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Class II major histocompatibility complex molecules of murine dendritic cells: Synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture

(epidermal Langerhans cells/dendritic cell differentiation/T-cell sensitization)

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ABSTRACT **Dendritic cells (DCs), such as Langerhans cells (LCs) of the epidermis and the DCs of lymphoid organs such as spleen, are potent antigen presenting cells. DCs express high levels of major histocompatibility complex (MHC) class II molecules, but, partly because of the low numbers of primary DCs in any tissue, there has been no detailed study of the biochemistry of their class II molecules. This information may be needed to help explain recent findings that DCs process native protein antigens when freshly isolated from epidermis and spleen. Processing ceases during culture, yet a strong accessory function for activating resting T cells develops. We studied immunoprecipitates of DC class II and invariant chain (I_i) molecules by two-dimensional gel electrophoresis. We found that (*i*) freshly isolated LCs synthesize large amounts of class II and I_i polypeptides; (*ii*) I_i molecules that are known to be involved in antigen processing display an unusually large number of sialic acids in fresh LCs; (*iii*) with culture, class II and I_i synthesis decreases dramatically and has virtually ceased at 3 days; and (*iv*) the turnover of class II in pulse/chase experiments is slow, being undetectable over a 12- to 32-hr culture period, whereas the turnover of I_i is rapid. We conclude that MHC class II molecules of DCs do not seem to be qualitatively unique. However, the regulation of class II and I_i expression is distinctive in that biosynthesis proceeds vigorously for a short period of time and the newly synthesized class II remains stably on the cell surface, whereas I_i turns over rapidly. This may enable DCs to process and retain antigens in the peripheral tissues such as skin and migrate to the lymphoid organs to activate T cells there.**

Dendritic cells (DCs) represent a system of abundantly major histocompatibility complex (MHC) class II-expressing leukocytes. They occur in nonlymphoid organs, blood, afferent lymph, and lymphoid tissues (1). Two states of differentiation can be distinguished (2, 3). "Immature" DCs, exemplified by freshly isolated epidermal Langerhans cells (LCs), are weak stimulators of resting T cells both in the allogeneic mixed leukocyte reaction (4, 5) and in polyclonal responses such as oxidative mitogenesis, concanavalin A mitogenesis, and anti-CD3 mitogenesis (6). These fresh LCs (7, 8) as well as fresh DCs from spleen (9), however, are efficient in processing native protein antigens for MHC class II-restricted presentation to presensitized peptide-specific T cells (*in vivo*-primed T cells, clones, T–T hybridomas). Upon 1–3 days of culture in macrophage- or keratinocyte-conditioned medium, or in culture medium supplemented with granulocyte/macrophage colony-stimulating factor, their functional properties become inverted. "Mature" DCs lose the capacity to process exogenous antigens but at the same time acquire the ability to sensitize resting T cells (2, 3). The loss of processing function is paralleled by the loss of acidic organelles such as endosomes (10), which are known as the compartments where class II encounters processed antigen (11–13).

Since MHC class II molecules are the essential elements of antigen processing and presentation, we studied in detail the composition and the chemical nature of class II polypeptides and associated invariant chains (I_i) in the course of DC maturation/differentiation in culture.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Charles River Wiga, Sulzfeld, F.R.G., and were used between 6 and 10 weeks of age.

Cells. Fresh [day 0 LCs (LCd0)] and cultured [day 1 and day 3 LCs (LCd1 and LCd3)] LCs were prepared and enriched from ear skin as described (4, 14, 15). DCs were obtained from spleen (16). Thioglycollate-elicited peritoneal macrophages were induced to express MHC class II by recombinant murine interferon γ (Stratech, London) (17). In addition, a macrophage line (P388D1) and a pre-B-cell line (18-81) were used.

