THE EXPRESSION OF A γ INTERFERON–INDUCED PROTEIN (IP-10) IN DELAYED IMMUNE RESPONSES IN HUMAN SKIN

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IFN-γ is a lymphokine synthesized and secreted by stimulated T lymphocytes. One of the important functions of IFN-γ is the activation of mononuclear phagocytes so that they are capable of killing a wide spectrum of intracellular pathogens (reviewed in reference 1). This process requires from 24 to 48 h and is associated with the enhanced production of reactive oxygen intermediates, a finding that may be explained by the greater affinity of the membrane oxidase for NADPH (2). More recently we have found that IFN-γ rapidly induces the expression of genes in macrophages and other cell types (3). One of these genes has been sequenced and codes for a 10-kD secreted protein (IP-10) with significant homology to a family of chemotactic and mitogenic proteins, including the α-platelet granule proteins platelet factor 4 (PF4)[1] and β-thromboglobulin (βTG).

The preparation of a polyclonal rabbit antibody against recombinant IP-10 (4) has now allowed us to examine the in situ formation of this protein in human tissues. For this purpose we have used a number of human conditions in which delayed-type hypersensitivity is either expressed or not expressed in the skin. For the former, we have used a classic purified protein derivative of tuberculin (PPD) reaction as well as the response to rIFN-γ injected into the skin (5–7). In addition, the polar forms of leprosy reflect a more chronic state in which tuberculoid lesions contain all the components of a delayed-type hypersensitivity (DTH) reaction, whereas lepromatous lesions lack the important helper T cell phenotype (8). Using immunocytochemistry we have now followed the temporal expression of two IFN-γ induced molecules, HLA DR (Ia) and IP-10, during immune response in the cells of the human dermis and epidermis.

Materials and Methods

The Generation of a DTH Response to PPD. After informed consent, we evaluated the delayed response to 5 U of PPD in 65 lepromatous leprosy patients from New Delhi, India (a high endemic area for tuberculosis). The study group included 45 lepromatous (LL) and 20 borderline lepromatous leprosy patients (BL) (9). The leprosy patients were examined in collaboration with Drs. A. K. Sharma and R. S. Mishra, at the Department of Dermatology, Safdarjung Hospital, New Delhi. Clinical diagnosis was accompanied by

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[1] *Abbreviations used in this paper:* αMEM, α-modified Eagle's medium; βTG, β-thromboglobulin; HUVE, human umbilical cord endothelial cells; PF4, platelet factor 4; PPD, purified protein derivative of tuberculin.

a histopathological diagnosis established by Dr. I. Nath of the Department of Pathology All India Institute of Medical Sciences, New Delhi. Antigen was injected intradermally into the skin of the back. Two 4-mm punch biopsies, one from the PPD-injected site and one from an uninjected adjacent site, were taken at 18, 41, 68–72, and 96 h, and at 1 and 2 wk after injection from lepromatous and borderline lepromatous patients. The biopsies were fixed as described below and transported to the United States for further processing.

Leprosy Patients. After informed consent, skin biopsies from 10 untreated patients from Brazil with various forms of leprosy were collected and examined. The Brazilian patients were examined in collaboration with Dr. E. N. Sarno of the Department of General Pathology, Hospital de Clinicas, Universidade do Estado do Rio de Janeiro. Clinical diagnosis was accompanied by a histopathological diagnosis established by Dr. E. N. Sarno according to the Ridley-Jopling classification (9).

Fixation and Processing of Cutaneous Biopsies. Skin biopsies were fixed in paraformaldehyde (3%), lysine (0.075 M), and sodium-*m*-periodate (0.01 M) in PBS for 4 h at 4°C as described by McLean and Nakane (10–11). The biopsies were incubated in PBS containing sucrose (10%) and digitonin (0.05 mM) and then serially suspended in graded solutions of sucrose (15–25%). The biopsies were stored in PBS with sucrose/glycerol (25 and 5%, respectively) until frozen.

