

# Novel Responses of Human Skin to Intradermal Recombinant Granulocyte/Macrophage-Colony-stimulating Factor: Langerhans Cell Recruitment, Keratinocyte Growth, and Enhanced Wound Healing

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## Summary

Recombinant granulocyte/macrophage-colony-stimulating factor (rGM-CSF), prepared from Chinese hamster ovary (CHO) cells and *Escherichia coli*, was administered to 35 patients with the borderline and polar lepromatous forms of leprosy by the intradermal and subcutaneous routes at doses of 7.5–45.0  $\mu\text{g}/\text{d}$  for 10 d. With each of these doses and routes, increases in the number of circulating eosinophils were noted. After the intradermal injection, the local skin sites demonstrated zones of roughening and micronodularity that appeared within 24–48 h and persisted for more than 6 d. Reinjection of sites led to enhanced areas of epidermal reaction. GM-CSF prepared from CHO cells was a more potent inducer of this effect. GM-CSF given by the subcutaneous route, at higher doses, failed to initiate these changes. At the microscopic level, the epidermis became thickened (+75%) with increased numbers and layers of enlarged keratinocytes. These contained increased numbers of ribosomes and prominent nucleoli, and were imbedded in a looser meshwork of the zona Pellucida. The modified keratinocytes remained MHC class II antigen negative throughout the course of the response. A major change in the dermis was the progressive accumulation of CD1<sup>+</sup>, Birbeck granule-positive cells. These Langerhans were recognizable at 48 h after intradermal injection and reached maximum numbers by 4 d. During this period the number of epidermal Langerhans cells remained relatively constant. No increment in dermal Langerhans cells occurred when GLM-CSF was injected by the subcutaneous route. No appreciable increase in the numbers of T cells and monocytes was noted, and granulocytes and eosinophils were largely present within the dermal microvasculature. 4-mm punch biopsies taken from injected sites and adjacent controls were compared in terms of the rapidity of wound healing. 22 of 26 sites demonstrated more rapid filling and hemostasis, whereas four were equivalent to controls. We conclude that rGM-CSF, when introduced into the skin, leads to enhanced keratinocyte growth, the selective recruitment of Langerhans cells into the dermis, and enhanced wound healing of the prepared site. There was no evidence of an enhanced cell-mediated response to *Mycobacterium leprae*, and bacillary numbers remained unchanged.

Our previous studies have dissected some of the cellular and molecular events associated with cell-mediated immunity in humans (1). These have focussed largely on delayed-type cutaneous responses, after the introduction of antigens or recombinant lymphokines (2). One of the prominent changes noted in the epidermis, overlying an antigen-generated response, was an increase in epidermal thickness as well as keratinocyte size and numbers (3). The effector molecules associated with this event were then explored using cultured keratinocytes (4). These studies indicated that IL-3 and GM-

CSF possessed stimulatory effects on keratinocyte growth in vitro. GM-CSF also has other effects on cutaneous cells and is a major factor in potentiating the APC capacity of Langerhans cells and inducing their cell division (5, 6). In addition, GM-CSF is a well-known stimulant of myeloid cells in the marrow and a modest activator of circulating monocytes (7). These studies prompted us to examine the effects of rGM-CSF when introduced into the skin of patients with severe leprosy. We now report on the novel effects of GM-CSF on keratinocytes and Langerhans cells in situ.

## Materials and Methods

**Patient Population.** 35 leprosy patients, 7 females and 28 males, (13–55 yr old, 29 with polar lepromatous leprosy, and 6 with borderline lepromatous leprosy (8), were selected for GM-CSF administration. Patients were seen at the Clinical Branch of the Leonard Wood Memorial Center for Leprosy Research (Cebu City, Philippines). Patients in reaction were excluded from the study. Written consent was obtained from all patients.

Patients had been treated from <1 to 21 mo with multidrug therapy with rifampin (600 mg/d) clofazimine (300 mg/mo and 50 mg/d), and dapsone (100 mg/d). All chemotherapy was continued during the study. Six sites (two ear lobes, two on the arms, and two lesions) were evaluated by slit smear for bacterial index (B.I.)<sup>1</sup> (9, 10) determinations at the start of the study and 21 d (20 patients) or 11 d (15 patients) after the first injection. The B.I. of patients tested ranged from 2.0 to 5.8 at the start of the study (mean  $\pm$  SD at the start, 4.5  $\pm$  0.85).

**Human GM-CSF.** Two preparations of recombinant human (rhu)GM-CSF were tested (Sandoz Pharma Ltd./Schering-Plough Ltd., Basel, Switzerland). The first was rhuGM-CSF protein produced in CHO cells (glycoprotein) (GMC 89-107) (batch y 026 0188). Vials contained 0.150 mg of lyophilized protein as calculated by amino acid analysis, or the corresponding 0.216–0.217 mg of glycoprotein. Vials were reconstituted with 2 ml of preservative-free sterile water, to give a stock solution of 75  $\mu$ g/ml (isotonic, pH 6.7–6.8) with a specific activity of  $8 \times 10^6$  chronic myelocytic leukemia (CML) U/mg protein. The second was rhuGM-CSF protein produced in *Escherichia coli* (CSF 39-300) (batch U028 0689) with a specific activity of  $2 \times 10^8$  U/mg acute myelocytic leukemia (AML). Vials contained 0.150 mg of lyophilized protein were reconstituted in 0.75 ml as above to give a stock solution of 200  $\mu$ g/ml. Reconstituted GM-CSF was used within 2 h. Intradermal injections were given in 100- $\mu$ l volume while subcutaneous injections were given in a volume of 200  $\mu$ l.

**Experimental Design.** Collaborative arrangements were made with Sandoz Pharma Ltd./Schering-Plough Ltd. (Basel, Switzerland) to supply rhuGM-CSF. The material used for the first clinical trial was the recombinant protein prepared from CHO cells whereas the *E. coli* product was used in the second trial. Both clinical trials were carried out in conjunction with physicians and investigators working in The Leonard Wood Memorial Hospital (Cebu City, Philippines), under the direction of Dr. Gerald Walsh. In the first trial carried out in April 1990, intradermal injections of either 7.5  $\mu$ g (10 patients) or 15.0  $\mu$ g (10 patients) of the CHO product were administered daily for 10 d to a total of 20 patients. A second trial in March 1991 used the *E. coli* material at a dose of 45  $\mu$ g given subcutaneously for 10 d to 15 patients. During the course of this systemic trial, intradermal injections of 10 and 20  $\mu$ g were also used. Nine patients received 10- $\mu$ g intradermal injections on day 1 of the study and all 15 patients received two 20- $\mu$ g intradermal injections into the same site on day 6 and 7 of the study. All injections were given into the skin of the back of the patients. Before, during, and after the administration of GM-CSF, blood samples were collected for the analysis of circulating cells and routine blood chemistries. Injected sites were evaluated for macroscopic changes and sampled by 4-mm punch biopsies 24–144 h after cytokine administration, prepared for immunocytochemistry, transmission electron microscopy, and histopathology. The biopsy site was scored for wound healing.

