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*J Immunol* 1998; 161:4033-4041; ; http://www.jimmunol.org/content/161/8/4033

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Murine Langerhans Cells Cultured Under Serum-Free Conditions Mature into Potent Stimulators of Primary Immune Responses In Vitro and In Vivo

Alena Kočíková,* Andrea Kolesarić,* Frieder Koszik,† Georg Stingl,* and Adelheid Elbe-Bürger2*

The ability of Ag-pulsed dendritic cells (DC) to induce primary immune responses has led them to be used for vaccination purposes. However, irrelevant Ags (e.g., FCS) can also be taken up by DC during their isolation and culture and then presented in vivo. To circumvent this, we have established a serum-free (SF) culture system. Murine epidermal cell (EC) suspensions were prepared with and without FCS and cultured for 3 days either in SF or FCS-containing medium. In spite of the lower Langerhans cell (LC) yields under SF conditions, both SF- and FCS-cultured LC (SF-cLC, FCS-cLC) underwent a similar maturation process, as evidenced by a similar increase in the cell surface expression of MHC class II and of costimulatory molecules. The further observation that SF-EC cultures elaborated comparable amounts of granulocyte-macrophage (GM)-CSF as FCS-cultured EC, but were relatively impaired in their IL-1α and TNF-α production, supports the role of GM-CSF in LC maturation and, less so, in LC survival. Functionally, freshly isolated SF-LC compared with FCS-LC in their Ag-processing capacity. Three-day-cultured SF-LC were as potent stimulators of polyclonal T cell responses and of the primary allogeneic MLR as FCS-cLC, but were relatively poor activators of naive, syngeneic CD4+ T cells. In vivo, hapten-modified SF-cLC induced a contact hypersensitivity response similar in magnitude and kinetics to that evoked by FCS-cLC. Our data show that, in the absence of serum and exogenous cytokines, LC mature into potent activators of T cell responses and could thus be a valuable cellular source for DC-based immunotherapy. The Journal of Immunology, 1998, 161: 4033–4041.

Dendritic cells (DC) are bone marrow–derived APC that are present in small numbers in both lymphoid and nonlymphoid tissues. One of their key functions in the regulation of immune responses is their ability to prime naive T cells (1). Langerhans cells (LC) are specialized skin DC residing within the epidermis and are crucial for the induction of immune responses against Ags introduced or newly generated in the skin (2, 3). After in vitro and in vivo perturbation of their microenvironment, LC undergo a process of maturation, as evidenced by changes in their phenotype and function. They down-regulate CD32 and F4/80, up-regulate MHC class I, MHC class II, and CD44 Ags, and de novo express CD80 and CD86 costimulatory molecules. Functionally, they reduce their Ag-processing potency and increase their ability to stimulate naive T cells (4–6). This strong immunostimulatory capacity makes cultured LC (cLC) attractive candidates for vaccination purposes. In fact, evidence exists that LC are capable of inducing protective tumor (7–10) and microbial (11–13) immunity. However, while LC in culture process and present the relevant immunizing Ag, they also have the ability to take up other undefined Ags being present in the culture medium, such as those of FCS. In vivo, this may result in the presentation of irrelevant peptides, causing activation of nonspecific and/or potentially autoreactive T cells and, thus, may preclude the use of LC for immunotherapy.

In this study, we have made an attempt to establish culture conditions that would allow the generation of immunostimulatory LC in the absence of serum and, by doing so, to gain further information about the factors mediating and securing LC maturation and survival.