Immunocytochemical Staining of Sections. The biopsies were embedded in OCT compound and frozen at −20°C. 6–8-μm sections were cut on a cryostat and applied to gelatin coated multiwell slides (Carlson Scientific Inc., Peotene, IL). The sections were dried overnight at 37°C, rehydrated in PBS and incubated with mouse mAbs or rabbit anti–rIP-10 antibodies followed by biotinylated horse anti–mouse Ig or goat anti–rabbit Ig and then avidin-biotin peroxidase complexes (Vector Laboratories, Inc., Burlington, CA). The reaction product was developed with 0.8 mg/ml 3-amino-9-ethylcarbazole in 0.02 M Tris buffer, pH 7.6, and 0.015 H_2O_2. The controls for IP-10 binding were: (*a*) normal rabbit serum, (*b*) absence of primary antibody and (*c*) antiserum against the 22-amino-acid COOH-terminal peptide of IP-10, which does not precipitate the intact molecule from induced cells (4). The controls for mAbs included the omission of the primary antibody. None of the controls gave any staining (see figures). Sections were counterstained with hematoxylin.

Monoclonal Antibodies. Mouse anti–human mAbs were used for the identification of specific cell types. OKT6 (anti-thymocyte and Langerhans' cells) was obtained from Ortho Diagnostics Systems Inc. (Raritan, NJ) (12). 9.3F10 (anti-HLA class II) was produced in this laboratory (13). The cell line 63D3 (anti-monocyte/macrophage) was obtained from American Type Culture Collection (Rockville, MD) (14). Leu-1 (anti–pan-T lymphocyte), Leu-2a (anti–suppressor/cytotoxic T lymphocyte), and Leu-3a (anti–helper T lymphocyte) were obtained from Becton Dickinson Monoclonal Centers, Inc. (Mountain View, CA) (15–16). Adjacent sections were evaluated for specific cell staining with a Nikon Microphot-FX light microscope.

Cell Culture. A primary keratinocyte cell line isolated from human foreskin was obtained from Clonetics Corporation (Boulder, CO) and maintained in a defined keratinocyte growth medium (17). Experiments were carried out on fourth-passage cells. The human fibroblast cell line FS4 was grown in α-modified Eagle's medium (αMEM) with 10% FCS and antibiotics. Human endothelial cells were isolated from umbilical cord veins (18) and grown in M199 medium supplemented with 20% heat-inactivated human serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All the experiments were performed on second-passage human umbilical cord endothelial cells (HUVE). PBMC were isolated from venous blood fractionated on a Ficoll-Hypaque gradient. Monocytes were isolated from these PBMC by Percoll gradient fractionation (19) and maintained in αMEM supplemented with 10% heat-inactivated autologous human serum or FCS, penicillin, and streptomycin.

All induction experiments were performed in the regular cell growth medium, using cells just before reaching confluence. Monocytes were induced at 10^6 cells/ml. The IFN-

γ was always diluted into medium containing 10% serum, except for keratinocytes where serum was omitted.

γ-Interferon. The IFN used in in vitro studies was highly purified recombinant protein synthesized in *Escherichia coli* generously provided by Genentech, Inc., So. San Francisco, CA. The endotoxin levels were determined in a Limulus amoebocyte lysate assay before shipping. The human rIFN-γ had a specific activity of $2–4 \times 10^7$ U/mg as determined in a human lung carcinoma A549 inhibition assay using the encephalomyocarditis virus. The lyophilized excipient and rIFN-γ (sp act, 2×10^7 U/mg protein) used in in vivo studies were obtained from Genentech, Inc., and diluted to a final concentration of 100 μg/ml.

Immunoprecipitation. Cells were lysed in PBS containing 1% NP-40, 0.2 U/ml aprotinin, 1 mM PMSF, and 0.1% diisopropylfluorophosphate (Sigma Chemical Co., St. Louis, MO) and prepared for immunoprecipitation as described (4). Affinity-purified antibodies were added to clarified cell lysates and cell supernatants to a final concentration of 0.5 μg/ml. The solution was incubated at room temperature for 4–16 h. Antigen-antibody complexes were precipitated by incubation with protein A–Sepharose, washed in buffer and detergent solution, and released from the beads by incubation at 100°C for 2 min in 2× PAGE sample buffer. 15% SDS-PAGE gels were run as described in the legend to Fig. 3. The densitometric scan (Spectrophotometer; Beckman Instruments, Inc., Fullerton, CA) was done on an autoradiogram exposed for 42 h for keratinocytes, 3 d for endothelial cells, 6 d for monocytes, and 3 wk for fibroblasts. These exposure times were selected to scan gels that were in the linear range of the x-ray exposures. Relative density units were calculated from the computerized area below the peak multiplied by the length of exposure of the gels in hours and standardized to 1 U for fibroblasts.

