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Antigen Processing by Epidermal Langerhans Cells Correlates with the Level of Biosynthesis of Major Histocompatibility Complex Class II Molecules and Expression of Invariant Chain

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Summary

Two prior studies with a small number of T cell lines have shown that the presentation of native protein antigens by epidermal Langerhans cells (LC) is regulated. When freshly isolated, LC are efficient antigen-presenting cells (APC), but after a period of culture LC are inefficient or even inactive. The deficit in culture seems to be a selective loss in antigen processing, since cultured LC are otherwise rich in major histocompatibility complex (MHC) class II products and are active APC for alloantigens and mitogens, which do not require processing. We have extended the analysis by studying presentation to bulk populations of primed lymph node and a T-T hybrid. Only freshly isolated LC can be pulsed with the protein antigens myoglobin and conalbumin, but once pulsed, antigen is retained in an immunogenic form for at least 2 d. The acquisition of antigen, presumably as MHC-peptide complexes, is inhibited if the fresh LC are exposed to foreign protein in the presence of chloroquine or cycloheximide. The latter, in contrast, improves the efficacy of antigen pulsing in anti-Ig–stimulated B blasts. In additional studies of mechanism, we noted that both fresh and cultured LC endocytose similar amounts of an antigen, rhodamine-ovalbumin, into perinuclear granules. However, freshly isolated LC synthesize high levels of class II MHC molecules and express higher amounts of the class II–associated invariant chain. Fresh LC are at least 5–10 times more active than many other cell types in the level of biosynthesis of MHC class II products. These findings provide a physiologic model in which newly synthesized MHC class II molecules appear to be the principal vehicle for effective antigen processing by APC of the dendritic cell lineage. Another APC, the B lymphoblast, does not appear to require newly synthesized MHC class II molecules for presentation.

The solution by Bjorkman et al. (1) of the crystal structure of MHC class I molecules, and the evidence from many laboratories that small peptides are stimulatory for T cells (2–11), have provided direct evidence that MHC-peptide complexes are the ligand recognized by the TCR. Nonetheless it remains difficult to monitor the formation of MHC-peptide complexes on living APC, and to study the mechanism of complex formation.

The principal approach for studying mechanisms of presentation is to use inhibitory compounds. For class II MHC products, agents like chloroquine that neutralize intracellular acidic compartments (12) block presentation of many protein antigens (13). A low pH may be required for proteolysis by acid hydrolases (13), association of peptides with class II (14), or release of the invariant chain from its complex with class II (15). The inhibitor of protein synthesis, cycloheximide (CHX)[1], also blocks class II presentation but only by peritoneal cells (16) and not by a B cell line (17). The site of action of CHX is unclear. It may be at the level of MHC class II biosynthesis, as suggested by analogy with the requirements for presentation by class I MHC products. Brefeldin A, which interferes with the egress of proteins from the rough

[1] *Abbreviations used in this paper:* CHX, cycloheximide; EC, epidermal cells; LC, Langerhans cells; RER rough endoplasmic reticulum; rho-ova, rhodamine ovalbumin; SpWMb, sperm whale myoglobin.

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endoplasmic reticulum (RER) (18), blocks presentation (19). Townsend et al. (20), by studying a variant cell line that fails to express class I at the cell surface, found that exogenous peptides can direct the assembly of newly synthesized β_2 microglobulin–class I heterodimers before the brefeldin-sensitive point in protein export. So for both class I and II, newly synthesized MHC products may be an important site for charging these molecules with peptides.

An alternate approach to unraveling the requirements for processing and presentation would be to identify physiologic situations in which the efficacy of these events is regulated. One such possibility has arisen in studies of antigen presentation by dendritic cells. When freshly isolated from both epidermis (21, 22) and spleen (23), dendritic cells can present native proteins. However, after a day in culture, presentation of proteins markedly decreases. This change is not due to a loss of antigen-presenting MHC products, because the levels of these products increase in the cultured Langerhans cells (LC), and the LC present peptide fragments. Cultured LC are fully capable of inducing T cell growth and DNA synthesis as well, since they are the most active accessory cell yet identified for stimulating responses to alloantigens and mitogens. The latter do not require de novo antigen processing. Therefore, it appears that cultured LC are selectively deficient in the mechanism for processing antigens into MHC-peptide complexes, but in contrast express all the additional machinery for stimulating T cells.

Here we analyze some of the potential sites at which processing may differ between fresh and cultured APC. One variable, endocytic activity, does not seem to diminish in culture, but two other variables change markedly. The freshly isolated LC actively synthesize MHC class II products, and these LC express high levels of invariant chain. Cultured LC are negative in both regards. Based on these findings and the sensitivity of presentation by fresh LC to CHX, we propose that newly synthesized MHC products are quantitatively an important venue for antigen processing and presentation in dendritic cells. This is not the case for the APC function of stimulated B lymphoblasts.

Materials and Methods

Animals. Female CD_2F_1 mice were purchased from The Trudeau Institute (Saranac Lake, NY) and used at 8–12 wk of age.

Reagents. Antigens used in this study were sperm whale myoglobin (SpWMb) and conalbumin (Sigma Chemical Co., St. Louis, MO). A myoglobin peptide, representing amino acids 106–118 with an additional NH_2-terminal tyrosine residue, was synthesized by The Rockefeller University Sequencing Facility. Chloroquine and CHX (Sigma Chemical Co.) were used at the doses indicated. All cells were cultured in RPMI 1640 medium supplemented with antibiotic, antimycotic, 2 mM glutamine, 50 μM β-ME, and 5–10% heat inactivated fetal calf serum.

Antigen-presenting Cells. Epidermal cells (EC) were prepared from ear epidermal sheets as described (24). Briefly, fresh LC were enriched from bulk epidermal cells by treatment with anti-thy-1 and complement followed by trypsin. This depletes ~90% of the keratinocytes, so that the preparations contain 10–15% Ia^+ LC. LC were enriched from cultured EC by flotation on BSA columns (24). These preparations contained 30–80% Ia^+ LC.

Anti-Ig blasts were prepared as described (25). Briefly, spleen cells were depleted of red cells (ammonium chloride lysis), T cells (mAb and complement), and adherent cells (passage over Sephadex G-10). The purified B cells were cultured with Sepharose-coupled goat anti-mouse Ig antibodies for 48 h. The blasts were harvested by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation.