Materials and Methods

Animals

BALB/c (H-2d), C3H/HeN (C3H) (H-2b), and C57BL/6 (H-2b) inbred mice of both sexes (6–12 wk old) were obtained from Charles River Wiga, Sulzfeld, Germany and bred and maintained in the Vienna International Research Cooperation Center animal facilities, Vienna, Austria. All animal procedures were approved by the Austrian Ministry of Science, Transporta-

Culture media (CM)

RPMI 1640 supplemented with 25 mM HEPES, 50 mg/ml gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 mM 2-ME, and 1× antibiotic-antimycotic solution (Life Technologies, Grand Island, NY), either serum-free (SF-CM) or containing 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria) (FCS-CM), was used. In some experiments, SF-CM was supplemented with 2% of the serum replacement product TCM (ICN Biomedicals, Eschwege, Germany) (TCM-CM), which is characterized by a low and defined protein content and the absence of growth factors, cytokines, and steroid hormones.
Epidermal cells (EC)  
Ears from BALB/c and C3H mice were washed in 70% ethanol, dried, split, placed dermal side down on a 1% tryptsin-PBS solution, and incubated for 35 (thin ear side) or 45 min (cartilage ear side) at 37°C. Epidermal sheets were peeled from the underlying dermis, floated in RPMI 1640 medium (with or without FCS) containing DNase (200 μg/ml DNase I; Sigma, St. Louis, MO) and mechanically agitated over a stainless steel mesh. Resulting cell suspensions were filtered through a cell strainer (70 μm; Falcon, Lincoln Park, NJ), washed three times, and then cultured (1.5 × 10⁵ cells/ml) in SF- or FCS-CM in 75-cm² flasks (Costar, Cambridge, MA) at 37°C. After 3 days of culture, nonadherent EC were harvested by vigorous pipetting, and dead cells were largely eliminated by density gradient centrifugation (Lymphocyte-M; Cedarlane Laboratories, Hornby, Ontario, Canada). Viability of both SF- and FCS-EC was >80% (n = 13) as determined by trypan blue exclusion. After phenotypic analysis of small aliquots, the two populations of cultured EC were adjusted to equal numbers of LC (SF-LC, FCS-LC) and used as stimulator cells of vitro and in vivo immune responses.

Cell lines  
The CTLL-2 cell line obtained from the American Type Culture Collection (ATCC, Manassas, VA; TIB 214) was maintained in FCS-CM supplemented with 1% culture supernatant of the IL-2-producing murine myeloma cell line X63Ag8–653, kindly provided by Dr. F. Melchers (Basel Institute of Immunology, Basel, Switzerland). The OVA-specific T cell hybridoma E8 was provided by Dr. N. Romani (Department of Dermatology, University of Innsbruck Medical School, Innsbruck, Austria).

Antibodies  
FITC-conjugated mAbs RM4-4 (anti-CD4), M1/69 (anti-CD24), 7D4 (anti-CD25), 3/23 (anti-CD40), IM7.8.1 (anti-CD44), 30F11.1 (anti-CD45), RA3-6B2 (anti-CD45R/B220), 3E2 (anti-CD54), 16-10A1 (anti-CD80), and GL1 (anti-CD86) were purchased from PharMingen (San Diego, CA). Tissue culture supernatant of the hybridoma NLDC-145 (anti-DEC-205) was obtained from Serotec (Oxford, U.K.). Hybridomas GK1.5 (anti-CD4, TIB 207), 53-6.72 (anti-CD8, TIB 105), J11d.2 (anti-CD24, TIB 183), IM7.8.1 (anti-CD44, TIB 235), M5/114.15.2 (anti-I-A¹-K¹-E¹-E², TIB 120), and RA3-3A1/6.1 (anti-CD45R/B220, TIB 146) were obtained from ATCC. Hybridoma anti-β2m (anti-CD25) and ZB6.2D8 (anti-CD44) were kindly provided by Dr. E. M. Shevach (National Institute of Allergy and Infectious Diseases, Bethesda, MD). FITC-conjugated mouse anti-β2m, goat anti-rabbit IgG, goat anti-mouse IgM, and rabbit anti-goat IgG were obtained from BioGenex (San Ramon, CA), phycocyanin-conjugated mouse anti-I-A¹-D¹ (0.25 μg/ml DNase I; Sigma, St. Louis, MO) and then incubated (10 7 cells/ml) with 2,4,5-trinitrobenzenesulfonic acid (TNCB, Sigma, St. Louis, MO) in PBS/1% FCS/0.1% NaN₃ and serially incubated with FITC-conjugated first-step Ab, washed again, and exposed to an appropriately diluted FITC-conjugated second-step reagent.