Results

A rabbit polyclonal antiserum raised against the IFN-γ-induced protein IP-10 (4) has been used in this study to demonstrate the expression of the peptide in DTH responses of human skin. Since the peptide is first expressed in the basal layer of the epidermis, the immunoperoxidase reaction was developed with 3-amino-9-ethylcarbazole, which gives a red stain easily distinguished from the brown color of melanin granules.

Staining of Normal Skin and Skin from the Lesions of Lepromatous Leprosy Patients. When normal human skin was examined for the expression of IP-10 and Ia antigen, no staining of the keratinocytes or any of the dermal macrophages, fibroblasts, or endothelial cells was observed. $T6^+$ Langerhans' cells of the epidermis, which express Ia antigen constitutively, did not stain for IP-10 (not shown).

Examination of biopsies from the skin lesions of lepromatous leprosy patients revealed a dermal accumulation of parasitized macrophages, often with a very foamy appearance, and a few lymphocytes. These cells expressed Ia antigen as did the $T6^+$ Langerhans' cells of the epidermis (Fig. 2*A*). However, no staining for IP-10 was observed in the cells of the dermis or the epidermis (Fig. 1*A*).

The DTH Response to PPD Injection. The intradermal injection of 5 U of PPD in previously sensitized lepromatous patients provided us with an excellent model for the study of delayed immune responses in humans. Temporal examination of the reaction site revealed a local accumulation of T lymphocytes and monocytes (Kaplan, G., A. Nusrat, S. Laal, et al., manuscript in preparation). A majority of the T cells that infiltrated the reaction site were of the OKT4/Leu-3a subset.

Staining of tissue sections of biopsies taken from 18 h to 2 wk after PPD injection enabled us to analyze the kinetics and persistence of expression of both

IP-10 and Ia antigen. In the dermis the induction of low levels of IP-10 was observed in scattered monocytes, fibroblasts, and endothelial cells by 18 h and even more prominantly by 41 h after PPD injection (Fig. 1). Staining persisted for at least 1 wk and usually subsided completely by 2 wk. The in vivo expression of IP-10 in these cells confirmed in vitro studies showing the induction of IP-10 after exposure of monocytes and endothelial cells to rIFN-γ (4, 6). Ia antigen, a molecule known to be induced by IFN-γ, was also expressed by 18 h on the newly infiltrating cells in the dermis.

Striking results were observed in the epidermis. By 18 h after the injection of PPD, single cells in the basal layer of the epidermis were clearly staining for IP-10 (Fig. 1*B*). At 41 h all the cells of the basal layer were stained (Fig. 1*C*). With time, stained cells were observed higher up in the epidermis until by about 1 wk all the keratinocytes were staining (Fig. 1*D*). The expression of Ia antigen paralleled the results for IP-10. At 18 h, fewer basal keratinocytes stained for Ia than for IP-10 (Fig. 2*B*). By 41 h many of the keratinocytes were Ia^+ (Fig. 2*C*) and by 72 h to 1 wk the entire epidermis was positive (Fig. 2*D*). Staining for Ia antigen usually subsided by 2 wk (not shown).

The staining of the keratinocytes for IP-10 appeared much more intense than the staining of the cells in the dermis. While the staining for IP-10 was clearly in the cytoplasm of the cells, Ia antigen was membrane associated. The changes in antigen expression were clearly restricted to the site of the DTH response.

The Response to Intradermal Injection of rIFN-γ. We have previously reported (7) that intradermal injection of low doses of rIFN-γ into the lesions of lepromatous leprosy patients mimics the DTH responses to antigen. In our present studies we have examined the effect of a single injection of 10 μg of rIFN-γ on the expression of IP-10 and Ia antigen. The local response to such treatment has generally been lower in intensity and duration than the response to 5 U of PPD.

