PRESENTATION OF EXOGENOUS PROTEIN ANTIGENS BY DENDRITIC CELLS TO T CELL CLONES

Intact Protein Is Presented Best by Immature, Epidermal Langerhans Cells

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Prior studies on the function of dendritic cells have emphasized their ability to stimulate resting T lymphocytes in such systems as the MLR and polyclonal responses to mitogens like sodium periodate (1-6). However, dendritic cells isolated from the epidermis (Langerhans cells [LC])[1] a nonlymphoid tissue, are weak accessory cells (7, 8). These LC must be cultured for 1-3 d with the cytokine granulocyte/macrophage CSF (GM-CSF) to become active stimulators of the MLR and polyclonal T cell mitogenesis (9, 10).

Here we have studied the efficacy of different types of dendritic cells to present a protein, myoglobin, to T cell clones for which immunogenic peptide fragments have been defined (11). We will show that the capacity of the dendritic cell population to present protein varies inversely with stimulating activity in the MLR. Freshly isolated epidermal LC actively present intact myoglobin, while cultured LC and spleen dendritic cells do *not*. These findings suggest that dendritic cell function involves two components that develop in sequence: a presentation step in which antigens are picked up in tissues like skin, and a sensitization activity in which dendritic cells acquire the capacity to induce a response in resting T lymphocytes, presumably as the dendritic cells migrate to the draining lymphoid tissues like spleen and lymph node.

Materials and Methods

Mice. 6-10-wk-old C57BL/6, BALB/c x DBA/2 [CxD2]F_1, A, and B6.H-2k mice were purchased from The Trudeau Institute, (Saranac Lake, NY) and CBA/J mice were from The Jackson Laboratories, (Bar Harbor, ME). Mice of both sexes 6-10 wk old, were used with similar results.

Culture Medium. This was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Hazelton Systems, Inc., Aberdeen, MD), 50 μM 2-ME and 20 μg/ml gentamicin.

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[1] *Abbreviations used in this paper:* GM, granulocyte/macrophage; LC, Langerhans cell.

Reagents. Sperm whale myoglobin was used directly from the manufacturer (Sigma Chemical Co., St. Louis, MO; Fluka, Ronkonkoma, NY). Synthetic peptides, representing amino acids 110–124, 110–121, 102–118, 69–84 were provided by Drs. J. Berzofsky (National Institutes of Health, Bethesda, MD) and J. Rothbard (Imperial Cancer Research Fund, London, UK).

Antigen Presenting Cells. Spleen cells were depleted of erythrocytes by lysis with 0.83% ammonium chloride in most experiments, and irradiated with 3,000 rad ^{137}Cs before use. Dendritic cells were low density spleen adherent cells that were depleted of B cells and macrophages by rosetting with antibody-coated RBC (12) and irradiated with 900 rad. LC were isolated from mouse ear skin and enriched by a panning method as described (9). They were irradiated with 900, 1,500, or 3,000 rad as indicated in the Results, but the dose of irradiation did not influence accessory function. In some experiments, we used heterogeneous epidermal suspensions, i.e., LC contaminated with keratinocytes, to verify that the panning procedure did not alter the results observed with enriched LC. The A20 BALB/c H-2^d B cell lymphoma line was provided by Dr. Jay Berzofsky, NIH. These cells were treated with 50 μg/ml mitomycin *c* (Sigma Chemical Co.) for 75–85 min and washed three times before use.

T Cell Clones. These were described elsewhere (11), and included one that was restricted to I-E^d (clone 11.3.7) and one restricted to I-A^d (clone 11.12.8). To maintain the clones, the cells were restimulated every 11–18 d with irradiated H-2^d spleen cells plus 5–10 μM myoglobin, generally one spleen equivalent and 10^6 cloned cells in a 25-cm^2 flask (No. 25100; Corning Glass Works, Corning, NY). For APC assays, the cloned T cells were taken 11–18 d after boosting, isolated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and 2×10^4 T cells were stimulated with graded doses of APC in a final volume of 0.2 ml medium in flat-bottomed 96-well plates (No. 3596; Costar, Cambridge, MA). At 44–48 h, the cultures were pulsed with 4 μCi/ml [^{3}H]TdR for 16 h (6 Ci/mmol; ICN Radiochemicals, Irvine, CA). All data are means of triplicates in which the standard deviations were <10% of the mean.