All APC were exposed to 900 rad from a cesium source immediately before coculture with T cells. The APC either were added directly with antigen to T cells (continuous exposure), or the APC were "pulsed" with antigen for 12–16 h before washing and addition to T cells in the absence of additional antigen. The final volume was 200 μl in flat-bottom microwells.

T Cells. The T cell hybridoma 11.3.7eH2 was obtained by fusion of the SpWMb-specific, I-E^d–restricted T cell clone 11.3.7.e (26) with the BW5147 TCR-α^-/β^- cell line (27). 3 d before fusion, the T cell clone was restimulated as previously described (26) in the presence of 5 U/ml of human rIL-2 (Cetus Corp, Emeryville, CA). After removal of dead cells on a Ficoll-Paque gradient 2–10 $\times$ 10^6 cells were fused with 40 $\times$ 10^6 BW5147 cells as described (28). The 11.3.7eH2 T cell hybridoma (referred to hereon as 11.3.7) was assayed for antigen specificity by IL-2 production in response to a series of SpWMb peptides and syngeneic APC. The antigen fine specificity of the T cell hybridoma was indistinguishable from the 11.3.7e clone. Stimulation of the T-T hybridoma cells was monitored by assaying the conditioned media, harvested at 24 h after culturing with APC, on the IL-2–dependent CTLL line. CTLL were plated at 5 $\times$ 10^3 per well and pulsed with 0.5 μCi of [^{3}H]TdR during the last 4 h of a 24-h assay. Data shown were obtained with a subsaturating dose of 25 μl of test supernatant.

Primary populations of antigen-primed LN cells were obtained from popliteal and brachial LN 10 d after priming the footpads with 100 μg of protein in CFA. The LN cells were not used directly, because of the high "background" proliferation (syngeneic MLR) that occurs when dendritic cells are mixed with primary T cells in the absence of antigen (29). Instead, as described elsewhere (23), the LN cells were expanded in vitro with protein antigen in the presence of 0.5% heat-inactivated mouse serum. 10–12 d later, the cultures were harvested and floated on Ficoll-Paque columns to isolate viable cells. The latter were used as responders at a dose of 3 $\times$ 10^4 per microtest well in the presence of Click's medium with antibiotics, 50 μM β-ME, and 0.5% mouse serum.

Pinocytosis of Rhodamine-conjugated Ovalbumin. Pinocytosis by LC was monitored after exposure to 0.1 mg/ml rhodamine-ovalbumin (rho-ova), (Molecular Probes, Inc., Eugene, OR) at 0–16 h or 50–66 h, which were the conditions used to pulse APC in all of our presentation experiments. No uptake could be observed with only a 2-h exposure to rho-ova. After washing, the epidermal cells were spun onto slides, stained with M5/114 mAb to mouse Ia antigens plus FITC mouse anti-rat Ig to identify the LC, and examined by two-color fluorescence microscopy.

Radiolabeling of LC and Immunoprecipitation. Cells were methionine starved for 1 h in methionine-free media and then labeled with 1 mCi [^{35}S]methionine (Trans ^{35}S-label; ICN Radiochemicals, Irvine, CA) for 4 h at 37°C supplemented with 2% dialyzed FCS. After washing in FCS-containing medium, the cells were lysed in 1% NP-40. Alternatively, 2 $\times$ 10^6 LC were surface radioiodinated with 3 mCi of $Na^{125}I$ (1 Ci-37GB1; New England Nuclear, Boston, MA) by the lactoperoxidase/glucose oxidase method, washed extensively, and lysed in 1% NP-40. The 10,000-*g* postnuclear extracts obtained from either of these lysates were

precleared twice with 50 μg/ml normal rat Ig and rabbit anti-rat Ig, adsorbed to protein A-Sepharose, and twice again with anti-rat Ig adsorbed to protein A-Sepharose. The precipitates were washed sequentially with Dulbecco's modified PBS (PD) three times, high salt buffer (0.6 M NaCl, 12.5 mM KPO_4, pH 7.4, 0.02% NaN_3), mixed detergent buffer (0.05% NP-40, 0.1% SDS, 0.3 M NaCl, 10 mM Tris, pH 8.6) and PD before suspension and boiling in SDS-PAGE running buffer containing 50 mM dithiothreitol. Eluates were resolved in 10% polyacrylamide slab gels using the buffer system of Laemmli (30). Gels were stained with Coomassie blue to detect standard. Gels of ^{35}S samples were enhanced before drying and exposure to Fuji x-ray film at −70°C with Cronex-Hi-Plus intensifying screens (Dupont Co., Wilmington, DE).

The antibodies used for immunoprecipitations were anti-Ia ($I\text{-}A^{b,d,q}$, $I\text{-}E^{d,k}$; 50% culture supernatant from hybridoma M5/114.15.1; ATCC TIB 120; reference 31); anti-$I\text{-}A^{b,d}$ (B21.2, ATCC TIB 229; reference 32); anti-$I\text{-}E^{d,k}$ (14-4-4S, ATCC HB 32); NLDC145 to interdigitating cells (33); and rabbit anti-mouse invariant chain (Ii) 99-112 peptide antibody that was provided by Dr. V. Quaranta (Scripps Clinic, La Jolla, CA). Class II molecules and invariant chains also were identified by immunoperoxidase labeling of cytospun epidermal cell suspensions with M5/114 and In-1 (34) mAb, provided by Dr. N. Koch, (Institut für Immunologie and Genetik Deutsches Krebsforschungszentrum, Heidelberg, FRG) followed by POX-mouse anti-rat Ig (Boerhinger Mannheim Diagnostics, Inc., Houston, TX).

Results

Antigen Presentation by Fresh and Cultured LC to Primed Lymph Node T Cells. Prior studies with a small number of T cell lines (21, 22) had indicated that freshly isolated, but not cultured, EC could present native proteins. We used bulk populations of LN T cells to see if this marked decrease in protein presentation was generally applicable. First, protein antigens were added directly to the EC-T cell cocultures, and DNA synthesis was measured 3 d later as an index of the T cell response. It was evident that freshly isolated epidermal populations could present either myoglobin or conalbumin to LN T cells, but epidermal cells that had been cultured for 1–3 d were inactive (Fig. 1, *top*). Cultured EC did present peptide fragments, however, and thus were similar to spleen dendritic cells (Fig. 1, *top*).