Intradermal administration of the excipient had no effect on the cellular composition or phenotype of the cells (Figs. 1*E* and 2*E* compared to Figs. 1*A* and 2*A*). Neither IP-10 nor Ia antigens were expressed. However, a single injection of 10 μg of rIFN-γ induced the expression of IP-10 in the basal cells of the epidermis by 18 h (not shown) in a similar way to that observed with PPD. Very little if any IP-10 was observed in the dermal cells at this time. By 72 h many of the basal keratinocytes stained for IP-10 but in most patients the cells of the upper epidermis were not stained (Fig. 1*F*). IP-10 expression persisted in some cells for up to 4–5 d and was reduced or totally absent from most cells by 6–7 d. In some patients that gave a larger reaction to intradermal rIFN-γ (30–40 mm of induration) the IP-10 expression was more intense and persisted for at least 7 d, more like the response to PPD.

The expression of Ia antigen was also induced by intradermal injection of 10 μg of rIFN-γ but not by excipient alone (Fig. 2, *E* and *F*). By 18 h after injection a few patches of keratinocytes of the basal layer of the epidermis were Ia^+ (not shown). At 41 h extensive staining was observed and by 72 h the entire epidermis was often Ia^+ (Fig. 2*F*). Ia antigen expression persisted for at least 1 wk and had disappeared by 2–3 wk. In general, the staining for Ia appeared somewhat later than that observed for IP-10, but by 41 h it was more intense, appeared on more cells of the epidermis and dermis, and persisted for longer.