Primary MLR. T cells were nylon wool-nonadherent spleen and lymph nodes that were generally depleted of Ia^+ cells with mAb and complement. 3×10^5 T cells were added per well, and the MLRs were pulsed at 56-72 h as above.

Results

Spleen and A20 Lymphoma Cells, but not Mature Dendritic Cells, Present Myoglobin to a Class II-restricted T Cell Clone. The two standard APC populations that are used to study presentation of native proteins to mouse T cell clones and hybrids are bulk spleen cells and the A20 lymphoma cell line. These were used to present myoglobin to an MHC class II (I-E^d)-restricted clone 11.3.7 (11) and compared with two sources of dendritic cells: spleen and cultured epidermal LC.

Both spleen and A20 presented myoglobin, but strikingly, the dendritic cells were weak and sometimes inactive (Table I). Even a dose of 6×10^4 dendritic cells did not elicit a significant response, whereas 10^3 cells elicit a strong allogeneic MLR (see below). All of the populations could present a peptide fragment of myogloblin, corresponding to amino acids 110–124 (Table I), although in many subsequent experiments, presentation of peptide by dendritic cells was not as strong as spleen. Previously it had been shown that the 110–124 peptide is 1–3 times more effective on a molar basis than myoglobin for stimulating the clone (11), and we confirmed this (data not shown). Presentation of peptide by dendritic cells required that they be obtained from mice of the I-E^d MHC haplotype, i.e, H-2d, but not H-2b or H-2a (Table I). In all cases, the proliferative response decreased threefold or more if either the dose of APC or antigen was reduced threefold (not shown). We conclude that spleen dendritic cells and cultured LC are quantitatively very weak at presenting myoglobin to this T cell clone, in spite of their potent accessory function in primary immune responses (7).

TABLE I

A Comparison of Different Cell Types as APC for a Myoglobin-specific T Cell Clone

Exp.	APC	Number of APC	Proliferation of clone 11.3.7			
			No antigen	Myoglobin	110–124	69–84
			cpm × 10^{-3}			
1	None	–	0.4	0.8	0.6	0.5
	Spleen cells	4×10^5	4.3	12.6	31.7	7.4
	A20 B cell lymphoma	5×10^4	5.2	51.9	73.4	5.5
	Spleen dendritic cells	2×10^4	0.4	0.5	7.6	0.8
	Cultured H-2^d LC	1.5×10^4	8.9	8.9	43.9	10.3
	Cultured H-2^a LC	1.5×10^4	9.1	9.8	9.0	9.8
2	None	–	0.3	0.4	0.4	0.4
	Spleen cells	5×10^5	11.5	64.8	133.9	5.4
	A20 B cell lymphoma	3×10^4	3.1	40.1	68.7	5.1
	Spleen dendritic cells	2×10^4	0.9	2.9	78.8	1.7
3	Spleen cells	3×10^5	3.4	19.6	19.8	-
	H-2^d dendritic cells	3×10^4	0.5	2.6	43.3	-
	H-2^b dendritic cells	7×10^4	3.4	5.6	4.6	-

Different populations of APC were tested for presentation of myoglobin (10 μM) or the indicated peptides (3 μM) to the I-E^d-restricted clone 11.3.7. Significant antigen-dependent proliferative responses are underlined. Cultured LC were 3-d epidermal cell suspensions that were enriched to >70% purity by floating the nonadherent epidermal cells on albumin columns.

