Interferon-inducible Protein 10 as a Possible Factor in the Pathogenesis of Cutaneous T-Cell Lymphomas

Andreas H. Sarris, Danai Dalani, Rose Ulmer, Mary Crow, Hal E. Broxmeyer, Michael Reiss, Nikos Karasavvas, Andrew D. Zelenetz, William Pugh, Fernando Cabanillas, Albert B. Deisseroth, and Madeleine Duvic

Departments of Hematology [A. H. S., F. C. I., Medical Specialties (D. D., M. C., M. D.), and Pathology [W. P.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; Department of Dermatology, The University of Texas Health Sciences Center Medical School, Houston, Texas 77030 [R. U., M. D.]; Departments of Medicine and Microbiology/Immunology and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202 [H. E. B.]; Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06510 [M. R., A. B. D.]; and the Lymphoma Service, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [N. K., A. D. Z.]

ABSTRACT

Human IFN-γ-inducible protein 10 (IP-10), a C-X-C chemokine secreted by IFN-γ-stimulated keratinocytes, is chemotactic for normal CD4-positive lymphocytes and inhibits the proliferation of early subsets of normal and of leukemic hemopoietic progenitors. Cutaneous T-cell lymphoma (CTCL) is an indolent lymphoproliferative disorder of CD4-positive lymphocytes that remain confined to the skin for many years before visceral dissemination. Because IFN-γ mRNA was detected in the epidermis of CTCL lesions, we decided to investigate the role of IP-10 in the epidermotropism of CTCL by determining its expression in normal skin and in CTCL lesions. Using purified recombinant IP-10 (rIP-10) or a recombinant fusion protein between IP-10 and the φ10 protein of phage T7, we generated rabbit antisera that recognized and neutralized rIP-10. Immunoperoxidase staining of normal epidermis demonstrated normal IP-10 immunostaining. In one patient who had matching biopsies performed before and after treatment, IP-10 was overexpressed before treatment but was normally expressed at remission. The in vitro proliferation of primary normal human keratinocytes was inhibited in a dose-dependent manner by rIP-10. These results suggest that IP-10 plays a role in the epidermotropism of CTCL. Additional work is needed to determine whether IP-10 stimulates or inhibits CTCL proliferation. A better understanding of the growth controls operating in CTCL may be useful in the development of curative strategies for this disorder.

INTRODUCTION

CTCL is an indolent lymphoproliferative disorder usually preceded by prolonged premalignant dermatitis. CTCL is almost invariably a disease of mature postthymic CD4-positive memory lymphocytes that have a cerebriform nucleus and rearranged T-cell antigen receptor genes. Pathologically, the diagnosis of CTCL is based on the presence of architectural atypia (band-like lymphoid infiltrate in the upper dermis and sterile Pautrier’s microabscesses in the epidermis) and of cytological atypia, i.e., the infiltrating lymphocytes appear malignant and have cerebriform nuclei (1). Either conservative measures such as treatment with topical alkylating agents, UV light, low-dose methotrexate, and spot radiotherapy or a combination of intensive chemotherapy and total skin electron-beam radiotherapy can induce remissions that are, however, not durable in most patients (2). The disease remains limited to the skin for many years before it extends to lymph nodes and viscera or transforms to large cell lymphoma (3).

IP-10 belongs to the chemokine family that includes small secreted basic proteins of approximately 7–10 kDa with chemotactic properties against neutrophils, monocytes, and lymphocytes (4). Human IP-10 was cloned from a cDNA library of U-937 cells treated with IFN-γ (5) but can also be induced by lipopolysaccharide, TNF-α, IFN-α, IL-1α, and IL-6 (6–11). Highly purified rIP-10 inhibits the proliferation of early subsets of normal hemopoietic progenitors (12–14) and of leu-