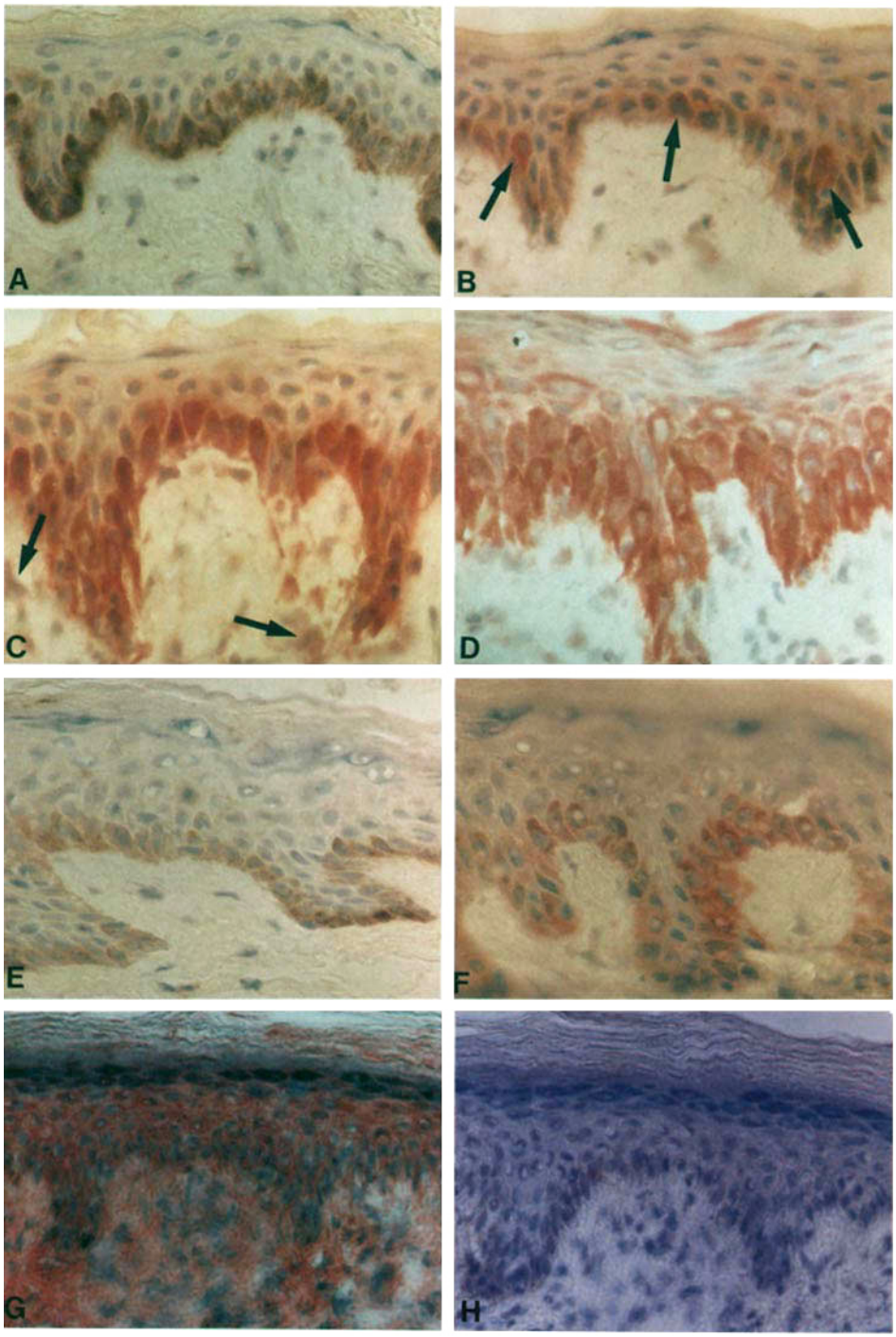

FIGURE 1. Immunohistochemical localization of IP-10 in human skin. (*A*) The skin of a lepromatous leprosy patient stained for IP-10. No red stain is seen in the dermis or the epidermis. (*B–D*) The delayed immune response to intradermal injection of PPD in lepromatous leprosy patients. (*B*) 18 h after administration of antigen, individual cells of basal layer of the epidermis are staining (*arrows*). (*C*) 41 h after antigen administration, all the cells of the basal layer of the epidermis are staining. Some red stain can also be seen in association with cells in the dermis (*arrows*). (*D*) 7 d after administration of antigen, most of the cells of the epidermis and some of the dermal cells are staining. (*E*) Skin lesion of lepromatous patient injected intradermally with 100 μl excipient alone (no rIFN-γ control) and biopsied 72 h after injection. No red stain is observed. (*F*) Skin lesion of lepromatous patient injected intradermally with 10 μg rIFN-γ in 10 μl of excipient and biopsied 72 h later. Some of the cells of the basal layer of the epidermis are staining. (*H–G*) Lesion of untreated tuberculoid leprosy patient stained for IP-10. (*G*) the cells of the epidermis and the dermis are intensely stained. (*H*) no primary antibody control, no red stain is observed. Magnifications: *A–F*, × 320; *G–H*, × 200.