Freshly Isolated Epidermal LC Actively Present Myoglobin. Given the above findings, we reasoned that there might be a stage in the life history of the dendritic cell where it could pick up protein antigens and then carry these antigens to T cells, as in draining lymphoid organs. There is evidence that dendritic cells can migrate to lymphoid organs from tissues like skin and gut via the afferent lymph (6, 13). We therefore compared freshly isolated epidermal LC with cultured LC for their capacity to present myoglobin to T clones. It is known that fresh LC are relatively weak accessory cells for primary T cell responses but do present antigens to sensitized T lymphoblasts (8), which may be comparable to chronically stimulated T cell clones like 11.3.7.

In fact, freshly isolated LC were extremely active at presenting myoglobin (Table II). 2×10^4 LC were more active than 1.5×10^5 spleen cells. Similar results were obtained if the LC were partially enriched, using anti-Thy-1 mAb and complement to deplete most keratinocytes, or were highly enriched by panning (Table II, Exps. A and B). H-2^d LC but not H-2^k LC presented myoglobin. LC that were cultured for 1 d could still present myoglobin, but LC that were cultured for 2–3 d were weak or inactive (Table II). However, the cultured LC were active in presenting peptide fragments (Table II).

Contrasting findings were made when these same APC populations were evaluated as stimulators of the primary MLR. The day 3 cultured LC and splenic dendritic cells were the most active stimulators, while the fresh LC were weak (Fig. 1).

Fresh LC Present Myoglobin to an I-A-restricted Clone. To extend the findings to a T cell clone that is restricted to I-A, the other major class II locus of the mouse, we studied clone 11.12.8 (11), which recognizes an epitope that includes glu 109, an immunodominant moiety for presentation of myoglobin on I-A^d molecules. Freshly

Table II
Freshly Isolated Epidermal LC Are Strong APC

Exp.	APC	Dose of APC	Proliferation of clone: No antigen	Myoglobin protein	Myoglobin peptide
				cmp × 10^{-3}	
A	LC, day 0	2×10^4	0.5	72.2	79.1
		5×10^3	0.4	12.0	14.3
	LC, day 1	2×10^4	1.4	19.1	50.4
	LC, day 3	2×10^4	1.0	1.0	80.3
		5×10^3	0.5	0.4	12.1
	Spleen	5×10^5	11.7	118.8	118.4
B	LC, day 0	2×10^4	0.7	63.2	51.6
		6×10^3	0.6	38.6	18.0
	LC, day 1	2×10^4	0.4	56.0	92.0
		6×10^3	0.4	25.3	26.1
	LC, day 3	2×10^4	1.0	6.2	91.5
		6×10^3	1.3	2.1	34.7
	Spleen	5×10^5	3.0	108.7	161.7
		1.5×10^5	0.9	24.5	47.0

Two experiments in which H-2^d LC were isolated after different times in culture and used as APC to present myoglobin protein (10 μM) and peptide (3 μM 110–124 in *A*; 20 μM 102–118 in *B*) to clone 11.3.7. In Exp. A, the day 0 LC were enriched by panning; the day 1 LC were enriched by culturing epidermal cells and depleting the nonadherent cells of residual Thy-1^+ keratinocytes; and the day 3 LC were enriched by floating on dense albumin columns. In Exp. B, all of the APC were treated with anti-Thy-1 and complement and the LC were panned with antileukocyte mAb. Not shown are data where day 0 LC from an MHC-inappropriate strain (CBA, H-2^k) were found to be inactive (0.5×10^{-3} cpm), which was the same result obtained with no added APC.

isolated LC were again very active APC, while cultured LC were inactive (Table III). Both types of LC could present the 102–118 peptide but not 110–121 (Table III).