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1 The abbreviations used are: CTCL, cutaneous T-cell lymphoma; IP-10, IFN-γ-inducible protein 10; rIP-10, recombinant IP-10; TNF-α, tumor necrosis factor α; IL, interleukin; IPTG, isopropyl-1-thio-β-D-galacto-pyranoside; HTLV, human T-cell lymphotropic virus; CI, confidence interval; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein; ICAM, intercellular adhesion molecule; PF-4, platelet factor 4; LFA-1, lymphocyte function-associated antigen 1; Gro-α, melanoma growth stimulating factor α.
kemic progenitors from untreated patients with acute myelogenous leukemia (15). The inhibitory activity of rIP-10 is temporally associated with increased cAMP levels and with the inhibition of Raf-1 kinase in the factor-dependent leukemic cell line MO7e (16). Human rIP-10 inhibits angiogenesis in vivo (17) and is chemotactic for human monocytes and CD4-positive lymphocytes both in vitro (18) and in vivo (19) but is inactive for neutrophils (20, 21). The chemotactic activity of IP-10 may account for its in vivo T-cell-dependent antitumor activity (22).

We were intrigued by the report that human CTCL epidermis contains IFN-γ mRNA (23), which can induce keratinocytes to secrete IP-10 (24). We subsequently presented evidence implicating IP-10 secretion by keratinocytes in the epidermotropism of CTCL (12). We now confirm and extend these observations by using new antisera and studying a larger group of patients, and in addition, we investigate the role of IP-10 in keratinocyte proliferation. Finally, we discuss these findings in relation to a model for the pathogenesis of CTCL that may be used as a basis for the systematic laboratory investigation of cytokine loops and the development of curative strategies for CTCL.

**PATIENTS AND METHODS**

**Proteins, Cytokines, and Chemokines.** The sources of the cytokines and chemokines we used were reported previously (12, 13). MCDB153-LB powdered medium, hydrocortisone, ethanolamine, phosphoethanolamine, supplementary amino acids, and triiodothyronine were purchased from Sigma Chemical Co. (St. Louis, MO). Epidermal growth factor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), insulin and transferrin were obtained from Collaborative Research (Bedford, MA), and bovine pituitary extract was obtained from Hammond Cell Technology (Alameda, CA).

**Antigens.** Human rIP-10 purified from baculovirus or *Escherichia coli* was used to generate antisera AS522 as described previously (12, 13). AS522 antisera was generated by immunizing rabbits with rIP-10, a recombinant fusion protein between the amino-terminal part of the pI10 protein of bacteriophage T7 and IP-10. To generate this fusion protein, we digested plasmid pET-3xa (25) with *NdeI* and calf intestinal phosphatase and ligated to it an equimolar mixture of the oligonucleotides TATGCATCACCATCACCATCACGC and TAGCGTGATGTGATGGTGATGCA, designed to insert an initiating methionine, followed by six histidines and an alanine at the amino-terminal end of pI10. The mixture was transformed into *E. coli* strain HMS174, and several transformants were sequenced to confirm the presence of only one insert in the correct orientation and to exclude cloning artifacts. One clone (AHS-1) was selected for additional expression studies. Direct DNA sequencing confirmed the insertion of the expected sequences in AHS-1 and predicted the expression of a mutant pI10 protein starting with an initiating methionine followed by six histidines, an inserted alanine residue, and finally by the open reading frame encoding the first 257 residues of the pI10 protein of bacteriophage T7. This is followed by a *BamHI* site that allows in-frame insertion of DNA sequences at the carboxy-terminal end and by a termination codon.

The cDNA encoding IP-10 (including the signal sequence) was then cloned in frame into the *BamHI* site of AHS-1 to generate a fusion gene coding for 265 residues of the modified rIP-10 protein followed by the 98 residues of IP-10, including the signal sequence. The DNA region of the fusion between pI10 and IP-10 was sequenced to exclude cloning artifacts. Lysozyme containing the cytokines and chemokines we used were reported previously (26, 27). Because 4IP-10 was a high-value protein, which can induce keratinocytes to secrete IP-10 (24).

**Generation and Characterization of Antisera.** The generation and characterization of AS522 have been described previously (12). To generate AS526, the rIP-10 fusion protein (500 µg) was emulsified with Freund’s complete adjuvant and injected intradermally in rabbits, who were given 250-µg boosters emulsified in incomplete Freund’s adjuvant every 2 weeks. Sera were stored frozen at −20°C in small aliquots and thawed and diluted in PBS containing 0.02% sodium azide before use. The method of detecting antibodies against rIP-10 by ELISA, by Western blotting, and by neutralization of bioactive rIP-10 using colony assays for normal hemopoietic progenitors has been described (12, 13).

