or cLC with chloroquine abrogated their Ag-presenting function in the stimulation of A.E7, the cytochrome *c*-specific T cell clone, suggesting that endosomal processing is essential for the presentation of intact pigeon cytochrome *c* by both fLC and cLC (Fig. 2). In comparison to fLC, cLC induced a very vigorous proliferative response by allogeneic T cells (Fig. 3) and exhibited markedly enhanced class II MHC expression (Fig. 4). Because of results with Ia^d cLC (see below) we determined the effect of further culture of C3H LC (beyond 3 days) on their class II MHC expression and function. cLC were cultured for an additional 2 and 4 days. Although there was a small gradual decrease in class II MHC expression with increasing time (beyond 3 days) in culture (Fig. 4), the cLC maintained their soluble protein Ag-presenting functions (Fig. 5*A*) as well as their alloantigen-presenting functions (Fig. 5*B*).

cLC from BALB/c mice do not stimulate T cell clones and hybridomas in presence of intact protein Ag. In contrast to our findings with C3H mice, cLC from BALB/c mice could not stimulate the myoglobin-specific T cell clone, TK.G4 in the presence of the intact protein Ag (Fig. 6*A*) although they exhibited enhanced class II MHC expression (mean fluorescence intensity of fLC was 325 compared to that of cLC that was 1032) and vigorous alloantigen-presenting function (Fig. 6*B*). This poor stimulatory function of BALB/c cLC in the presence of the intact protein Ag myoglobin was also observed when the intact protein Ag hen egg lysozyme-specific T cell hybridoma Hd-1.AC5 was used (Fig. 7*A*). Because BALB/c cLC present the relevant HEL peptide 106-129 to Hd-1.AC5, we conclude that they are unable to process intact protein Ag (Fig. 7*B*).

cLC from C3H mice stimulate the HEL-specific T cell hybridoma in presence of intact protein HEL. Inasmuch as BALB/c cLC did not stimulate Hd-1.AC5, the HEL-(and I-E^d) specific T cell hybridoma, we determined whether C3H cLC could process and present HEL to HEL- (and Ia^k) specific T cell hybridomas. Indeed, cLC from C3H mice were as efficient as fLC at inducing IL-2 production from these cells (Fig. 8).

cLC from Ia^k-bearing mice can, but cLC from Ia^d-bearing mice cannot, stimulate T cell clones or hybridomas in presence of intact protein Ag. To determine whether the differences observed between cLC from C3H and BALB/c mice were related to MHC or background genes, we studied additional strains of H-2^d and H-2^k mice. When we used other Ia^k expressing cLC (from AKR and B10.A mice) we found that they can process and present native pigeon cytochrome *c* and HEL to protein-specific T cell clones and hybridoma (Fig. 9). However, Ia^d expressing cLC (from DBA.2 mice) did not stimulate the myoglobin- or HEL-specific T cell clone TKG.4 or hybridoma Hd-1.AC5 in the presence of the intact protein Ag (Fig. 10). Furthermore, cLC from BALB.k mice (which differ from BALB/c only at H-2) efficiently processed and presented native ovalbumin and pigeon cytochrome *c* to protein-specific T cell hybridomas (Fig. 11).

Ag-dose response of T cell clones. When the concentrations of intact protein Ag were varied, cLC induced as

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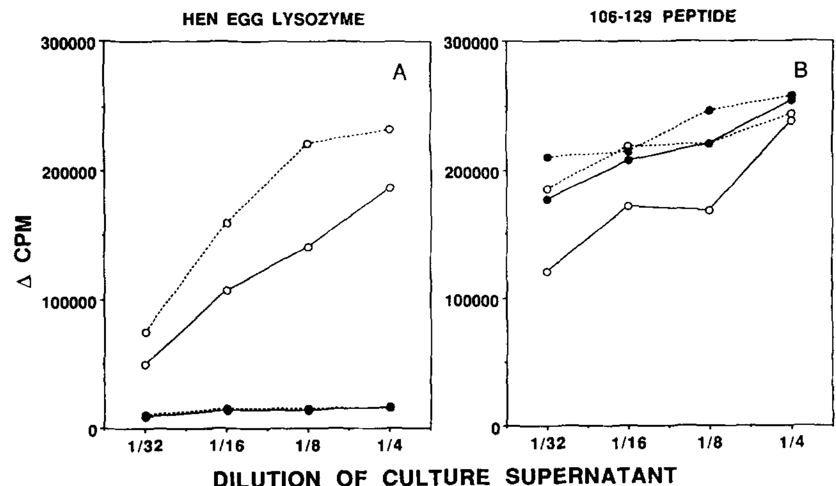