Immunolabeling Procedures. Epidermal sheets were prepared as described (18). Single cell suspensions were attached to multiwell microscopic slides coated with poly-L-lysine (50 μg/ml; type VII; Sigma). Cells were briefly fixed with 4% paraformaldehyde. Antibody and washing solutions contained 0.02% saponin (Sigma) to enhance penetration. Monoclonal antibody (mAb) In1 (19), rat IgG2b anti-murine invariant (I_i) chain, reacting with intracytoplasmic I_i31 and I_i41 chains (20) was used. Binding of In1 was visualized with biotinylated anti-rat immunoglobulin (not cross-reactive with mouse immunoglobulin; Vector, Burlingame, CA) followed by streptavidin fluorescein isothiocyanate (Amersham). To identify LCs, this incubation sequence was extended by rat immunoglobulin to block free anti-rat binding sites, and tetramethylrhodamine B isothiocyanate-conjugated anti-I-$A^{b,d}$ (B21-2; TIB229 from the American Type Culture Collection).

Metabolic Labeling. Cells were washed three times in methionine-free RPMI 1640 medium (GIBCO). Cells (1×10^6) were resuspended in 60 μl of methionine-free RPMI 1640 medium and 100 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (SJ204; Amersham) was added. With partially enriched

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Abbreviations: EC, epidermal cell; DC, dendritic cell; I_i, invariant chain; LC, epidermal Langerhans cell; LCd0, freshly isolated LC; LCd1, 1-day cultured LC; LCd3, 3-day cultured LC; MHC, major histocompatibility complex; mAb, monoclonal antibody.

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LCd0, up to 40 × 10^6 total epidermal cells (ECs) were incubated in proportional volumes and radioactivities. After 90–120 min at 37°C, the tubes were filled up with 15 ml of warm culture medium and incubated for another hour. Then cells were washed and lysed. For pulse/chase experiments, 30 × 10^6 ECs (containing 6 × 10^6 LCd0) and 30 × 10^6 macrophages were labeled with 40 μCi per 1 × 10^6 ECs in 200 μl for 15 min at 37°C. After three washes in complete medium, ECs were cultured as described above for 12 and 32 hr. Macrophages (15 × 10^6) were cultured for the same periods in Teflon beakers.

Cell-Surface Iodination. Cell-surface proteins were labeled by the lactoperoxidase method (21).

Immunoprecipitation and Two-Dimensional Gel Electrophoresis. This was done as described in detail previously (22), using mAb In1 (see above), rat mAb 17/227, anti-I-A^d/E^d, and rat mAb NLDC-145, anti-DC-specific antigen (23). On two-dimensional gels, proteins were separated by charge using nonequilibrium pH gradient gel electrophoresis in the first (horizontal) dimension and by size using reducing NaDodSO$_4$/PAGE in the second (vertical) dimension. To achieve a semiquantitative comparison between the various cell populations, equal numbers of class II-expressing cells were labeled and immunoprecipitated and gels were scanned by densitometry (Laser Densitometer, Molecular Dynamics, Sunnyvale, CA).

Functional Assays. Oxidative mitogenesis assays (4, 14) and antigen-specific hybridoma assays (8, 10) were done as described.

RESULTS

Functional Properties of Immature and Mature DCs. Freshly isolated LCs are weak stimulators of resting T cells (4) but excellent processors of native protein as determined with peptide-specific T-cell clones (7) or hybridomas (refs. 8 and 10; Table 1). After 2–3 days of culture, LCs selectively lose processing capacity; in the same time, however, they become much more efficient in stimulating resting T cells (Table 1).

Expression of MHC Class II Molecules and Associated I_i on DCs. It has been shown that immature DCs—i.e., resident LCs—express substantial levels of MHC class II antigens (4, 16, 24). We inspected epidermal sheets by immunohistochemistry for the presence of class II and associated I_i. Both molecules are coexpressed in LCs but are not detectable in surrounding keratinocytes (Fig. 1 *A* and *B*).

Upon culture of LCs, the surface expression of MHC class II molecules is rapidly (i.e., within hours) increased up to 10-fold (4, 24, 25) and resembles class II expression on splenic and thymic DCs. In contrast, the expression of intracellular I_i chain decreased upon 3-day culture of LCs as visualized with mAb In1 (Fig. 1 *C–H*; ref. 8). Equally low amounts of cytoplasmic I_i were immunostained in spleen DCs (data not shown).