**Patients and Biological Specimens.** Patients referred to The University of Texas M. D. Anderson Cancer Center were included in this study if the diagnosis of CTCL was confirmed by a combination of all available data, including clinical presentation, microscopic examination of paraffin-embedded tissue, immunophenotypic analysis, and detection of clonal T-cell antigen receptor gene rearrangements by Southern blotting, and if frozen punch-biopsy specimens were available for immunocytochemistry. The criteria used at our institution for the distinction of CTCL from lymphomatoid papulosis have been described previously (28). The appropriate signed informed consents were obtained before all biopsies, as required by institutional guidelines. Clinicopathological staging of CTCL was determined according to the National Cancer Institute criteria in a conference with participation of hematologists, pathologists, radiotherapists, and radiologists (29).

**Immunocytochemical Analysis.** Immunoreactive IP-10 was detected on frozen punch biopsies as described previously (13). Sections of normal human epidermis were incubated with normal rabbit sera (negative control) and with AS526 (positive control). At least one section derived from a 6-mm CTCL punch biopsy was stained and examined in its entirety under low-power (×10) and high-power objectives (×50 or ×100) in a nonblinded fashion by both A. H. S. and M. D. The final scoring of all specimens was performed by a joint examination of all specimens by A. H. S., M. D., and D. D. Normal skin or CTCL lesional biopsies demonstrated no immunoperoxidase staining.
Expression of recombinant φIP-10 and characterization of antiserum AS526. A. induction and φ10 or φIP-10. Transformed BL21(DE3) cells were grown to an absorbance of 0.7 before the addition of IPTG to a final concentration of 0.4 msi. The indicated times, an aliquot was withdrawn, boiled in sample butter, analyzed by SDS-PAGE, and stained with Coomassie Blue. M, molecular weight markers, with sizes in thousands shown to the left. Numbers at the top of each lane, elapsed time (in h) after the addition of IPTG. φ10, cells transformed with vector AHS-1. φIP-10, cells transformed with AHS-1 containing the fusion gene φIP-10. B. characterization of AS526. Left panel, silver stain; right panel, Western blot of two duplicate gels. AS526 and AS522 were used at a dilution of 1:1000 as described previously (12). The source of the specimens is indicated at the top of each lane. M, molecular weight marker proteins. Lane 1, supernatant of keratinocytes induced by IFN-α for 24 h; Lane 2, supernatants of S9 cells infected with recombinant baculovirus A221 encoding full-length IP-10 (12). Lane 3, E. coli BL21(DE3) cells transformed with pET-3d coding for rIP-10 (12) were grown to an absorbance of 0.7 and then induced with 0.4 mM IPTG. Two h later, an aliquot of the culture was analyzed by SDS-PAGE. Lane 4, same as Lane 3, but developed with AS522. Lanes 1–3 were developed with AS526. The immunoreactive band around M, 18,000 in Lanes 3 and 4 represents dimeric IP-10 (M. Crow and A. H. Sarris, unpublished observations). The numbers on the left indicate the molecular weights of the marker proteins in thousands. Right, the position of full-length secreted rIP-10 without the signal sequence is indicated.

Detection of native rIP-10 by ELISA. Highly purified bioactive rIP-10 was used with antisera AS522 or AS526, which were diluted 1000-fold. Chemokines, overlapping signals generated by 10 ng of recombinant MCP-1, recombinant MIP-1α, MIP-1β, recombinant MCP-2, recombinant MCP-3, RANTES, recombinant IL-8, recombinant GRO-α, and C-10.

Neutralization of the biological activity of rIP-10 by excess AS522 or AS526. The results are expressed as the means of triplicate determinations. Bars, SE. SLF, steel factor; GM-CSF, granulocyte macrophage colony-stimulating factor.
Table I

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* NCI, National Cancer Institute.

a Immunoreactivity was determined by staining with AS522 or AS526 diluted 200–400-fold in several different experiments.

b Stage could not be determined because CTCL coexisted with diffuse large cell lymphoma.

c SS, Sézary syndrome. Patients 21–29 had Sézary syndrome characterized by malignant lymphocytes in the peripheral blood and simultaneous cutaneous patches, plaques, or tumors.

d Western blot analysis revealed epidermotropism and sterile Pautrier’s microabscesses in all nine patients with Sézary syndrome.

