Properties of lymph-borne (veiled) dendritic cells in culture

I. MODULATION OF PHENOTYPE, SURVIVAL AND FUNCTION: PARTIAL DEPENDENCE ON GM-CSF

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SUMMARY

Lymph-borne dendritic cells (L-DC) collected from the thoracic duct of rats following mesenteric lymphadenectomy are derived from the small intestine. We have cultured these cells *in vitro* and examined their survival and phenotypic and functional changes. L-DC survive poorly in culture in normal media (< 50% overnight) but survival can be markedly increased by supplementation with Con A-stimulated spleen cell supernatant or recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) but not by recombinant IL-1, IL-2, IFN-γ or by an IL-3-rich supernatant. The effects of GM-CSF are blocked by a specific antiserum. L-DC display heterogeneity for some surface markers, cytoplasmic inclusions and enzyme reactivity. After 16–48 hr culture the pattern of expression is markedly different. The numbers of Thy-1-positive L-DC and the amount of Thy-1 expressed increases, as do the numbers of L-DC expressing OX48 antigen. All L-DC remain Ia positive, but the proportion expressing the iC'3b receptor, non-specific esterase or cytoplasmic DNA inclusions decreases to almost zero. In contrast to Langerhans' cells, fresh L-DC are potent stimulators of an allogeneic mixed leucocyte reaction (MLR) but their potency is considerably increased after 16 hr culture. Also in contrast to Langerhans' cells, the increase in potency is not affected by culture with CAS and is thus unlikely to be dependent on GM-CSF. The changes described in L-DC properties could be related to their role as antigen-presenting cells.

INTRODUCTION

The lymphoid dendritic cell (DC) appears to be uniquely able to present antigen to unstimulated T lymphocytes (reviewed by Steinman *et al.*, 1986) but little is known of its *in vivo* life history and physiology. Strongly Ia positive cells with dendritic morphology are present in most peripheral tissues (Hart & Fabre, 1981) but their properties, as exemplified by Langerhans' cells, differ from those of splenic DC (Schuler & Steinman, 1985). Some peripheral DC can undergo further differentiation. Schuler & Steinman (1985) cultured Langerhans' cells and showed that while Ia expression increased, expression of complement and Fc receptors and F4/80 antigen (Austyn &

Gordon, 1981) decreased. Concomitantly, Langerhans' cells came to resemble DC functionally.

We and others have shown that rat lymph-borne dendritic cells (L-DC) are heterogeneous in terms of morphology, enzyme cytochemistry and surface markers (Pugh, MacPherson & Steer, 1983; MacPherson & Pugh, 1984; Fossum, 1984). This could represent different lineages of L-DC, different stages in maturation or maturation in distinct microenvironments, for example Peyer's patch or lamina propria.

To explore the regulation and significance of L-DC heterogeneity we have cultured L-DC under different conditions, quantified their survival and investigated changes in their phenotype and function. In this report we examine the requirements for optimal L-DC survival in culture and show that the expression of several potentially significant markers, including Thy-1, is differentially modulated in culture. We also show that a major factor responsible for L-DC survival and phenotypic modulation in culture is granulocyte–macrophage colony-stimulating factor (GM-CSF). We have also compared the ability of fresh and cultured L-DC to stimulate a mixed leucocyte reaction (MLR).

Abbreviations: BSA, bovine serum albumin; CAS, supernatant from spleen cells cultured with concanavalin A; CR3, receptor for inactivated third complement component; DC, lymphoid dendritic cell; FCS, fetal calf serum; GM-CSF, granulocyte–macrophage colony-stimulating factor; [³H]TdR, tritiated thymidine; IFN, interferon; IL-, interleukin; L-DC, lymph-borne (veiled) dendritic cell; mAb, monoclonal antibody; MLR, mixed leucocyte reaction; PBS, phosphate-buffered saline; PLL, poly-L-lysine; SPF, specific-pathogen free; SRBC, sheep erythrocytes; TDL, thoracic duct lymph cells; XTDL, thoracic duct lymph cells from mesenteric lymphadenectomized rats.

