

ACCESSORY AND STIMULATING PROPERTIES OF  
DENDRITIC CELLS AND MACROPHAGES  
ISOLATED FROM VARIOUS RAT TISSUES\*

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Numerous investigators have shown that responses of T lymphocytes to mitogens and antigens require accessory cells, which have generally been considered to be Ia<sup>+</sup> macrophages (1-7). We recently reported (8) that a distinctly different cell, the dendritic cell, functioned as the required accessory cell in periodate-induced responses of rat lymph node cells (LNC).<sup>1</sup> Clearly, an important issue is whether macrophages possess accessory activity or whether this property belongs exclusively to dendritic cells.

Contradictory findings have been reported concerning the role of macrophages in lymphocyte responses to antigens and mitogens; both helper effects (9-11) and suppressor effects (12-14) have been attributed to macrophages. In some other cases (15), studies have used macrophages "activated" by various stimuli, which results in functionally heterogeneous populations. Most frequently, the peritoneal cavity has been the source of macrophages as accessory cells, but nonlymphoid tissues have also provided cells with similar activities. Thus, it has been reported that liver Kupffer cells function as accessory cells for mitogen-treated lymphocytes (16-18) and that the skin "macrophage," the Ia-positive Langerhans cell, stimulate in a mixed skin cell-lymphocyte reaction (19, 20).

However, there is evidence that in preparations obtained from mouse spleen (21-23) and rat lymph node (8), dendritic cells are more potent than macrophages as accessories or stimulators. Also, Hart and Fabre (24) recently presented histological evidence that Ia<sup>+</sup> dendritic cells are present in many more rat organs and tissues than previously realized and suggested that dendritic cells might play a vital role in immune surveillance or self tolerance.

In an attempt to evaluate the relative contribution of macrophages and dendritic

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<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; EA, opsonized sheep erythrocytes; EC, epidermal cells; HBSS, Hanks' balanced salt solution; HD, high density; HIHS, heat-inactivated horse serum; LC, liver cells; LD, low density; LNC, lymph node cells; PBS, phosphate-buffered salt solution; PEC, peritoneal exudate cells; UV, ultraviolet light.

cells as accessories for periodate-induced responses, we isolated these cells from the same starting lymph node cell preparation. The nonadherent nature of the rat dendritic cell has allowed us to purify both cell types without significant cross contamination. Accessory activity copurifies entirely with dendritic cells. These cells also serve as potent stimulators of a mixed leukocyte reaction. Lymph node macrophages lack accessory or stimulator activity, despite the fact that both cell types carry Ia antigens on their surfaces.

In addition, we examined cell preparations from various rat tissues for accessory activity, and, where present, attempted to identify the accessory cell. We now report the presence of accessory activity in cell suspensions from rat spleen, peritoneal exudates, liver, and epidermis; in each case, the activity copurified with an Ia<sup>+</sup> cell that is identical to the rat lymph node dendritic cell in terms of its morphology and potency as an accessory cell. Macrophages from these tissues lacked accessory activity.

Although the exact relationships among dendritic cells in various anatomical sites remain to be elucidated, we found that a morphological marker of the Langerhans cell, the Birbeck granule (25), is also detectable in lymph node dendritic cells. Rat dendritic cells differ from macrophages in a number of other important respects, which strengthens the view that they form a distinct class of cells.

## Materials and Methods

*Animals.* 150–220 g male Lewis or Buffalo rats (Charles River Laboratory, Wilmington, MA) were used.

### *Preparation of Cell Suspensions*

**LYMPHOID CELLS.** The preparation of cells from spleen and from cervical and mesenteric lymph nodes was previously described (26).

**PERITONEAL EXUDATE CELLS (PEC).** Cells were harvested by injecting 10 ml cold phosphate-buffered salt solution (PBS) into the peritoneal cavity. The abdomen was then massaged and the cell suspension drained through an 18-gauge needle resulting in a recovery of  $1.3 \times 10^7$  cells per animal on average.

**LIVER CELLS (LC).** Single cell suspensions, rich in Kupffer cells, were prepared essentially as described (27, 28). After perfusion of rat liver with a 0.05% collagenase solution (Worthington Biochemical Corp., Freehold, NJ), the heterogeneous cell preparation was centrifuged at low *g*-force to remove parenchymal cells. Cells remaining in the supernatant were washed in Hanks' balanced salt solution (HBSS) and used as the initial cell preparation, which contained an average of  $2.7 \times 10^8$  cells per liver.

**EPIDERMAL CELLS (EC).** Animals were shaved and the skin cleaned with ethanol. Body wall skin sections were taken, and epidermal cell suspensions were prepared using a slightly modified method of Steinmuller and Wunderlich (29). Skin pieces were floated dermal side down on the surface of a 1% trypsin solution (Sigma Chemical Co., St. Louis, MO) in PBS. After 2 h incubation at 37°C, the skin was blotted on dry filter paper, soaked in 0.025% DNase I (Sigma Chemical Co.), and the dermis removed. Cells from the basal layer of the epidermis were dispersed by rubbing it with a glass rod and filtered through cotton to remove debris and hair. The cells were washed in HBSS containing 10% heat-inactivated horse serum (HIHS; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and used as the initial cell preparation in which an average of  $3.5 \times 10^6$  cells/cm<sup>2</sup> skin was recovered.

**Fractionation of Cells in Bovine Serum Albumin (BSA) Gradients.** Cells were suspended at up to  $1 \times 10^8$  cells/ml in a dense BSA solution ( $\rho = 1.085$  g/cm<sup>3</sup>) prepared according to Steinman and Cohn (30), overlaid with 1 ml lower density BSA solution ( $\rho = 1.048$  g/cm<sup>3</sup>), and centrifuged at 10,000 *g* for 30 min at 4°C. Low density (LD) cells at the interface and high density (HD) sedimenting cells were collected separately and washed. Total recovery of cells ranged from 70–80%.

