Brief Definitive Report

GRANULOCYTE/MACROPHAGE COLONY-STIMULATING FACTOR AND INTERLEUKIN 1 MEDIATE THE MATURATION OF MURINE EPIDERMAL LANGERHANS CELLS INTO POTENT IMMUNOSTIMULATORY DENDRITIC CELLS

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Dendritic cells (DC) are leukocytes specialized to initiate primary T cell–dependent, immune responses (1). DC in lymphoid organs and lymph derive from the bone marrow (2). Important developmental events might also occur in other sites. Evidence comes from studies of murine epidermal Langerhans cells (LC) (3, 4). These Ia^+ cells are weak accessory cells for the sensitization phase of several responses, but progressively increase stimulatory capacity during epidermal cell (EC) culture. The function, morphology, and surface markers of cultured LC come to resemble lymphoid DC. By studying LC that have been enriched by a negative selection approach, we have defined a major role for granulocyte/macrophage colony-stimulating factor (GM-CSF) for the immunologic maturation of LC in culture. This maturation seems to be independent of the capacity of GM-CSF to maintain LC viability and high levels of Ia.

Materials and Methods

Animals. BALB/c (H-2^d) and C3H/He (H-2^k) mice (6–12 wk old of both sexes) were from the Versuchstierzucht, Himberg, Austria.

Culture Medium. We used RPMI 1640/10% FCS (56°C, 0.5 h; Seromed, Biochrom KG, Berlin, FRG), 1 mM L-glutamine, 5×10^{-5} M 2-ME, and 50 μg/ml gentamicin sulfate.

Identification of LC. We used mAbs to I-A or I-E antigens to stain cytospins or cell suspensions; the latter was monitored by flow cytometry (FACStar; Becton Dickinson & Co., Oxnard, CA). The mAbs were anti-I-$E^{k,d}$ (clone 14-4-4S, mouse IgG2a, HB32; from American Type Culture Collection [ATCC], Rockville, MD) followed by FITC-F$(ab')_2$ goat anti–mouse Ig (Grub; Scandic, Vienna, Austria), or FITC–anti-I-$A^{b,d}$ (clone B21-2, rat IgG2b, TIB229; ATTC). Controls were FITC–anti-mouse IgM or FITC–anti-Thy-1 (both rat IgG2b mAb; Becton Dickinson & Co.).

LC Enrichment after 72-h EC Culture (72-h LC). BALB/c ear EC suspensions (3, 4) were cultured for 24 h. Nonadherent cells were removed, cultured for 48 h, and floated on dense albumin columns. The floating fraction contained all the LCs at 40–60% purity (3).

LC Enrichment after 12-h EC Culture (12-h LC). BALB/c EC (viability >85%) were treated with anti–Thy-1 mAb (TIB 99; ATTC) and low-tox-M rabbit complement (Cedarlane Labs, Hornby, Ontario) at 3×10^6/ml. This removed dendritic Thy-1^+ ECs and most keratinocytes, which expressed low amounts of Thy-1 (5). The suspension (viability 15–20%) was washed twice with cold PBS without Ca^{++} and Mg^{++}, treated at

This work was supported by a grant from the Austrian Fonds zur Foerderung der Wissenschaftlichen Forschung (project No. P6170M) and the Austrian Ministry of Science.

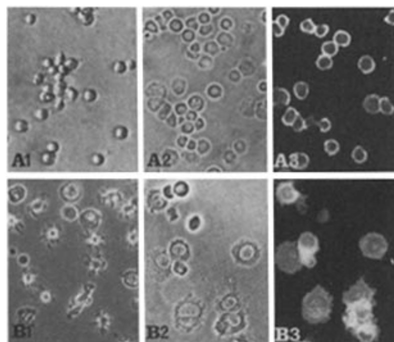

FIGURE 1. Cytology and expression of Ia by 12-h LC before (*A*) and after (*B*) 60-h culture in rGM-CSF. The LC were examined by phase contrast (*A1*, *B1*; × 300), by phase contrast after cytospinning (*A2*, *B2*), and by anti-Ia immunofluorescence (reference 3) (*A3*, *B3*; × 400).