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MATERIALS AND METHODS

Animals

Rats were SPF PVG(RT1c) or DA(RT1a) strains bred in the

MRC Cellular Immunology Unit. Mesenteric lymphadenectomy and thoracic duct cannulation were carried out as described elsewhere (Pugh *et al.*, 1983). Some rats were gamma-irradiated with 5 Gy from a cobalt source immediately before cannulation.

Cell separation

Metrizamide separation. Thoracic duct lymph cells from mesenteric lymphadenectomized rats (XTDL) were overlaid over 15% Metrizamide (Nyegaard, Oslo) in phosphate-buffered saline (PBS) containing 0·1% bovine serum albumin (BSA) and centrifuged at 400 *g* for 30 min. The interface cells contained 60–80% L-DC. The majority of contaminating cells were B lymphoblasts.

Rosetting. (Mason, 1981) XTDL were incubated with a 'cocktail' of monoclonal antibodies for 60 min at 4°. After washing they were rotated with SRBC coated with rabbit anti-mouse IgG (a kind gift from Dr A. F. Williams, Sir William Dunn School of Pathology), and subsequently separated by centrifugation over 15% Metrizamide.

In some experiments antibody-coated XTDL were incubated with magnetic beads coated with anti-mouse Ig (Dynal, Wirral, Cheshire), and labelled cells separated using a magnet.

Using either technique, the frequency of residual contaminating cells was less than 5%.

Immunocytochemistry

Washed cells in PBS with 5% BSA were cytocentrifuged onto glass slides coated with poly-L-lysine (100 *µg*/ml; Sigma, Poole, Dorset) and air dried. In some experiments cells were allowed to sediment onto poly-L-lysine-coated slides (PLL preps).

After fixation for 10 min in ethanol at 4°, preparations were washed and stained with mouse anti-rat monoclonal antibodies (mAb) (kind gifts from Dr A. F. Williams). Second-layer antibody was peroxidase-conjugated rabbit anti-mouse Ig (Dako, High Wycombe, Bucks; P161), at a dilution of 1:30–1:50, containing 5% rat serum. Reaction product was visualized using diaminobenzidine (1 mg/ml) and 0·005% H_2O_2. To assess staining, slides were coded and scored on a 0–3+ scale.

Culture of L-DC

Whole XTDL or XTDL depleted of different cell populations were cultured in 96-well plates or small Teflon beakers. Media were either RPMI-1640 supplemented with 10% fetal calf serum (FCS) or 5% rat serum, or a serum-free medium, Hb1012 (NEN, Boston, MA). L-DC survived at least as well in Hb102 as in RPMI.

Culture supplements

Concanavalin A (Con A)-stimulated spleen cell supernatant (CAS) was used. Rat spleen cells were cultured in RPMI-1640 or Hb102 for 48 hr in the presence of 1 *µg* Con A. Recombinant human interleukin-1 (IL-1) was a kind gift from Dr P. T. Lomedico, Hoffman-La Roche (Geneva, Switzerland). Recombinant human IL-2 was a kind gift from Dr R. Grosclaude, Biogen, Geneva, Switzerland. Recombinant IL-3 was a kind gift from Dr V. Kindler, University of Geneva, Switzerland. Recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF) was a kind gift from Biogen. Rabbit antiserum to GM-CSF was a kind gift from Dr D. Y. Mochizuki, Immunex, Seattle (Mochizuki *et al.*, 1986).

Table 1. Survival of L-DC in culture

| Separation | Supplement | Surviving L-DC (% of input) (1 SD) | |
		24 hr	48 hr
(a)			
Metrizamide	Nil	38·4 (10·7)	22·4 (16·1)
Metrizamide	10% CAS	65·1 (7·8)	50·2 (10·5)
(b)			
Metrizamide	Nil	29·9	ND
Metrizamide	10% CAS	55·9	ND
Rosetting	Nil	12·5	ND
Rosetting	10% CAS	54·0	ND

(a) L-DC enriched from XTDL by centrifugation over 15% Metrizamide were cultured in Hb102 or RPMI-1640+5% rat serum with or without the addition of 10% CAS. At intervals viable L-DC were counted. The table shows the average survival from seven experiments at 20–24 hr and four experiments where the cultures were continued for 44–48 hr.