*Mitogen Treatment and Culture Conditions.* Lymphocytes were stimulated by treatment with 1.2 mM sodium periodate (Fisher Scientific Co., Springfield, NJ) in ice-cold PBS at  $1 \times 10^7$  cells/ml (26, 31). Cell concentrations were determined with a Coulter counter (model ZBI; Coulter Electronics, Inc., Hialeah, FL). Cells were cultured in RPMI 1640 containing 100  $\mu\text{g}/\text{ml}$  of streptomycin and 100 U/ml of penicillin (Associated Biomedic Systems, Inc., Buffalo, NY) supplemented with 10% HIHS. Mitogen-treated lymphocytes were dispensed in triplicate to Microtest II plates (3040; Falcon Plastics, Div. of Becton, Dickinson & Co., Oxnard, CA) at 0.1 ml/well ( $5 \times 10^5$  cells). Increasing numbers of different cell subpopulations were added to restore the mitogen response of accessory cell-depleted HD-LNC, and each well was adjusted to 0.2 ml culture medium. Cultures were maintained at 37°C in a humidified atmosphere of 7% CO<sub>2</sub> in air. Cell proliferation was measured by incorporation of [<sup>3</sup>H]dT 4 h before harvest at 48 h, using a solution of low specific radioactivity (31). The presented data are corrected for the background response (no added accessory cells), which usually was below 400 cpm. The standard deviation was seldom higher than 15% and ranged between 6–10%. In our assay, [<sup>3</sup>H]deoxythymidine (dT) incorporation and lymphocyte proliferation are directly proportional to the number of accessory cells added to  $5 \times 10^5$  periodate-treated responder cells. One unit of accessory activity is defined as the number of cells producing a response of 2,000 cpm above the control response.

For mixed leukocyte cultures,  $\gamma$ -irradiated stimulator cells included Lewis HD cells, adherent LNC, and purified dendritic cells. Syngeneic Lewis or allogeneic Buffalo HD-LNC were used as responder cells at a concentration of  $5 \times 10^5$  cells/well. Stimulator cells were added in increasing numbers to responder cells in a final volume of 0.2 ml/well. Proliferation was determined daily for 6 d.

*Selective Depletion of Subpopulations.* Adherent populations were removed from cell suspensions by culturing  $1 \times 10^8$  cells/dish in 100-mm plastic tissue culture dishes (3003; Falcon Plastics) for 2 h at 37°C. Nonadherent cells were then collected by tilting and swirling each dish. The adherent cells were removed with a rubber policeman, and >60% remained viable for at least 120 h, as checked by trypan blue exclusion. In some experiments, unfractionated LNC were cultured in increasing numbers in Microtest plates for 2 h. Nonadherent cells were washed off by flushing each well three times with 0.3 ml HBSS. Remaining adherent cells were then assayed for their accessory cell activity.

To separate FcR<sup>+</sup> and FcR<sup>-</sup> cells, cell suspensions were mixed with opsonized sheep erythrocytes (EA) in a ratio of 1:50, pelleted, and incubated at 4°C for 30 min. To preserve the rosettes, the pellet was then gently resuspended in dense BSA solution and fractionated (8, 21). Erythrocytes in the FcR<sup>+</sup> population were lysed by ammonium chloride. In those experiments where nonrosetting HD cells were used as responder lymphocytes, EA-rosetted HD cells were fractionated on Ficoll/Hypaque (LSM; Litton Bionetics, Kensington, MD). Nonrosetting cells at the interface were then collected and washed with HBSS before mitogen treatment.

Surface IgG<sup>+</sup> cells were removed from cell suspensions by the panning technique of Wysocki and Sato (32). Rabbit anti-rat IgG (N. L. Cappel Laboratories Inc., Cochranville, PA) was diluted 1:10 with normal rabbit globulin (Microbiological Associates, Bethesda, MD). Polystyrene petri dishes (8-757-12; Fisher Scientific Co., Pittsburgh, PA) were coated with 5 ml of antibody diluted to a protein concentration of 40  $\mu\text{g}/\text{ml}$ . The percentage of IgG<sup>+</sup> cells in the binding and nonbinding fractions was determined with fluorescein-labeled goat anti-rat IgG (ICN Pharmaceutical Inc., Cleveland, OH).

*Purification of Dendritic Cells.* The purification of rat dendritic cells has been described previously (8). Because rat lymph node dendritic cells are not adherent, the procedure of Steinman and Cohn (30) was modified. Briefly, LD cells were  $\gamma$ -irradiated (1,000 rad, Co source) and the nonadherent cells cultured for  $\geq 1$  d. Nonadherent cells were collected and subjected to BSA fractionation to remove dense and dead cells. The LD cells were collected and EA-rosetted. Nonrosetting cells were recovered, containing 85–92% dendritic cells with long motile cytoplasmic projections and a yield of 0.01–0.1% of the starting LNC preparation (Fig. 1). The contaminating cells were large lymphocytes that survived the irradiation.

*Nonspecific Esterase Staining.* For the identification of mononuclear cells and macrophages in LNC suspensions, the staining procedure for nonspecific esterase described by Koski et al. (33) was used. Cells were resuspended in 5–10  $\mu\text{l}$  HIHS, and smears were prepared on coverslips.

After the staining procedure, cells containing intensely red-stained granules were considered esterase positive.

*Indirect Immunofluorescence.* To characterize surface molecules,  $1 \times 10^6$  washed cells were suspended in 20  $\mu$ l of mouse monoclonal antibody against rat Thy-1.1 or Ia-antigens (OX7, OX3; Sera-Lab, Sussex, England) diluted 1:2 in PBS containing 3 mM sodium azide. After 60 min on ice, the cells were washed twice and reincubated with 30  $\mu$ l of fluorescein-conjugated goat anti-mouse IgG (Litton Bionetics) diluted 1:10 in PBS containing sodium azide. The labeled cells were then viewed with a 63  $\times$  oil immersion lens on a Zeiss microscope with epifluorescence optics (Carl Zeiss, Inc., New York). Control cells were incubated with the second antibody only.