**Histopathology.** A part of each biopsy specimen was fixed in

neutral buffered 10% formalin overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological diagnosis and acid-fast staining of enumeration of *Mycobacterium leprae*.

**Immunocytochemistry.** Biopsy specimens were fixed in PBS containing 3% paraformaldehyde, 75 mM lysine, and 10 mM sodium metaperiodate (11), and were processed as described (12). Frozen sections were stained with mouse mAbs as described (12) to identify cell types and phenotypes.

**mAbs.** Mouse mAbs were used for the identification of specific cell types. Antibodies Leu-4, Leu-2a, and Leu-3a (CD3, CD4, and CD8 anti-T cells) (13, 14) and Leu-M5 (CD11c, anti-monocyte/macrophage) (15) were obtained from Becton Dickinson and Co. (Mountain View, CA). Antibody OKT6 (CD1, anti-Langerhans cells) (16) was obtained from Ortho Pharmaceutical Corp. (Raritan, NJ) and antibody 9.3F10 (anti-MHC class II antigen) was produced in this laboratory (17).

**Electron Microscopy.** A part of each biopsy was processed for transmission EM studies. Biopsies were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, pH 7.4, for 16 h at 4°C, postfixed in 2% OsO<sub>4</sub> for 1 h at 4°C, stained in block for 1 h with uranyl acetate in 70% ethanol, dehydrated in increments with ethanol, and embedded in epon blocks. Sections were stained with lead citrate and examined with a transmission electron microscope (JEM 100CX; Jeol USA, Peabody, MA).

**PBMC Phenotyping.** Cells were analyzed by flow cytometry. The buffer used throughout consisted of PBS containing 2% FCS, 0.1% NaN<sub>3</sub>. PE-conjugated mAbs reactive with CD3, CD4, CD8, CD56, and CD26 (NKH1) CD11c (monocytes), CD1 (Langerhans cells),  $\gamma/\delta$  T cells, and CD20 (B cells) were obtained from Coulter Immunology (Hiialeah, FL). Cells were stained at 4°C for 1 h. After incubation, the cells were centrifuged, the supernatant removed, and 1 ml of PBS with 0.4% paraformaldehyde was added. The cells were stored at 4°C until analyzed by flow cytometry (Ortho Diagnostic Systems, Inc.). At least 2,000 cells were analyzed in each preparation, and the percentage of positively stained cells was calculated.

**Determination of Epidermal Thickness.** Epidermal thickness was evaluated by direct examination of hematoxylin-stained sections using a computer-based image digitizing system (Southern Micro Instruments, Inc., Atlanta, GA). The distance from the epidermal-dermal junction to the outer surface of the epidermis was measured at equal intervals along the length of the sections. At least 25 measurements were made per section. Results were expressed as the ratio of mean thickness of the GM-CSF-injected site to the mean thickness of the control site for each patient.

**Evaluation of Langerhans Cell Numbers in the Epidermis and Dermis.** Sections were evaluated for specific cell staining with a Nikon Microphot-FX light microscope. The numbers of CD1<sup>+</sup> cells of the epidermis and the dermis were evaluated by direct counting of the stained cell bodies (with nuclei) per  $\times 40$  microscopic field. The total area of epidermis and upper dermis was examined on each section, CD1<sup>+</sup> cells counted, and the mean and standard deviation of the mean calculated. At least four different sections, with 10 adjacent  $\times 40$  microscopic fields from every biopsy, were counted.

**Cytokine Determinations.** Plasma samples were collected before and after GM-CSF injection and stored at  $-70^\circ\text{C}$  until used. GM-CSF and TNF- $\alpha$  concentrations were determined by specific ELISA (Endogen, Boston, MA). ELISAs were performed exactly as specified by the manufacturers. The limits of detection were 4–6 pg/ml.

**PCR-assisted RNA Amplification.** Total RNA preparation and cDNA were prepared as described (18). Lymphokine-specific primers

<sup>1</sup> Abbreviation used in this paper: B.I., bacterial index.

for GM-CSF, IL-1 $\beta$ , IL-2, -3, -4, -5, -6, -8, TNF- $\alpha$ , and TNF- $\beta$ , as well as  $\beta$ -actin (3'), were prepared and provided by S. Ehlers as described (19).

**Wound Healing.** Biopsy sites were evaluated daily by three independent investigators, and the extent of healing (drying, rounding of the wound edge, formation of granulation tissue, and keratinocyte growth) was evaluated.

## Results

**Clinical and Laboratory Results.** The administration of GM-CSF from mammalian or prokaryotic cell origin was not associated with systemic symptoms in any of the 35 patients injected, except for a slight elevation in fever (a maximum of 39°C) in seven patients. No alterations in blood chemistries or urinalysis were noted during and after the trial. The levels of circulating WBC are presented in Table 1 and 2. After the intradermal administration of 7.5 or 15  $\mu$ g of CHO GM-CSF to patients (10 per group), a small increment in the total WBC count was noted. About half the increment was due to a threefold increase in the number of eosinophils

(Table 2) and the rest was accounted for by enhanced number (~20% increase) of neutrophils. The numbers of eosinophils returned to preinjection levels by 21 d while those of neutrophils remained slightly elevated. No differences were observed between the patients injected with 7.5 or 15  $\mu$ g of CHO GM-CSF (Table 2). After the subcutaneous administration of 45  $\mu$ g of *E. coli* GM-CSF to 15 patients, a smaller increment in total WBC counts was noted, the majority of which was also accounted for by the increase in eosinophils. The response of eosinophils to the intradermal and subcutaneous injection of GM-CSF is seen in Table 2. In each case there was a 2–2.5-fold increase in the percentage of eosinophils in the absence of other significant changes.

Phenotypic analysis of PBMC with mAbs and FACS<sup>®</sup> analysis showed no differences in the distribution of cell types before and immediately after the administration of GM-CSF. ELISAs of serum GM-CSF and TNF- $\alpha$  were uniformly negative during the experiment. Finally, PCR amplification of PBMC mRNAs failed to show the induction of GM-CSF message and revealed no significant changes in messages for IL-1, IL-6, or IL-8.

**Table 1.** Influence of rGM-CSF on Levels of Circulating Leukocytes

	Total no. of leukocytes per mm <sup>3</sup> of blood				
	Day 0	Day 11	Day 11-0	Day 21	Day 21-0
A. WBC*	9,577 <sup>†</sup>	12,550	2,973	11,292	1,715
Neutrophils	5,966	7,128	1,162	7,413	1,447
Lymphocytes/monocytes	2,978	3,287	309	3,274	296
Eosinophils	589	1,995	1,406	575	-14
B. WBC	11,720	12,650	930	NT	NT
Neutrophils	7,700	7,754	54	NT	NT
Lymphocytes/monocytes	2,930	2,972	42	NT	NT
Eosinophils	809	1,632	823	NT	NT

A, 7.5 or 15  $\mu$ g recombinant CHO GM-CSF given intradermally daily for 10 d; or B, 45  $\mu$ g recombinant *E. coli* GM-CSF given subcutaneously daily for 10 d.