(b) L-DC were enriched either by centrifugation or by rosetting with SRBC coated with a cocktail of monoclonal antibodies, W3/25 (anti-CD4), OX8 (anti-CD8), OX12 (anti-Ig light chain) and OX52 (anti-T cells), prior to culture with 10% CAS.

Indirect binding assays

Cytospin preparations of L-DC and other cell types were fixed in cold ethanol, and incubated with mAb under saturating conditions. After washing they were incubated at 4° for 60 min with affinity-purified rabbit anti-mouse Ig (a kind gift of Dr A. F. Williams), iodinated by the Chloramine-T method, and prepared for autoradiography.

To assess labelling quantitatively, grains overlying an arbitrary area of a cell, including most of the cell body, defined by an eyepiece graticule, were counted. Background counts were estimated by counting an adjacent area of blank slide.

Mixed leucocyte reaction (MLR)

Cells were cultured in 96-well plates in RPMI-1640 supplemented with 10^{-5}M mercaptoethanol and 5% FCS or rat serum. Responder cells were DA (RT1ᵃ) TDL or lymphnode cells and stimulators were Metrizamide-separated PVG (RT1ᶜ) XTDL irradiated with 10 Gy. After 72 hr, cultures were pulsed with 0·5 *µ*Ci tritiated thymidine ([³H]TdR) (Amersham, Bucks) and harvested 16 hr later using an Ilacon (Tonbridge, Kent) harvester.

Monoclonal antibodies

The specificities of the antibodies used are referred to in the text. Full details of all mouse anti-rat monoclonals used are given in Jefferies (1988).

RESULTS

Survival of L-DC in culture is regulated by GM-CSF

Fresh XTDL or Metrizamide-separated L-DC were cultured for 5–72 hr and viable L-DC counted. Overnight recovery was usually less than 70% for whole XTDL and 40% for separated

Table 2. Effects of defined mediators on LC-DC survival

Addition	Surviving L-DC (% of input)			
	Exp. (a)	(b)	(c)	(d)
Nil	34·4	25·0	27·1	38·6
CAS 10%	ND	73·7	53·1	63·6
rIL-1 10 U/ml	ND	ND	ND	27·0
rIL-2 100 U/ml	ND	18·5	ND	ND
WEHI Sup. 10%	ND	ND	ND	27·3
rGM-CSF 100 U/ml	66·4	ND	54·8	ND
rGM-CSF 100 U/ml + antibody	27·2	ND	ND	ND
rGM-CSF 10 U/ml	60·1	ND	ND	ND
rGM-CSF 10 U/ml + antibody	26·0	ND	ND	ND
r IFN-γ 50 U/ml	ND	ND	ND	29·5

L-DC, separated over Metrizamide, were cultured in Hb102 supplemented with recombinant molecules or supernatants as shown. 20–24 hr later viable L-DC were counted. Because of variation between experiments the results shown represent individual experiments but similar results have been obtained for each supplement on all of at least four occasions.

Table 3. RNA synthesis by fresh and cultured L-DC

Cell type	Fresh L-DC	Cultured L-DC	Cultured L-DC + 10% CAS
Grain count (average ± 1 SD)	18·50 ± 13·6	21·2 ± 11·5	38·4 ± 15·8

L-DC were enriched from XTDL by centrifugation over Metrizamide and cultured for 20 hr in Hb102 with or without 10% CAS. Fresh and cultured cells were incubated for 60 min with 10 μCi [³H]UdR, washed and cytospin preparations autoradiographed. Grains were counted over 50 cells from each sample.

L-DC. The addition of normal TDL or cells from the pellet of separated XTDL did not improve recovery of separated L-DC after culture, suggesting that decreased survival was due to damage in the separation procedure.