*UV Inactivation of Dendritic Cells.* A  $\gamma$ -irradiated cell suspension containing 85% dendritic cells from lymph nodes was used. Cells were resuspended at  $2.5 \times 10^5$  cells/ml in RPMI 1640 without serum and kept in suspension in a magnetic stirrer. Ultraviolet light (UV) was administered with a bank of two FS-15 fluorescent tubes (Sylvania Electric Products Inc., Salem, MA). The tube-to-target distance measured 4 cm. At time zero and different times of irradiation, aliquots were directly transferred to wells, and mitogen-treated HD-LNC were added. No change in the viability of the irradiated cells was observed with trypan blue.

## Results

*Accessory Cell Activity of Different LNC Subfractions.* We previously reported (8) that fractionation of LNC in discontinuous BSA gradients yields two fractions of cells having different roles in oxidative mitogenesis. One, termed HD cells, does not respond to periodate treatment even though >90% of the recovered LNC are in this fraction. However, periodate-treated HD cells do respond in a dose-dependent manner upon addition of small numbers of LD cells from the minor fraction (<10% of the recovered LNC) that collects at the interface between light and dense BSA solutions. We demonstrated that most if not all of the accessory activity in the low density fraction resides in a subpopulation of cells described as "dendritic." To determine whether other cells also possessed significant accessory activity, we assayed each of the subfractions collected during purification of dendritic cells (Fig. 1); the results (Table I) are presented as the average of two experiments.

The simple one-step fractionation in a discontinuous BSA gradient resulted in >90% of the applied accessory activity being recovered with 6% of the LD cells, which represents a 16-fold enrichment over that of the unfractionated LNC. HD cells had no detectable accessory activity.

LD cells consisted primarily of lymphocytes, blast cells, and macrophages. Shortman et al. (34) reported that the accessory activity of macrophages is not impaired after  $\gamma$ -irradiation, and we similarly found that LD cells function normally after irradiation. Therefore, LD cells were irradiated, cultured for 2 h, and subfractionated into adherent and nonadherent cells. Only the nonadherent population had accessory activity (Table I); it should be noted that the adherent population almost completely lacked accessory activity, even though most of its cells were macrophages.

More cells adhered during a 16-h culture period; no accessory activity was detected despite the presence of macrophages. Virtually all of the accessory activity was recovered with the nonadherent population, of which 11% survived irradiation and overnight culture. Because only viable cells function as accessories, a considerable enrichment in accessory activity per cell resulted from the removal of the radiosensitive cells. This can be seen in the results obtained after an additional fractionation of the nonadherent cells in BSA. The HD population contained most of the fractionated nonadherent cells, but only 3% were viable, and accessory activity was nil. In contrast,

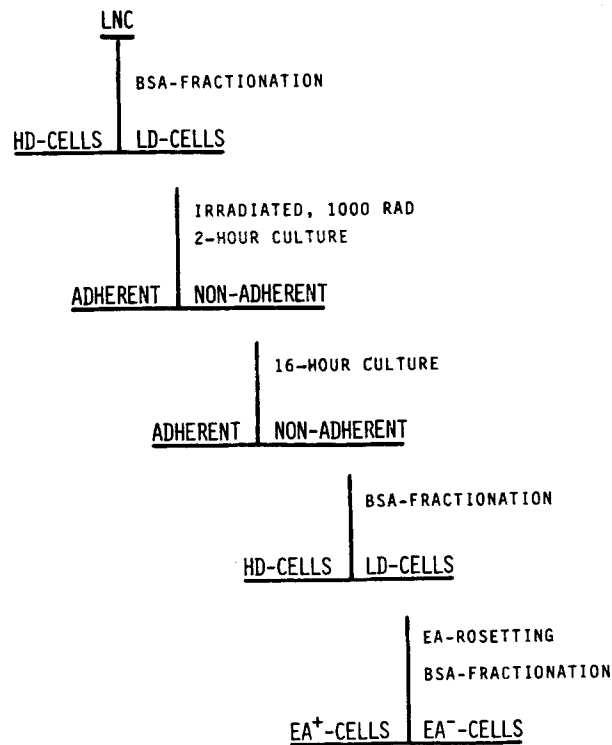


FIG. 1. Flowchart of the procedure for purification of dendritic cells.

TABLE I  
Accessory Activity of Fractionated Lymph Node Cells

Step	Procedure	Fraction	Cell recovery		Accessory activity*	
			%		mU/cell	% recovered
1		LNC	100		0.0024	100‡
2	BSA	HD	70		<0.001	—
		LD	5.9		0.037	93
3	2 h culture	ad <sup>+</sup>	0.03		<0.001	—
		ad <sup>-</sup>	4.9		0.04	83
4	16 h culture	ad <sup>+</sup>	0.01		<0.001	—
		ad <sup>-</sup>	4.3		0.039	72
5	BSA	HD	2.7		<0.001	—
		LD	0.1		1.65	70
6	EA-rosetting	EA <sup>+</sup>	0.05		0.16	3.4
		EA <sup>-</sup>	0.05		3.0	65

\* Cell populations were assayed for accessory activity in presence of  $5 \times 10^6$  periodate-treated HD-LNC.

‡ The total accessory activity was  $2.9 \times 10^3$  units.

nearly all of the accessory activity resided in the minor population of LD cells, of which >95% were viable. A 600-fold enrichment of accessory activity per cell over the starting LNC was obtained.

Of the cells present in the LD population, 75% were identified as dendritic cells and 2.3% as macrophages. To reduce further the contamination by macrophages, this

preparation was incubated with opsonized sheep erythrocytes and subfractionated into rosetted (EA<sup>+</sup>) and nonrosetted (EA<sup>-</sup>) cells. The results in Table I show that nearly all of the accessory activity was recovered with the nonrosetted population in which the frequency of macrophages was <0.1%. Rat dendritic cells do not form EA-rosettes, but they represented 3.9% of the cells that sedimented with the rosetted population, possibly as a result of physical entrapment. These "contaminating" dendritic cells most likely accounted for the accessory activity measured in this fraction.

Despite losses of dendritic cells in the rosetting step, a final activity of 3 mU/cell was obtained, which represents greater than a 1,200-fold enrichment in a preparation that contained 85% dendritic cells. Overall, 65% of the initial accessory activity was recovered in only 0.05% of the starting LNC.