Flow cytometry analyses  
For two-color analyses, cells (3 × 10⁶/sample) were resuspended in cold PBS/1%FCS/0.1%NaN₃, and serially incubated with FITC-conjugated mAb directed against selected mouse Ags and phycoerythrin-conjugated anti-MHC class II mAb (30 min, 4°C). For control purposes, cells were incubated with isotype-matched mAbs. Stained cells were analyzed using a FACScan flow cytometer equipped with LYSYS II software (Becton Dickinson, Mountain View, CA). Dead cells were excluded by 7-aminoactinomycin D (Sigma) uptake.

For the analyses of cytolytic Ags, cells were fixed with paraformaldehyde (0.25% in PBS) for 10 min at room temperature, washed and permeabilized with saponin (0.3% saponin in PBS/1%FCS, Sigma) for 10 min at room temperature. After washing, cells were incubated with the non-conjugated first-step Ab, washed again, and exposed to an appropriately diluted FITC-conjugated second-step reagent.

T cell proliferation assays  
T cells were prepared from mesenteric lymph nodes using Ab- and C-mediated lysis as described previously (14). Briefly, cell suspensions were passed through nylon wool columns, and nonadherent cells were treated with a mixture of the following mAbs: 3C7, 7D4, J11d.2, M5/114.15.2, IM7.8.1, RA3-3A1/6.1 for 30 min at 4°C. Subsequently, cells were incubated with Low-tox-M rabbit C (Cedarlane Laboratories) for 45 min at 37°C. Of these cells, 97 to 99% were CD2⁺ as determined by flow cytometry. To purify CD4⁺ or CD8⁺ T cells, mAbs 3.168.81 or GK1.5 plus B6.2D8 were added to the Ab mixture. The purity of both CD4⁺ and CD8⁺ T cells was 97 to 98%. All proliferation assays were performed in the presence of FCS-CM in 96-well round-bottom microtiter plates. SF- and FCS-CM (10⁻¹ to 10⁻³/ml) were cultured with either purified unfractionated or OVA-specific, CD4⁺ T cells, or CD8⁺ T cells (10⁵/ml) and, for control purposes, alone. In some experiments, SF- and FCS-CM were cocultured with naive, syngeneic CD4⁺ or CD8⁺ T cells in the presence of either Con A (Pharmacia Biotech, Uppsala, Sweden; 2.5 μg/ml and 5 μg/ml for CD4⁺ and CD8⁺ T cells, respectively) or soluble anti-CD3ε mAb (clone 145-2C11, 0.1 μg/ml and 1 μg/ml for CD4⁺ and CD8⁺ T cells, respectively). Proliferation was assessed by [3H]Thymidine (Amersham, Arlington Heights, IL) incorporation (37 kBq/well) added during the final 12 h of culture. Data are expressed as mean cpm ± SD of triplicate cultures.

For secondary T cell proliferation assays, naive T cells (H-2b; 2 × 10⁵/well) were first stimulated with allogeneic FCS-CM (H-2b; 10⁵/well) in 24-well culture plates. Four days later, cells were harvested, washed and rested for 2 days in FCS-CM. Following density gradient (Lympholyte-M) centrifugation, dead cells were removed, and viable cells (10⁵/well) were restimulated with SF- or FCS-CM (H-2b; 10⁵/well). Proliferation was determined after 2, 3, and 4 days of culture.