High Rate of I_i and Class II Biosynthesis in Immature DCs and Its Decrease upon Their Maturation in Culture. Although class II and I_i genes are located on different chromosomes and their structural genes are not related under immune-activated conditions, their expression is tightly co-regulated (26). Two-dimensional separation of metabolically labeled class II and I_i immunoprecipitates reveals that freshly isolated LCs synthesize large amounts of class II and I_i polypeptides (Fig. 2 *A* and *F*). When LCs were cultured for different times and their biosynthesis was analyzed, profound changes were observed. The rate of synthesis of both class II and I_i molecules dropped sharply (Fig. 2 *B–D*; ref. 8). After 1 day of culture, this became evident and within 3 days LCs had virtually stopped class II and I_i production. Spleen DCs synthesize considerably less class II and I_i than fresh LCs, albeit not as little as LCd3 (Fig. 2*E*). The down-regulation of class II/I_i production in cultured LCs was selective, because the same cells actively synthesized another protein—namely, the DC antigen immunoprecipitated by mAb NLDC-145 (8, 23) (data not shown).

Unusual Expression and Maturation of I_i in Immature LCs. The I_i gene encodes two polypeptides—I_i31 and I_i41 (27). In B cells, I_i41 usually represents 10% or less of I_i (19, 28) (Fig. 3*E*). In freshly isolated LCs, however, the proportion of I_i41 in relation to I_i31 is unusually high (Fig. 3*A*). Densitometric analysis of the gels depicted in Fig. 3 gave I_i31/I_i41 ratios of 1.3 (43% I_i41) for fresh LCs, 3.4 (23% I_i41) for spleen DCs, 2.3 (30% I_i41) for macrophages, and 10.4 (9% I_i41) for B cells.

Upon intracellular transport of class II and I_i polypeptides to trans-Golgi compartments, their glycan side chains are sialylated (29). I_i chains from freshly isolated LCs acquire an unusual number of sialic acids (Fig. 3*A*; up to 17 acidic spots), which increase the molecular weight of I_i31 up to 45,000. Similarly, I_i41 is intensely sialylated. As a consequence of sialylation, some I_i chains are more acidic than class II α chains (Fig. 3*A*, far right). Upon maturation of LCs, the

Table 1. Reciprocal expression of antigen processing and T-cell sensitizing capacities in fresh and cultured LCs

Antigen presenting cells	Dose of antigen presenting cells						
	10^4	3 × 10^3	10^3	3 × 10^2	10^2	3 × 10^1	10
Hybridoma assay	Proliferation of CTLL-2						
A. fLCs −	6.2	5.4	5.9	5.1	5.0	5.4	5.2
B. fLCs + myo	129.3	172.9	199.3	145.6	121.1	52.1	17.8
C. cLCs −	1.0	1.5	2.5	1.3	2.8	2.1	2.1
D. cLCs + myo	2.7	1.0	2.0	2.4	2.3	1.9	1.6
Oxidative mitogenesis	Proliferation of T cells						
E. 0-hr LCs	—	44.9	15.5	5.9	2.7	—	—
F. 12-hr LCs	—	45.9	19.3	6.5	2.2	—	—
G. 32-hr LCs	—	180.4	144.0	52.8	15.6	—	—
H. 12-hr MAs	—	8.6	4.2	2.1	1.6	—	—
I. 32-hr MAs	—	8.7	4.9	3.0	2.4	—	—

The hybridoma assay was as follows. Antigen processing capacity was determined by measuring interleukin 2 production of myoglobin-peptide-specific hybridoma 11.3.7 by means of CTLL-2 indicator cells (rows A–D). Note that freshly isolated LCs (fLCs) efficiently activate the hybridoma in the presence of sperm whale myoglobin protein (myo) (row B). Cultured LCs (cLCs) have lost this capacity (row D). Oxidative mitogenesis was as follows. LCs and peritoneal macrophages (MAs) cultured for 0, 12, and 32 hr were used to stimulate periodate-modified resting T cells. Note that 12-hr LCs (row F), which express levels of class II equivalent to those of 32-hr LCs (row G) (5, 24), are as weak stimulators as are freshly isolated LCs (row E). Class II-positive macrophages do not activate resting T cells (rows H and I). This experiment was done in parallel to the labeling experiment in Figs. 4 and 5. Results are expressed as cpm × 10^{-3}.

