Communication

Induction of Interferon-γ Inducing Factor in the Adrenal Cortex*

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Interferon-γ inducing factor (IGIF) is a recently identified cytokine also called interleukin-1y (IL-1y) or interleukin-18 (IL-18). Its biological activity is pleiotropic, and, so far, it has been shown to induce interferon-γ production in Th1 cells, to augment the production of granulocyte-macrophage-CSF, and to decrease that of interleukin-10 (IL-10). We first detected newly synthesized IGIF mRNA by differential display in the adrenal gland of reserpine-treated rats and then isolated two transcripts by reverse transcription polymerase chain reaction. They were identified as rat IGIF on the basis of the high homology with mouse: 91% at both the nucleotide and the amino acid level.

Subsequently, we investigated the effects of stress on IGIF mRNA levels and found that acute cold stress strongly induced IGIF gene expression. In situ hybridization analysis showed that IGIF is synthesized in the adrenal cortex, specifically in the zona reticularis and fasciculata that produce glucocorticoids. The presence of IGIF mRNA was also detected in the neurohypophysis although induction by stress was not significant. Our results call for more attention to the role of the adrenal gland as a potential effector of immunomodulation and suggest that IGIF itself might be a secreted neuroimmunomodulator and play an important role in orchestrating the immune system following a stressful experience.

A growing number of findings support the idea that the nervous, endocrine, and immune systems form an integrated network of fundamental importance for the homeostasis of the organism (1). Communication among these systems is possible because they share ligands and receptors previously thought to be tissue-specific.

Interferon-γ inducing factor (IGIF) is a newly identified cytokine first isolated from Kupffer cells of mice injected with Propionibacterium acnes and challenged with lipopolysaccharide (LPS) to induce toxic shock (2–4). The names of interleukin-1y (IL-1y) and of interleukin-18 (IL-18) also have been proposed on the basis of homology with the structure of human interleukins-1 (5) and of IGIF’s peculiar activity (6), respectively. Mouse IGIF has been shown to induce interferon-γ production by Th1 cells, to stimulate natural killer cell (NK) proliferation, and to mediate inflammatory tissue damage (2). Further studies with human IGIF (6) showed this cytokine increased the production of granulocyte macrophage-colony-stimulating factor and decreased that of interleukin-10 (IL-10). Taken together, these data imply that IGIF is a potent cytokine involved in the cell-mediated immune response and might serve as an antimicrobial and antigen agent.

Here we report the unanticipated localization of IGIF mRNA in rat adrenal cortex and neurohypophysis. Interestingly, IGIF mRNA is strongly induced in the adrenal cortex after cold stress or reserpine treatment, in the absence of any exogenous infectious or inflammatory challenge. These findings suggest IGIF might play a major role as neuroimmunomodulator.

EXPERIMENTAL PROCEDURES

All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University Medical College. Male Sprague-Dawley rats weighing 300–500 g from Charles River Breeding Laboratories (Boston, MA) were used. For the pharmacological treatment, they received subcutaneous injections of reserpine (Sigma), 10 mg/kg in 20% ascorbic acid, or an equivalent volume of the vehicle, 4 h prior to anesthesia and perfusion. Cold stress was given by placing the animals, still in their cage, at 4 °C for 4 h. Access to food and water was free, and the light/dark cycle was maintained at 12 h/12 h.

For tissue collection animals were decapitated after treatment and adrenal glands were rapidly dissected, frozen in liquid nitrogen, and stored at −80 °C until extraction of RNA.

Differential Display—Differential display was performed as described previously using the RNAImage system (GenHunter Corp., Brookline, MA) (7). In brief, mRNA was extracted using the PolyATtract System 1000 (Promega) from adrenal gland of reserpine or vehicle-treated animal dissected 4 h after the injection. The RNA was then treated with RNase-free DNase I (GenHunter Corp.) and reverse-transcribed using the three one-base anchored oligo(dT) strategy (8). PCR was performed in the presence of [α-35S]dATP and oligonucleotides specifically designed for differential display (7). PCR conditions were 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 2 min, and extension at 72 °C for 30 s; the last extension step at 72 °C was prolonged for 5 min. Radiolabeled reaction products were subjected to electrophoresis on a 6% denaturing polyacrylamide/urea gel. The differently expressed PCR products were excised from the gel, reamplified by PCR and subcloned into the pCR-TRAP cloning vector (GenHunter Corp.). Inserts were sequenced by dideoxynucleotide sequencing.