Comparable results were obtained when epidermal cells were pulsed with antigen, washed, and then cocultured with sensitized T cells. If the protein was administered from day 0–1 or day 0–3 of culture, the pulsed cells triggered a T cell response (Fig. 1, *bottom*). The effective dose of epidermal LC was only 1% of the required dose of spleen cells, and the maximal response was greater (not shown, but see below). If the protein was administered from day 0–1 of culture, and then the cells were washed and cultured 2 d before testing for APC function, the pulsed EC remained active (Fig. 1, *bottom*). However, if the protein was administered for the last 2 d of a 3-d epidermal culture, the LC acquired little or no presenting function (Fig. 1, *bottom*). Therefore, only freshly isolated LC seem to be able to acquire protein antigens in an immunogenic form. Once antigen is captured, it is efficiently retained, since the cells can be washed and cultured for 2 d before use as APC.

One explanation for the different capacities of fresh and cultured LC to present protein antigens was that the fresh EC were selectively contaminated with cell types other then LC, capable of processing the myoglobin and then providing

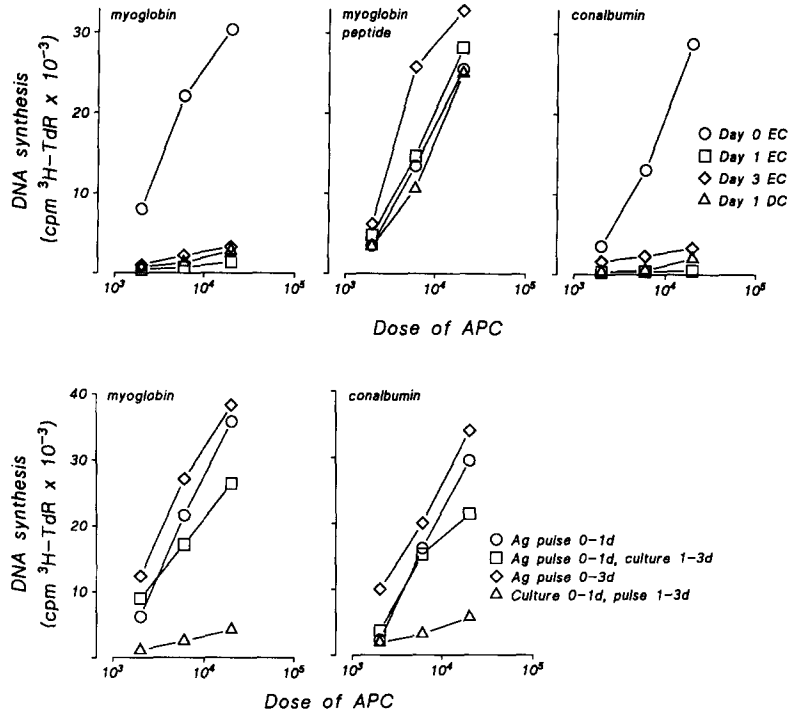

Figure 1. Freshly isolated but not cultured LC present myoglobin and conalbumin to antigen-primed lymph node cells. (*Top*) Four populations of APC were irradiated and added in graded doses of Ia^+ cells to a constant dose of 3 × 10^4 LN T cells that had been primed to myoglobin or conalbumin (see Materials and Methods). The APC were spleen dendritic cells (*DC*), fresh epidermal cells (EC, 10–15% Ia^+), EC cultured for 1 d (30–40% Ia^+), and EC cultured for 3 d (40–60% Ia^+). Shown here are DNA synthesis data when the APC and T cells were cultured in the continuous presence of 5 μM protein or 0.5 μM myoglobin peptide (residues 105–118). Not shown are the lack of responses (<1,500 cpm) when antigen was omitted, or when the inappropriate protein was added (i.e., myoglobin to conalbumin-primed T cells or vice versa). (*Bottom*) Epidermal cells were cultured with myoglobin or conalbumin at 100 μg/ml as indicated in the key. The cells were enriched by flotation on dense BSA columns, and washed several times before being added in graded doses of Ia^+ cells to antigen-primed T cells. Not shown are the lack of responses when myoglobin-pulsed APC were cultured with conalbumin-primed T cells and vice versa.

Table 1. *Fresh Keratinocytes Do Not Provide Significant Amounts of Peptides for Presentation by Langerhans Cells*

T cells	APC	[^{3}H]TdR Incorporation				
		DNA synthesis by T cells		DNA synthesis by CTLL		
		Without/with conalbumin	Without/with myoglobin	–	SpWMb	Myo p106–118
		cmp × 10^{-3}		*cpm* × 10^{-3}		
LN	d0 EC	3.0/28.4	4.0/30.4			
	d0, Ia$^-$ EC	0.5/0.6	0.3/0.4			
	d3 EC	3.0/4.7	3.3/4.5			
	Mix of d0, Ia$^-$ EC + d3 EC	1.5/5.9	3.1/6.1			
11.3.7 hybridoma	d0 EC			2.4	12.5	14.4
	d0, Ia$^-$ EC			2.4	1.2	2.3
	d3 EC			2.1	1.1	16.9
	Mix of d0, Ia$^-$ EC + d3 EC			2.6	1.0	7.4

* T cells were obtained from lymph nodes that had been primed with either conalbumin or myoglobin in CFA (see Materials and Methods). The T cells were cultured with different populations of APC ± the specific protein at 100 μg/ml. The EC populations, except when treated with α-Ia and complement, contained 10^4 Ia$^+$ LC. ^{3}H-TdR was added to measure DNA synthesis at 60–72 h.
‡ 5 × 10^4 11.3.7 hybridoma T cells were cultured with 3 × 10^3 of different populations of APC ± native SpWMb or myoglobin peptide 106–118. 24-h culture supernatant was assayed for CTLL growth activity.

peptides for presentation by the LC. This was addressed by mixing Ia$^-$ fresh epidermal cells with cultured EC and testing if the former provided myoglobin peptides that could be presented to lymph node T cells or T-T hybrids. Mixtures of Ia$^-$ epidermal cells and cultured EC did not present myoglobin (Table 1).