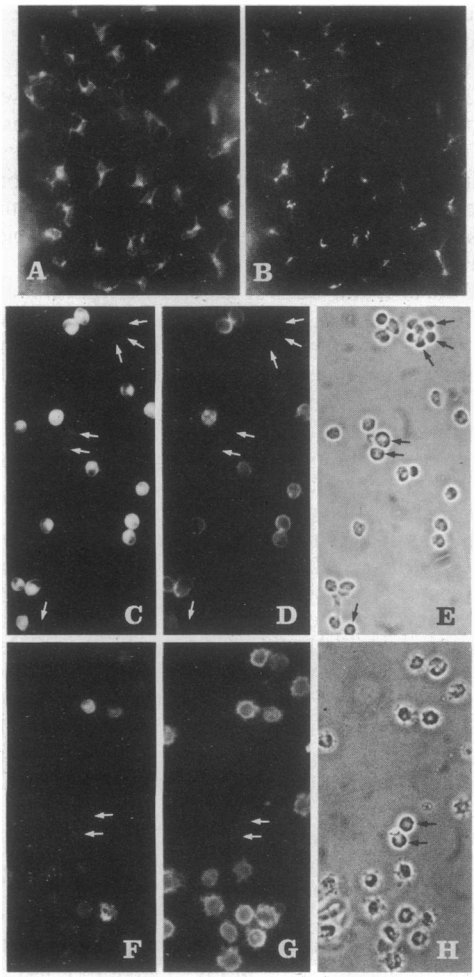

FIG. 1. Expression of I_i molecules by murine epidermal LCs. Epidermal sheets (*A* and *B*) were double-stained with mAb In1, anti-I_i (*A*), and mAb B21-2, anti-I-$A^{b,d}$ (*B*). Note that all class II-expressing cells also bear I_i and vice versa. Enriched populations of fresh (*C–E*) and 3-day-cultured LCs (*F–H*) attached to poly-L-lysine-coated slides were double-stained with mAbs In1 (*C* and *F*) and B21-2 (*D* and *G*). The expression of I_i chains decreases with culture and is almost absent in LCs cultured for 3 days (*C* vs. *F*), whereas class II expression increases (*D* vs. *G*). Note that all photographs were exposed and developed identically to allow for a semiquantitative comparison of fluorescence intensities. Arrows, keratinocytes. (*E* and *H*) Corresponding phase-contrast pictures. (×100.)

number of sialic acid residues bound per I_i molecule decreases. Cultured LCs as well as spleen DCs, a macrophage, and a B-cell line carry 9–11 acidic spots per I_i31 chain as opposed to up to 17 in freshly isolated LCs (Fig. 3 *B–E*). The molecular weight of the most sialylated I_i31 chains of cultured LCs remains below 40,000 and their negative charge resembles that of class II α chains. Class II molecules were always less sialylated (three or four acidic spots) than I_i chains (Fig. 3). It is emphasized that they, when opposed to actin as an internal reference standard, did not strongly vary in their charge (i.e., sialylation) between LCd0, LCd1, spleen DCs, macrophages, and B cells.

Comparison of Molecular and Functional Properties of Immature and Mature DCs. The exclusive functional property of mature DCs to sensitize resting T cells was frequently attributed to the nature of class II molecules on these cells (30, 31). However, biochemical analysis of surface radioiodinated LCs (data not shown) revealed no unusual pattern of surface class II molecules of mature DCs as compared to macrophages or B cells. Alternatively, fresh LCs were metabolically pulse labeled and chased for 12 or 32 hr to ensure