Cloning and Probe—Rat IGIF was isolated by RT-PCR from the adrenal gland of a reserpine-treated animal. Two μg of total RNA were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of oligo(dT)12-18. The cDNA obtained was amplified with a mouse specific 5′-primer (5′-ACAAATGGGCTCCATGTA-3′) and a rat specific 3′-primer (5′-AGTGGAACATCAGATTTATCG-3′). The amplification was performed in 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; the last extension step at 72 °C was prolonged for 5 min. The amplified cDNA was purified from agarose gel, subcloned in pCR-TRAP cloning vector, and sequenced by the dideoxynucleotide method.

Rat IGIF probe was obtained by PCR using the pCR-TRAP clone containing the subcloned rat IGIF as template. The primers used for the amplifications were the same 3′-primer utilized for the cloning and a new 5′-primer internal to the isolated fragment: 5′-ACGTGACAAACGGCAGTAACCGG-3′. PCR conditions were the same as described above.
The amplified fragment (437 bp in length) was purified from agarose gel, labeled with $[^35S]dATP$ using the random primer method, and used as a probe for in situ hybridization.

**In Situ Hybridization—**In situ hybridization was performed as described (9). In brief, animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline containing 0.5% sodium nitrate and 10 units/ml heparin sulfate followed by cold formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The tissue was immersed in the fixative for 1 h and stored in 30% sucrose overnight. Free floating sections (40 μm thick) were placed in vials containing 2 ml of cold formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The sections were fixed in Kodak fixer, counterstained with cresyl violet, and dehydrated, and coverslipped.

Sections were washed in serial dilutions of SSC at 48°C starting with 2× SSC, ending with 0.1× SSC. After a 15-min wash in 0.05 M phosphate buffer, hybridization was carried out overnight at 48°C. The sections were subsequently dipped in Kodak NTB-2 emulsion and exposed for 2 weeks. After developing in Kodak D-19 developer at 16°C, slides were exposed to Kodak XAR-5 film at 4°C. Slides were subsequently dipped in Kodak NTB-2 emulsion and exposed for 2 weeks. After developing in Kodak D-19 developer at 16°C, slides were exposed to Kodak XAR-5 film at 4°C. The sections were then mounted, dehydrated, and coverslipped.

**RESULTS**

**Differential Display and Subcloning—**We performed differential display analysis on mRNA from reserpinized and vehicle-treated rat adrenal gland RNA. The samples were collected from two animals 4 h after the injection of reserpine (10 mg/kg) or vehicle solution (20% ascorbic acid), and the reactions were done using different primer combinations as described under “Experimental Procedures.” The reactions that generated PCR products exhibiting a differential profile were repeated, and the product exhibiting a differential profile were repeated, and the product was excised from the gel and reamplified. The amplified fragment (437 bp in length) was purified from agarose gel, labeled with $[^35S]dATP$ using the random primer method, and used as a probe for in situ hybridization.

**In Situ Hybridization—**In situ hybridization was performed as described (9). In brief, animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline containing 0.5% sodium nitrate and 10 units/ml heparin sulfate followed by cold formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The tissue was immersed in the fixative for 1 h and stored in 30% sucrose overnight. Free floating sections (40 μm thick) were placed in vials containing 2 ml of cold formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The sections were fixed in Kodak fixer, counterstained with cresyl violet, and dehydrated, and coverslipped.

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**TRAP**

TRAP, single colonies were analyzed for the presence of the insert, and positive clones were sequenced.

**IGIF Identification and Isolation—**The sequence of the subcloned cDNA, about 200 bp in length, was submitted to GenBankTM, EMBL, DDBJ, and PDB data bases for homology comparison and found to have 90% homology to the 3′ end of mouse mRNA for IGIF precursor polypeptide (accession number D49949). The isolated cDNA corresponded to the last 80 bp of the mouse IGIF coding region and the remaining to the 3′ untranslated region.