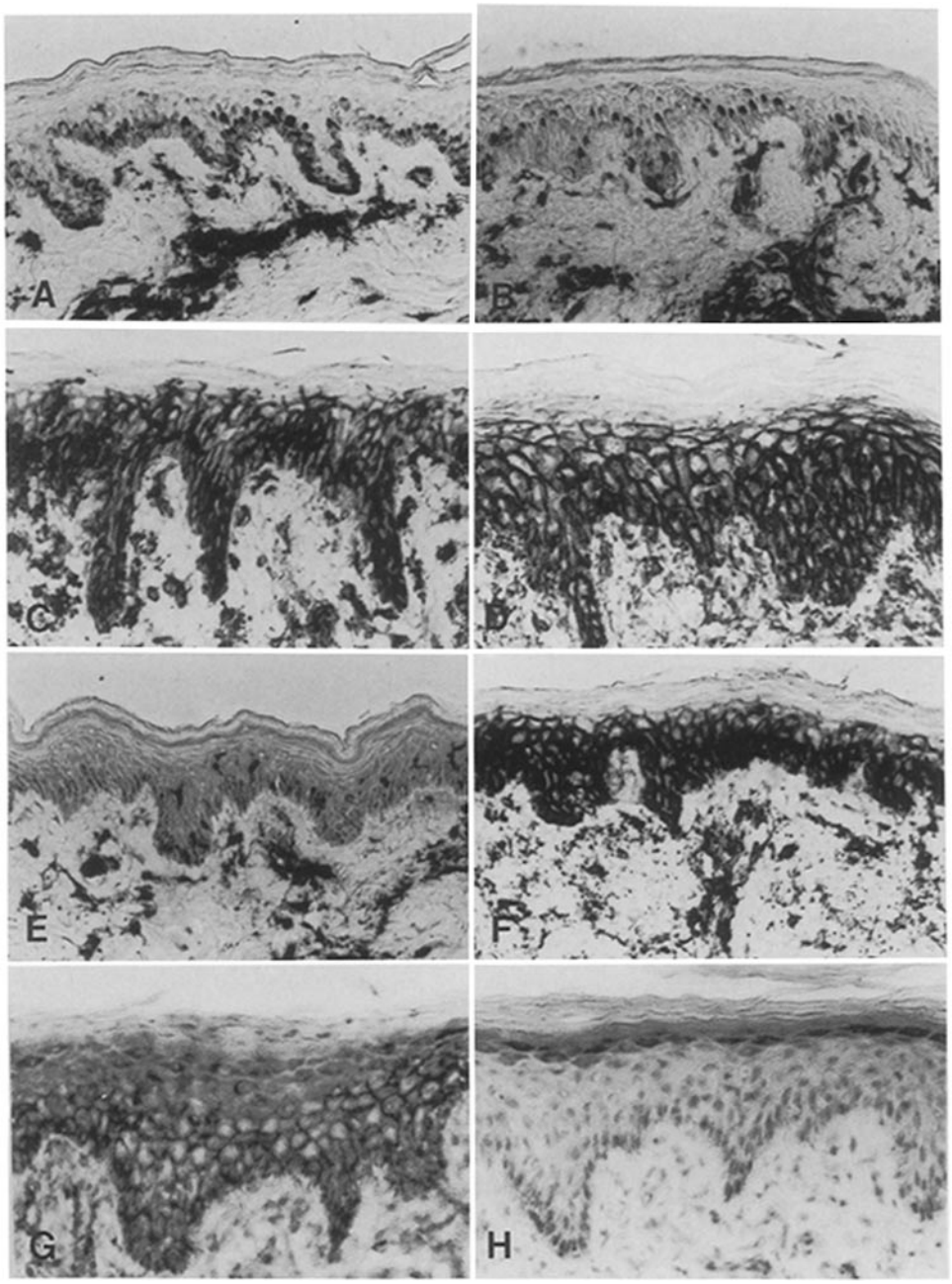

FIGURE 2. Immunohistochemical localization of Ia antigen in human skin. (*A*) The skin of a lepromatous leprosy patient stained for Ia antigen. The cells of the dermis are all staining for Ia antigen. The Langerhans' cells are the only cells staining in the epidermis, no stain is seen on the keratinocytes. (*B–D*) The delayed immune response to intradermal injection of PPD in lepromatous leprosy patients. (*B*) 18 h after administration of antigen, the cells of the dermis are staining and the majority of the cells of the basal layer of the epidermis are not yet staining. (*C*) 41 h after antigen administration, almost all the cells of the epidermis are staining. (*D*) 7 d after administration of antigen, most of the cells of the epidermis and the dermis are staining. (*E*) Skin lesion of lepromatous patient injected intradermally with 100 μl excipient alone (no rIFN-γ control) and biopsied 72 h after injection. No epidermal stain is observed. (*F*) Skin lesion of lepromatous patient injected intradermally with 10 μg rIFN-γ in 100 μl of excipient and biopsied 72 h later. All the cells of the epidermis are staining. (*H–G*) Lesion of untreated tuberculoid leprosy patient stained for Ia antigen. (*G*) The cells of the epidermis and the dermis are intensely stained. (*H*) No primary antibody control, no stain is observed. Magnification, × 200.