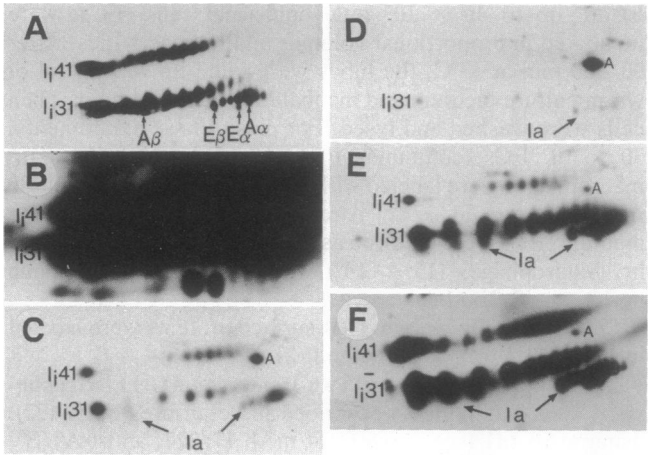

FIG. 2. Biosynthesis of class II and invariant chain polypeptides in LCs and DCs. LCd0 (*A*, *B*, and *F*), LCd1 (*C*), LCd3 (*D*), and spleen DCs (*E*) were metabolically labeled for 1 hr with [^{35}S]methionine and lysates were immunoprecipitated with anti-class II (*A–E*) and anti-I_i (*F*) mAbs. To demonstrate the decreasing intensities of the two-dimensional pattern, the gels in *B–E* were exposed to x-ray films for 25 days. In addition, the two-dimensional gels in *A* and *F* were exposed for 1 day. Class II chains are denoted with Aβ, Eβ, Eα, Aα (*A*) or with Ia (*C–F*). I_i41 and I_i31 are coprecipitated invariant chains. Note the marked decrease in biosynthesis upon culture of LCs (*B–D*). Assignment of the spots was done by using defined class II/I_i transfectants (22). A, actin.

expression of radiolabeled molecules on the cell surface (32). The same populations were also used to study antigen-presenting cell function. Anti-class II immunoprecipitates of 12-hr chased LCs (i.e., functionally immature LCs; Table 1) or 32-hr chased LCs (i.e., functionally mature LCs; Table 1) gave virtually identical two-dimensional gels (Fig. 4 *A* and *B*). There was also no striking qualitative difference to class II of peritoneal macrophages (Fig. 4).

Turnover of MHC Class II and I_i Molecules. This was studied in pulse/chase experiments of metabolically labeled cells. Class II molecules of LCs were more stable than those of macrophages: after a 12- or 32-hr chase period, the amounts of precipitable class II molecules were similar in LCs indicating that they had not decreased during this

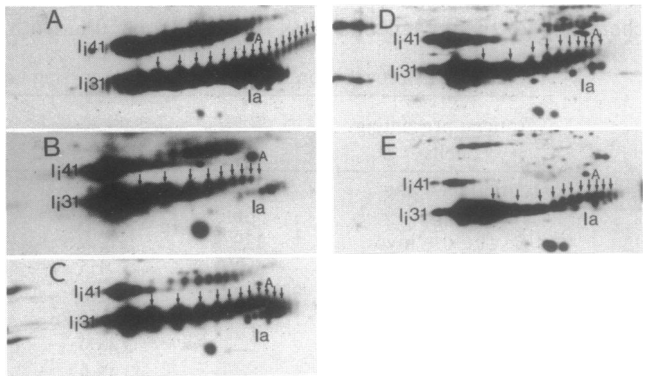

FIG. 3. Glycosylation patterns of I_i. Two-dimensional gels of I_i immunoprecipitates were exposed to x-ray films for various times to obtain similar intensities of invariant chains. (*A*) LCd0, 2 days. (*B*) LCd1, 25 days. (*C*) Spleen DC, 25 days. (*D*) Macrophage line P388D1 [preincubated with interferon γ (50 units/ml) to induce class II molecules], 20 days. (*E*) Pre-B-cell clone 18-81H6 (class II-positive variant of the 18-81 cell line), 10 days. The number of acidic spots of the I_i31 chain is marked by arrows. The ratio of I_i41/I_i31 is highest in fresh LCs (*A*) and lowest in B cells (*E*). Bands visible on the far left of *C* and *D* are ^{14}C molecular weight standards of M_r 27,000 and 46,000. Ia, class II molecules; A, actin.