\* n = 20.

<sup>†</sup> Means for all patients.

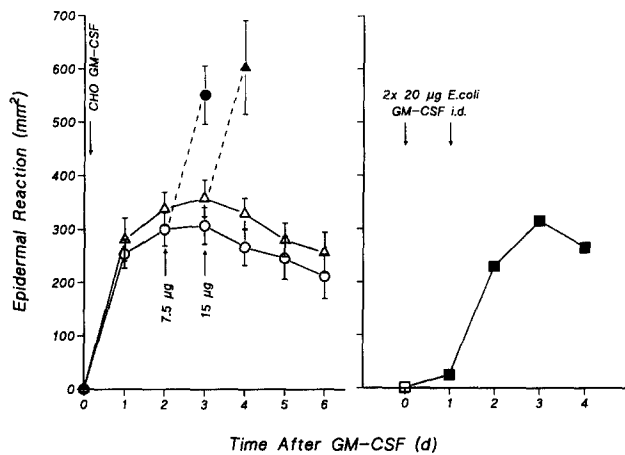
<sup>‡</sup> n = 15.

**Table 2.** Influence of rGM-CSF on the Levels of Circulating Eosinophils

Type of cytokine	Dose	Eosinophils as percent of total cells		
		Day 1	Day 11	Day 21
A. CHO GM-CSF	7.5 $\mu$ g i.d.* /d for 10 d	5.4	13.8	6.4
	15.0 $\mu$ g i.d.* /d for 10 d	6.7	17.1	5.3
B. <i>E. coli</i> GM-CSF	45 $\mu$ g s.c. <sup>‡</sup> /d for 10 d	6.9	12.9	N.T.

\* n = 10.

<sup>‡</sup> n = 15.



**Figure 1.** The local cutaneous response to intradermal GM-CSF injection. The area (in mm<sup>2</sup>) of modified epidermis in response to cytokine injection is shown for CHO GM-CSF (left) (20 patients) and *E. coli* GM-CSF (right) (15 patients). An early (24-h) response to a single 7.5-µg (O) and 15-µg (Δ) CHO GM-CSF injection peaked at 3 d and then subsided slowly. Reinjection of the site at 2 d (7.5 µg) and 3 d (15 µg) led to a consistent increase in the area of epidermis modified by the cytokine (filled symbols). The first 20-µg intradermal dose of *E. coli* GM-CSF (□) did not induce epidermal changes. Reinjection of the site (20 µg; filled symbols) induced a local response that peaked 2 d later and then subsided slowly. Results are expressed as the mean area of response for all patients injected  $\pm$  1 SD.

*The Local Cutaneous Response to Intradermal Injection of rGM-CSF: Macroscopic Changes.* The intradermal injection of GM-CSF at doses of 7.5–20.0 µg/0.1 ml of diluent was followed by a consistent series of events, the extent and kinetics of which depended upon the type of the GM-CSF and the dose used. In all cases injection sites exhibited slight erythema and no significant swelling or induration. In this sense the response differed markedly from the results with the intradermal injection of both IFN-γ and IL-2 (12, 20). Within 24 h, the surface of the skin of the responsive site showed roughening in a circular zone up to 200 mm in diameter. Within the next 24 h, examination of the area with a hand lens illustrated the presence of many sites of micro-papillation contained within the roughened area and not associated with hair follicles. At 6 d this zone was scaly, as the keratinized layers were desquamated, leaving normal appearing skin.

The areas of the epidermal response are demonstrated in Fig. 1, which compares the responses to rGM-CSF obtained from CHO cells and *E. coli*. It appears that on a weight basis, the CHO preparation (Fig. 1, left) is more potent and leads to a prompt (24-h) modification of the epidermis. The reinjection of the site at 48 h (7.5 µg) or 72 h (15 µg) after the first injection led to a consistent increase in the area of the responsive site with accentuation of the changes noted above.

With the *E. coli* material little response occurred at 24 h with 10 µg (not shown) or 20 µg (Fig. 1, right), but upon reinjection of the 20-µg sites, the typical cutaneous modifications noted with the CHO-derived GM-CSF ensued. It was of interest that little or no epidermal change occurred when 45 µg of the *E. coli* GM-CSF was injected subcutaneously

for a 10-d period. In only 4 of 170 sites examined was there a slight roughening of the overlying epidermis after subcutaneous injection.

We conclude that rGM-CSF has the ability of modifying the epidermis only when introduced in close proximity to this compartment.

*Microscopic Changes in the Epidermis.* Striking changes were noted in the epidermis of responsive sites when biopsies were examined histologically. Within 24 h after CHO GM-CSF injection, enlargement of keratinocytes was apparent and the thickness of the epidermis was increased (Fig. 2). Quantitative analysis of the biopsies indicated that the epidermis had increased in thickness by a mean of 75% by 24 h and remained thickened for ~120 h (Fig. 3). These effects were not observed as clearly after *E. coli* GM-CSF injection into the skin. The fine structure of the epidermis will be discussed in a later section.