Ag-processing assay  
The processing activity of SF- and FCS-CM (H-2b) was measured using the OVA-specific, MHC class II (I-Eb)–restricted T cell hybridoma E8 (15). The activation of hybridoma cells was determined by measuring IL-2 production in a bioassay using the IL-2-dependent CTL-L-2 cell line (15, 16). Briefly, freshly prepared SF- and FCS-CM (1.5 × 10⁵ cells/ml) were pulsed with OVA (0–2 mg/ml, Sigma) for 24 h at 37°C. Cells were then harvested and washed, and enriched for LC by density gradient centrifugation. The number of stimulator cells in both SF- and FCS-CM was adjusted to equal numbers of LC (SF-LC, FCS-LC). OVA-pulsed and, for control purposes, unpulsed SF- and FCS-CM (5 × 10⁵/well) were cultured either with E8 hybridoma cells (10⁵/well) or alone in 96-well flat-bottom microtiter plates. Supernatants were harvested after 24 h and added to the CTLL-2 cells (6 × 10⁵/well) at a final dilution of 1:3. After incubation for 36 h at 37°C, [3H]Thymidine incorporation was determined as described for T cell proliferation assays. In other experiments, SF- and FCS-CM (5 × 10⁵/well) were cocultured with E8 hybridoma cells (10⁵/well) in the presence of graded concentrations of OVA (0–1250 μg/ml). Supernatants were harvested after 24 h and the IL-2 content was assessed as described.

Assessment of cytokine production in EC cultures  
SF- and FCS-EC were prepared and cultured as described above. At 10, 24, 48, and 72 h, supernatants were harvested and stored at −20°C until use. Cytokine concentrations were determined by ELISA according to the manufacturer’s instructions for GM-CSF, IL-1α (Endogen, Cambridge, MA), and TNF-α (Genzyme, Cambridge, MA).

Hapten modification of cells  
SF-, TCM- and FCS-CM were washed twice in HBSS without Ca²⁺ and Mg²⁺ and then incubated with 2,4,5-trinitrobenzenesulfonic acid (TNBS, Sigma) at a concentration of 5 mM (pH 7.2) for 10 min at 37°C. Cells were then washed three times in a 10-fold volume excess of SF and 10% FCS-containing RPMI 1640 medium for SF-LC/TCM-LC and FCS-LC, respectively. Trypan blue exclusion revealed >70% viable cells (n = 5).

Sensitization and elicitation of contact hypersensitivity (CHS)  
BALB/c mice were anesthetized i.p. with tribromoethanol (2.5% in isotonic saline, 350 μl per mouse, Aldrich, Steinheim, Germany). As a positive control, mice were painted on dry shaved abdominal skin with 50 μl of 2% trinitrochlorobenzene (TNCB; TCI, Tokyo Kasei, Tokyo, Japan) in a 1:1 acetone and olive oil carrier solution (epicutaneous control group). Other groups of mice were injected with syngeneic, trinitrophenyl (TNP)-modified or unmodified SF-, TCM- and FCS-CM (graded numbers: 10⁻³ to 10⁻⁶ LC/mouse) in 100 μl HBSS with Ca²⁺ and Mg²⁺ s.c. into the tail base. Five days later, all groups of mice including a naive group (mice receiving ear challenge only) were challenged on the dorsal and ventral surfaces of their right ears with 20 μl of 0.5% TNCB. Ear thickness was measured before challenge and at 24, 48, and 72 h after challenge with an engineer’s micrometer (Hahn und Kolb, Stuttgart, Germany). The value measured before challenge was subtracted from the value assessed after challenge.
Experimental groups consisted of four mice each. Ordinary one-way ANOVA was used to compare experimental and control groups.

Results

LC undergo similar phenotypic changes in SF as in serum-containing EC cultures

Single cell suspensions, containing 2 to 3% MHC class II⁺ cells, were prepared from mouse ear epidermis and cultured for 3 days either in SF- or FCS-containing medium. In both cultures, cells with cytoplasmic extensions were already detectable at 24 h, and their dendritic morphology was more pronounced after 48 h of culture (Fig. 1). After 3 days, nonadherent EC were harvested from both cultures, and cell numbers and viabilities were assessed and compared. While the cellular recoveries from SF and FCS cultures were 48% and 71% (BALB/c) or 66% and 79% (C3H), respectively, the numbers of viable EC were essentially equal under both culture conditions in both mouse strains. Among viable EC recovered, the percentage of dendritically shaped cells in SF cultures was approximately half of that encountered in FCS cultures (Table I). Immunofluorescence staining revealed that these dendritically shaped cells, but not other EC, expressed high levels of MHC class II molecules and, thus, represent LC (data not shown). When we cultured the EC in RPMI 1640 supplemented with the serum replacement product TCM, recovery and viability of both LC and keratinocytes were not different from those observed in SF cultures, suggesting that TCM does not provide the survival factor(s) for LC or keratinocytes (data not shown).