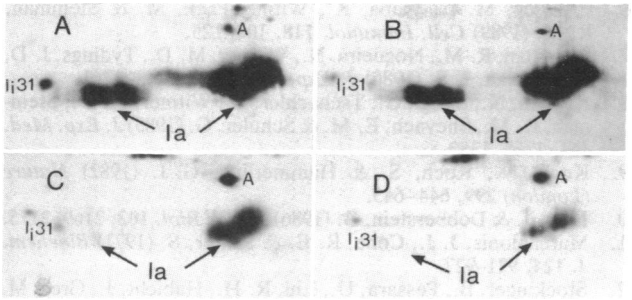

FIG. 4. Pulse/chase labeling of class II molecules in LCs and peritoneal macrophages. Freshly isolated LCs (*A* and *B*) and peritoneal macrophages (*C* and *D*) were pulse labeled for 15 min with [^{35}S]methionine and subsequently cultured for 12 hr (*A* and *C*) or 32 hr (*B* and *D*). Class II polypeptides (Ia and arrows) were immunoprecipitated with mAb 17/227. Note that only low amounts of I_i are present because of its rapid turnover and dissociation from class II. A, actin.

interval (Fig. 4 *A* and *B*). In contrast, markedly less class II was precipitated from macrophages after a 32-hr chase than after a 12-hr chase, suggesting a shorter half-life (Fig. 4 *C* and *D*). Densitometric scans of the gels in Fig. 4 demonstrated a 55% reduction of precipitable material (i.e., class II molecules) in macrophages over a period of 20 hr but no reduction in LCs. I_i molecules showed a different turnover. In freshly prepared LCs, they are synthesized in extremely large amounts as compared to other cell types (Fig. 2 *B–E*). The amount of I_i decreased sharply during the chase period, which is consistent with a short half-life of I_i (compare Fig. 5 *A* and *B* with Fig. 2*F*). It is remarkable that in LCs—as opposed to macrophages—I_i41 was relatively more stable than I_i31. Although initially more I_i31 than I_i41 was made by fresh LCs (Fig. 2*F*), most of the I_i31 had disappeared after a pulse/chase of 12 or 32 hr, whereas I_i41 remained precipitable with In1 (Fig. 5 *A* and *B*). In macrophages, neither chain was precipitable after the chase (Fig. 5 *C* and *D*).

DISCUSSION

DCs can efficiently activate resting T lymphocytes and are therefore key antigen presenting cells (1). It is unclear whether and how this capacity relates to the nature of MHC class II molecules. Also, the phenomenon of DC maturation/differentiation in culture (loss of antigen processing capacity and acquisition of stimulatory function) (3) needed to be studied in detail at the level of class II biochemistry. This report confirms and extends recent work by Puré *et al.* (8) by using two-dimensional analyses and by focusing on structural features of the invariant chain.

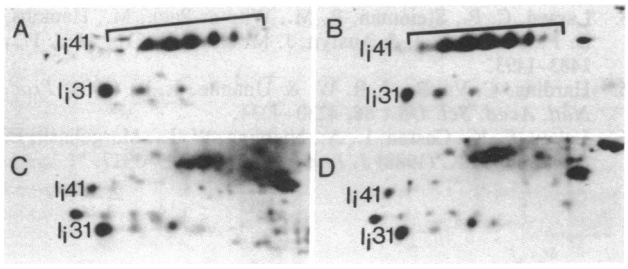

FIG. 5. Pulse/chase labeling of invariant chains in LCs and peritoneal macrophages. Cells were prepared and labeled as in Fig. 4. I_i chains were immunoprecipitated with In1. In both cultured LCs (*A* and *B*) and in macrophages (*C* and *D*) only marginal amounts of sialylated I_i31 chains and small amounts of unsialylated precursors are present. This indicates a high turnover rate of I_i31. In contrast, sialylated I_i41 in LCs is more abundant than I_i31, although synthesis of I_i31 in fresh LCs exceeds that of I_i41. Associated class II is hardly detectable. A spot to the left of I_i41 and I_i31 of macrophages is distinct from I_i chains and was not identified.