Rat IGIF coding region was isolated from the adrenal gland by reverse transcriptase-PCR. Two distinct PCR products were visible: a very abundant band and a faint shorter one. This pattern was reproduced even when a different primer combination was used (data not shown). Their sequence overlap with that of the fragment derived from differential display so that it was possible to reconstitute the whole sequence (Fig. 1). They appear to be two different isoforms of the same molecule identified as rat IGIF on the basis of the high homology with mouse IGIF. The longer transcript shows 91% homology to mouse IGIF. The longer transcript shows 91% homology to mouse IGIF.
laris and fasciculata that synthesize glucocorticoids. No mRNA was detected in the medulla where reserpine is known to act. The level of transcription returned to basal within 24 h of the end of the cold exposure. The presence of IGIF mRNA was also detected in the posterior lobe of the pituitary gland (Fig. 2F) although the differences in the levels of transcripts do not appear significant in the various conditions.

**DISCUSSION**

The presence of IGIF mRNA was discovered by differential display in the adrenal gland of reserpine-treated rats. This early evidence was confirmed with the isolation of a rat IGIF by reverse transcriptase-PCR from the adrenal gland of an animal injected with reserpine. Two PCR products were identified. They appear to be two isoforms (IL-18 and IL-18a) of the same molecule, although further characterization might be required to confirm it. On the basis of the structural homologies observed between mouse IGIF and human interleukins-1 (5), it is curious to notice that the 19 amino acids missing in the short isoform correspond to the amino-terminal peptide encoded by exon 7 of human IL-1α and human IL-1β.

Subsequent in situ hybridization analysis then clearly showed the localization of IGIF transcripts in the zona reticularis and fasciculata of the adrenal cortex and confirmed the pattern of induction after reserpine treatment. Reserpine, which causes catecholamine depletion and blocks their reuptake, also induces evident sickness when injected. Since it is known that the pharmacological and the biochemical effects of reserpine on the adrenal glands resemble that of stress (10–12), we investigated the possibility that IGIF induction was actually due to the stressful condition the treated animals undergo. In situ hybridization analysis was repeated comparing the adrenal glands of reserpine-treated, vehicle, control, and cold-stressed animals.

Cold stress, given as exposure to 4 °C for 4 h prior to anesthesia, is indeed sufficient to stimulate IGIF mRNA transcription in the same areas of the adrenal cortex. The observation that the mRNA levels return to basal within 24 h of the cold stress suggests that IGIF transcription is a rapid response to stress probably accounting for an immediate rather than for an adaptive response. Mouse IGIF is believed to be produced as precursor and secreted after cleavage of the leader peptide at aspartate in amino acid position 35 (36 in rat form) mediated by an interleukin-1β convertase-like enzyme (ICE-like). Interleukin-1β convertase mRNA, and its catalytic activity has been found in adrenal and pituitary glands of rats treated with LPS (13), and IGIF protein has been detected in the supernatant of cultured Kupffer cells after stimulation with LPS (4). IGIF could be secreted from the adrenal and pituitary glands following a stressful experience and act as neuroimmunomodulator or require the presence of at least a second stimulus, such as an infectious agent.

IGIF mRNA was found in Kupffer cells and macrophages where its level does not appear to change even after stimulation with infectious and inflammatory agents (4, 6). We report here for the first time the observation of a pattern of IGIF mRNA induction, notably following a stressful experience and, very interestingly, localized in the adrenal gland. IGIF and glucocorticoids seem to be both synthesized by the same cells of the adrenal cortex, induced by stress, and yet appear to have different functions on the immune system. The production by the adrenal gland of molecules with protective effects against stress has long been postulated, and the anti-inflammatory properties of the glucocorticoids are seen as a means to prevent immune system overreaction (14). The small amount of data available on the biological activity of IGIF show, on the contrary, its properties as proinflammatory agent and stimulator of the cell-mediated immune response, thus its ability to enhance the immune functions in a situation of potential danger. In this view, it would be of great interest to investigate the role of IGIF in relation to autoimmune diseases.

The exact effects that IGIF would exert on the immune system in this context and its physiological meaning remain to be investigated. Certainly the role of the adrenal gland as effector of immunomodulation needs to be reconsidered and deserves great attention.

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