Figure 7. cLC from BALB/c mice cannot process and present intact protein Ag but can present the relevant peptide to the HEL-specific, I-E^d-restricted T cell hybridoma Hd1-AC5. Varying numbers (6,000—*continuous line* or 20,000—*interrupted line*) of fLC (*open circles*) or cLC (*closed circles*) from BALB/c mice were incubated with the hybridoma in the presence of 300 μg/ml HEL (*A*) or 50 μg/ml of the 106-129 peptide (*B*). The supernatants that were generated after 24 h were cultured with CTLL, and [^{3}H]-TdR incorporation by CTLL was used as an index of IL-2 production by the hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from the culture without Ag was less than 1000 cpm.

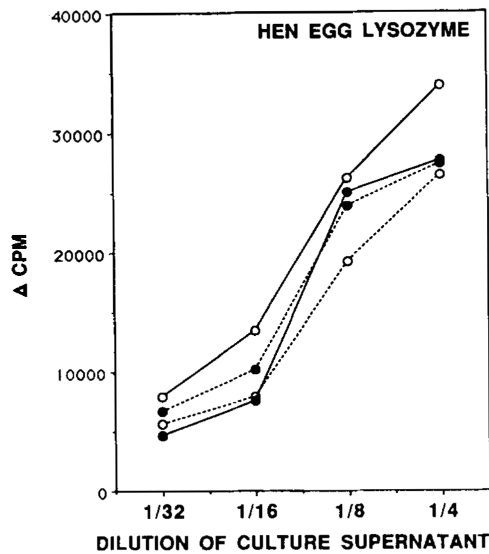

Figure 8. cLC from C3H mice can process and present HEL to the HEL-specific I-A^k-restricted T cell hybridoma, C10.9. Varying numbers (6,000—*continuous line* or 20,000—*interrupted line*) of fLC (*open circles*) or cLC (*closed circles*) from C3H mice were incubated with the hybridoma in the presence of 100 μg/ml HEL. The supernatants that were generated after 24 h were cultured with CTLL in various dilutions, and [^{3}H]-TdR incorporation by CTLL was used as an index of IL-2 production by the hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from the culture without antigen was less than 1000 cpm.

much proliferation as fLC at high concentrations but induced less proliferation of cytochrome *c*-specific, I-E^k-restricted T cell clones at lower concentrations of intact protein Ag (Fig. 12*A*). In contrast, when Ia^d cLC were used with varying concentrations of myoglobin, there was virtually no T cell proliferation induced when 1 μM of Ag was used. Although some T cell proliferation was induced with cLC at higher concentrations of intact protein, compared to fLC, these Ia^d cLC were very poor inducers of T cell proliferation (Fig. 12*B*).

cLC from (C3H × BALB/c) F_1 mice stimulate Ia^k- but not Ia^d-restricted T cell clones and hybridomas. To determine whether the difference in Ia^k and Ia^d presentation of Ag would be retained when the same cLC from (Ia^d × Ia^k) F_1 mice were used, we assessed the Ag-presenting function of these cLC using various Ia^d- and Ia^k-restricted T cell clones. We found that these cLC could not stimulate cLC from Ia^d-restricted T cells but stimulated the Ia^k-restricted T cell clones very effectively (Fig. 13).

Highly purified cLC from C3H mice can process and present intact protein antigen to pigeon cytochrome c-specific T cell clone. We next determined whether it is the cLC or keratinocytes that process protein Ag for presentation by Ia^k cLC. Purified cLC (96% Ia^+, 4% keratinocytes) still stimulated the pigeon cytochrome *c*-specific T cell clone in the presence of intact protein (Fig. 14). On a per cell basis, these purified cLC were virtually as efficient as the enriched cLC (34%) population at performing this function.