To verify the specificity of peptide presentation, we tested the same peptides and APC using clone 11.3.7, which responds to 110–121 but only weakly to 102–118. Both

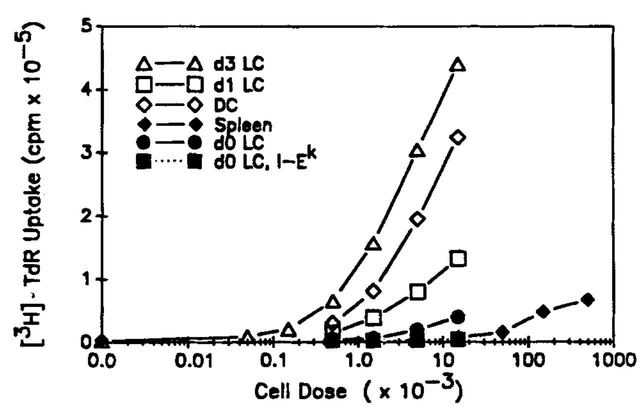

Figure 1. The MLR stimulating activity of fresh and cultured LC, spleen dendritic cells (DC), and unfractionated spleen. Graded doses of the indicated H-2^d stimulator populations were added to 3×10^5 Ia^-, nylon wool-nonadherent $CD4^+$ T cells from allogeneic H-2^k mice. DNA synthesis was measured at 78–90 h. These same preparations were used to present myoglobin in Table II, Exp. B.

TABLE III

Fresh and Cultured LC Differ in the Capacity to Present Proteins and Alloantigens

APC		T cell proliferation								
		Clone 11.3.7 (I-E^d)				Clone 11.12.8 (I-A^d)				Primary T
Type	n	No antigen	Myoglobin	102–118	110–121	No antigen	Myoglobin	102–118	110–121	Alloantigen
	$\times 10^{-4}$		$cpm \times 10^{-3}$				$cpm \times 10^{-3}$			
LC, day 0	6	2.2	58.5	11.7	33.0	0.6	264.2	236.3	0.5	14.0
	2	0.7	15.0	3.4	11.7	0.4	203.7	91.8	0.4	3.0
	0.6	0.4	3.9	1.9	3.2	0.3	36.1	13.5	0.4	2.2
LC, day 3	2	1.2	2.1	2.6	30.0	0.5	1.2	70.0	0.5	142.4
	0.6	0.4	0.7	0.8	9.6	0.5	0.7	55.2	0.5	85.3
	0.2	–	–	–	–	–	–	–	–	40.7
Spleen	60	0.8	147.0	28.4	263.0	0.5	236.6	152.6	0.9	28.3
	20	0.3	13.0	5.9	31.4	0.4	53.9	32.7	–	9.0
	6	0.4	0.8	0.5	2.6	0.3	3.1	2.4	–	2.1

Langerhans cells (LC) from H-2d BALB/c × DBA/2 mice were enriched from fresh or 3-d cultured epidermal suspensions using a panning protocol. The LC were compared with spleen for their capacity to present myoglobin protein (10 μM) and the indicated myoglobin peptides (10 μM) to clones 11.3.7 and 11.12.8, and to stimulate a primary MLR in B6.H-2^k purified T cells. The cultures with clones were pulsed at 48–66 h with 4 μCi/ml [^{3}H]TdR, and the allo MLR cultures were pulsed at 72–90 h. Significant, antigen-dependent proliferative responses are underlined.

fresh and cultured LC presented peptide fragments to clones in an appropriately specific manner (Table III).

MLR stimulation was evaluated with these same populations of APC. Again cultured LC were more than 30 times as active as MLR stimulators than freshly isolated LC, and more than 300 times more active than spleen (Table III). Therefore the capacity of an APC to present a protein antigen to a sensitized T cell, and the capacity to stimulate a primary immune response, represent distinct physiological entities that develop in sequence when epidermal LC are cultured.

Discussion

Presentation and Sensitization Steps in T Cell Stimulation. It is known that lymphoid dendritic cells and cultured epidermal LC are powerful accessory cells for the triggering of T lymphocytes, and are 10–30 times more active than populations of freshly isolated LC (7–10, 14). We now find that the opposite holds true for the presentation of protein antigens to T cell clones. Fresh LC are far more active than cultured LC (Tables I–III).

These sets of findings at first seem inconsistent, but they can be interpreted if one considers that APC can serve two broad functions, antigen presentation and T cell sensitization, and that these may be independently regulated in dendritic cells and possibly other types of APC.