*Studies with Adherent Cells.* Although the above results indicated that adherent cells lacked accessory activity, three other approaches were used to examine this further. First, LNC were cultured in increasing numbers, up to  $4.8 \times 10^6$  cells/well, in the Microtest plates used to assay accessory activity. 2 h later, nonadherent cells were removed, and generally 0.1% of the LNC were found to remain adherent. To these adherent LNC, periodate-treated HD-LNC were added. Despite the presence of increasing numbers of adherent cells per well, only a very slight increase in [<sup>3</sup>H]dT incorporation above the control cultures was observed (Table II). Second, adherent LNC were collected from culture dishes and tested for both accessory activity and a possible synergistic activity with nonadherent LD cells. As seen in Fig. 2, no accessory activity was detected, and the adherent cell preparation neither enhanced nor inhibited the accessory activity of a constant number of LD cells. Finally, residual macrophages in our lymphocyte preparations could possibly act in conjunction with dendritic cells in T cell responses. Thus, macrophages were removed from HD cells by adherence and EA-rosetting. When the periodate-induced response of the macrophage-depleted HD cells was compared with the response of unfractionated HD cells in the presence of increasing numbers of purified dendritic cells, no significant difference was observed. These data, taken together with those of the experiments

TABLE II  
*Accessory Activity of Adherent LNC*

Number of LNC added*	[ <sup>3</sup> H]dT incorporation‡
$\times 10^{-6}$	<i>cpm</i>
0.15	209 ± 9
0.30	412 ± 30
0.60	265 ± 14
1.20	211 ± 29
2.40	301 ± 10
4.80	376 ± 19

\* Different numbers of LNC were cultured in microtiter plates as indicated. After 2 h, nonadherent cells were removed and periodate-treated HD-LNC were added to the remaining adherent cells. At all levels of added LNC, ~0.1% remained adherent.

‡ A background response of 78 cpm for periodate-treated HD-LNC has been subtracted.

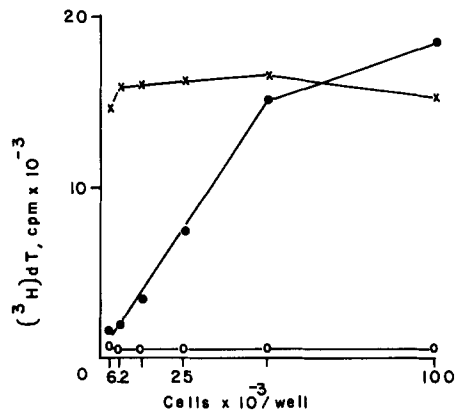


FIG. 2. Accessory activity of adherent LNC. LNC were cultured in petri dishes to prepare adherent cells. Adherent cells were collected and added to periodate-treated HD-LNC with (X) or without (O) 10% LD-LNC. A titration of the accessory activity of LD-LNC is included for comparison (●).

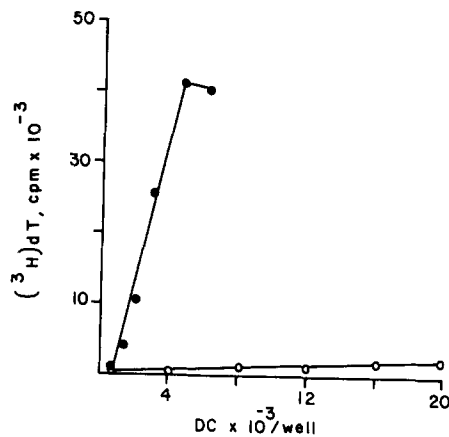


FIG. 3. Accessory cell-dependent periodate response of  $\text{IgG}^+$  and  $\text{IgG}^-$  HD-LNC. Responder LNC were prepared by panning on IgG-coated petri dishes.  $\text{IgG}^+$  (80% positive, O) and  $\text{IgG}^-$  (97% negative, ●) cells were treated with periodate, and purified accessory cells (85% dendritic cells) were added.

above, strongly suggest that resident lymph node macrophages neither inhibit nor enhance periodate-induced lymphocyte stimulation.

*Identification of the Responding Cell.* Previous investigations in mouse and guinea pig have established that only T lymphocytes respond to periodate treatment (4, 5, 35). As similar studies have not been done in rat, it was important to determine whether T cells are also the responders in dendritic cell-mediated stimulation. LNC were fractionated on a BSA gradient. The starting cell suspension contained 18%  $\text{IgG}^+$  cells, of which 92% were recovered in the HD fraction. The HD cells were then fractionated into  $\text{IgG}^+$  and  $\text{IgG}^-$  populations by the "panning" method of Wysocki and Sato (32). Repeated panning yielded a total recovery of 60% of the cells applied. When the  $\text{IgG}^+$  population (>80% positive by fluorescence analysis) was treated with periodate and cultured with varying numbers of purified dendritic cells, only small responses were observed, even with high numbers of dendritic cells (Fig. 3). In marked

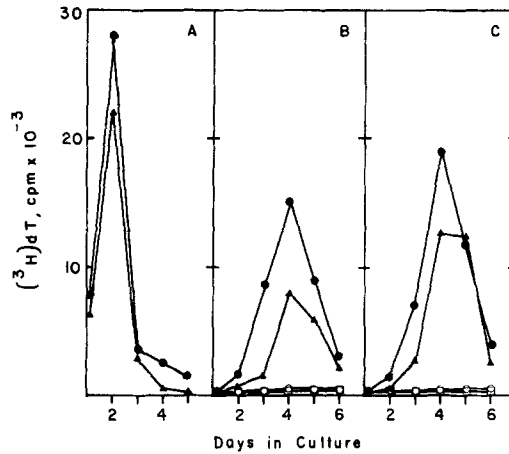


FIG. 4. Comparison of proliferation kinetics in three types of dendritic cell-mediated responses. A, proliferation of Lewis HD-LNC after periodate treatment in presence of 2,500 ( $\blacktriangle$ ) or 5,000 ( $\bullet$ ) purified dendritic cells. B, syngeneic mixed leukocyte reaction. Stimulator cells were purified,  $\gamma$ -irradiated Lewis dendritic cells at cell concentrations indicated above. As responder cells, Lewis HD-LNC ( $5 \times 10^5$  cells/well) were used. C, one-way allogeneic mixed leukocyte culture. To  $5 \times 10^5$  Buffalo HD-LNC,  $\gamma$ -irradiated Lewis dendritic cells were added as described above. 5,000 adherent LNC ( $\square$ ) or HD-LNC ( $\circ$ ), both  $\gamma$ -irradiated, were included as stimulators.

contrast, the  $\text{IgG}^-$  population (containing 3%  $\text{IgG}^+$  cells) responded extremely well (Fig. 3). These results indicate that the T cell is the predominant if not the exclusive responding cell in rat.