Ag-pulsed fLC from BALB/c mice that are cultured are able to very effectively induce proliferative response in TKG.4, myoglobin-specific T cell clone. As cLC from BALB/c mice were unable to process native protein but could present the relevant peptide to cloned T cells (see above), we determined whether, if LC are exposed to native protein overnight and then cultured, they could induce a T cell proliferative response several days later. When LC were pulsed overnight with the intact molecule and then cultured for 1 or 3 days, they very effectively induced a proliferative response in the T cell clone (Fig. 15). As before, 1- or 3-day BALB/c cLC were unable to process and present intact protein Ag.

DISCUSSION

Epidermal Langerhans cells are capable of functioning as APC in the activation of T cells for three different kinds of Ag-alloantigens, haptens, and soluble protein Ag. LC may interact with T cells within the epidermis or more likely, they migrate to either the dermis and to the regional lymph nodes where they activate memory or naive T cells. Recent evidence indicates that, after application of haptens to the epidermis, there is activation of a subpopulation of LC which exhibit enhanced APC function (30). As well, there are data which strongly suggest that LC migrate from skin, via lymphatics, to regional

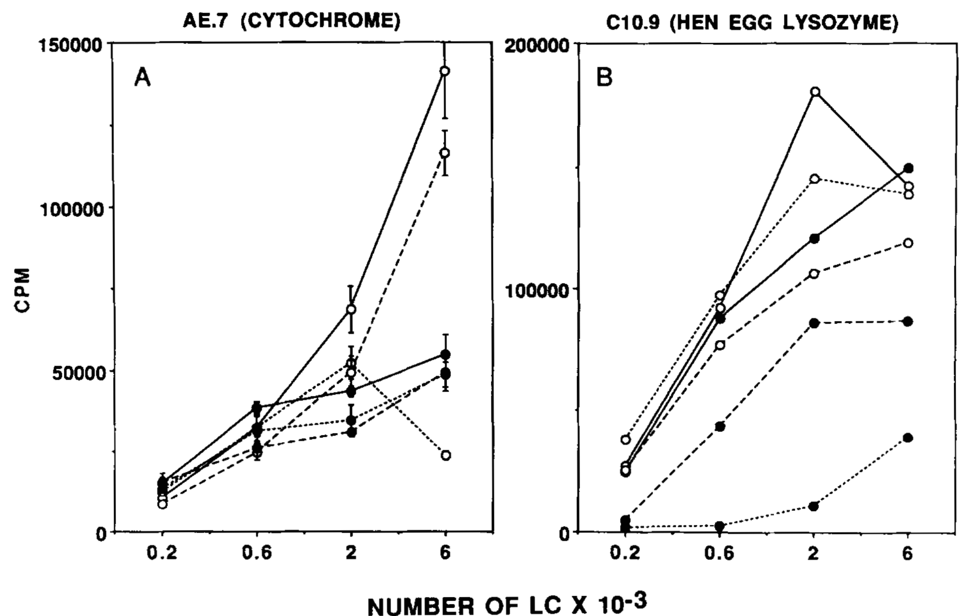

Figure 9. cLC from Ia^k-bearing mice can process and present cytochrome c and HEL to a specific T cell clone or hybridoma. The cytochrome *c*-specific, I-E^k-restricted T cell clone, A.E7, was stimulated with different numbers of fLC (*open circles*) and cLC (*closed circles*) from C3H mice (- - -) AKR mice (———), or B10.A mice (-----) in the presence of 50 μg/ml of pigeon cytochrome *c* (*A*). [^{3}H]-TdR incorporation was used as an index of T cell proliferation. [^{3}H]-TdR incorporation by the clones cultured with fLC or cLC and without Ag was less than 10,000 cpm. The HEL-specific, I-A^k-restricted T cell hybridoma, C10.9 was also stimulated with different concentrations of fLC and cLC from C3H, AKR, or B10.A mice in the presence of 100 μg/ml HEL (*B*). The supernatants (diluted 1/4) that were generated after 24 h were cultured with CTLL, and [^{3}H]-TdR incorporation by CTLL was used as an index of L-2 production by the hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from the culture without antigens was less than 1000 cpm.