Fresh and Cultured LC Accumulate Comparable Levels of a Rhodamine Conjugate of Ovalbumin. Another possible reason for the lack of presentation by cultured EC was a loss in the ability to take up exogenous antigen for intracellular processing. The endocytic activity of LC is known to be weak in situ (35), but we were able to detect uptake in vitro by

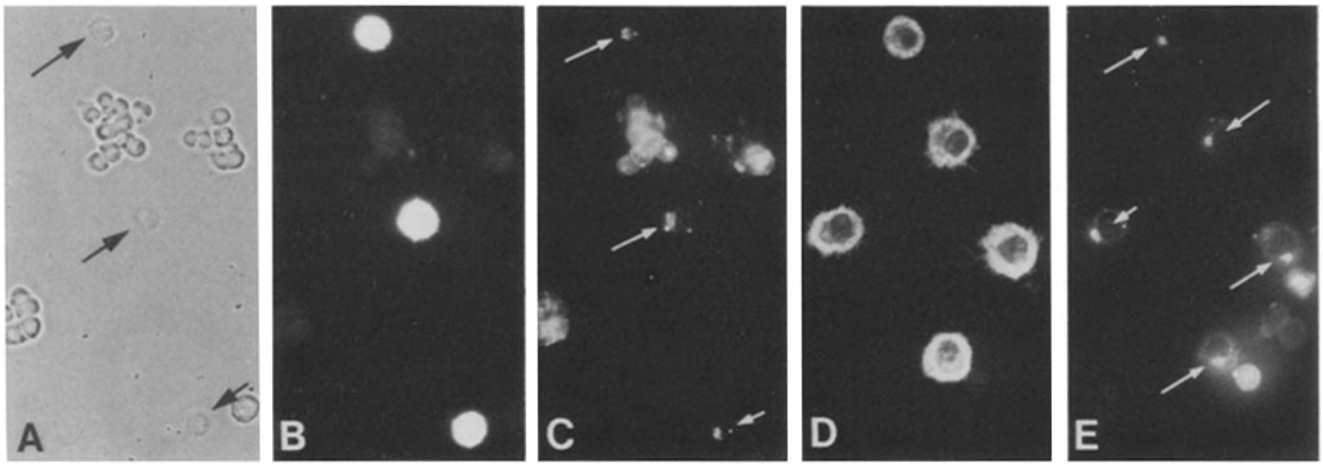

Figure 2. Pinocytosis by LC after exposure to 100 μg/ml rho-ova at 0–16 h (*A–C*) or 50–66 h (*D*, *E*) of culture. After washing, the EC were spun onto slides and stained with M5/114 mAb to mouse Ia antigens followed by FITC mouse anti-rat Ig. ×300. (*A*) Phase contrast with the three LC arrowed. (*B*) Anti-Ia stain (FITC optics) to identify the LC. (*C*) rho-ova binding (RITC optics) showing binding to keratinocytes and uptake into perinuclear granules in each LC (*arrows*). Little or no labeling of LC was observed with a 2-h exposure to tracer, but the keratinocytes did label and even at 4°C. (*D*) Anti-Ia stain (FITC optics) of a cultured epidermal preparation. (*E*) rho-ova uptake (RITC optics) by cultured LC into perinuclear granules (*arrows*). The extent of LC labeling with rho-ova is minute relative to what is observed with peritoneal macrophages, which are so active in pinocytosis that the entire cell is brightly fluorescent (23).

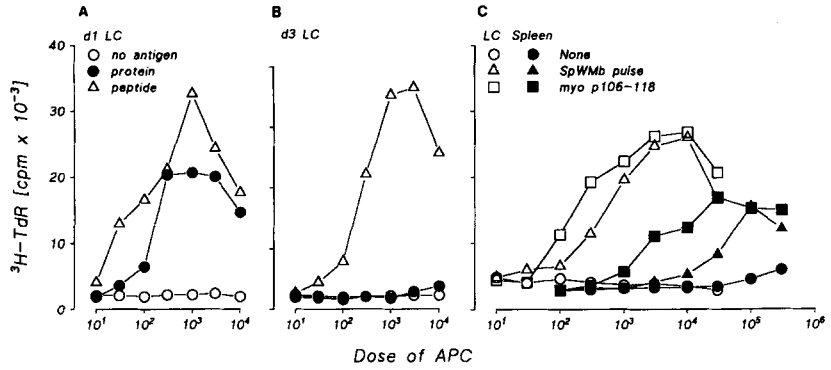

Figure 3. Antigen presentation to the 11.3.7 T-T hybridoma. Fresh (*A*) or 2-d cultured (*B*) LC were cultured for 16 h in the presence (*closed circle*) or absence (*open circles*) of 100 μg/ml intact SpWMb. After 16 h the cells were harvested and washed, and the indicated numbers of Ia$^+$ cells were cocultured with 5 × 10^4 11.3.71 T hybridoma cells. LC that had not been pulsed with myoglobin were also tested for their capacity to present the 106–119 peptide at 100 μg/ml (*triangle*). In *C*, LC (*open symbols*) are compared with bulk spleen cells (*closed symbols*) for presentation of protein administered as a 16 h pulse (*triangles*) or peptide administered continuously (*squares*). Presentation was monitored by assaying 25 μl of a 24 h culture supernatant on the IL-2–dependent CTLL line. Other doses of supernatant were tested for growth factor but are not shown.

using rho-ova as a tracer and fluorescence microscopy. Both fresh (Fig. 2, *A–C*) and cultured (Fig. 2, *D* and *E*) LC identified by two-color fluorescence with FITC anti-Ia and morphology, appeared to internalize the protein into perinuclear granules to a comparable extent. Ia$^-$ keratinocytes bound substantial amounts of rho-ova (Fig. 2 *C*), but this was not immunogenic (Table 1).