In the next series of experiments, we investigated whether the isolation and culture of EC in the absence of FCS influences the phenotype of LC. Flow cytometry analyses revealed high expression of CD24, modest levels of CD45 and MHC class II, but no CD80 or CD86 Ags on LC freshly isolated in the presence (FCS-LC) or in the absence (SF-LC) of FCS (Fig. 2A). After 24 h of culture, both SF- and FCS-LC up-regulated MHC class II Ags and expressed moderate levels of CD40, CD80, and CD86 Ags (data not shown). After 3 days of culture, EC were enriched for LC by density gradient centrifugation, resulting in 20 to 38% (SF conditions) and 23 to 50% (FCS conditions) of cells exhibiting the morphologic features of LC (n = 13). By flow cytometry, both SF- and FCS-LC exhibited a similar increase in the cell surface expression of MHC class II, CD25, and of the costimulatory molecules CD40, CD54, CD80, CD86, as well as a similar decrease in the expression of CD24 and CD45 molecules (Fig. 2B). Identical phenotypic changes were observed in 3-day-cultured SF- and FCS-LC from BALB/c mice (data not shown). In contrast to FCS-LC, SF-LC expressed slightly less of DEC-205 and CD44 molecules in C3H mice (Fig. 2B) and considerably less of these molecules in BALB/c mice (data not shown). We conclude that serum-free EC culture conditions promote a phenotypic metamorphosis in the LC population, similar to that induced by a serum-containing milieu, but are somewhat impaired in their capacity to maintain LC viability.

LC cultured under SF conditions stimulate T cell proliferation in vitro

Using the allogeneic MLR as read-out system, we first investigated whether SF-cLC are capable of inducing a primary T cell response. Repeated experiments showed that, over the entire range of stimulator cell concentrations (10⁵–10⁶ LC/well), SF-LC isolated and cultured from two different mouse strains (C3H, BALB/c) induced a vigorous T cell response comparable to that evoked by FCS-cLC (Fig. 3, A and B). The primary T cell alloresponse induced by both SF- and FCS-cLC peaked on days 3 to 4 and markedly declined on day 5 of culture (data not shown). We further observed that SF-cLC are as potent stimulators of allogeneic T cells in a secondary MLR as are FCS-cLC, both inducing a peak of the response on days 2 to 3 (data not shown).

In contrast to the equal allostimulatory potency of SF- and FCS-cLC, the ability of SF-cLC to stimulate proliferation of naive, syngeneic CD4⁺ T cells was weaker than that of FCS-cLC (Fig. 3, C and D). The differences in the proliferative responses elicited by SF-cLC vs FCS-cLC were particularly pronounced at high stimulator cell numbers (Fig. 3D). The T cell proliferation peaked on days 4 to 5, for both SF- and FCS-cLC, and decreased on day 6 (Fig. 3C).

We next investigated whether SF-cLC can function as stimulators in polyclonal T cell responses. As can be seen in Figure 3, E and F, we found that both SF- and FCS-cLC are equally potent accessory cells in Con A- and anti-CD3-driven T cell activation. Not only the magnitude but also the kinetics of the proliferative response induced by SF-cLC compared with those induced by FCS-cLC, with a peak occurring on day 3 and on days 2 to 3 for CD4⁺ and CD8⁺ T cells, respectively. In conclusion, these data

<table>
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<th>Mice</th>
<th>Culture Conditions</th>
<th>Number of Isolated Cells/Ear</th>
<th>EC Numbers Before Culture</th>
<th>Cellular Recovery After 3 Days of Culture</th>
<th>Numbers of Viable EC After 3 Days of Culture</th>
<th>Numbers of Viable LC After 3 Days of Culture</th>
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<td>BALB</td>
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<td></td>
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<td>1.8–2.1 × 10⁶</td>
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<td>1.2–1.5 × 10⁶</td>
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<td>19.9 × 10⁶ (66%)</td>
<td>3.6 × 10⁶ (12.0%)</td>
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<tr>
<td></td>
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<td>1.5–1.9 × 10⁶</td>
<td>30 × 10⁶ (100%)</td>
<td>23.6 × 10⁶ (79%)</td>
<td>3.8 × 10⁶ (12.7%)</td>
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</table>