10^6 cells/ml for 10 min at 37°C with 0.1% trypsin and 30 μg/ml DNase I (Sigma Chemical Co., St. Louis, MO), and washed again. This resulted in a viable (>90%) suspension with ~10% LC. 5–10 × 10^6 cells were plated in 100-mm petri dishes (No. 3003; Falcon Labware) in 15 ml medium. At 12 h, nonadherent cells were removed and floated on dense albumin. The low-density fractions were >95% viable and 40–55% LC. Typically, we started with 200–250 × 10^6 EC from 150 ears and obtained 1–1.6 × 10^6 12-h LCs.

Culture of 12-h LC. 10^5 12-h LC were plated in 16-mm wells (3424; Costar Europe, Badhoevedrop, The Netherlands) in 1 ml medium ± 30% vol/vol keratinocyte-conditioned medium or purified cytokines. After 1–6 d the cells were removed, wells were rinsed twice with 1 ml medium, and cells were washed twice by centrifugation in 3 ml medium. We determined viable yields and the percentage of LC and tested accessory function.

Keratinocyte-conditioned Medium (KCM). Adherent keratinocytes, remaining from the preparation of 72-h LCs (above), were cultured for 2 d. The medium was centrifuged, filtered (0.2 μm), aliquoted, and stored frozen at −80°C.

Cytokines. Murine rIL-1α was provided by Dr. P. LoMedico, Hoffmann-La Roche, Inc., Nutley, NJ (6), and used at 50 U/ml (7). Purified natural IL-3 (Genzyme, Boston, MA) was used at 50–200 CFU/ml. rGM-CSF, >4 × 10^7 CFU/mg (8), was supplied by Dr. S. Gillis, Immunex Corp., Seattle, WA. It had maximal activity at 0.5 ng/ml and was used at 2 ng/ml. Dr. Gillis also provided rabbit antiserum to murine GM-CSF (8) used at 1% vol/vol.

T Cell Proliferative Assays. To test the stimulatory capacity of LC for unprimed T cells, we used the primary MLR and the polyclonal response of periodate-modified T cells ("oxidative mitogenesis") (3).

Results

The Survival of 12-h LCs Depends on GM-CSF. 12-h LC were medium-sized, Ia-rich cells (Fig. 1). Upon further culture, the number of viable LC dropped to ~10% after 2.5 d unless KCM (30% vol/vol) or rGM-CSF (2 ng/ml) was added (Table I and Fig. 2). With these additives, viability dropped more slowly, comparable to prior observations in bulk EC cultures (3). The effect of KCM on viability was blocked by 1% vol/vol anti-GM-CSF (8), but not by 1% pooled control serum, and was overcome by excess GM-CSF (Table I). rIL-1 and IL-3 did not maintain viability (Table I). Cultured LC did not proliferate, since yields did not change if the LC were exposed to 900 rad ^{60}Co before culture (not shown) as was observed in bulk EC cultures (3). When 12-h LC were cultured for 60 h in the presence of KCM, rGM-CSF, or rGM-CSF plus rIL-1-α, the levels of Ia did not increase further but the LC enlarged and developed many cytoplasmic processes and veils (Figs. 1 and 3). This appearance was identical to LC that had matured in 72-h bulk cultures (3).