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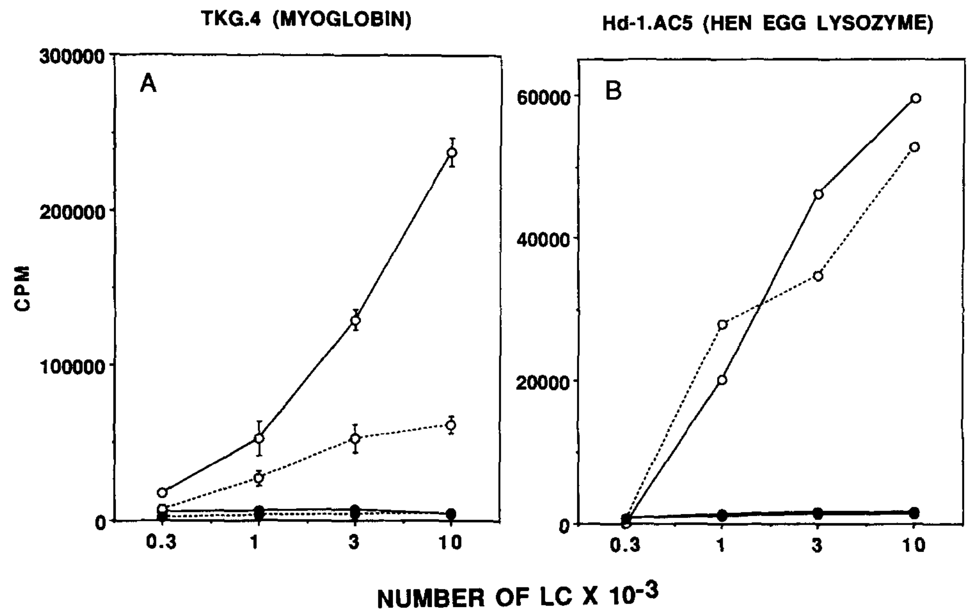

Figure 10. cLC from Ia[d]-bearing mice cannot process and present myoglobin or HEL to an Ag-specific T cell clone or hybridoma. The myoglobin-specific, I-A[d]-restricted T cell clone, TK.G4, was stimulated with different numbers of fLC (*open circles*) and cLC (*closed circles*) from BALB/c mice (——) or DBA.2 mice (- - -). T cell clones were cultured with or without 2 μM myoglobin (*A*). [^{3}H]-TdR incorporation was used as an index of T cell proliferation. [^{3}H]-TdR incorporation of the clones cultured with fLC or cLC without Ag was less than 1000 cpm. The HEL-specific, I-A[d]-restricted T cell hybridoma Hd1-AC5, was stimulated with different numbers of fLC and cLC from BALB/c or DBA.2 mice in the presence of 300 μg/ml HEL (*B*). The supernatants (diluted 1/4) that were generated after 24 h were cultured with CTLL, and [^{3}H]-TdR incorporation by CTLL was used as an index of IL-2 production by the hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from the culture without Ag was less than 1000 cpm.