Presentation of Protein by Fresh EC to T-T Hybridoma Cells Is Sensitive to Chloroquine and Cycloheximide. To evaluate the effects of inhibitory compounds, we used as a homogeneous source of T cells the T-T hybrid 11.3.7eH2. After a 16-h pulse with 100 μg/ml of native myoglobin, day 1 EC (Fig. 3 *A*) but not 3 d cultured EC (Fig. 3 *B*) were potent stimulators of IL-2 release from 11.3.7eH2. Similarly, fresh EC but not cultured EC could present myoglobin protein when it was added continuously to the APC-T hybrid cocultures (data not shown). However, the identical preparations of 1- and 3-d cultured EC could present the myoglobin peptide 106–118 to the T-T hybrid (Fig. 3, *A* and *B*). The efficiency of EC as APC was much greater than that of spleen cells (Fig. 3

Table 2. *Effect of Chloroquine and CHX on the Presentation of Native Myoglobin and a Dominant Peptide Fragment to the 11.3.7.1 Hybridoma*

Exp.	APC	Chloroquine	[^{3}H]TdR incorporation			Exp.	APC	CHX	[^{3}H]TdR incorporation		
			No Ag	Protein pulse	Peptide				No Ag	Protein pulse	Peptide
				cpm × 10^{-3}						*cpm* × 10^{-3}	
1	d1 LC	–	2.4	8.5	34.7	2	d1 LC	–	5.9	42.6	48.5
		+	1.8	1.0	29.4			+	5.4	9.5	23.8
				(12)*	(85)					(22)	(49)
	d3 LC	–	2.2	0	29.1		d3 LC	–	6.7	0	11.8
		+	2.8	0	31.5			+	6.4	0	14.3
					(108)						(121)
							B blasts	–	0.6	2.7	7.0
								+	2.9	10.8	8.9
										(400)	(127)

* Number in parentheses are percent of control.

Fresh or 2.5-d cultured, thy-1$^-$ epidermal cells, or 2-d anti-Ig activated B blasts, were either untreated or pulsed with 100 μg/ml SpWMb for 16 h in the presence or absence of 2.5 μg/ml CHX or 100 μM chloroquine. After 16 h the cells were harvested, washed, added in graded doses to 5 × 10^4 T-T hybridoma cells, and IL-2 release was measured at 24 h. In the indicated column, 2 μM myoglobin peptide 106–118 was added directly to the cocultures of APC that had not been pulsed with antigen, and T hybridomas. Shown here are the IL-2 bioassay results with 10^3 LC in Exp. 1 and 3 × 10^3 LC or 5 × 10^4 B blasts in Exp 2. In addition we monitored the effects of drug treatment on the expression of surface MHC class II molecules using the M5/114 mAb, FITC mouse anti-rat Ig, and a FACScan instrument. Chloroquine did not significantly reduce expression of surface MHC class II products, but CHX had a significant effect. Specifically, the mean fluorescence intensity in arbitrary units was 202 for fresh LC; 1,069 for LC cultured 16 h without antigen or CHX; 1,018 for LC cultured 16 h with antigen only; 513 for LC cultured 16 h with CHX only; and 404 for LC cultured with antigen and CHX.