Supernatants from Con A-stimulated spleen cell cultures (CAS) consistently improved L-DC survival (Table 1). Differences in survival were apparent by 5 hr in culture, and lasted at least 72 hr.

The effect of CAS on L-DC survival could have been direct or via an effect on a contaminating cell population. To investigate this, XTDL were depleted of T and B lymphocytes by rosetting or with magnetic microspheres. Cytospin preparations stained with OX12 (anti-Ig light chain) or OX19 (anti-T cell, CD5) showed less than 5% contaminating lymphocytes after separation. Table 1 shows that depletion of these cell populations did not alter the effect of CAS on L-DC survival, and thus it is unlikely that CAS was acting on L-DC indirectly. No cells capable of phagocytozing opsonized SRBC were detected after separation suggesting efficient depletion of macrophages also.

To identify the active factor in CAS, recombinant cytokines were assayed for their ability to promote L-DC survival

Table 4. Expression of Thy-1 by fresh and cultured L-DC; immunocytochemistry

	L-DC alone	L-DC + allogeneic lymphocytes
Thy-1 + L-DC input	0·78 × 10⁶ (45·6%)	0·65 × 10⁶ (35·7%)
Thy-1 + L-DC recovered	1·11 × 10⁶ (86·0%)	1·21 × 10⁶ (93·8%)
Increase in Thy-1 + L-DC	43·2%	87·6%

L-DC were enriched to 78% by centrifugation over Metrizamide and cultured alone or with a 10-fold excess of allogeneic lymphocytes in Teflon beakers in RPMI-1640 + 5% rat serum. After 48 hr they were harvested, viable L-DC counted and cytospin preparations stained for Thy-1 using MRC OX7. Preparations were coded prior to counting. The figures in parentheses give the percentages of total L-DC that were Thy-1 positive.

(Table 2). Murine IL-1 sometimes had a minor effect when added to Metrizamide-separated XTDL, but this was abolished if Fc receptor-bearing cells were removed by rosetting (data not shown), suggesting an indirect effect via contaminating macrophages. Human IL-2, murine IL-3 or supernatant from WEHI cell cultures had no effect. Rat interferon-gamma at 50 U/ml was also without effect.

In several experiments, murine rGM-CSF, at 50 or 100 U/ml, was as effective as CAS in promoting L-DC survival. In three experiments, up to 95% of the effects of GM-CSF were blocked by a specific anti-GM-CSF antiserum, showing that GM-CSF was a major factor promoting the survival of L-DC *in vitro*.

Increased L-DC survival is accompanied by increased RNA synthesis

L-DC cultured in medium supplemented with CAS appeared larger than fresh cells and many possessed conspicuous nucleoli. Fresh L-DC or L-DC cultured for 16 hr with or without 10% CAS as supplement were incubated with tritiated uridine ([³H]UdR) and cytospin preparations autoradiographed. Grain counts over L-DC (Table 3) showed that the incorporation of uridine into RNA was similar in fresh L-DC and L-DC cultured without CAS, but that CAS stimulated a marked increase, signifying a stimulation of RNA and presumably protein synthesis.

Cultured L-DC modulate expression of other surface markers

Approximately 50% of fresh L-DC was stained by the mAb MRC OX7, specific for rat Thy-1.1. After overnight culture more than 95% of L-DC were positive, and the intensity of staining was increased. In contrast, all L-DC remained Ia positive with no change in staining intensity.

The increased proportion of Thy-1-positive cells was not due to selective loss of negative cells. Table 4 shows that there is an absolute increase in the numbers of positive cells of approximately 80% after 16 hr culture.