*Rat Dendritic Cells as Potent Stimulator Cells in Syngeneic and Allogeneic MLR.* It has been shown by Steinman and Witmer (21) that mouse dendritic cells act as potent stimulator cells in an allogeneic MLR. Recently, Nussenzweig and Steinman (22) reported that purified mouse dendritic cells will also stimulate syngeneic T lymphocytes in a syngeneic MLR. We assessed the role of rat dendritic cells from the same preparation in three types of reactions; periodate-induced mitogenesis (Fig. 4A), syngeneic (Fig. 4B), and allogeneic (Fig. 4C) MLR. The syngeneic response of Lewis dendritic cells with Lewis HD-LNC and the allogeneic response (Lewis dendritic cells with Buffalo HD-LNC) both peaked between 96 h and 120 h. The magnitude of each response was proportional to the number of dendritic cells added as stimulators. The periodate-induced proliferation of Lewis HD-LNC, which is dendritic cell dependent, reached a maximum of 28,000 cpm at 48 h. At this time, the contribution of the syngeneic MLR was  $<2,000$  cpm. Neither  $\gamma$ -irradiated HD cells nor macrophages were able to serve as stimulator cells in these responses (Fig. 4B and 4C).

*Characteristics of Rat Dendritic Cells.* A consistent observation is that accessory cells express Ia antigens (1, 6, 36-38). Through the use of a monoclonal antibody specific for rat Ia antigens (39), in immunofluorescence studies we found that  $>95\%$  of the purified dendritic cells gave a very strong positive reaction. In contrast, 75% of the adherent macrophages were only weakly positive. As control, a monoclonal antibody directed against rat Thy-1.1 (40) gave a very positive staining with rat thymocytes but did not stain dendritic cells or macrophages. Mouse spleen macrophages and dendritic cells have also been reported to lack this antigen (41). The anti-Ia antibody



did not stain IgG-negative HD-LNC but gave a weak positive staining with IgG-positive HD-LNC.

The presence of nonspecific esterase and phagocytic capability are frequently used as markers for cells of the monocyte-macrophage lineage. We found that 82% of adherent LNC were positive for the esterase marker, whereas dendritic cells were negative. Dendritic cells also lacked the ability to phagocytose carbon particles under conditions in which macrophages were phagocytic.

Metabolically active dendritic cells were required as accessories for responses to periodate because UV-irradiated dendritic cells lacked this function (Table III). Heat-inactivated dendritic cells (5 min at 60°C) also failed to serve as accessory cells.

*Comparison of the Accessory Activity of Dendritic Cells from Spleen and Lymph Node.* We previously reported (8) that dendritic cells possessing accessory activity could be purified from rat spleen cell preparations in higher yield than from LNC. We now report that the accessory activity of nonadherent, nonrosetting preparations of dendritic cells from both tissues is identical on a per cell basis (2–5 mU/cell). Addition of similar numbers of dendritic cells from either source produced similar proliferative responses for periodate treated HD-LNC. Dendritic cells from rat thymus have also been found to have accessory activity comparable to lymph node dendritic cells (42).

*Peritoneal Exudate Preparations.* The peritoneal cavity is the most common source of macrophages. We found that freshly isolated PEC had little accessory activity regardless of whether cells were added directly to the assay (Table IV) or were first cultured in microtest plates for 2 h to assay the adherent population only (Table V). Of the adherent cells, some 65% stained positively for nonspecific esterase, and a similar percentage phagocytosed carbon particles.

To identify the accessory cell, PEC were fractionated as described under Materials and Methods. After centrifugation on a discontinuous BSA gradient, 65% of the PEC floated, whereas only 17% sedimented. The minor HD-PEC population was enriched in mast cells and had no accessory activity. In contrast, LD-PEC did exhibit accessory activity, but only at the lowest numbers tested (Table IV). The adherent population present in the LD cell fraction also lacked accessory activity.

Further fractionation of the LD-PEC yielded an EA<sup>-</sup>, nonadherent population

TABLE III  
Effect of UV Irradiation on Purified Dendritic Cells\*

Time of irradiation	[ <sup>3</sup> H]dT incorporation
<i>min</i>	<i>cpm</i>
0	25,090 ± 1,740
0.15	21,400 ± 1,630
0.3	14,640 ± 1,580
0.6	13,980 ± 970
1.2	11,160 ± 870
2.4	8,410 ± 910
4.8	6,670 ± 720
9.6	2,670 ± 230
19.2	810 ± 70

\* A dendritic cell fraction (80% dendritic cells) was UV-irradiated as described in Materials and Methods. Aliquots were taken at indicated times of irradiation and added to periodate-treated HD-LNC. A background response with HD-LNC of 387 cpm has been subtracted.

TABLE IV  
Accessory Activity of PEC Populations

Cells added per culture	<sup>3</sup> H]dT incorporation with				
	PEC	LD-PEC	AD <sup>+</sup> LD-PEC*	EA <sup>-</sup> -PEC‡	EA <sup>+</sup> -PEC
3,125§	100 ± 13	1,900 ± 162	198 ± 37	2,062 ± 279	187 ± 15
6,250	237 ± 28	2,366 ± 84	247 ± 72	4,145 ± 309	331 ± 93
12,500	411 ± 37	419 ± 57	317 ± 31	8,655 ± 565	595 ± 67
25,000	563 ± 63	63 ± 12	433 ± 85	16,251 ± 282	989 ± 89
50,000	1,281 ± 137	—	219 ± 57	20,339 ± 589	875 ± 40

\* LD-PEC were cultured in petri dishes for 2 h. Nonadherent cells were removed and adherent cells (AD<sup>+</sup>) recovered using a rubber policeman. The viability was 65%.