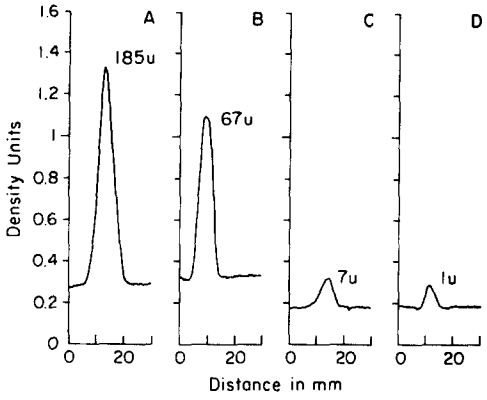

FIGURE 3. Inducible secretion of the IP-10 protein from human cells: immunoprecipitation analysis of primary fibroblasts, monocytes, endothelial cells, and keratinocytes. The cells were biosynthetically labeled with [^{35}S]methionine and then used for immunoprecipitation analysis as described in Materials and Methods. ~10^6 cells were used for each immunoprecipitation. The fibroblasts samples were analyzed by 15% SDS-PAGE under reducing conditions and the monocyte, endothelial cell, and keratinocyte samples were analyzed by 15% SDS-PAGE under nonreducing conditions. The autoradiograms were scanned, the area below the IP-10 peak was evaluated, and the relative density units were calculated as described in Materials and Methods. (*A*) Keratinocytes, gel exposed for 42 h; (*B*) endothelial cells, gel exposed for 3 d; (*C*) monocytes, gel exposed for 6 d; (*D*) fibroblasts, gel exposed for 3 wk.

IP-10 and Ia Expression in the Lesions of Tuberculoid Leprosy and Cutaneous Leishmaniasis. Patients with the tuberculoid form of leprosy, undergoing chronic DTH reactions in the epidermal lesions, have large numbers of T lymphocytes of the helper phenotype in their lesions (8) and express Ia antigen on the keratinocytes overlying the lesions (5). In these patients, the infiltrating cells of the dermis and the keratinocytes of the epidermis stained intensely for IP-10 (Fig. 1 *G*). This staining is in keeping with continued local production of IFN-γ by dermal T cells. In contrast to tuberculoid leprosy, the lesions of lepromatous leprosy show the absence of T helper lymphocytes and Ia antigen expression on keratinocytes of the epidermis and do not express IP-10 in the skin.

A similar observation was made when the chronic lesions of cutaneous Leishmaniasis were examined (not shown).

The Inducible Expression of IP-10 in Keratinocytes In Vitro. The strong staining reaction of keratinocytes as compared with other cell types suggested that keratinocytes might be a major producer of IP-10. To investigate the relative synthetic capacity of these cells we established in vitro cultures of human keratinocytes. The secretory and protein processing patterns were evaluated in parallel cultures of human monocytes, fibroblasts, endothelial cells, and keratinocytes. The cells were either left untreated or induced with 100 U/ml of rIFN-γ for 4 h and then biosynthetically labeled with [^{35}S]methionine for 10 h. The cell lysates and supernatants were immunoprecipitated using the anti-IP-10 antibodies and were analyzed by SDS-PAGE, and the amount of IP-10 immunoprecipitated was quantitated by gel scanning. The four cell types examined reveal a similar pattern of induction, secretion, and processing of the IP-10 protein (4). Keratinocytes secreted 185 density units as compared with endothelial cells (67 U), monocytes (7 U), and fibroblast (1 U) (Fig. 3). All the cells produced IP-10 only in the presence of rIFN-γ. ~94% of synthesized IP-10 was secreted by all cell types tested.

Discussion

Our knowledge of the stimulation of helper T cells and the concordant production of lymphokines comes largely from in vitro studies. In this milieu, incubation of resting T cells in the presence of antigen and the potent accessory cell, the dendritic cell or its equivalent, leads to the generation of the macrophage activating lymphokine IFN-γ. This and other soluble products are synthesized in proportion to the number of sensitized T cells that undergo blastogenesis. In the present in vivo experiments we have generated all the known components of this reaction within the confines of the human dermis. After the introduction of antigen into the dermis, large number of T cells of the helper phenotype emigate from the vasculature and, with monocytes, form the primary elements of the delayed immune response. In addition, $T6^+$ Langerhans' cells, presumably from the circulation, enter the lesion and could serve as potent accessory cells (20–23). As a result, lymphokines are produced and influence many cells in the dermal and epidermal environment. Our ability to monitor the production of lymphokines depends largely upon their modulation of other cells. In the case of IFN-γ we observe the prompt and extensive expression of class II MHC determinants on the surface of overlying keratinocytes, a process already described in some detail (5, 24–25). We assume that dermal T cells are the source of IFN-γ and that the lymphokine diffuses from the DTH reaction inducing Ia expression. Other responses to IFN-γ such as macrophage activation are less easy to observe, although the subsequent fate of intracellular *Mycobacterium leprae* to an evoked DTH reaction is currently under study.