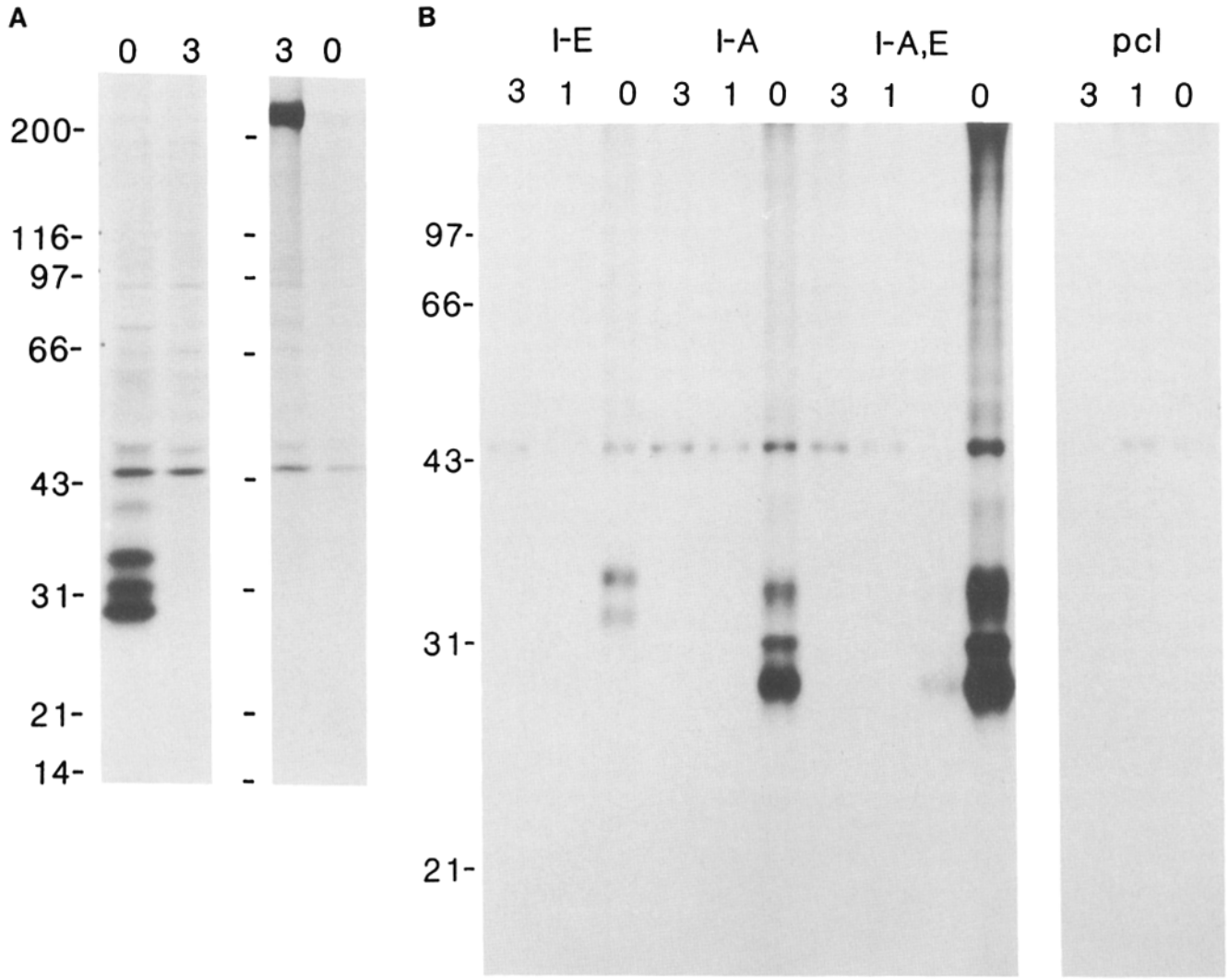

Figure 4. High level synthesis of MHC class II molecules by freshly isolated epidermal LC and its downregulation by day 1 and day 3 of in vitro culture. (*A*) Lysate from ~10^6 freshly isolated (lane *0*) or 3-d cultured (lane *3*) epidermal LC from CxD_2F_1 H-2^d mice were reacted with anti-Ia (*left*) or NLDC145 anti-interdigitating cell mAb (*right*). Immune complexes were precipitated with rabbit anti-rat Ig adsorbed to protein A–Sepharose. The gel was exposed for 2 d. (*B*) I-A and I-E were analyzed separately by precipitation with anti–I-$A^{b,d}$ (B21-2), anti–I-$E^{d,k}$ (14-4-4S), or anti–I-A and I-E (M5/114) as above. Epidermal cells were either labeled immediately after isolation (lanes *0*) or after 1 or 3 d of culture (lanes *1* and *3*, respectively). The final preclears for each lysate are shown in lanes marked *pcl*.

C). The optimal number of epidermal LC was routinely 1–3 × 10^3 per 5 × 10^4 T hybridoma cells, while for spleen cells it was 1–3 × 10^5, and the maximal response was always greater with EC. At supraoptimal numbers of APC, responses decreased.

Chloroquine was tested as an inhibitor of presentation. When fresh LC were pulsed with myoglobin in the presence of chloroquine, presentation was markedly reduced (Table 2, Exp. 1). Peptide presentation by the chloroquine-treated cells was not inhibited.

During the first 12–18 h in culture, the level of MHC class II molecules on the LC surface increased at least fivefold (36, 37). This process is CHX sensitive (36). We therefore tested if CHX could block presentation. At a dose of 2.5 μg/ml, CHX reduced [^{3}H]leucine incorporation into protein by 85–95% during a 12-h labeling. Correspondingly, the increase in surface Ia expression during this time was only 2-fold rather than a 5–10 increase observed in control cells not treated with CHX (see legend to Table 2). The small increase in Ia observed in the presence of CHX might reflect class II molecules that were synthesized before CHX could exert its effect, e.g., in a preformed intracellular pool of Ia. Presentation of myoglobin by antigen-pulsed, CHX-treated, day 1 LC was markedly reduced (Table 2). There was also a partial reduction in the ability of CHX-treated day 1 LC to present peptide, possibly due to a reduction in surface class II, but there was no reduction in peptide presentation by CHX-treated day 3 LC (Table 2).

When another APC, the activated anti-Ig blast, was tested, CHX did not reduce presentation of myoglobin to the T-T

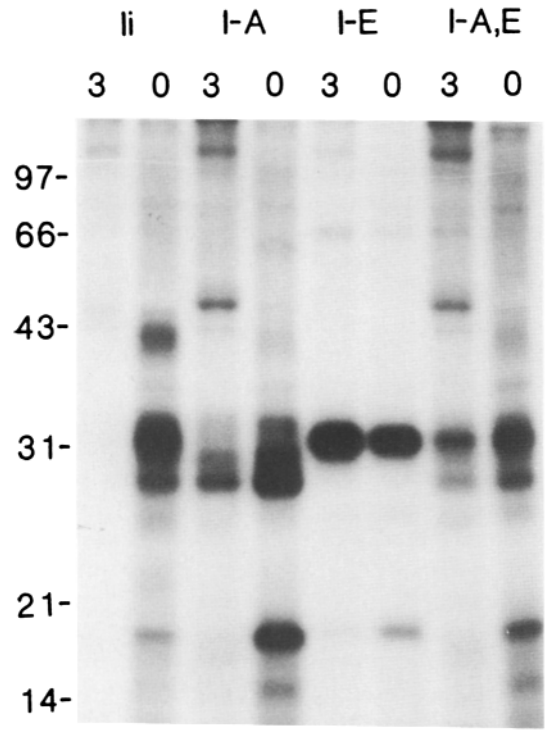

Figure 5. Freshly isolated and 3-d cultured LC both express high levels of surface class II but surface invariant chain is only detected on fresh LC. Lysates from 2 × 10^6 radioiodinated LC were precipitated with anti-Ia mAb as in Fig. 3. Invariant chain (*Ii*) was precipitated with a rabbit anti-mouse invariant chain 99-112 peptide antibody.

hybrid, and typically enhanced it (Table 2). The results with the anti-Ig blasts are consistent with those reported for the A20 B cell lymphoma where presentation was CHX resistant (17). The LC behave more like peritoneal cells studied by Jensen (16) in being CHX sensitive.

High Rate Synthesis of MHC Class II Molecules in Fresh LC and Its Selective Downregulation During In Vitro Culture. To test if class II molecules were potentially the site of the CHX effect, we studied their biosynthesis in [^{35}S]methionine-labeled cultures. Fresh LC exhibited a remarkably high rate of synthesis of Ia, allowing for detection in the relatively small numbers of fresh LC that were available for such studies (Fig. 4 *A*). The level of incorporation of label into Ia antigens in fresh LC was greater than in 5–10-fold as many cells of other cell types labeled in parallel, including 2-d anti-Ig blasts, fresh peritoneal exudate cells, low density spleen cells, and 1 d cultured spleen dendritic cells (data not shown). In contrast, Ia synthesis was not detectable in 3-d cultured LC (Fig. 4 *A*, *left panel*, lane *0* vs. *3*). Additional studies compared LC labeled at 0–4, 20–24, and 68–72 h of culture. The synthesis of both I-A and I-E MHC class II products was no longer detectable by the first day of culture (Fig. 4 *B*). This is consistent with the loss of APC function of LC by day 1 of culture (above).