RESULTS

To facilitate the purification of antigens, we generated a new bacterial expression vector by adding 6 histidine residues to the amino terminus of the 610 protein in vector pET-3xa. The addition of IPTG induced rIP-10 and rIP-10, which after 2–3 h of induction accounted for 20–30% of stainable bacterial protein (Fig. 1A).

The insoluble fusion protein rIP12IP-10 was solubilized in guanidine hydrochloride and rapidly purified to homogeneity by nickel-agarose affinity chromatography under denaturing and reducing conditions that are expected to eliminate protein-protein interactions. The purification was completed within 2 days, with a yield of approximately 60 mg from 1 liter of bacterial culture, which is 5–10 times higher than that of rIP-10 isolated from the same bacterial expression system (12). The in-frame incorporation of Met (His)6Ala in the amino-terminal of the rIP10 fusion protein was confirmed by limited amino-terminal sequencing of the purified protein (data not shown).

Although purified rIP-10 was insoluble in aqueous buffers, it remained soluble in 4 M urea, allowing emulsification in Freund’s adjuvant, subsequent injection into rabbits, and generation of antiserum AS526. Western blotting demonstrated that AS526 and AS526 diluted 200–400-fold in several different experiments. The subjects of this study were 29 previously untreated patients with CTCL who had clinical skin involvement and pathologically proven epidermotropism (Table 1). None of them were seropositive for HIV-1/2 or for HTLV-I/II. The National Cancer Institute stage was I in 12 patients, II in 7 patients, and IV in 9 patients and could not be determined in 1 patient. Circulating Sézary cells and epidermotropic lymphocytes were seen in the cutaneous biopsies of all nine patients with stage IV disease and Sézary syndrome even when not clinically apparent.
Fig. 4 Immunoperoxidase staining of IP-10 in frozen sections from skin biopsies of CTCL lesions. A and B, normal skin stained with AS522 and AS526, respectively. C, clinically normal skin from a patient with CTCL. D, E, and F, CTCL lesions. In D and E, arrowheads, locations of Pautrier’s microabscesses; arrows, malignant lymphocytes in the dermis. F, sweat gland in a lesion of a patient with CTCL, showing IP-10 overexpression of the ductular epithelial cells but not in the surrounding lymphoid infiltrate (arrows). C, D, E, and F were stained with AS526. All panels were photographed with a ×10 objective.

Fig. 5 Immunoperoxidase staining of IP-10 in frozen sections from skin biopsies of CTCL lesions. Arrowheads, lymphocytes infiltrating the epidermis either singly or in groups (Pautrier’s sterile microabscesses). Panels were photographed with ×10 (A) or ×100 (B, C, and D) objectives.
174 IP-10 as a Possible Factor in the Pathogenesis of CTCL

Effect of rIP-10 concentration on the day 4 cell count.

A. Cells treated with rIP-10 (50 ng/ml), and both medium and IP-10 were replaced every other day. At 48-h intervals, cells were detached by trypsinization and counted with a Coulter counter (model ZM; Coulter Electronics, Luton, United Kingdom). Statistically significant differences in cell numbers between cells treated with rIP-10 and vehicle only were noted after 4, 6, 8, and 10 days (P < 0.05 by Student’s two-tailed t test). The results shown are the mean and SE of three independent experiments. B. The effect of rIP-10 concentration on the day 4 cell count.

**Fig. 6** Effect of rIP-10 on the growth of cultured normal primary human keratinocytes. A, cells were plated in 24-well dishes at a density of 10^4 cells/well. Forty-eight h later, the cells were treated with rIP-10 (50 ng/ml), and both medium and IP-10 were replaced every other day. At 48-h intervals, cells were detached by trypsinization and counted with a Coulter counter (model ZM; Coulter Electronics, Luton, United Kingdom). Statistically significant differences in cell numbers between cells treated with rIP-10 and vehicle only were noted after 4, 6, 8, and 10 days (P < 0.05 by Student’s two-tailed t test). The results shown are the mean and SE of three independent experiments. B. The effect of rIP-10 concentration on the day 4 cell count.