Cytospin preparations of fresh and cultured L-DC, and of fresh thymocytes, were incubated with MRC OX7 and subsequently with ¹²⁵I-rabbit anti-mouse Ig under saturating conditions. Grain counts after autoradiography showed an increase in the proportion of positive cells and an increase in the average

Table 5. Expression of Thy-1 by fresh and cultured L-DC; indirect binding assay

Cell type	Fresh L-DC	Cultured L-DC	Thymocytes
Average grain count (range)	6·20 (0–54)	59·7 (7–176)	10·52 (1–21)
Average site number per cell	$0·59 \times 10^6$	$5·62 \times 10^6$	1×10^6
Maximum site number per cell	5×10^6	18×10^6	2×10^6

L-DC (78% pure) were prepared from XTDL by centrifugation over Metrizamide and cultured in RPMI + 5% rat serum for 48 hr in Teflon beakers. Cytospin preparations of L-DC before and after culture and of fresh thymocytes were labelled under saturating conditions with MRC OX7 or MRC OX21 and subsequently with ^{125}I-labelled rabbit anti-mouse Ig. Autoradiographs were prepared and grains counted over 50 cells of each type using an eye-piece graticule.
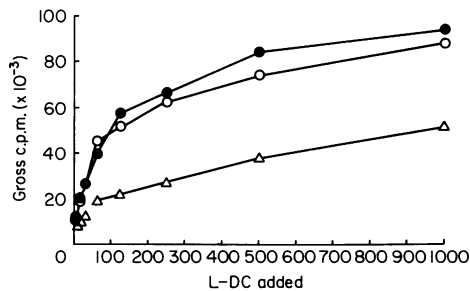

Figure 1. L-DC were enriched by centrifugation over metrizamide and either used immediately (fresh; △) or cultured for 16 hr in Hb102 with (C+CAS; ○) or without (C-CAS; ●) 10% CAS supplement. Graded numbers of viable L-DC were added to 5×10^5 responder cells and the cultures harvested 4 days later. The results are expressed as gross c.p.m. Each point represents the mean of quadruplicate cultures.

amount of antigen expressed per L-DC (Table 5). Comparison of grain counts overlying thymocytes (known to express $\sim 10^6$ Thy-1 sites per cell; Mason & Williams, 1980) labelled with MRC OX7 under identical conditions, showed that cultured L-DC expressed an average of $5·62 \times 10^6$ sites per cell (about a five-fold increase) with a maximum site number of 18×10^6.

In contrast, the expression of Ia did not change for at least 48 hr in culture and nor did culture of L-DC in the absence of CAS lead to a decrease in Ia expression (data not shown).

MRC OX42 recognizes the iC′3b (CR3) receptor on L-DC (Robinson, White & Mason, 1986). Approximately 40% of fresh L-DC was positive for OX42 but after culture for 20–48 hr the frequency of positive cells fell to 5–10% and the intensity of staining also decreased.

MRC OX48 recognizes a 95,000 MW molecule of unknown function present on activated lymphocytes (Paterson et al., 1987). 5–10% of fresh L-DC expressed this marker but after 20 hr culture over 95% were positive. The kinetics of expression of OX48 are similar to those for the IL-2 receptor (see accompanying paper).

Cultured L-DC show decreased non-specific esterase and cytoplasmic DNA inclusions

60–70% of fresh L-DC were positive for non-specific esterase (NSE) and 10–20% for Feulgen-positive (DNA) cytoplasmic inclusions. After 16 hr culture only 10–20% were NSE-positive and no cytoplasmic DNA inclusions were seen. Strongly NSE-positive debris, not present in fresh preparations, was present, suggesting that the loss of strongly positive cells might be due to selective cell death. The loss of cytoplasmic DNA could be due to intracellular digestion.

Culture of L-DC increases MLR stimulatory potency

Cultured Langerhans' cells are much more potent stimulators of a MLR than fresh cells (Schuler & Steinman, 1985). Metrizamide-separated L-DC were cultured for 16 hr with or without 10% CAS and tested as stimulators in a MLR. In four experiments (a representative one is shown in Fig. 1) L-DC cultured for 16–24 hr were considerably more potent than fresh cells as stimulators. This increase in stimulatory capacity was not dependent on the presence of CAS.

DISCUSSION

L-DC represent an intermediate stage in the DC lineage, migrating from peripheral tissues to lymph nodes where at least some become interdigitating cells in the paracortex (Fossum, 1988). L-DC are a phenotypically heterogeneous population (MacPherson & Pugh, 1984) and the primary purpose of this investigation was to investigate the significance of this heterogeneity.