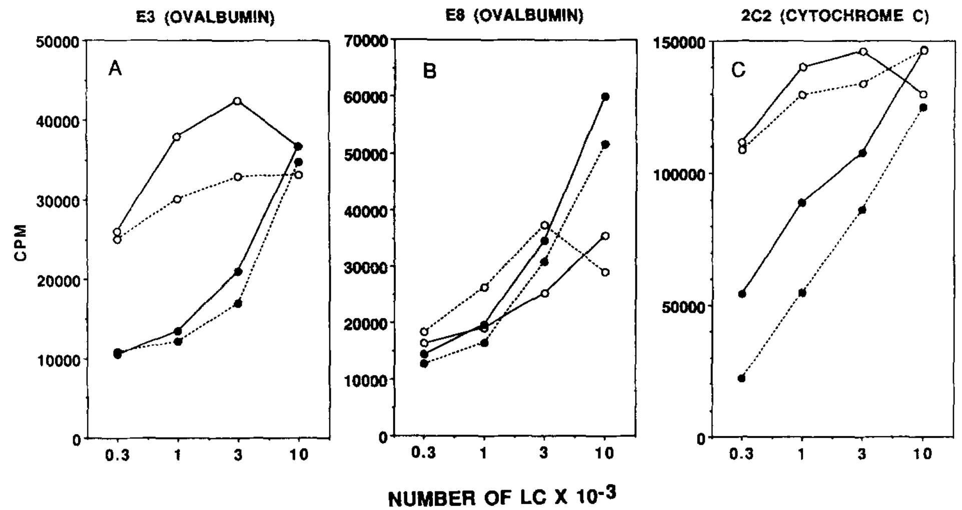

Figure 11. cLC from BALB.k (H-2[k]) mice can process and present protein Ag to T cell hybridomas. The OVA-specific, Ia[k]-restricted T cell hybridoma, E3 (*A*), the ovalbumin-specific Ia[k]-restricted T cell hybridoma, E8 (*B*), and the pigeon cytochrome *c*-specific I-E[k]-restricted T cell hybridoma, 2C2 (*C*), were stimulated with different concentrations of fLC (*open circles*) or cLC (*closed circles*) from C3H (——) or from BALB.k (- - -) mice in the presence of 1 mg/ml OVA or 2.5 mg/ml cytochrome *c*. The supernatants (diluted 1/4) that were generated after 24 h were cultured with CTLL, and [^{3}H]-TdR incorporation by CTLL was used as an index of IL-2 production by the hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from the culture without antigens was less than 1000 cpm.

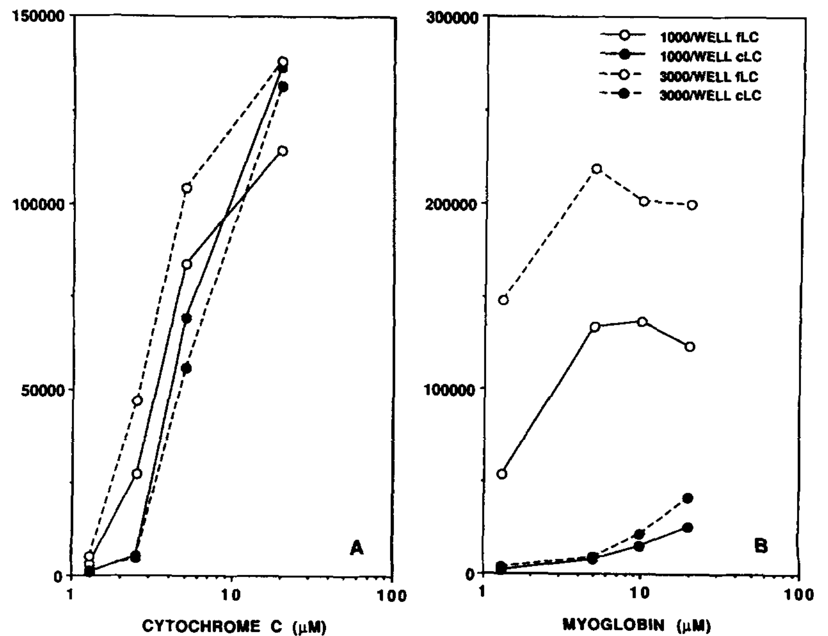

Figure 12. Ag dose-response curve in the stimulation of the pigeon cytochrome *c*-specific I-E[k]-restricted clone A.E7 (*A*) or the myoglobin-specific I-A[d]-restricted clone TK.G4 (*B*). Varying concentrations of intact protein Ag, were added to either fLC or cLC which were cocultured with the syngeneic T cell clone.