TABLE I
The Survival of 12-h LC Depends on GM-CSF

Additives to culture medium (concentration)	Recovery of viable LCs after 60 h in culture
None	9.0 ± 2.8 (14)
rGM-CSF (2 ng/ml)	59.0 ± 11.6 (14)
rIL-1-α (50 U/ml)	9.7 ± 4.5 (5)
IL-3 (50 CFU/ml)	7.1 ± 2.1 (5)
rGM-CSF + rIL-1	66.6 ± 14.1 (14)
rGM-CSF + IL-3	60.0 ± 11.3 (5)
KCM (30% vol/vol)	60.2 ± 13.0 (8)
KCM + anti-GM-CSF (1% vol/vol)	10.0 ± 5.0 (3)
KCM + anti-GM-CSF + rGM-CSF (100 ng/ml)	52.0 ± 11.3 (3)
rGM-CSF (100 ng/ml)	63.0 ± 14.7 (3)

10^5 enriched 12-h LC were cultured in 1 ml medium ± additives. After 60 h, viable LC yields were determined and expressed relative to the number of LC at the start (100%). Data are means ± SD with the number of experiments in parentheses.

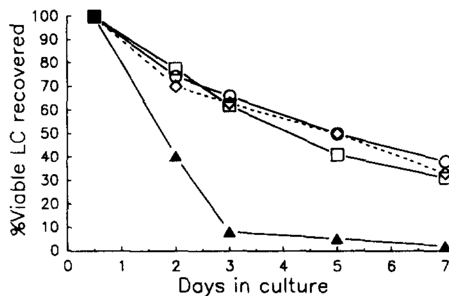

FIGURE 2. Survival of 12-h LC during culture (see Table I) in medium alone (▲), KCM (◇), rGM-CSF (□), rGM-CSF + rIL-1 (○). Data are means of four experiments.

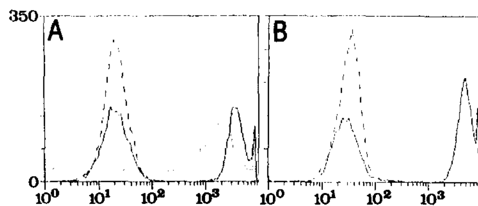

FIGURE 3. Cell surface expression of Ia antigens on DC. The cells were stained with a saturating dose of FITC-mAb to I-$A^{b,d}$ (clone B21-2) or with an isotype-matched FITC anti-mouse IgM control (*A, B*; *dashed line*). The cells were enriched 12-h LC (*A*; *solid line*; 45% Ia^+), spleen DC (*A*; *dotted line*), and 12-h LC that had been cultured for 60 h in 2 ng/ml rGM-CSF and 50 U/ml rIL-1 (*B*; *solid line*; 54% Ia^+). Similar results were obtained with an mAb to I-E (clone 14-4-4S; not shown).

GM-CSF and IL-1 Mediate Increased Stimulatory Activity of Cultured LC. Since GM-CSF was the only purified factor that supported the viability of 12-h LC, we studied its impact on accessory function and tested combinations of GM-CSF with other cytokines. In both the oxidative mitogenesis assay and the MLR, enriched 12-h LC showed some stimulatory activity. At limiting doses, the activity was <10% of that observed with 3-d cultured LC (Table II). This confirms prior work where 12-h LC were inactive when enriched to >95% purity but had some activity if only partially enriched (4). After 60 h with rGM-CSF or KCM, LC increased at least sixfold in activity, but this was still weaker than LC enriched from companion bulk EC cultures (Table II, Fig. 4). Addition of anti-GM-CSF

TABLE II

The Maturation of Epidermal LC In Vitro Is Mediated by the Combined Action of GM-CSF Plus IL-1