Biochemistry of Class II and I_i Molecules of Immature DCs—Significance for Antigen Processing. LCs *in situ* or freshly isolated from the epidermis have some capacity to endocytose (8, 33, 34). They are equipped with acidic organelles like endosomes (10), the necessary cellular compartments for antigen processing (11, 12). The high rate of class II synthesis gives immature DCs the possibility to charge a large number of class II molecules with immunogenic peptides. The concomitant abundant synthesis of I_i, which is also involved in antigen processing (22, 26), would ensure that the peptide binding groove (35, 36) of all *de novo*-synthesized class II molecules is protected until the class II/I_i complex reaches the organelle where it encounters the antigen, presumably the acidic endosome (11, 12). The unusually high degree of I_i sialylation in fresh LCs gives these polypeptides a strong negative charge. This could promote trafficking/targeting and protease stability of the class II/I_i complexes, thereby facilitating their entry into the acidic processing organelles (27, 37). The significance of the high I_i41/I_i31 ratio is unknown at present. Taken together, these biochemical features may explain the extraordinary antigen-processing capacity of immature DCs as compared to other types of antigen presenting cells (7–9, 33).

Biochemistry of Class II and I_i Molecules of Mature DCs—Significance for T-Cell Sensitization. The cellular mechanism responsible for the unique capacity of mature DCs to sensitize resting T cells is presumably their ability to bind T cells in an antigen-*in*dependent fashion (38). It has not been possible so far to block this type of DC–T-cell interaction with antibodies to defined adhesion molecules (39–41). Moreover, the high density of class II molecules on mature DCs does not account for this binding property (5). Therefore, one may either postulate a hitherto unknown "clustering molecule" or, alternatively, obvious differences in class II molecules. Addressing the latter possibility, analysis of two-dimensional gels did not reveal such differences between class II molecules of antigen presenting cells that can (cultured LCs, spleen DCs) or cannot (fresh LCs, macrophages, B cells) bind T cells antigen independently. Low sialylation of surface class I and II has been discussed to be responsible for the immunostimulatory power of mature DCs (30, 31). However, we found no differences of class II sialylation patterns of immature and mature DCs and of B cells. Also, neuraminidase treatment of fresh LCs (F.K., N.R., and G.S., unpublished data) and macrophages (42) did not endow these cells with a T-cell sensitizing capacity. Thus, the putative molecule responsible for the initial antigen-independent binding of mature DCs to resting T cells remains unknown. Our data make it seem unlikely that it is merely a modification of class II.

Regulation of Class II and I_i Synthesis. Using flow cytometry it was shown that the increase in class II expression upon culture of LCs was not dependent on the cytokines granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α (14). Also, interleukin 4 and/or interferon γ do not appear to be responsible cytokines because the up-regulation of class II expression occurs also in the absence of these factors (14). Cytokine effects on the synthesis of class II/I_i as measured by metabolic labeling of DCs have not yet been investigated.

LC Culture as a Physiologic Model for Antigen Presentation *in Vivo*. There is evidence that antigen-laden LCs can migrate from the skin to the draining lymph node (43, 44). It is tempting to speculate that before or during this migration LCs shut off class II/I_i synthesis, thereby preventing the possibility that by continuous processing the cells displace those immunogenic peptide/class II complexes that they have

formed while still in the epidermis. Indeed, both types of mature DC studied, cultured LCs and spleen DCs, have virtually stopped making class II and I_i. Their I_i molecules (Figs. 1 and 5) as well as their acidic organelles (10) rapidly decreased. In contrast, class II molecules were relatively stable in LCs as compared to murine B-cell lines and macrophages (45, 46). This very slow turnover of class II would make DCs well suited to carry antigenic peptides bound to class II to the lymphoid organs over a period of several days and efficiently present them to the T cells there. Indeed, it was recently shown that antigen-pulsed DCs can retain immunogenic peptide for at least 2 days *in vitro* (8) and *in vivo* (33).

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