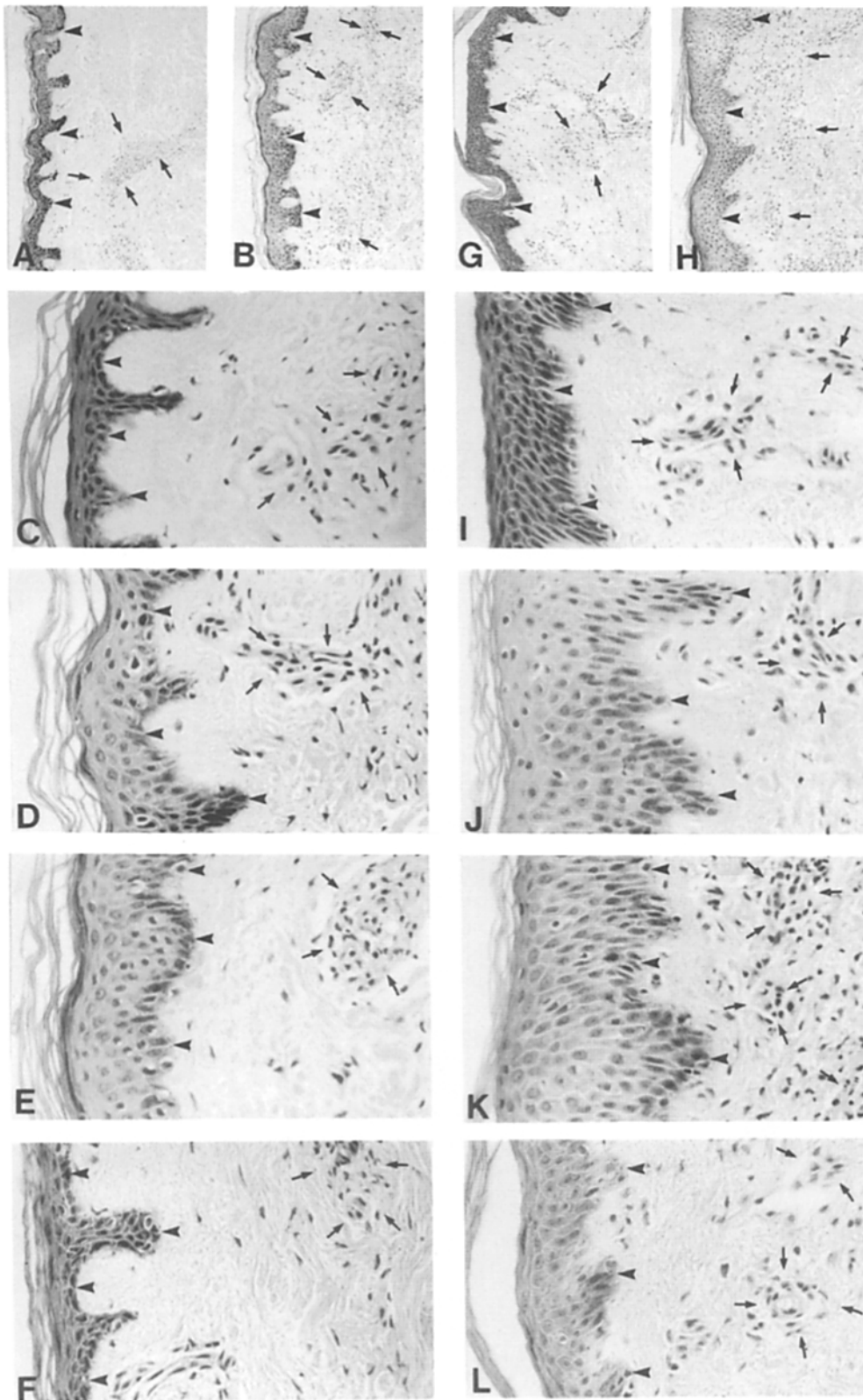
*Histopathology and Bacillary Indices of Skin Biopsies and Slit Smears.* Biopsies obtained from control and injected skin sites during the course of the intradermal and subcutaneous administration of rGM-CSFs were used to evaluate the histopathology and B.I. of the skin in these patients. Control specimens showed the usual sparse mononuclear cell infiltrate of lepromatous leprosy, the majority of cells being macrophages with varying numbers of acid-fast bacilli (21). The epidermis was thin and the rete were often shallow with only one to two layers of rounded, basal cells. Only a small amount of additional cellular infiltrate was noted during the course of the GM-CSF injections (Fig. 2). This consisted of scattered eosinophils and neutrophils and small accumulations of perivascular mononuclear cells; in some patients these sites resembled the lesions of erythema nodosum leprosum without the vascular changes. Little else was noted in the dermis. The B.I.s of the patients evaluated before and after completion of a 10-d course of cutaneous injections of GM-CSF are shown in Table 3. No significant changes in the bacterial load were noted during this period in contrast to results obtained with PPD antigen, rIFN-γ, and rIL-2 (22–24). We conclude that locally administered GM-CSF fails to modify the mononuclear phagocyte population of the dermis and the number of intracellular leprosy bacilli.

*Immunocytochemistry of the Cutaneous Site.* Immunohistological staining of the dermal and epidermal cellular components are illustrated in Fig. 4. The basal, lepromatous infiltrate

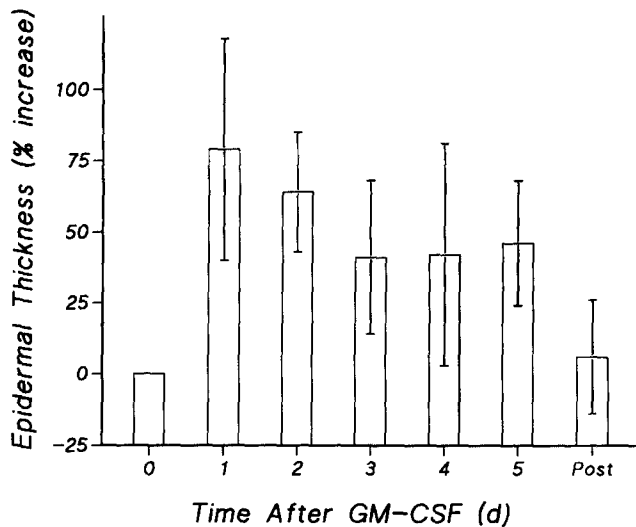
**Table 3.** B.I. in Response to GM-CSF

Type of cytokine	No. of patients	B.I.*	
		Pre	Post
CHO GM-CSF	20	4.47 $\pm$ 1.06	4.43 $\pm$ 1.16
<i>E. coli</i> GM-CSF	15	4.54 $\pm$ 0.69	4.30 $\pm$ 0.83

\* Mean B.I.  $\pm$  1 SD of the mean.



**Figure 2.** Effect of CHO GM-CSF on the histological appearance of the skin. Light micrographs of sections of biopsies obtained from two representative patients are shown. A comparison of a preincubation control site (A and C) to a 24-h postcytokine administration site (B and D), a 96-h postcytokine administration site (E), and an 11-d control (F) are shown for patient no. 5. A preinjection control site (G and I), 24-h post- (H and J), and 48-h postcytokine (K), and an 11-d control (L) are shown for patient no. 8. A thickening of the epidermis (arrowheads) is observed already by 24 h (D and J). No systemic enhanced thickness is observed (F and L compared with C and I, respectively) after subcutaneous injection. GM-CSF injection did not significantly enhance the dermal infiltration by leukocytes (small arrows). A, B, G, and H,  $\times 25$ ; C-F and I-L,  $\times 200$  (H+E staining).



**Figure 3.** Effect of CHO GM-CSF on epidermal thickness. The thickness of the epidermis was evaluated for all 20 patients before (0 d), 1–5 d after cytokine injection, and in control site obtained from uninjected sites at the end of the study (11 d postinjection). These sites enabled us to evaluate possible systemic effects at sites removed from cytokine administration. The mean  $\pm$  1 SD of all preinjection sites (0 d) was  $53 \pm 9 \mu\text{m}$ . Results are expressed as mean percent increase in thickness relative to preinjection controls  $\pm$  1 SD.

was MHC class II positive in both control and injected sites, and consisted of parasitized macrophages and some CD3<sup>+</sup> T cells (not shown). The major changes in the dermis were the accumulation of CD1<sup>+</sup> Langerhans cells (Fig. 4 F). Initially, at 24 h after a single injection of CHO GM-CSF, a few cells were scattered randomly in the upper portion of the dermis. With the passage of time, and peaking at  $\sim$ 72–96 h, aggregates of CD1<sup>+</sup> cells were observed in a clearly perivascular location. Similar results were observed with *E. coli* GM-CSF although the numbers of dermal cells were lower (Fig. 5). After a second injection of GM-CSF into the same site, the response was increased and the total number of cells accumulating in the dermis was higher. Quantitative analysis of this dermal population demonstrates a 350–700% increase in the number of CD1<sup>+</sup> cells in the dermis in response to the local injection of *E. coli*-derived GM-CSF. As in the case of the epidermal thickening, the CHO preparation appeared more potent on a weight basis (not shown).