SF- and FCS-EC were prepared from BALB/c and C3H mice and cultured for 3 days. Thereafter, nonadherent EC were harvested, washed, and counted in a hemocytometer. Viability of EC was determined with trypan blue. LC were enumerated by their dendritic morphology and their numbers were calculated from the numbers of viable EC. Viability of both SF- and FCS-cLC was >98%. Results are representative of three experiments for each mouse strain.
FIGURE 2. Phenotypic comparison of LC isolated and cultured under SF and FCS conditions. A, Freshly prepared EC (C3H) were stained and analyzed by flow cytometry as described in Materials and Methods. B, After 3 days of culture, nonadherent EC were enriched for LC by density gradient, washed, stained, and analyzed. In all experiments, stained cells were gated for viable cells, and 7,000 events per sample were acquired. Dot blots represent results of two (A) or three (B) independent experiments.
FIGURE 3. Comparative assessment of the immunostimulatory potential of SF- and FCS-cLC in vitro. A and B, SF- and FCS-cLC (A, 10^3/well; B, graded numbers) from C3H (A) and BALB/c (B) mice were cultured with naive, allogeneic T cells (C57BL/6, 10^5/well) in 96-well round-bottom microtiter plates. Proliferative responses were determined on days 3 and 4 (A and B) of culture. Data are representative of three experiments. T cells as well as LC did not proliferate when cultured with medium alone (<400 cpm). C and D, SF- and FCS-cLC (C, 5 x 10^3/well; D, graded doses) from C3H mice were cultured with naive, syngeneic CD4^+ T cells (10^5/well) in 96-well round-bottom microtiter plates. In C, proliferative responses were determined on days 4 to 6 and in D, on day 5 of culture. CD4^+ T cells and cLC incubated with medium alone did not proliferate (<500 cpm). Results are representative of three experiments. E and F, SF- and FCS-cLC (10^3/well) from C3H mice were cocultured with naive, syngeneic CD4^+ or CD8^+ T cells (10^5/well) in the presence of either Con A (2.5 μg/ml and 5 μg/ml for CD4^+ and CD8^+ T cells, respectively) or soluble anti-CD3 mAb (clone 145-2C11, 0.1 μg/ml and 1 μg/ml for CD4^+ and CD8^+ T cells, respectively) in 96-well round-bottom microtiter plates. Proliferative responses were determined on days 2 to 4 of culture. Data shown are representative of two experiments. CD4^+ and CD8^+ T cells did not respond to any of the stimuli when cultured in the absence of LC (CD4^+ T cells + Con A = 126 ± 12 cpm, CD4^+ T cells + anti-CD3 = 269 ± 50 cpm, CD8^+ T cells + Con A = 119 ± 34 cpm, CD8^+ T cells + anti-CD3 = 415 ± 187 cpm).
CTLL-2 cells were cultured for 36 h, and [3 H]TdR was added to the cul-
vested and added to IL-2-dependent CTLL-2 cells at a final dilution of 1:3.
in 96-well flat-bottom microtiter plates for 24 h. Supernatants were har-
show that SF-cLC, similar to FCS-cLC, are exceedingly potent
Ag-processing capacity of LC isolated under SF conditions.
Freshly prepared SF- and FCS-EC (C3H) were cultured in the presence of
OVA (1 mg/ml) for 20 h. The nonadherent cells were harvested, washed,
and enriched for LC by density gradient centrifugation. SF- and FCS-LC-
OVA (5 × 10^5/well) were cocultured with E8 hybridoma cells (10^5/well) to
produce IL-2 when cultured with unpulsed LC (E8 + SF-LC = 2374 ± 360 cpm, E8 + FCS-LC = 1853 ± 215 cpm).