Two findings indicated that the marked decrease in Ia biosynthesis during culture was selective. Another surface antigen that is recognized by the mAb NLDC145 (33) was synthesized more actively by day 3 than day 0 LC (Fig. 4 *A*,

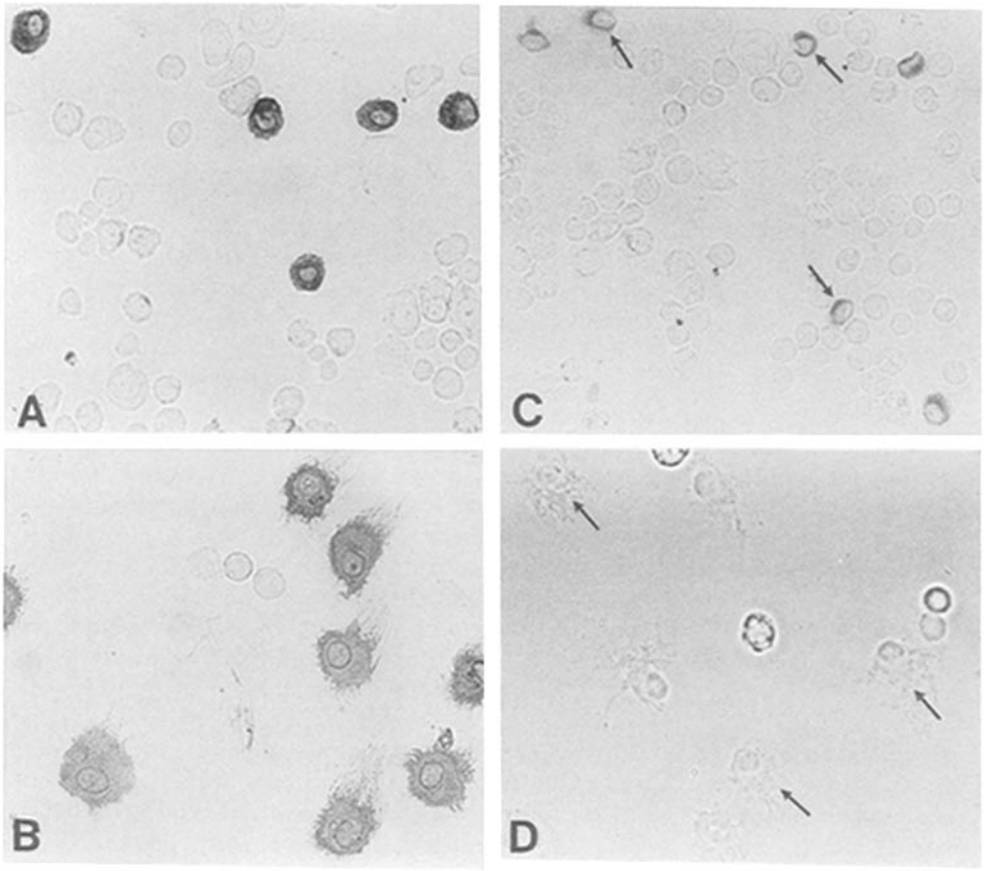

Figure 6. Expression of class II MHC products and associated invariant chain in LC: immunoperoxidase staining of acetone-fixed cytospin preparations. LC in the fresh epidermal cells (*A* and *C*) stain with both anti-MHC class II (M5/114 mAb; *A*) and anti-invariant chain (In-1; *C*) mAb. LC in the cultured epidermal cells (*bottom*) stain strongly with anti-MHC class II (*B*), but not detectably with anti-invariant chain (*D*). ×300.

right), and there was comparable labeling of nonspecific bands in the immunoprecipitates, particularly one at 45 kD (Fig. 4 *A*).

To verify that the preparations of day 3 cultured EC expressed typical class II MHC products on the cell surface, we did surface radioiodination studies. The levels of radioiodinated surface Ia precipitated from cultured LC was at least comparable to the level of Ia precipitated from cultured LC (Fig. 5), in spite of the loss of Ia biosynthesis by day 1 of culture. This indicates that at least a significant proportion of the Ia synthesized by freshly isolated LC is stably expressed for at least 2–3 d.

MHC Class II–associated Invariant Chain (Ii) Expression in LC. The invariant chain has been implicated in antigen presentation by fibroblast cell lines that have been transfected with both invariant and MHC class II genes (38). Immunoprecipitation of Ia with a rabbit anti–mouse invariant chain peptide antibody strongly suggests that invariant chain was present on the surface of freshly isolated, but not cultured, LC (Fig. 5, *left lanes*).

We also examined the expression of invariant chain using the mAb In-1, which reacts with the cytoplasmic domain of the protein (34). Cytospin preparations showed strong staining by In-1 in the cytoplasm of most of the fresh LC (Fig. 6 *C*). Only a small proportion of cultured LC were stained by the In-1 and even in the positive cells the staining was less intense than in the fresh LC (Fig. 6 *D*). The cultured LC were stained intensely with mAb to the class II heterodimers (Fig. 6, *A* and *B*). Under the same experimental conditions, we detected low levels of Ia associated invariant chain in B cells and greater levels in anti-Ig activated B lymphoblasts (our unpublished observations).

Discussion

The Distinct Antigen-presenting Features of Epidermal LC. LC are the dendritic APC of the epidermis (24, 39–41). When freshly isolated, LC can present exogenous protein antigens to T cell clones (21, 22) and are 100 times more effective than a standard population of APC, spleen cells (21) (Fig. 3). Within 1 d in culture, LC lose the ability to present native protein. We find that this downregulation occurs for two proteins and for primary populations of responding T cells (Fig. 1). In addition, after an exposure to protein, the LC can retain the antigen in immunogenic form for at least an additional 2 d in culture (Fig. 1, *bottom*). This differs dramatically from the reported loss of immunogen from antigen-pulsed B cells within 8–12 h (42). To our knowledge no other APC, except spleen dendritic cells (23), display comparable traits of potency, downregulation of protein presentation, and prolonged antigen retention.

Perhaps the downregulation of antigen processing and the prolonged retention of antigens are related. If LC were to generate antigenic peptides continuously for days, without any new synthesis of MHC products (Fig. 4, and see below), previously acquired peptides might be displaced. The physiologic role of LC may be to carry antigens via the lymph to T cells in the draining lymphoid tissue (40, 43). These events may require a period of days in which antigens that are picked up in the skin have to be retained for presentation to T cells that recirculate through lymph nodes.