Immunohistochemical staining of normal skin with AS522 and AS526 demonstrated immunoreactive IP-10 in basal keratinocytes (Fig. 4, A and B) with a distribution similar to that previously reported for normal skin (13, 24). Staining of normal skin with AS526 revealed that basal keratinocytes stained strongly, whereas suprabasal keratinocytes stained very faintly. There was no staining in the absence of primary serum or with nonimmune rabbit serum (data not shown). In CTCL lesions, the epidermis overlying the malignant lymphoid infiltrate was often hyperplastic but was normal over clinically uninvolved skin. Immunoperoxidase staining revealed increased IP-10 expression in basal as well as suprabasal keratinocytes (Fig. 4, D–F, and Fig. 5, A–D) in 28 of 29 CTCL patients, for an overall frequency of 97% and 95% CI of 82–100% (Table 1). Immunoreactive IP-10 was undetectable in the malignant lymphocytes (Fig. 4, D–F, and Fig. 5, A–D) of 26 patients and was detected in the lymphocytes of 3 patients for an overall frequency of 10%, with 95% CI of 2–27%. As shown in Table 1, expression of IP-10 was limited to the lymphocytes of patients with Sézary syndrome, in which it was detected in three of nine patients for a frequency of 33% and 95% CI of 8–70%. In the one patient who had had biopsies of the same lesion at presentation and after therapy-induced remission, the IP-10 immunoreactivity was initially increased but was normal after treatment (Table 1, patient 6). Clinically uninvolved skin from patients with CTCL exhibited normal IP-10 immunostaining.

Culture of primary human keratinocytes in the presence of rIP-10 resulted in inhibition of proliferation that was evident after 2 days of exposure to rIP-10 (Fig. 6A) and was statistically different by Student’s two-tailed t test. When day 4 counts were examined as a function of the concentration of rIP-10, a dose-response effect was seen with an apparent plateau at concentrations higher than 12.5 ng/ml (Fig. 6B). This dose-response curve is very similar to that obtained by others (12, 13) with normal hemopoietic progenitors and confirmed in this study (Fig. 3) as well as the dose-response reported with leukemic progenitors (15) and the factor-dependent cell line M07e (16), suggesting that a similar receptor is operative in all these systems.

**DISCUSSION**

We used bacterial protein expression systems to facilitate the purification of appropriate recombinant proteins to generate and characterize two rabbit antisera that specifically recognized and neutralized rIP-10. These antisera recognized only rIP-10 in supernatants of cultured keratinocytes and did not cross-react with the other chemokines (recombinant RANTES, recombinant MCP-1, recombinant MIP-1α, and recombinant MIP-1β), which are known to be chemotactic for lymphocytes (32). Immunoperoxidase staining of punch biopsies with these antisera revealed qualitative and quantitative differences in the patterns of IP-10 immunoreactivity between CTCL and normal skin. The IP-10 immunoreactivity was more intense in CTCL lesions and extended to the suprabasal keratinocytes, which were often hyperplastic, forming a thickened epidermis over the lymphoid infiltrates that are known to contain both benign and malignant lymphocytes. Although IP-10 can be secreted in vitro by monocytes and by activated lymphocytes (33), it was detected in the malignant lymphocytes of 10% of all patients with CTCL but in 33% of the subgroup with Sézary syndrome. Therapy-induced remission resulted in resolution of the epidermal hyperplasia and in normalization of IP-10 immunoreactivity in areas of prior cutaneous lesions.

The known in vitro (18) and in vivo (19) chemotactic activity of rIP-10 for normal CD4-positive lymphocytes and the localization data presented here provide a basis for the epider-
motropism and the multiple skin relapses of CTCL. In addition, the increased expression of IP-10 only by the keratinocytes overlying the malignant lymphoid infiltrates (13) suggests that an inducer of IP-10 synthesis is secreted by CTCL cells. The inhibition of keratinocyte proliferation by highly purified rIP-10 is similar to that of MIP-1α (34).