While LC are very efficient in processing native protein Ags when
freshly isolated from the dermis, they lose this ability during vitro
culture (5). To determine whether LC, when isolated and
pulsed with a soluble protein Ag in the absence of FCS, would still
have the ability to process and present this Ag, we pulsed freshly
isolated SF-EC as well as FCS-EC with OVA for 20 h. The nonadherent and
nonadherent cell populations harvested from E8 hybridoma cells failed to produce IL-2 when cultured with unpulsed LC
and [3 H]TdR was added to the cul-
tures 12 h before harvest. Data are representative of three experiments. E8 hybridoma cells failed to produce IL-2 when cultured with unpulsed LC (E8 + SF-LC = 2374 ± 360 cpm, E8 + FCS-LC = 1853 ± 215 cpm).

ag processing defect exhibited by both SF- and FCS-cLC may be a
consequence of reduced amounts of proinflammatory cytokines
detected in SF cultures. Indeed, TNF-α has been shown to signif-
cantly up-regulate the expression of panCD44 on cultured LC
(26). CD44 and DEC-205 molecules are significant for LC func-
tion, the first being important for their migratory and sensitizing
capacity (27), and the latter being a receptor involved in their
Ag-processing machinery (28). Interestingly, neither the Ag-pro-
cessing and the immunostimulatory function nor the migratory ca-
pacity of SF-cLC were adversely affected by the lower expression
of CD44 and DEC-205. It is not clear whether the low levels of
these molecules expressed on SF-cLC sufficed to secure these

**FIGURE 4.** Ag-processing capacity of LC isolated under SF conditions. 
Freshly prepared SF- and FCS-EC (C3H) were cultured in the presence of
OVA (1 mg/ml) for 20 h. The nonadherent cells were harvested, washed,
and enriched for LC by density gradient centrifugation. SF- and FCS-LC-
OVA (5 × 10^5/well) were cocultured with E8 hybridoma cells (10^5/well) to
produce IL-2 when cultured with unpulsed LC (E8 + SF-LC = 2374 ± 360 cpm, E8 + FCS-LC = 1853 ± 215 cpm).

**Hapten-modified SF-cLC sensitize for CHS**

Because LC play a critical role in the induction of the immune
response to reactive haptens (23), we used the CHS model to de-
termine whether SF-cLC are functional in vivo. Naive BALB/c mice were injected s.c. with syngeneic, TNP-modified or unmod-
ified SF-, TCM- and FCS-cLC and challenged with the same hap-
ten 5 days later. Results obtained in three independent experiments showed that TNP-modified SF- and FCS-cLC induced a CHS
response similar in magnitude and kinetics to that elicited by TNP-
modified FCS-cLC and by epicutaneous sensitization (Fig. 7, A and B). The response peaked at 24 h and declined 48 h after chal-
lenge (Fig. 7B). Remarkably, as few as 10^4 of TNP-modified SF-
cLC were able to immunize for CHS with the same potency as
equal numbers of TNP-modified FCS-cLC (Fig. 7C). Similar re-
sults were obtained in C3H and C57BL/6 mice, although the mag-
nitude of the CHS response was considerably lower than in
BALB/c mice (data not shown). These data show that LC cultured
under SF conditions are potent stimulators of cell-mediated im-
munity in vivo.

**Discussion**

In this study, we have shown that LC cultured in the absence of
FCS acquire a mature phenotype similar to that achieved under
SF conditions. Whether this phenotype corresponds to the termi-
nally mature, IL-10-resistant phenotype of DC (24, 25) remains to
be investigated. Our finding that SF-cLC express slightly lower
levels of CD44 and DEC-205 molecules than FCS-cLC may be a
consequence of reduced amounts of proinflammatory cytokines
detected in SF cultures. Indeed, TNF-α has been shown to signif-
cantly up-regulate the expression of panCD44 on cultured LC
(26). CD44 and DEC-205 molecules are significant for LC func-
tion, the first being important for their migratory and sensitizing
capacity (27), and the latter being a receptor involved in their
Ag-processing machinery (28). Interestingly, neither the Ag-pro-
cessing and the immunostimulatory function nor the migratory ca-
pacity of SF-cLC were adversely affected by the lower expression
of CD44 and DEC-205. It is not clear whether the low levels of
these molecules expressed on SF-cLC sufficed to secure these
functions or whether other molecules with similar properties became involved.