As mentioned, the epidermal keratinocytes remain MHC class II antigen negative throughout this series of events (Fig. 4). This is in clear distinction to the induction of keratinocyte class II antigen after the keratinocyte growth subsequent to the intradermal administration of antigen, IFN- $\gamma$ , and IL-2 (12, 20, 25). In addition, the number of epidermal CD1<sup>+</sup> Langerhans cells remains relatively constant throughout the post-GM-CSF injection period. Although a minor decrement is noted in epidermal Langerhans cell numbers (Fig. 5), this cannot account for the numbers accumulating in the dermis and suggests a vascular origin for the dermal cells.

**Electron Microscopy of the Cutaneous Sites.** Transmission electron micrographs of the dermal and epidermal populations are illustrated in Figs. 6 and 7. No striking alterations were noted in the extent or composition of the underlying lepromatous infiltrate and organisms continued to be found in multibacillary vacuoles in macrophages. Both neutrophils and eosinophils were apparent throughout thin sections of GM-CSF-injected sites. Although some were present in the tissues, the majority were within what appeared to be dilated elements of the microvasculature located in the upper dermis (Fig. 6, A and B). Endothelial cells of these vessels were not appreciably altered in contrast to the effects of IL-2 (24).

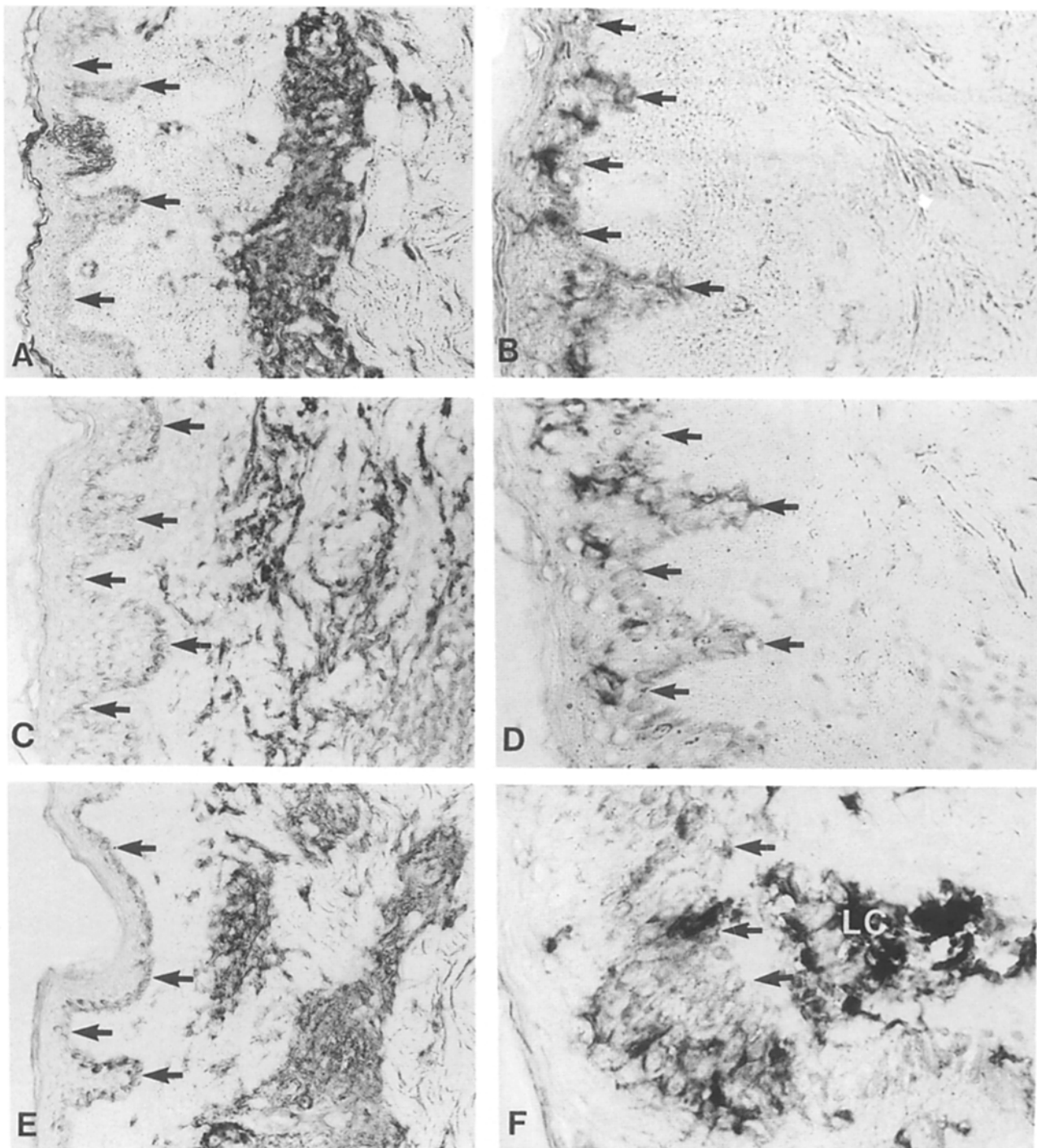
The novel dermal elements were typical Langerhans cells that conformed to the distribution of CD1<sup>+</sup> cells described by immunocytochemistry. These cells were elongated with a typical chromatin pattern and contained within their cytoplasm paddle shaped, Birbeck granules (Fig. 6, C–F). The number and distribution of cytoplasmic organelles of the dermal cells was identical to that observed in the Langerhans cells of the epidermis.

The keratinocyte population of GM-CSF-injected sites was markedly altered (Fig. 7). Many layers of more spherical cells were visible that contained within them increased numbers of free ribosomes. Although tight junctional zones were clearly present (Fig. 7 F), it was obvious that the keratinocytes were more loosely associated and demonstrated prominent intercellular spaces (Fig. 7, B, D, and F). This was in contrast to the tightly coupled keratinocytes of the adjacent control sites (Fig. 7, A, C, and E). Mitotic figures were not noted in the specimens examined at either the light or transmission EM level. Nucleoli were prominent in the larger nuclei of the GM-CSF-exposed keratinocyte.