We proposed a model (13) for the pathogenesis of CTCL based on the trafficking of normal lymphocytes (Ref. 35; Fig. 7) in which epidermal keratinocytes, malignant lymphocytes, cytokines, and IP-10 play crucial roles. Skin-homing memory lymphocytes have been imprinted to return to the place where they first encountered antigen (36). CTCLs that arise by malignant transformation of memory CD-4 positive lymphocytes obviously retain this tropism. The nature of the antigen causing skin tropism is unknown, but there are indications that it may be staphylococcal superantigens (37). The first step in the homing cascade is the binding of cutaneous lymphocyte-associated antigen to E-selectin, which is present on dermal endothelial cells in delayed hypersensitivity and in the chronic inflammatory reactions that often precede frank CTCL (35). This binding causes the lymphocytes to attach to the surfaces of luminal endothelial cells and is followed by the second step, lymphocyte activation, which occurs as the loosely bound lymphocytes encounter IP-10 secreted by endothelial cells or keratinocytes (22, 38). Lymphocyte activation by IP-10 is presumed to cause conformational changes in cell-surface integrins and to allow strong interaction with adhesion molecules present on the surfaces of endothelial cells, which is followed by extravasation.

Fig. 7 The cytokine loops in CTCL. Interactions of keratinocytes, malignant lymphocytes involving secreted cytokines, IP-10, and various vascular adhesion molecules and ICAMs. CLA, cutaneous lymphocyte antigen; VCAM, vascular endothelial adhesion molecule. +, stimulation; −, inhibition; VLA, very late activation. The ligand for cutaneous lymphocyte-associated antigen 1 in the dermis and epidermis is not known, and this is indicated by ? on the surface of the keratinocyte. The line crossing the membrane of the CTCL cells represents the putative chemokine receptor for IP-10, which has not been cloned yet. The presence of IFN-γ and TNF-α in CTCL lesions has been demonstrated by reverse-transcription PCR and immunocytochemistry (51). The chemotaxis of CTCL to epidermis can be accomplished if IFN-γ or the other IP-10 inducers (TNF-α, IL-1α, or IL-6) are secreted by the reactive and/or the malignant lymphocytes.
and the binding of lymphocyte integrins to ICAMs in the dermis and epidermis. The attraction and retention of lymphocytes in the dermis or epidermis are presumed to be caused by the secretion of IP-10 by keratinocytes in CTCL lesions, which in turn may be induced by IFN-γ (23, 39, 40), IL-1α (41), and TNF-α (42), which have been detected in CTCL lesions. Epidermal LFA-1 and ICAM-1 have also been detected in epidermotropic CTCL (40, 43) but not in nonepidermotropic Sézary syndrome (40).

The stimulus that sustains the secretion of IFN-γ, IL-1α, and TNF-α is unknown. However, the tax/rex region of HTLV-1 is an intriguing candidate because it can immortalize T-cells (44) and can also induce the IFN-α promoter in lymphoid cells (45). Thus, in HTLV-1-associated CTCL, the tax gene may cause IFN-γ secretion and the overexpression of selectins by CTCL cells (46), thus causing the secretion of IP-10 by endothelial cells and keratinocytes and, in this manner, causing the observed epidermotropism. It is unclear whether tax is responsible for IFN-γ and TNF-α expression in most patients with CTCL, who have no serological evidence of HTLV-I infection detectable by routine immunological methods. However, this mechanism may operate in some of these patients, in whom the tax gene has been detected by PCR amplification (47–50).

The relatively frequent expression of IP-10 by lymphocytes of patients with Sézary syndrome may explain the systemic circulation of these malignant lymphocytes by abolishing the chemotactic gradients of IP-10. However, the persistent epidermotropism seen in these patients suggests that either the resulting IP-10 levels are not high enough to mask chemotactic gradients or that other chemotactic cytokines contribute to the observed epidermotropism.

Our model emphasizes the interactive participation of both keratinocytes and malignant lymphocytes and provides a framework for systematic investigation of the roles played by cytokines, IP-10 (and possibly other chemokines), selectins, integrins, and other adhesion molecules in the biology of CTCL. We hope that an improved understanding of the growth of CTCL will help in the identification of new therapeutic agents with novel mechanisms of action, which are sorely needed because at present there is no curative treatment for most patients with CTCL.

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