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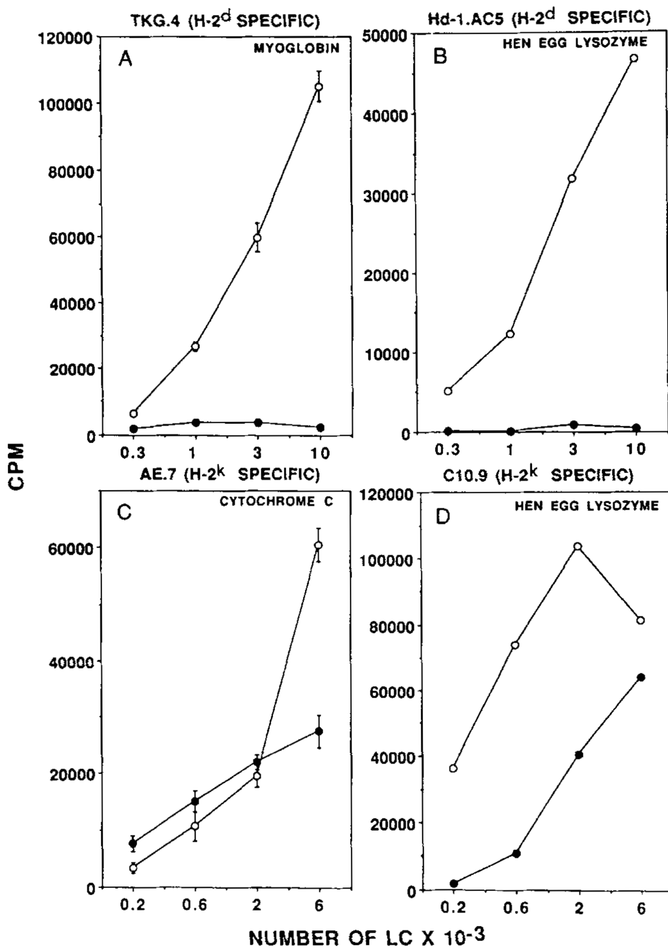

Figure 13. cLC from (C3HxBALB/c) F_1 mice can process and present intact protein Ag to Ia^k-restricted T cell clone or hybridoma, but cannot process intact proteins for presentation to an Ia^d-restricted T cell clone or hybridoma. The myoglobin-specific, I-A^d-restricted T cell clone TK.G4, (*A*), HEL-specific, I-A^d-restricted T cell hybridoma Hd-1.AC5, (*B*), cytochrome *c*-specific, I-E^k-restricted T cell clone A.E7, (*C*), and HEL-specific, I-A^k-restricted T cell hybridoma, C10.9 (*D*), were stimulated with different numbers of fLC (*open circles*) and cLC (*closed circles*) from F_1 mice in the presence of intact protein Ag. [^{3}H]-TdR incorporation was used as an index of T cell proliferation. [^{3}H]-TdR incorporation of these clones cultured with fLC or cLC without Ag was less than 10,000 cpm. The supernatants (diluted 1/4) that were generated after 24 h with the hybridomas were cultured with CTLL, and [^{3}H]-TdR incorporation by CTLL was used as an index of IL-2 production by hybridomas. [^{3}H]-TdR incorporation by CTLL cultured with the supernatant from the culture without Ag was less than 1000 cpm.