"Antigen presentation" generates the ligand that is recognized by the α/β heterodimer of the clonally specific portion of the TCR for antigen-MHC. Presentation typically requires that the antigen be denatured or processed in some way such that the antigen or antigen fragment (15–19) can associate with a peptide binding groove on the external aspect of the MHC product (20). It is not possible to quantitate the amount of presented antigen directly. Instead, one measures the capacity of an APC to stimulate a T cell ("accessory function"). However, it is likely that the relative deficiency of cultured LC and spleen dendritic cells to stimulate myoglobin-specific clones involves a lesion in myoglobin processing and presentation since these same cultured LC do present peptide fragments and actively stimulate T cells in the MLR (Fig. 1, Table III).

"Sensitization" refers to the events that are required in addition to antigen presentation to make T cells begin to produce their differentiated products, lymphokines and cytolysins, and to become more responsive to growth factors. The distinction between antigen presentation and sensitization became evident in studies of the MLR. It was found that dendritic cells were active MLR stimulators. Other cells like macrophages and B lymphocytes were less active but did not lack antigen, since they could present transplantation antigens in an MHC-restricted way to T lymphoblasts that had first been sensitized by dendritic cells (21, 22). Likewise, freshly isolated LC could stimulate T blasts but not resting T cells (8).

Once a T cell becomes an IL-2-responsive lymphoblast, it effectively binds and responds to many types of APC, even those fixed with aldehydes (21). Chronically stimulated T cell clones correspond in functional respects to lymphoblasts, since both are recently stimulated with antigen and are IL-2 responsive. Therefore, long-term clonal populations of T cells are valuable models for monitoring presentation or "antigenicity" but they do not necessarily provide information on "immunogenicity," that is, the sensitization requirements for resting T cells.

In the accompanying paper (14) evidence also was presented for a dissociation of presentation and sensitization functions in the response to the mitogen anti-CD3. In the anti-CD3 model, Fc receptors on the LC present anti-CD3 mAb as the ligand for the TCR. Freshly isolated LC likely have much more ligand (10 times more Fc receptors) but are much less active than cultured LC as sensitizing cells for the T cell proliferative response.

Mechanisms for Presentation of Proteins by Dendritic Cells. For antigen presentation by MHC class II molecules, peptide fragments of the original antigen might be generated either by extracellular (19) or by intracellular (15) proteolysis. Lymphoid dendritic cells and cultured epidermal LC show little endocytic activity (7, 23), at least as judged by the accumulation of tracer proteins and particulates. This minimal endocytic activity might account for the observed deficit in the ability to present myoglobin.

Freshly isolated LC, which do present myoglobin, may be capable of some endocytic activity. Wolff and Schreiner (24) documented uptake of horseradish peroxidase in situ after administration of protein subcutaneously. With freshly isolated LC, Schuler et al. (25) noted uptake of staphylococci (25), Takigawa et al. (26) noted uptake of lectins, and Hanau et al. (27) noted internalization of the mAb anti-CD1 and anti-HLA-DR. Since we study spleen dendritic cells after a day in culture, it is also possible that these cells in situ or freshly upon isolation, can exhibit enough endocytic function to present myoglobin.

An additional possibility would be that LC acquire antigens from extracellular proteolysis, as from proteases released during an inflammatory response in skin or from other cell types such as macrophages. Given the lack of presenting function by cultured lymphoid dendritic cells and epidermal LC, it would be possible to test this hypothesis by mixing macrophages of the inappropriate MHC and dendritic cells of the appropriate H-2^d MHC. We do know that keratinocytes, i.e., Ia^- epidermal suspensions, are unable to process myoglobin for the cultured LC. We also need to test in detail the efficacy of mature dendritic cells to present peptide fragments, i.e., to compare dendritic cells and other APC using a range of peptide doses and sequences. Our current experiments indicate that lymphoid dendritic cells are less effective than epidermal LC and bulk spleen in presenting peptides.