We have now carried the analysis of the delayed immune response further through our ability to localize other molecules induced by IFN-γ. The discovery that IFN-γ induces the early expression of IP-10 allowed us to produce specific antibodies against the molecule and subsequently to identify it in tissue sections by immunocytochemical means. Temporal analysis of the cells and immune states in which IP-10 is expressed form the major portion of the present manuscript. Both monocytes, macrophages, and endothelial cells are stained for IP-10 in the dermis after the first 24 h after administration of stimulant. Although largely a secretory product, sufficient product accumulates within the cell body to allow its identification in sections. To our surprise, the most extensive staining occurred in the keratinocytes of the epidermis. First observed within the replicating cells of the basal layer, staining moved centripetally as the new keratinocytes populated the upper layers and matured. The intense in situ staining of keratinocytes is consistent with in vitro immunoprecipitation studies in which keratinocytes synthesize and secrete more IP-10 than either HUVE, monocytes, or fibroblasts.

The elucidation of the primary amino acid sequence of IP-10 (3) has allowed a comparison to other known proteins. IP-10 is homologous to a newly emerging family of chemotactic and mitogenic proteins associated with inflammation and cell proliferation including PF4; connective tissue activating peptide III (CTAP III), an unprocessed form of βTG; and a Rous sarcoma virus–induced protein, 9E3. These homologies suggest that IP-10 may share some of the functional properties with other members of this family. PF4 is a potent chemoattractant for both granulocytes and monocytes (26). Peptide CTAP III is a growth factor for fibroblasts (27), and 9E3 is induced by the Rous Sarcoma virus (28). Such

activities are of obvious interest in terms of the inflammatory response but require more definitive experiments with IP-10. What is apparent, however, is that IFN-γ, the product of the T cell, may through its induction of IP-10 recruit a broad spectrum of "nonprofessional cells" involving them in the cell-mediated response. Such recruitment if coupled with growth promotion and/or chemoattraction would magnify the immune response by means of what we could consider a "secondary cytokine." Perhaps the large amount of keratinocyte IP-10 serves as an autocrine growth regulator or promotes the directional flow of specific epidermal populations such as the Langerhans' cell.

Summary

Our knowledge of the induction of new molecules by IFN-γ has led to the characterization of IP-10 and the preparation of a monospecific, polyclonal antibody. Using this reagent we have now examined inflammatory states occurring in human skin and used immunocytochemical staining for the expression of both Ia and IP-10 determinants.

After evoking a delayed-type response to purified protein derivative of tuberculin (PPD), we noted the presence of IP-10 in dermal macrophages and endothelial cells. Intense staining of the basal layer of epidermal keratinocytes was prominent at 41 h, and by 1 wk the entire epidermis was staining. The comparison of the amount of IP-10 secreted by keratinocytes vs. macrophages, fibroblasts, and endothelial cells revealed that keratinocytes were by far the major producers of this molecule. The expression of Ia occurred in conjunction with IP-10. The injection of rIFN-γ mimicked many of the features of the PPD response, including the expression of both Ia and IP-10 by epidermal keratinocytes. Coexpression was also found in the natural lesions of tuberculoid leprosy and cutaneous Leishmaniasis. However, it was absent in lepromatous leprosy, a state where activated T lymphocytes are not present.

We suggest that the local production of IFN-γ by T cells of the dermal infiltrate induces IP-10 formation in both the dermis and epidermis. IP-10 and Ia then serve as specific markers of immune IFN and its possible influence on effector cells of the cell mediated immune response.

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