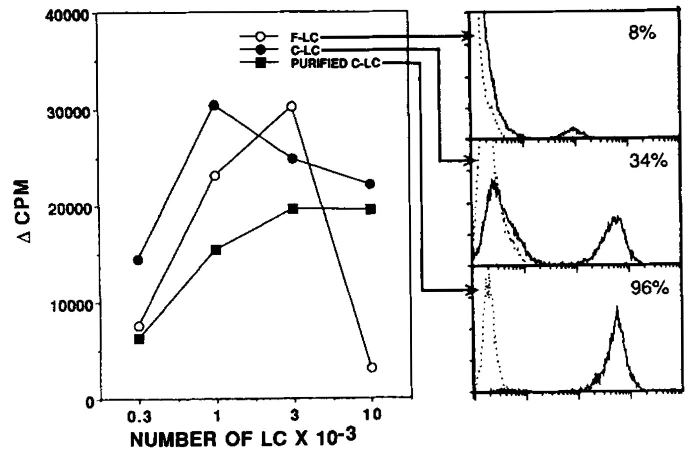

Figure 14. Purified cLC from C3H mice can process and present intact protein Ag to a specific T cell clone. The cytochrome *c*-specific, I-E^k-restricted T cell clone, A.E7, was cultured with different concentrations of fLC, cLC, and cLC (96%) purified by immunomagnetic beads. Δcpm [^{3}H]-TdR incorporation of the culture with Ag minus [^{3}H]-TdR incorporation of the culture without Ag was used as an index of T cell proliferation. Percentage of I-A^k-bearing cells of fLC, cLC, and purified cLC were determined by flow microfluorimetry. Percentages of cLC in each group are noted. Equal numbers of cLC were used in each culture.

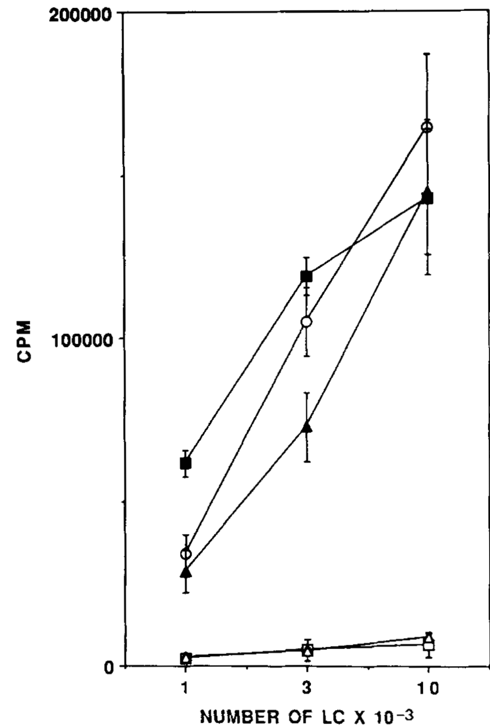

Figure 15. BALB/c LC that were pulsed in vitro with intact protein Ag immediately after being obtained from the skin can after 3 days of culture, stimulate a T cell clone. The myoglobin-specific, I-A^d-restricted T cell clone, TK.G4, was cultured with: fLC incubated with intact myoglobin throughout culture (*open circles*), 1 day cLC in the absence of Ag (*open triangles*), 3 day cLC in the absence of Ag (*open squares*), or with fresh LC that had been pulsed with the intact myoglobin for 18 h and then washed and cultured for 1 day (*closed squares*) or 3 days (*closed triangles*). [^{3}H]-TdR incorporation was used as an index of T cell proliferation.

lymph nodes and therein present Ag to T cells (10, 11).

Functional studies of LC in vitro have used single cell suspensions of epidermal cells with or without the use of LC enrichment techniques. These in vitro studies were performed exclusively with freshly prepared epidermal cell suspensions until Schuler and Steinman (9) described a short term culture system for LC that enhanced the alloantigen presenting abilities of these cLC. Subsequent studies have demonstrated that cLC exhibit markedly enhanced class II MHC Ag expression and serve as very effective presenters of haptens and soluble protein Ag (7, 8).

Differing experimental results have been reported regarding the ability of short term cLC to process and present intact protein Ag to specifically sensitized T cells (7, 8, 13). In an attempt to resolve this controversy, we tested the ability of fLC and cLC from different inbred strains of mice to process and present several intact protein Ag to a panel of T cell clones and T cell hybridomas. We used C3H and other Ia^k-bearing mice because our prior studies demonstrated processing and presentation of soluble protein Ag by these strains (7, 8); we also used BALB/c and other Ia^d-bearing mice because other investigators have been unable to demonstrate processing of intact protein Ag by cLC from BALB/c mice (13). We also used several different intact proteins for

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the generation of T cell responses. We found that, in contrast to Ia^d-bearing mice whose cLC could not process and present intact protein Ag, cLC from various Ia^k-bearing mice do process and present intact protein Ag. Even when using widely varying protein concentrations, the differences between cLC and fLC in Ia^k and Ia^d were strikingly different. By using relatively purified (96%) cLC, we demonstrated that it is the cLC and not the usually contaminating keratinocytes that actually process the intact proteins.