Lymphoid dendritic cells and cultured epidermal LC stimulate helper T cell–dependent antibody responses, while fresh LC are inactive (8, 28). There are several possibilities to account for the presentation function of dendritic cells in this situation: (*a*) The preparation of antigen (red cells or hapten-carrier conjugates) may have contained immunogenic fragments or "preprocessed" antigen; (*b*) the dendritic cells may have participated in a strong syngeneic MLR, which in turn may have induced a processing capacity; (*c*) sufficient processing may have occurred intra- or extracellularly to present to helper T cells; (*d*) The dendritic cell preparations may have contained a small subset of actively processing cells some of which had the features of fresh LC. A small subset might go unnoticed in our current studies in which relatively large doses of APC are needed to stimulate the T cell clones.

What Is a Transplantation Antigen? Major transplantation antigens are equated to allogeneic MHC molecules, but the latter may have to become occupied with peptides to be recognized by T lymphocytes (20). Since our data show that dendritic cells in tissues effectively form MHC-myoglobin complexes, we would suggest that the strong MLR stimulating activity of cultured epidermal LC, and most likely dendritic cells from lymph, blood, and lymphoid organs (29), reflects the fact that these cells *previously* have processed antigens *and* acquired the capacities of this lineage to initiate T cell responses like the MLR. This hypothesis can be tested by studying dual-reactive T cell clones that respond either to self-MHC plus protein or to allogeneic MHC. Only in the latter situation should spleen dendritic cells and cultured LC be active APC.

The Specialized Properties of Dendritic Cells as Presenting Cells. The specialized properties of dendritic cells for initiating the immune response have been reviewed elsewhere (30, 31) and include: high levels of MHC products; ability to cluster antigen-specific lymphocytes for days and to induce lymphokine production and functioning IL-2 receptors; capacity to home to the T-dependent regions of lymphoid organs; and mobilization from immature precursors in tissues in response to a specific cytokine, GM-CSF, which might be released as an early event after deposition of antigen.

It now appears that the handling of antigens by dendritic cells is also distinctive in that handling of intact proteins can be downregulated when the dendritic cells mature and/or leave a tissue to migrate to a lymphoid organ. It is of interest that LC also upregulate expression of MHC products some fivefold over the first 12–18 h of culture (32). This is the time that LC handle exogenous proteins, thereby providing an excellent means for charging the MHC products on tissue dendritic cells with the specific antigen that is deposited in that site. The epidermal dendritic cell, and possibly dendritic cells in other nonlymphoid organs, therefore are specialized to act as "sentinels" for the deposition of antigens that are to stimulate T cells, as suggested for contact allergens (33). As a result of the capacity to regulate the acquisition of antigen, the dendritic cells that emerge from nonlymphoid tissues (into the blood or lymph) primarily present proteins acquired there. By decreasing further processing, dendritic cells might not displace previously acquired peptides through the handling of additional materials, especially self proteins. During transplantation, this would provide a powerful source of transplant-derived antigens. During physiologic responses, the possibility of autoimmunity should also be reduced since dendritic cells would not be handling self proteins after they leave the site of antigen deposition.

Summary

The capacity of dendritic cells to present protein antigens has been studied with two MHC class II–restricted, myoglobin-specific, T cell clones. Spleen dendritic cells and cultured epidermal Langerhans cells (LC) presented native myoglobin weakly and often not at all. These same populations were powerful stimulators of allogeneic T cells in the primary MLR. Freshly isolated LC were in contrast very active in presenting proteins to T cell clones but were weak stimulators of the MLR. Both fresh and cultured LC could present specific peptide fragments of myoglobin to the clones. These results suggest that dendritic cells in nonlymphoid tissues like skin can act as sentinels for presenting antigens in situ, their accessory function developing in two phases. First antigens are captured and appropriately presented. Further handling of antigen then is downregulated while the cells acquire strong sensitizing activity for the growth and function of resting T lymphocytes. The potent MLR stimulating activity of cultured epidermal LC and lymphoid dendritic cells prob-

ably reflects prior handling of antigens leading to the formation of allogeneic MHC-peptide complexes.

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