‡ The EA<sup>-</sup>-population contained 60% dendritic cells.

§ The indicated numbers of  $\gamma$ -irradiated cell populations were added to periodate-treated HD-LNC. A background response of 250 cpm of HD-LNC alone has been subtracted.

TABLE V  
Accessory Activity of Adherent PEC

PEC added*	<sup>3</sup> H]dT incorporation
$\times 10^{-6}$	cpm
0.02	358 ± 41
0.04	509 ± 54
0.08	782 ± 93
0.16	1,289 ± 141
0.32	1,764 ± 278
0.64	1,452 ± 177
1.28	1,196 ± 202

\* Unfractionated PEC were cultured at the indicated numbers in Microtest plates for 2 h. Nonadherent cells were removed and periodate-treated HD-LNC were added to the wells. A background response of periodate-treated HD-LNC of 250 cpm has been subtracted.

(1.5% of the starting PEC), which had an accessory activity of 0.33 mU/cell (Table IV). 60% of the cells were morphologically similar to dendritic cells from lymph nodes and did not stain for nonspecific esterase. An additional 30% of the recovered cells showed a very weak, diffuse staining pattern, and 10% were neither dendritic nor esterase positive. The EA<sup>+</sup> population had no accessory activity. Thus, dendritic cells are present in LD-PEC and could account for the accessory activity found in this fraction.

We performed additional experiments to determine why LD-PEC have such a low level of accessory activity compared with that of the nonadherent EA<sup>-</sup> population. Addition of increasing numbers of LD-PEC to cultures containing both periodate-treated HD-LNC and lymph node dendritic cells resulted in a progressive inhibition of thymidine incorporation (Table VI). Thus, at least two distinct types of cells exist in LD-PEC; one, a dendritic cell, acts as an accessory, and a second, possibly a macrophage, inhibits the mitogenic response.

*Accessory Activity of Liver Cell Populations.* Kupffer cells of guinea pig and mouse liver have been reported to function as accessory cells in mitogen-induced T cell proliferation (16–18). We found only a low activity for the enriched population of nonparenchymal cells that was prepared by collagenase perfusion of rat liver and

TABLE VI  
Inhibition of Accessory Activity by Low Density PEC

LD-PEC added per culture*	[ <sup>3</sup> H]dT incorporation‡
	<i>cpm</i>
0	31,356 ± 1,848
195	30,083 ± 2,120
390	26,113 ± 783
780	24,875 ± 611
1,560	11,644 ± 798
3,125	2,467 ± 442
6,250	179 ± 91
12,500	63 ± 19

\* Each culture contained periodate-treated HD-LNC and  $6 \times 10^3$  lymph node dendritic cells to produce a high response. To these cultures, the indicated number of LD-PEC (67% of the unfractionated PEC) was added.

‡ A background response of periodate-treated HD-LNC of 198 cpm was subtracted from each culture.

TABLE VII  
Accessory Activity of Various Liver and Skin Cell Populations

Procedure	Fraction	Cell recovery	Accessory activity	Dendritic cells
		% of total	mU/cell	%
Liver cell populations				
Initial cell preparation	LC	100	0.018	—
BSA-fractionation	HD	44	0.004	—
	LD	8	0.096	—
EA-rosetting	EA <sup>+</sup>	2.6	0.081	15
	EA <sup>-</sup>	0.47	2.2	85
Skin cell populations				
Initial cell preparation	EC	100	0.002	—
BSA-fractionation	HD	60	<0.001	—
	LD	5	0.018	—
EA-rosetting	EA <sup>+</sup>	2.2	0.026	<1
	EA <sup>-</sup>	0.8	1.5	50

differential centrifugation of the isolated cells (Table VII). After fractionation in BSA, the LD population showed a fivefold increase in accessory activity, whereas the HD cells, composed mostly of parenchymal cells, had very low activity. After  $\gamma$ -irradiation and overnight culture of the LD cells, 92% nonadherent and 4% adherent cells were recovered, the latter mostly consisting of macrophages and fibroblasts that had no detectable activity. Of the nonadherent cells, the EA<sup>+</sup> population contained 15% dendritic cells, but the accessory activity was lower than expected, possibly due to the presence of 85% Fc receptor and nonspecific esterase-positive macrophages that could

inhibit the mitogen response. In contrast, the EA<sup>-</sup> population (85% dendritic cells) exhibited high accessory activity. 15% of the EA<sup>-</sup> population stained weakly for the esterase marker. Thus, 62% of the accessory activity was recovered with only 0.47% of the cells present in the initial preparation.

*Epidermal Dendritic Cells.* Because Ia-positive epidermal cells of Langerhans cells serve as stimulators for allogeneic lymphocytes (19, 20) and therefore have properties similar to lymphoid dendritic cells (21, 22), we wanted to determine whether both cell types shared other properties. Accordingly, trypsinized skin sections from the body wall were used to prepare a single cell suspension that was found to have little accessory activity for periodate-treated HD-LNC (Table VII). However, after fractionation on BSA gradients and overnight culture, the LD population exhibited a ninefold increase in activity, whereas the HD population had no detectable activity. The accessory activity of the LD cells did not change after  $\gamma$ -irradiation. Further fractionation yielded an adherent population with no accessory activity, a nonadherent EA<sup>+</sup> population exhibiting an accessory activity of 0.026 mU/cell, and a nonadherent EA<sup>-</sup> population (0.8% of the starting skin cells) that had an accessory activity of 1.5 mU/cell. Substantially more accessory activity was recovered in the EA<sup>-</sup> population than was present in the starting cell suspension, possibly because defects resulting from trypsinization were repaired during overnight culture (20, unpublished observation). Morphologically, one-half of the cells in the EA<sup>-</sup> population had the same appearance as lymph node dendritic cells; of these, >90% were found to bear Ia antigens. The unfractionated EC suspension contained only 1% Ia<sup>+</sup> cells. Functionally, epidermal and lymph node dendritic cells from the same animals had an accessory activity that correlated well with the percentage of dendritic cells present in both preparations (LNC: 85% dendritic cells, 2.3 mU/cell; EC: 50% dendritic cells, 1.5 mU/cell).