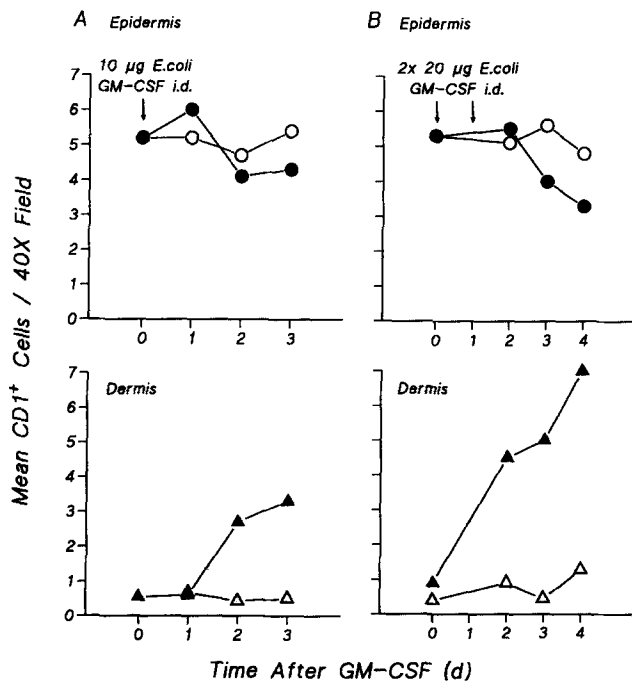
Samples obtained from patients who had received 45- $\mu\text{g}$  subcutaneous injections of *E. coli* GM-CSF, but no intradermal cytokine, failed to show any of the previous findings. This indicates the necessity of introducing GM-CSF into the local environment to obtain both the enhancement in Langerhans cell numbers and in keratinocyte growth promotion.

**The Enhanced Wound Healing of GM-CSF-injected Punch Biopsy Sites.** After the excision of skin by punch biopsies, the healing site was evaluated daily. For the majority of the study simultaneous biopsies were obtained from GM-CSF-injected sites that exhibited the gross changes noted in Fig. 1 and from adjacent, “normal” uninjected skin. During the course of our initial studies with CHO GM-CSF it was noted that the biopsy sites obtained from cytokine-prepared sites healed more rapidly than controls. More careful quantitation of this event was carried out with the *E. coli* product. The appearance and the rate of healing of these sites was evaluated on a daily basis in 26 pairs of biopsies from 15 patients.

The results of this analysis are shown in Fig. 8. Sites injected with *E. coli* GM-CSF were biopsied 24–96 h after the intradermal injection. Within 24 h of the biopsy, the GM-CSF-injected site was dry, lacked bleeding points, and was covered by a whitish opaque film. The control site was oozing blood which continued to stain the overlying dressings. Both sites had initially been apposed with a butterfly bandage.



**Figure 4.** Effect of CHO GM-CSF on keratinocyte phenotype and Langerhans cell distribution. Light microscopy micrographs of biopsies sectioned and stained for MHC class II antigen (A, C, and E) and CD1 antigen (B, D, and F) expression on cells of the epidermis (arrows) and the dermis. MHC class II antigen was not expressed on keratinocytes before cytokine injection (A), nor was it induced on keratinocytes by GM-CSF injection at any time from 24 h (C) to 5 d. Class II antigen was not expressed on control sites at any time up to 11 d (E) after cytokine injections. The infiltrating cells of the dermal lepromatous lesions were MHC class II antigen positive throughout. This infiltrate was not enhanced by GM-CSF injection. CD1 was expressed on Langerhans cells of the epidermis before (B) as well as 24 h (D) and 72 h (F) after cytokine injection. In addition, CD1<sup>+</sup> cells (LC) were observed in the dermis in perivascular clumps (F) after GM-CSF injection. A, C, and E,  $\times 150$ ; B, D, and F,  $\times 250$  (patient no. 5).



**Figure 5.** Effect of GM-CSF on CD1<sup>+</sup> Langerhans cell distribution. A single 10- $\mu$ g (A) or two 20- $\mu$ g (B) injections of *E. coli* GM-CSF given intradermally did not reduce the numbers of CD1<sup>+</sup> epidermal cells significantly (●) relative to control sites (○) (top). However, dose-dependent accumulation of CD1<sup>+</sup> cells in the dermis in response to GM-CSF (▲), as compared with control sites (△), was noted. Results are expressed as mean CD1<sup>+</sup> cells observed per  $\times 40$  microscopic field.

At 48 and 72 h the control sites continued to ooze. The cutaneous defect had vertical sides with sharp edges. In contrast, the GM-CSF-injected site became progressively shallower as epidermal components closed the lesion.

The enhanced process of healing was clearly evident in 22 of the 26 biopsy sites evaluated, whereas in 4 of 26, healing of the control and the injected sites were equivalent. These results were the composite analysis of three independent observers.

## Discussion

The present studies outline a series of novel findings that are the consequence of introducing rGM-CSF into the skin of patients with the lepromatous form of leprosy. Although this milieu already contains cellular elements that have been in the dermis for long periods of time, we see no a priori reason why these would influence the results as described in this report. Nevertheless, studies on normal skin should be carried out in the future. Neither of the major effects of the cytokine on Langerhans cell accumulation and on keratinocyte growth have been appreciated in studies after parenteral administration nor in transgenic mice (7, 26, 27). In both situations the influence of GM-CSF on the bone marrow and on the products of the committed stem cells have been most prominent. Similarly, in our studies, following both the in-

tradermal and subcutaneous routes, changes in the level of circulating leukocytes were evident. These, however, were restricted to an increase in the number of circulating eosinophils and some neutrophils without appreciable increments in the number of monocytes. This may be the consequences of both the low dose of the agent and the short time span of the experiment.