While cultured LC do not present native proteins, they are not deficient in many of the other properties required of APC. In fact, cultured LC express the highest levels of antigen-presenting MHC products among leukocytes (24, 36, 37), retain the ability to present peptide antigens to T cells (21) (Figs. 1 and 3), and are the most potent APC for foreign MHC molecules and mitogens (24, 44) that do not require de novo processing. Further studies are needed to evaluate the efficacy of peptide presentation by fresh and cultured LC, since we have only tested a single relatively high dose of 0.5 μM (Figs. 1 and 3). Nevertheless cultured LC represent a physiologic APC in which there appears to be a selective loss of the capacity to process exogenous proteins, thereby providing an important model for studies of mechanism.

The Endocytic Activity of LC. The presentation of proteins requires a chloroquine-sensitive step within acidic intracellular vacuoles to which antigens can gain access by endocytosis (13). Alternatively, proteins can be processed extracellularly (45). Antigen processing by fresh LC likely occurs intracellularly, since it is sensitive to chloroquine (Table 2).

Dendritic cells exhibit weak endocytic activity (46), at least relative to the level of endocytosis exhibited by macrophages. However, most of the endocytic activity in macrophages is directed to antigen scavenging and destruction rather than presentation (47). The uptake of antigens into dendritic cells may be directed more to presentation. In a sense, dendritic cells resemble B lymphocytes which can be very active APC and yet are remarkably less effective than macrophages in accumulating endocytic tracers (our unpublished observations).

rho-ova is one of the more sensitive tracers for detecting pinocytosis by dendritic cells (23) (Fig. 2). Uptake of agents like lucifer yellow, FITC-dextran, horseradish peroxidase, cascade blue ovalbumin, are all undetectable using the exposure conditions required for antigen pulsing; i.e., 0.1 mg/ml for 12–16 h (data not shown). Because of the low level of pinocytic activity, we have not been able to quantitate uptake rates in fresh and cultured LC.

Both fresh and cultured LC can internalize rho-ova (Fig. 2), so that uptake per se is not the explanation for differences in antigen processing. We have not tested the acidity of the intracellular vacuoles that were labeled with rho-ova. Therefore it remains possible that the pH of endocytic vacuoles in freshly isolated and cultured LC are significantly different, an observation recently made by Stossel et al. (48).

In most studies of antigen presentation in tissue culture, antigen uptake primarily occurs through bulk or fluid phase pinocytosis. Receptor-mediated uptake may enhance the efficiency of presentation. Fresh LC have Fc receptors, and these are downregulated during culture (24, 44). These Fc receptors might enhance the uptake and processing activity of freshly isolated LC as long as antibody is present. Also, we have observed uptake of horseradish peroxidase by fresh but not cultured LC using a dose of 1 mg/ml (our unpub-

lished observations). Perhaps freshly isolated LC selectively express mannose receptors and for this reason are able to capture some peroxidase and other glycoproteins by adsorptive uptake.

Role of Newly Synthesized Class II Products in Presentation of Exogenous Proteins. The importance of newly synthesized MHC molecules has been emphasized for presentation by class I products. Peptides derived from the cytoplasm (5), or perhaps from the external environment (20), likely associate with the forming class I-β_2 microglobulin heterodimer. It may be quantitatively inefficient for peptides to gain access to peptide binding grooves at the cell surface, because these grooves are already occupied. Our results provide a biologic model in which biosynthesis of class II MHC molecules also correlates with the efficacy of presentation. Immediately upon testing in vitro, incorporation of [^{35}S]methionine into class II is at least 5–20 times more active in LC than in any other cell, and within 20 h, biosynthesis effectively ceases. Likewise fresh LC are efficient at processing foreign proteins, and within a day activity drops markedly (Figs. 1 and 3). Perhaps processed foreign proteins and newly synthesized class II interact in a late endosome (49).

There is recent evidence that invariant chains, which are abundant in fresh but not cultured LC (Figs. 5 and 6), are required for presentation by L cells that have been transfected with class II genes (38). It has been hypothesized that an amphipathic α-helical region within the invariant chain occupies the class II peptide binding groove before entry of a foreign peptide (50). Occupancy by invariant chain may be important to protect the groove from peptides that are present in the secretory pathway, which may be the site in which peptides gain access to class I (5, 20). In an acidic endocytic compartment, the invariant chain could be displaced or degraded (15). Unoccupied, newly synthesized class II might be more accessible to exogenous peptide.

Presentation by class II products in epidermal LC is blocked by CHX. The sensitivity to CHX is similar to that described by Jensen for mouse peritoneal cells (16). Our studies implicate the class II heterodimer as the CHX-sensitive site, but the evidence is correlative at this time. We have attempted to selectively block class II biosynthesis with antisense oligonucleotides, but we are unable to do so (data not shown). Presumably, the oligonucleotides do not gain access to the cytoplasm, and/or class II mRNA is too abundant or too rapidly induced to be sensitive to inhibition by this method. It is only feasible to add oligonucleotides after the 5–6 h it takes to prepare the cells.

In contrast to LC, treatment with CHX does not block presentation by anti-Ig–activated B lymphoblasts, and in fact, CHX consistently enhances presentation (Table 2). The findings are consistent with similar results in a B cell lymphoma (17).

Regulation of Class II MHC Biosynthesis in Dendritic Cells. The mechanism of regulation of MHC biosynthesize in LC is unclear. The cytokine granulocyte/macrophage (GM)-CSF influences LC viability and function (51, 52), but we have been unable to block the upregulation of class II with anti-GM-CSF (our unpublished observations). The regulation in dendritic cells is likely to be different from other leukocytes. For macrophages (32) and B cells (53), T cell products like IFN-γ and IL-4 induce class II biosynthesis. Yet in our studies, we are using populations that are depleted of thy-1$^+$ T cells of either α/β or γ/δ variety.

The regulation of MHC expression on dendritic cells remains a critical unknown for the further analysis of the findings reported here, since as mentioned, it is not yet possible to selectively inhibit LC at the level of class II mRNA. By blocking the signals that upregulate class II, it may be possible to interrupt antigen presentation and immunogenicity at a very early step.

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