Birbeck granules have been described as a marker for Langerhans cells (25). Electron microscopic examination revealed the presence of these granules in both epidermal and lymph node dendritic cells (Fig. 5). The findings of our morphological and functional studies strongly suggest that the Langerhans cell and dendritic cell in rat belong to the same cell lineage.

### Discussion

We recently identified (8) rat dendritic cells as potent accessory cells in the mitogenic stimulation of rat T lymphocytes treated with either periodate or neuraminidase plus galactose oxidase. Owing to the linear response obtained when increasing numbers of accessory cells were added to a fixed number of mitogen-treated lymphocytes, we developed a dose-dependent assay that has enabled us to quantitatively monitor accessory activity during the fractionation of cells from a variety of rat tissues. For each tissue, only those fractions enriched in dendritic cells contained significant accessory activity (Tables I, IV, and VII). Other fractions, including those known to contain macrophages, generally had accessory activity below the level of detection, even though the assay is sensitive enough to measure the activity of <100 dendritic cells (8). On this basis, we conclude that the dendritic cell in a variety of rat tissues is the predominant and perhaps exclusive accessory cell for oxidative mitogenesis. In this regard, it is noteworthy that dendritic cells isolated from thymus (42), lymph node, spleen, liver, skin (this paper), as well as dendritic cells that develop in

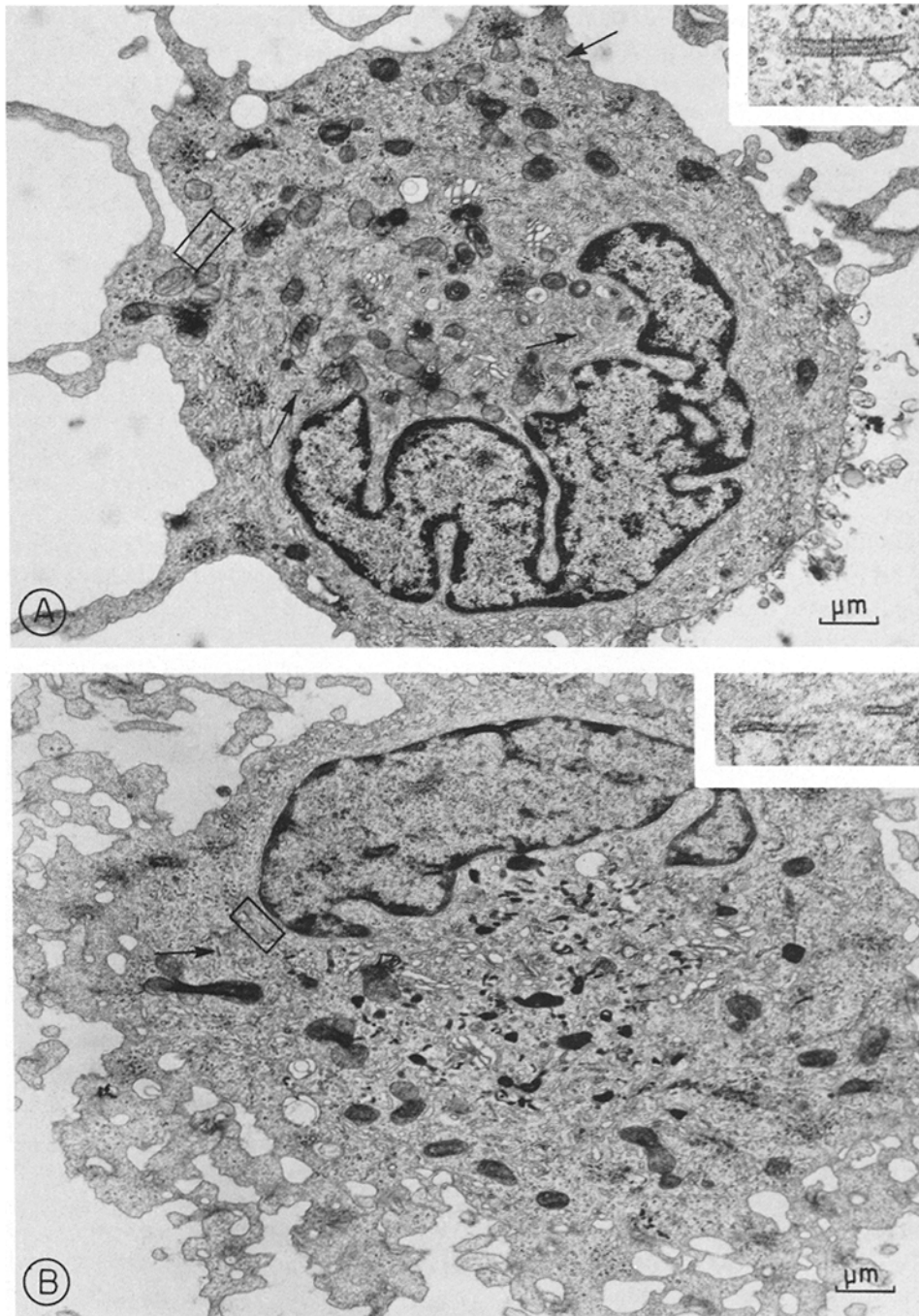


FIG. 5. Electron micrograph of a lymph node (A) and an epidermal (B) cell with dendritic morphology and several Birbeck granules. Cells were purified and prepared for electron microscopy (57) after 72 h in culture ( $\times 8,000$ ). The insets show one granule from each cell in greater detail ( $\times 55,000$ ).

vitro from functionally incompetent precursors in bone marrow and peripheral blood (in preparation), all have virtually the same accessory activity on a per cell basis.