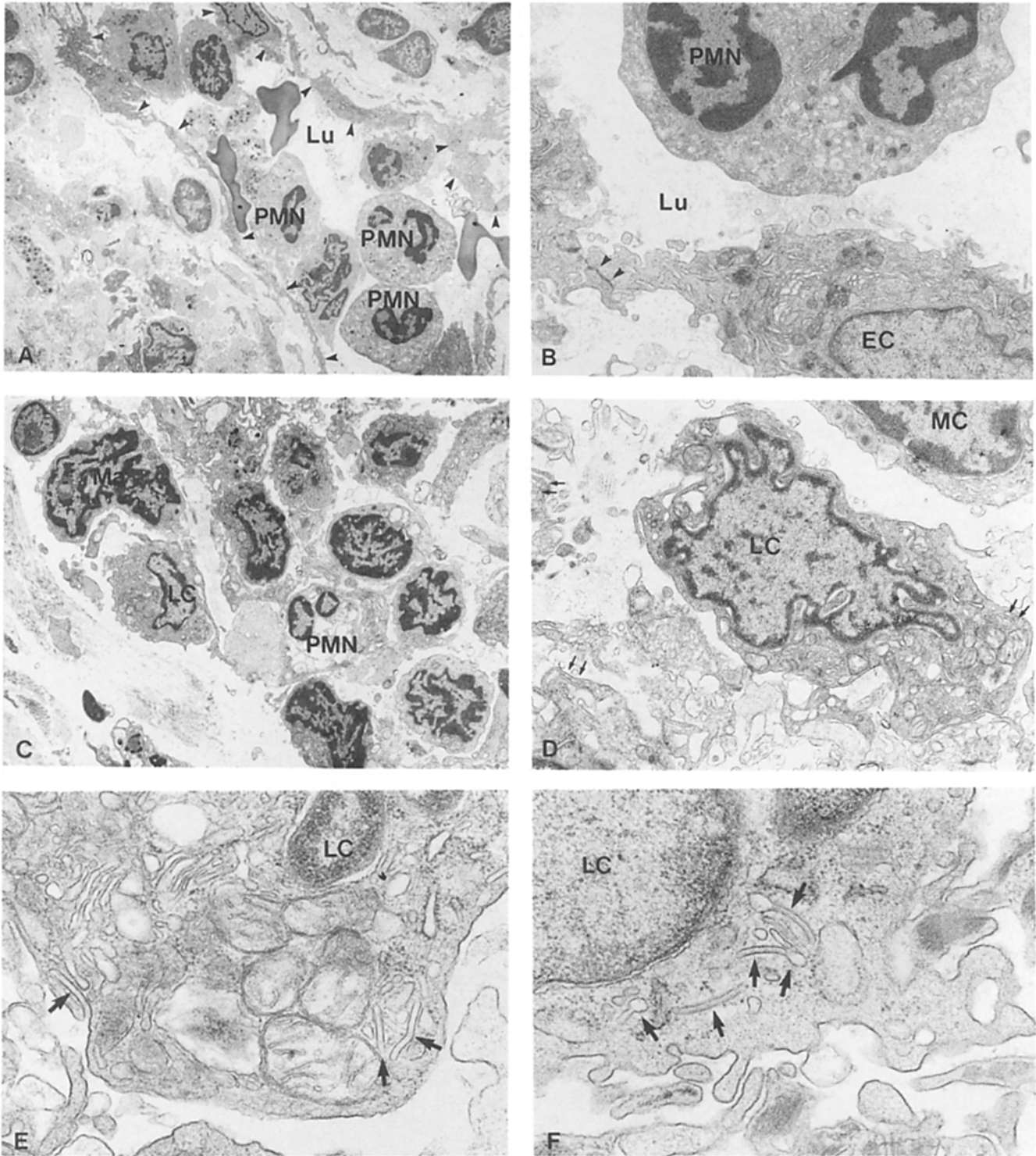
These results confirm and explain our prior observations on both the accumulation of CD1<sup>+</sup> Langerhans cells in the dermis (28) and on the rapid growth of keratinocytes that overly a delayed-type cell-mediated response (3). In the latter case these observations were retested in an *in vitro* environment. We tested the effect of selected secretory products of immune cells from a cell-mediated response on primary cultures of keratinocytes (4). One of the inducing agents, IFN- $\gamma$ , in fact, inhibited keratinocyte replication *in vitro*. In contrast, IL-3 and GM-CSF enhanced keratinocyte numbers *in vitro*.

The prompt stimulation of keratinocyte growth in the *in vivo* site is, to our knowledge, quite unique in the history of growth factors. Epidermal growth factors, insulin, cholera toxin, and platelet-derived growth factor have not modified the growth rate of keratinocytes *in situ* but are widely used to enhance growth of cells in culture. The effect in the patient requires a finite preparatory phase, lasts for 4–6 d, and allows differentiation, so that keratin synthesis, programmed cell death, and cell sloughing eventually ensue. As this growth takes place intercellular junctions are loosened and one might expect that the epidermis becomes more permeable to exogenous molecules. Another possibility is that we may be inducing the synthesis and release of keratinocyte GM-CSF or other molecules that extend the response. We are currently examining these questions by PCR analysis of keratinocyte mRNAs.

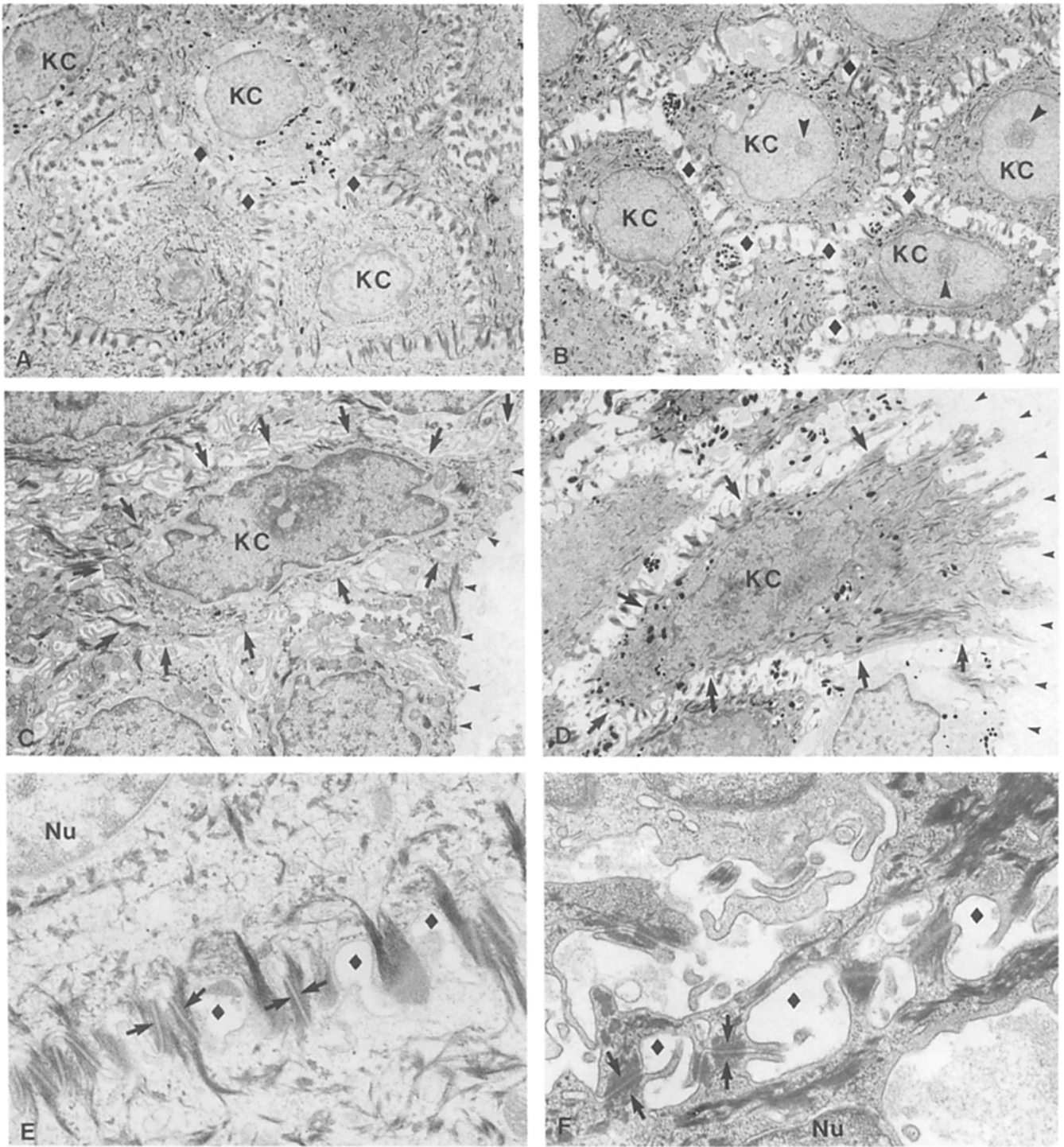
We believe that the growth stimulation is related to our observations on more rapid wound healing. Such a scenario would include the rapid influx of keratinocytes into the wound, initially serving a hemostatic function and gradually repairing the defect. The presence of granulocytes at the site might also enhance antibacterial effects. These observations, which are based on gross examination, will be supported in the future by more accurate evaluation carried out in normal skin. Even in the absence of more quantitative information it is nevertheless important to speculate on the possible roles of cutaneously administered GM-CSF in wound healing. These could include the preparation of skin sites for subsequent surgical procedures and its topical application for thermal and traumatic injuries.