Stimulator cells	Cytokines added to assays	Irradiation (900 rad)	[^{3}H]Thymidine uptake with stimulator doses of: Oxidative mitogenesis				Allogeneic mixed leukocyte reaction			
			1×10^4	3×10^3	1×10^3	3×10^2	1×10^4	3×10^3	1×10^3	3×10^2
			cpm $\times 10^{-3}$							
72-h LC (control)	0	+	235	171	102	36	341	351	160	76
	GM-CSF	+	238	175	105	32	297	232	179	79
	GM-CSF + IL-1	+	232	165	112	44	278	198	166	83
	KCM	+		ND			248	180	127	61
12-h LC	0	+	57	23	7	2	44	25	14	3
		−	57	23	9	2	33	17	11	4
	GM-CSF	+		ND			55	48	28	9
		−					57	32	20	9
	GM-CSF + IL-1	+		ND			131	90	63	21
		−					84	62	45	22
	KCM	+		ND			60	29	20	11
		−					43	27	22	10
12-h LC cultured 60 h in:										
KCM	0	+		ND			140	75	30	11
GM-CSF	0	+	198	134	63	24	146	99	34	13
GM-CSF + IL-1	0	+	243	190	102	47	233	149	70	28
72-h LC (companion)	0	+	268	215	116	47	281	156	81	38

12-h LC were tested for accessory function right away and again after 60 h of culture in medium supplemented with 30% vol/vol KCM, 2 ng/ml rGM-CSF, or rGM-CSF plus 50 U/ml rIL-1. Stimulatory activity was compared to 72-h LC isolated from control bulk EC cultures at the start of each bioassay. Where indicated, cytokines were added to the mitogenesis assays. Syngeneic BALB/c T cells were used for oxidative mitogenesis and allogeneic C3H for the MLR. Uptake of the former T cells was <2,000 cpm even when cytokines were added, while the MLR background of 600 cpm increased to 1,300 cpm with KCM and 5,500 cpm with GM-CSF ± IL-1.

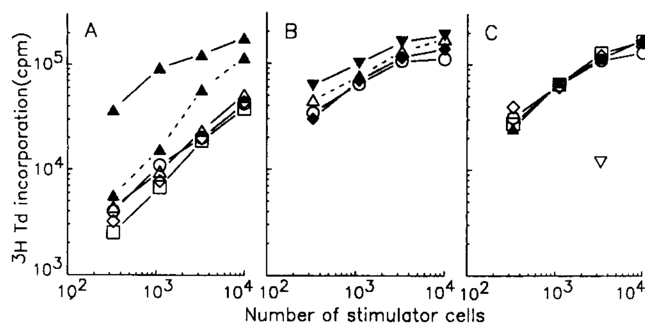

FIGURE 4. GM-CSF and rabbit anti-GM-CSF influence LC maturation but not accessory function during the oxidative mitogenesis assay. LC were enriched from 12-h EC. Part was tested for accessory function right away (*A*). Splenic DC, part of which had been pulsed with 50 U/ml rIL-1 overnight to enhance their function (7), were included for comparison (*A*). The other aliquot of 12-h LC was cultured in medium with the indicated supplements for 60 h, washed, and tested for accessory function (*B* and *C*). The stimulators were tested ±900 rad of irradiation (^{60}Co) with comparable results (not shown). T cells alone, ± supplements, took up <2,000 cpm. (*A*) Spleen DC (▲----▲), spleen DC pulsed with IL-1-α (▲—▲), 12-h LC (□—□), 12-h LC plus rGM-CSF added to the oxidative mitogenesis assay (○—○), 12-h LC plus 1% rabbit anti-GM-CSF added to the assay (△—△), 12-h LC plus 1% control rabbit serum in the assay (◇—◇). (*B*) 12-h LC cultured in rGM-CSF (○—○); rGM-CSF + rIL-1 (▼—▼); rGM-CSF + IL-3 (◆—◆); 12-h LC cultured in rGM-CSF, then 1% anti-GM-CSF added to the mitogenesis assay (△—△). (*C*) 12-h LC cultured in 30% KCM (□), KCM + anti-GM-CSF (▽), KCM + anti-GM-CSF + rGM-CSF at 100 ng/ml (▲), KCM + control serum (◇), rGM-CSF (○).

blocked the effect of KCM, and the few remaining cells had the activity of 12-h LC (Fig. 4*C*). 100 ng/ml rGM-CSF reversed the block of anti-GM-CSF.