In unperturbed murine skin in vivo, EC constitutively produce low amounts of cytokines, particularly IL-1α, IL-7, TGF-β, GM-CSF, and TNF-α (29), which presumably contribute to the homeostasis of the skin. When the epidermal microenvironment is perturbed, i.e., when EC are dissociated by proteases and then cultured in serum-containing media, EC rapidly increase their cytokine production (e.g., IL-1α, GM-CSF, and TNF-α) and create a milieu that promotes LC survival and their maturation into potent APC (17, 29). We have shown here that the presence of serum is not necessarily required to fully accomplish LC maturation in vitro but helps to secure LC survival in culture. We also observed that the absence of FCS in EC cultures does not affect GM-CSF production by keratinocytes but leads to a decreased secretion of IL-1α and TNF-α, most likely as a consequence of the observed death of basal keratinocytes being the main source of these two cytokines (21, 22). Thus, our data imply that GM-CSF is an indispensable LC maturation factor but does not suffice to keep LC alive. Concerning the role of IL-1α and TNF-α in LC survival, we found that neither the IL-1α-rich SF supernatants of the basal keratinocyte cell line Pam 212 (30, 31) nor the addition of murine TNF-α (100 pg/ml) to SF-EC cultures was able to improve LC yields (A. Kočíková, unpublished observations). Whether other factors known to promote the differentiation and/or survival of LC/DC, such as TGF-β1 (32–34) or TNF-related activation-induced cytokine (TRANCE) (35), play a role in our experimental system remains to be investigated.

It is known that FCS consists of cytokines, growth factors, hormones, and vitamins (36), but many of its components are still poorly defined. To avoid the presence of xenogeneic proteins in EC cultures, we have established SF conditions and have shown here that, even in the absence of FCS, LC can mature into potent Ag-presenting and accessory cells. Interestingly, in contrast to their allostimulatory capacity, SF-cLC were less effective stimulators of naive, syngeneic CD4+ T cells than FCS-cLC. One explanation of this finding could be that the FCS constituents may structurally resemble self-proteins and, thus, may induce T cell activation when presented in the context of MHC molecules on LC. The absence of potentially cross-reactive FCS proteins in LC/DC cultures may be a benefit for their clinical applicability, because autoreactive responses could be avoided. To the best of our knowledge, this is the first report on the culture of LC under SF conditions and supports the efforts of other researchers who have...
used autologous serum/plasma or cytokines instead of FCS in DC cultures (33, 34, 37–39).

Our results show that freshly isolated SF- and FCS-LC are equally potent in their capacity to process a soluble protein Ag. Thus, our SF system should allow us to investigate the particular steps of the DC/LC Ag-processing machinery in vitro without any influence of foreign proteins/peptides. This may provide a clue about how to preferentially introduce peptides into either the MHC class I or class II processing pathway in the APC and, thus, may be useful in the development of new immunizing strategies. We have preliminary data indicating that, at higher doses of OVA, the SF-LC are even better Ag-processing cells than FCS-LC, perhaps because the MHC molecules of LC are more accessible for loading with specific peptides under SF conditions (A. Kočíková, unpublished observations). As a consequence, one may assume that the Ag dosing can be more accurately accomplished under SF than under FCS conditions. This is important in view of the findings that high doses of soluble Ag lead to the differentiation into Th-like cells producing predominantly IFN-γ, while low doses of the same Ag can induce differentiation into Th2-like cells producing mainly IL-4 (40, 41). Thus, by modulating the amount of Ag under SF conditions, immune responses could be directed toward a Th1- or Th2-type in vivo. This might be useful for the therapy of diseases in which a particular T cell subpopulation is known to play a pathogenetically important role.

Acknowledgments

We thank Dr. M. Epstein for critically reading the manuscript, S. Olt for excellent technical assistance, and E. Berger for animal care.

References