Perhaps the most important and revealing experiments performed in this study are those that used ($Ia^k \times Ia^d$) F_1 mice in that cLC from these mice could process and present Ag for Ia^k plus X responsive T cells but not Ia^d plus X responsive T cells. How can this occur when the expression of Ia^k and Ia^d on the F_1 cLC is enhanced to the same magnitude? If the proteolytic enzyme pathways used for the degradation of intact proteins are different for each of the haplotypes, it is possible that some and not other of these enzymes would be lost during culture. However, there appears to be disagreement as to whether endocytic activities are altered even when one compares fLC to cLC from Ia^d mice (31, 32). More likely, however, is the possibility that there may be a difference in the dissociation of the invariant chain in cLC of each of these inbred strains. Thus, if dissociation of invariant chain from the class II MHC Ag of cLC from $H\text{-}2^d$ mice occurs very early or very late, there may be very poor association of the relevant peptide fragment with the appropriate class II MHC Ag and this would obviate activation of the primed T cells. However, if dissociation of invariant chain occurred at a different rate in $H\text{-}2^k$ cLC, association of the relevant peptide may proceed in a manner which allows for T cell recognition (33, 34). Indeed, very recent evidence indicates that in $H\text{-}2^d$ mice, the invariant chain is present on the surface of fLC but not cLC (31).

This same question regarding processing function of APC also relates to splenic dendritic cells. Romani et al. (13) have reported that splenic dendritic cells also function relatively (to fLC) poorly, in Ag processing. In contrast, Chain et al. (35) and Katz et al. (36) demonstrated that splenic dendritic cells efficiently process and present some intact protein Ag to some T cells. Significantly, these studies used different combinations of class II MHC haplotypes and intact protein Ag. Romani et al. (13) used Ia^d-restricted T cell clones and sperm whale myogloblin whereas the others (35, 36) used Ia^k-restricted T cells and keyhole limpet hemocyamin or Ia^b-restricted T cells and OVA. The finding, therefore, of differences in processing abilities of all types of APC may relate to the particular class II MHC Ag or haplotype.

One must ask why with their enhanced class II MHC expression, and with their extraordinary potency as alloantigen-presenting cells, these cLC are not superpotent protein APC, particularly because others have clearly demonstrated that Ag presentation to T cells is a function of Ia Ag levels (37–39). The reason for this is unknown. Our initial studies, using primarily haptens, suggested that these Ag were more effectively presented to T cells by cLC than by fLC; however, some experiments also indicated that the same was true for intact protein Ag. The studies reported herein indicate that despite the enhanced class II MHC expression and enhanced alloantigen-presenting function of $H\text{-}2^k$ cLC, the ability of these cells to present intact protein Ag was the same or less efficient than when fLC were used. Clearly, however, the cLC from Ia^k mice were able to process and present intact protein Ag such that these cLC have been used to induce primary sensitization of naive T cells to intact protein Ag in vitro (8). Data from others (31) and our preliminary data suggest that class II MHC turnover in cLC is far slower than in fLC and this may account, at least in part, for the finding that cLC are not superpotent APC for intact protein Ag (data not shown).

Ultimately, we are interested in knowing whether LC function in vivo in processing and presenting intact protein Ag. If LC are exposed to intact protein Ag in the skin, are they able to process the Ag and induce the activation of T cells? Although no in vivo experiments were performed in this study the probable answer to this question is "yes" for each of the strains tested. Thus, we found that, in vitro, if Ia^d or Ia^k fLC are pulsed with protein Ag, they are fully capable of inducing proliferation of cloned T cells. This is in keeping with the recent report of Puré et al. (31) and implies that although cLC may be considered the in vitro equivalents of LC that had migrated from the epidermis to the regional lymph nodes, they may do their processing in the skin or in transit to regional lymph nodes and exhibit the relevant class II MHC-peptide complex when they arrive in the regional lymph nodes where they activate T cells.

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