Attempts to culture L-DC *in vitro* were hampered by the low recoveries of viable L-DC after periods as short as 12–16 hr. That the low recoveries were due to L-DC death is suggested because the total number of dead and live cells recovered after short periods of culture was very similar to the total input into culture.

Mechanical trauma incurred during separation procedures may contribute to the poor survival of L-DC in culture but could not be the sole explanation. Firstly, even with minimal handling, less than 50% of L-DC survived overnight in standard media. Secondly the addition of CAS had a striking effect in increasing survival although, at best, less than 75% of L-DC survived short periods in culture. The effect of CAS could be reproduced by the addition of rGM-CSF but by no other purified factor we assayed, and this effect was efficiently blocked by an antiserum to GM-CSF.

It is not clear whether the relatively short survival of most L-DC in culture is artefactual or reflects a naturally short life span in lymph nodes. L-DC are rapidly turning over *in vivo* (Pugh et al., 1983) suggesting a short intra-nodal life span. If L-DC are important in the transport of antigen to lymph nodes, a rapid turnover of L-DC would facilitate the interaction of these cells and lymphocytes.

The major known sources of GM-CSF are activated T lymphocytes (Kelso & Metcalf, 1985) and stimulated macrophages (Thorens, Mermod & Vassali, 1987). In a stimulated lymph node it is very likely that GM-CSF is being actively secreted, and the action of GM-CSF in increasing L-DC survival could have a physiological role in facilitating T-cell activation by increasing the presentation of antigens borne by L-DC.

GM-CSF may have a physiological function in immune responses (Morrissey *et al.*, 1987), in that the size of an antibody response *in vivo* or *in vitro* could be increased by GM-CSF, which appeared to act both by increasing Ia expression on accessory cells and by stimulating IL-1 secretion by these cells. Our results suggest that Ia expression by L-DC is not affected by GM-CSF and Koide, Inaba & Steinman (1987) have shown that murine splenic DC are not able to secrete IL-1 but that their efficiency as accessory cells is stimulated by IL-1. As the splenic accessory cells used by Morrissey *et al.* (1987) would contain both macrophages and DC it is possible that both cell types are being affected by GM-CSF.

GM-CSF affects several other mature cell types, having chemokinetic and stimulatory effects on neutrophils (Weisbart *et al.*, 1985; Lopez *et al.*, 1983) and enhancing the antibody-dependent cytotoxicity of monocytes (Grabstein *et al.*, 1986). That GM-CSF is acting directly on L-DC is suggested by its promotion of L-DC survival after T-cell and macrophage depletion, by the rapidity of stimulation of IL-2 receptor expression (Macpherson, Fossum & Harrison, 1989) and by the lack of effect of other lymphokines and cytokines thus far tested. The survival of murine Langerhans' cells is similarly increased by GM-CSF (Witmer-Pack *et al.*, 1987; Heufler, Koch & Schuler, 1988).

Fresh Langerhans' cells are weak stimulators of the MLR but their potency increases by up to 10 times in culture (Schuler & Steinman, 1985). Fresh L-DC are potent stimulators (Mason, Pugh & Webb, 1981) but our results show that their potency is increased significantly in culture. In contrast to Langerhans' cells this increase appears independent of exogenous GM-CSF. This could reflect a difference in maturity, with only functionally mature L-DC being released into lymph, or could relate to the origin of some L-DC from Peyer's patches (Pugh *et al.*, 1983) where they may have been influenced by local cytokines.

The expression of several other markers is modulated on cultured L-DC. The expression of Thy-1 and OX48 are rapidly up-regulated but the function of these molecules is not known. C′R3 expression diminished in culture, as is also the case with murine Langerhans' cells (Schuler & Steinman, 1985).

It is not clear to what extent the effects on survival and phenotype seen in cultured L-DC reflect *in vivo* events, but our observations suggest that the L-DC is not an end-stage cell but that, as with Langerhans' cells, its properties may be modulated by cytokines in ways which may alter its biological functions as an antigen-presenting cell *in vivo*.

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