The selective accumulation of Langerhans cells in the dermis is of equal interest. As a result of Langerhans cell recruitment the sites contain cells capable of interacting with responsive T cells migrating through the dermis. It has already been shown (5, 6) that GM-CSF is a major potentiating factor in the antigen-presenting capacity of freshly isolated epidermal Langerhans cells. This report adds an additional parameter to GM-CSF Langerhans cells interactions. GM-CSF has not previously been shown to be chemotactic for these cells. The





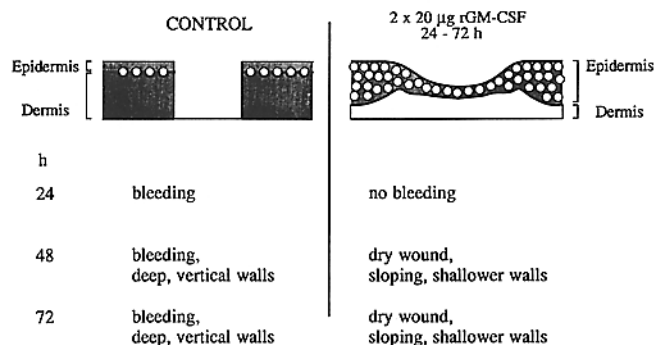
**Figure 6.** The effect of GM-CSF on the cells of the dermis. Transmission electron microscopy micrographs of CHO GM-CSF-injected sites. (A) A dilated element of the microvasculature is observed. The lumen (*Lu*) contains a number of erythrocytes and granulocytes (*PMN*). The wall of the vessel appears normal (*arrowheads*). Only a few cells are observed in the extravascular dermal space ( $\times 2,700$ ). (B) A close-up of a granulocyte (*PMN*) within the vasculature lumen (*Lu*). The endothelium (*EC*) appears normal and tight junctions (*arrowheads*) are intact ( $\times 12,200$ ). (C) A cellular infiltrate in the dermis containing a degranulated granulocyte (*PMN*), a Langerhans cell (*LC*), macrophages (*Ma*), and a few lymphoid cells ( $\times 4,500$ ). (D) A dermal Langerhans cell (*LC*) adjacent to a mast cell (*MC*). Birbeck granules are observed in the cytoplasm of the Langerhans cell (*arrows*) ( $\times 12,000$ ). (E and F) High magnification micrographs of dermal Langerhans cells (*LC*) with multiple Birbeck granules (*arrows*) ( $\times 28,000$ ).



**Figure 7.** The effect of GM-CSF on the cells of the epidermis. Transmission electron microscopy micrographs of CHO GM-CSF-injected (*B*, *D*, and *F*) and control (*A*, *C*, and *E*) sites. Controls: epidermal keratinocytes (KC) of control sites are closely associated (*arrows* in *C*) with limited extracellular space (◆) between the cells. Tight junctions are prominent between adjacent cells in *E* (*arrows*) (*A*,  $\times 4,300$ ; *C*,  $\times 9,000$ ; and *E*,  $\times 25,000$ ). GM-CSF sites: epidermal keratinocytes (KC) of GM-CSF-injected sites are loosely associated (*arrows* in *D*) with large spaces between them (◆). Keratinocyte nuclei are large and nucleoli are prominent (*arrowheads*). Tight junctions are prominent in *F* (*arrows*). *B*,  $\times 4,300$ ; *D*,  $\times 7,200$ ; and *F*,  $\times 25,000$ . Nu, nucleus, arrowheads in *C* and *F* mark the dermal epidermal junction.

### Healing of Control and GM-CSF Prepared Sites

4 mm Punch Biopsy



**Figure 8.** The effect of GM-CSF on wound healing. Biopsy sites (4 mm) were evaluated for 3 d for their extent of healing. Control sites (*left*) continued to bleed for longer and the walls of the wound remained vertical for longer than the GM-CSF-injected sites (*right*). Sites were injected with two 20-µg doses of *E. coli* GM-CSF with a 24-h interval and were biopsied 24–72 h after the second cytokine injection. By 5–6 d both types of wounds were relatively well healed.

present results suggest the induction of a selective chemoattractive stimulus. We suspect that Langerhans cell precursors enter the dermis from the circulation and then mature in the perivascular zone in large aggregates. Since no measurable number of CD1<sup>+</sup> cells is observed in the peripheral blood during GM-CSF injections, we suggest the presence of less mature circulating cells. We speculate that the normal production of GM-CSF by epidermal keratinocytes is the stimulus for the continual emigration of Langerhans cells into the differentiating keratinocyte layer. We also suspect that the emigration of Langerhans cells into delayed-type hypersensitivity sites as evoked by antigen, IFN-γ, and IL-2 is secondary to the local production of GM-CSF. Finally, the absence of MHC class II antigen on the GM-CSF-induced modified keratinocyte indicates the absence of appreciable amounts of IFN-γ released into this environment.

Although GM-CSF is widely considered to both enhance the number and functional properties of macrophage populations (7), little influence on this cell series was noted. In contrast to IFN-γ and IL-2 (12, 20), no effect of GM-CSF was noted evoking a local emigratory monocyte response, nor stimulating macrophages to destroy leprosy bacilli in these short-term studies.

We thank Sandoz Pharma Ltd./Schering-Plough Ltd. for its supply of GM-CSF. We are also indebted to the staff of The Leonard Wood Memorial Center and Eversley Childs Sanitarium (Cebu City, Philippines) and to Ms. Judy Adams and Marguerite Nulty for photographic and secretarial assistance.

This work was supported in part by Sandoz Pharma Ltd./Schering-Plough Ltd. and by the U.S. Public Health Service (A-22616-06).

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Received for publication 18 February 1992.

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