When 12-h LC were cultured in combinations of rGM-CSF and other factors, only rIL-1-α led to a further twofold enhancement of function (Table II, Fig. 4). The LC thereby acquired the same activity as LC that had matured in companion bulk EC cultures (Table II). Anti-GM-CSF did not block stimulatory activity after

the LC had matured (Fig. 4, *A* and *B*). Addition of rGM-CSF to the proliferative assays only led to a slight increase in oxidative mitogenesis and a larger increase in the MLR when 12-h but not 72-h LC were tested (Table II, Fig. 4). We conclude that the combination of GM-CSF and IL-1 mediates optimal maturation of LC in culture.

Discussion

Our findings that keratinocyte-derived cytokines (9–11) mediate the viability and function of LC are comparable to independent studies of Witmer-Pack et al. (12). There are four important differences in methodology and results. (*a*) Witmer-Pack enriched LC from fresh epidermal suspensions by panning with an antileukocyte mAb. This provided LC in higher purity (80%). We used negative selection from 12-h EC cultures to reduce any possibility that trypsinization and positive selection altered LC function. (*b*) By using oxidative mitogenesis as one assay of accessory function, we could show that the effect of GM-CSF was not simply due to improved viability. This polyclonal response was measured at 40 h, and GM-CSF was essential for LC viability during this interval. Yet GM-CSF did not significantly enhance the function of 12-h LC during the mitogenesis response (Fig. 4). Instead, LCs had first to mature in cytokine for 60 h before function increased markedly (Table II, Fig. 4). (*c*) Ia levels on LC increased markedly during the first day of culture (3, 13). By starting with 12-h LC, we noted a marked increase in accessory function with no change in surface Ia (Fig. 3). Therefore, our findings indicate that GM-CSF exerts a maturation effect on function, which is distinct from the maintenance of LC in a viable, Ia-rich state. (*d*) Finally, we tested combinations of GM-CSF and other cytokines and noted that IL-1-α led to a further increase in activity. Of interest is the finding that IL-1 enhances the function of spleen DC about threefold (7).

The induction of stimulatory function within the dendritic cell lineage presumably represents a control point in the afferent limb of immunity. GM-CSF and IL-1 are cytokines that are produced by many cell types upon a variety of stimuli, and seem suitable for fulfilling this control.

Summary

Freshly isolated, murine epidermal Langerhans cells (LC) are weak accessory cells for primary T cell–dependent immune responses, but increase their stimulatory capacity at least 20-fold progressively over a 3-d culture with keratinocytes. We have studied the mediators of LC maturation. LC enriched from 12-h epidermal cultures by negative selection do not survive when cultured for 60 h in standard medium. LC survive and show increased stimulatory capacity for oxidative mitogenesis and the primary MLR when 30% keratinocyte-conditioned medium is included. Of the three cytokines that are known to be produced by keratinocytes, only granulocyte/macrophage CSF (GM-CSF) maintains viability and increases stimulatory capacity. IL-1 alone does not keep LC alive, but further enhances the stimulatory activity when combined with GM-CSF. IL-3 has no effect. The increase in LC stimulatory capacity is not due to increased Ia antigen expression, which does not change between 12 and 60 h. Function is not simply due to improved viability, as GM-CSF does not enhance the function of 12-h LC

when added to the short-term oxidative mitogenesis assay. By generating LC with strong stimulating activity for resting T cells, GM-CSF and IL-1 may be critical in the sensitization phase of T cell–mediated immunity.

We thank Drs. S. Gillis and P. LoMedico for valuable reagents; Dr. H. Glassl for performing the flow cytometry; Dr. D. Geissler for evaluating cytokines in the marrow colony assay; and Drs. P. Fritsch, R. Steinman, and Z. Cohn for continued support.

Received for publication 21 October 1987 and in revised form 24 November 1987.

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