Our finding that rat lymph node macrophages lack accessory activity differs from the reports that macrophages in other species do function as accessory cells (43, 44). Additional experiments addressed to this point ruled out the possibility that nonviable or insufficient numbers of rat macrophages were tested. Moreover, rat lymph node macrophages did not affect the response between dendritic cells and periodate-treated lymphocytes (Fig. 2); thus, these macrophages do not have suppressive properties that could account for their inability to function as accessory cells. These cellular requirements are not unique to oxidative mitogenesis, because in another functional test, that of stimulation in an MLR, macrophages present in preparations of both high density LNC and adherent cells failed to stimulate under conditions in which dendritic cells were potent stimulators.

Several other aspects concerning the potential accessory activity of macrophages must be considered. The cells we studied were obtained from untreated rats. Thus, our results might not bear upon those obtained with activated macrophages. A large body of evidence supports an important role for Ia antigen in accessory or stimulatory activities, and it has been suggested that the amount of Ia expressed might be critical for these processes (45). Although the majority of macrophages are weakly Ia positive compared with dendritic cells, our qualitative results leave open the possibility that the density of Ia is not sufficiently high for these cells to function as accessories or stimulators. However, it is also possible that rat macrophages, regardless of their content of Ia, do not have these functions. In this regard, it might be of some significance that our macrophage preparations lack accessory and stimulatory activities under conditions in which dendritic cells functionally are so potent. Not only do these findings indicate that dendritic cell contamination of our macrophage preparations is insignificant, but they also raise the possibility that the macrophage preparations reported by others to have accessory or stimulatory activity do in fact contain some dendritic cells. This is an especially important consideration when high ratios of macrophages to lymphocytes are required to achieve optimum responses.

We recovered a high percent of the initial accessory cell activity in our dendritic cell preparations. However, our purification scheme involves  $\geq 1$  d in culture, during which a change in potency of accessory cells could occur. No differentiation of precursor cells into accessory cells was observed in long-term cultures of irradiated lymph node cells (unpublished observation).

Epidermal Langerhans cells from mouse, guinea pig, and man also carry Ia antigens (46) and function as stimulator cells for allogeneic lymphocytes. Tanaka and Sakai (20) made similar findings with rat epidermal cells and observed higher responses in mixtures of irradiated skin cells and allogeneic lymphocytes when adherent cells were removed from the responder cell preparation. In an extension of those studies, we found that the rat epidermal dendritic cell is Ia<sup>+</sup>, does not form rosettes with opsonized sheep erythrocytes, and has accessory activity. In contrast, the guinea pig Langerhans cell has been reported to bear Fc and C3 receptors (47).

Dendritic cells in skin and lymphoid tissues have identical morphological and functional features, which suggests that they belong to the same class of cells. This suggestion is further strengthened by the presence in lymph node dendritic cells of Birbeck granules, believed to be a morphological marker for epidermal dendritic

(Langerhans) cells. Such a finding may not be surprising in view of the report by Silberberg-Sinakin et al. (48) that antigen-bearing Langerhans cells migrate to lymph nodes. We are currently investigating whether dendritic cells in other tissues also possess Birbeck granules.

Our conclusion that dendritic cells differ from macrophages in morphology, surface properties, and immune regulatory functions is supported by our findings that (a) dendritic cells with accessory function are present in many rat tissues and organs, including those exposed to a variety of antigens such as spleen, liver, and skin; (b) important attributes of macrophages such as Fc receptors and ability to adhere and phagocytose are not features of rat dendritic cells; (c) macrophage preparations highly depleted of dendritic cells do not serve as accessory cells or are inhibitory; and (d) Ia antigens that play an important role in the induction of immune reactions are present on dendritic cells, and the biological function of these cells can be inhibited with a monoclonal antibody directed against Ia antigens (49). In addition, dendritic cells are present in cultures of the bone marrow and peripheral blood cells, sources of many cell types that participate in immune reactions, and their differentiation seems to be regulated by factor(s) released during a mitogen response of lymphocytes (manuscript in preparation). There is also evidence that follicular dendritic cells are involved in long-term antigen retention during immunity (50, 51).

More work remains to be done to elucidate the exact lineage of the dendritic cells we studied. Based on the information currently available, however, we feel that the interdigitating cell of lymph nodes (52, 53), large mononuclear cells found in the lymph of humans (54), rabbits (55), and pigs (56), and the Langerhans cells of human dermal tissue (54) could belong to the same lineage.

### Summary

Single cell suspensions of rat lymphoid and nonlymphoid tissues were fractionated on discontinuous gradients of bovine serum albumin into high density and low density subfractions. In general, accessory activity required for responses of periodate-treated T lymphocytes was recovered only in a low density population containing a small percent of the total fractionated cells from lymph nodes, spleen, liver, skin, and peritoneal exudates. Further purification always led to an increase of both accessory activity and number of dendritic cells present in nonrosetting and nonadherent populations. After purification, a high recovery of the total accessory activity was found in fractions that contained a high percentage of dendritic cells resulting in a more than 1,000-fold enrichment in accessory activity per cell. No other fraction obtained during the purification contained significant accessory activity. In all cases, macrophage-enriched populations lacked accessory cell activity. With the exception of peritoneal exudate cell preparations, which contained an inhibitory cell, the level of accessory activity in a given population was always found to be a function of the number of dendritic cells present.

Dendritic cells from all sources were nonadherent, nonphagocytic, radio-resistant, and nonspecific esterase negative. They expressed Ia antigens and lacked Fc receptors. Both epidermal and lymph node dendritic cells contain Birbeck granules, subcellular structures previously described only for Langerhans cells.

Accessory activity requires viable dendritic cells but is unaffected by 1,000 rad of  $\gamma$ -irradiation. However, ultraviolet irradiation abolished the activity of accessory cells.

The cells that responded to periodate were IgG-negative T cells, whereas IgG-positive B cells could not be stimulated under the same conditions. Only periodate-treated T cells and dendritic cells were needed for responses to occur; removal of virtually all macrophages from these purified preparations had no effect. Dendritic cells were also required as stimulators in mixed leukocyte cultures, whereas macrophages, even though Ia positive, were inert.

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