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Response of Serum Macrophage Migration Inhibitory Factor Levels to Stimulation or Suppression of the Hypothalamo-Pituitary-Adrenal Axis in Normal Subjects and Patients with Cushing's Disease

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Macrophage migration inhibitory factor (MIF) is a proinflammatory pituitary and immune cell cytokine and a critical mediator of septic shock. It has been reported that MIF is secreted in parallel with ACTH from the pituitary in response to stress or inflammatory stimuli. MIF release from immune cells is also induced rather than inhibited by glucocorticoids. It has therefore been suggested that MIF may be a novel counterregulatory hormone of glucocorticoid action that acts both as a paracrine and endocrine modulator of host responses. We have measured circulating MIF levels, using a human MIF ELISA, in normal subjects and patients under numerous pathophysiological conditions. Serum MIF was measured in normal subjects who underwent stimulation of the hypothalamo-pituitary-adrenal axis with an insulin tolerance test (n = 8), a CRH-stimulation test (n = 5), a short synacthen test (n = 5), and following a low-dose dexamethasone suppression test (n = 6). We also sampled from a peripheral vein and both inferior petrosal sinuses before and after CRH stimulation in four patients with a histologically proven diagnosis of Cushing's disease. Immunostaining of the pituitary tumors for MIF was also performed. In normal subjects serum MIF levels did not rise in parallel with cortisol during the insulin tolerance or CRH test or after administration of synthetic ACTH. In all subjects cortisol levels became undetectable after the low-dose dexamethasone suppression test, and no consistent change was observed in serum MIF levels during the test. In patients with Cushing's disease, there was no basal central-to-peripheral gradient in MIF, and no consistent changes occurred in serum MIF levels in either the left or right inferior petrosal sinus after CRH stimulation; however, immunostaining of the surgically removed pituitary tumors from the same patients showed strong staining for both ACTH and MIF. These results show that in humans acute modulation of the hypothalamo-pituitary-adrenal axis does not significantly alter circulating MIF levels. In addition, ACTH-secreting pituitary tumors that express MIF do not release MIF either spontaneously or in response to CRH stimulation, and there is no gradient for MIF in the venous drainage of the pituitary. Our study suggests that the pituitary gland is not the major contributor to circulating MIF; an autocrine or paracrine role for pituitary-derived MIF is more likely. (*J Clin Endocrinol Metab* 87: 1834–1840, 2002)

GLUCOCORTICOIDS ARE IMPORTANT physiological modulators of fuel metabolism that also maintain fluid and electrolyte balance and preserve the integrity of the cardiovascular system (1). In addition, they are powerful suppressors of the immune system. The antiinflammatory and immunosuppressive actions of corticosteroids provide a protective mechanism against the possible cardiovascular collapse that might otherwise be induced by the unconditioned release of immune mediators associated with the immune response. In the setting of severe inflammatory stress, the cortisol production rate markedly increases, protecting the organism from overreaction of the immune system and localizing its activity in time and space (2). However, a novel endocrine system has recently been described that may in turn act to counterregulate the potent immunosuppressive and antiinflammatory effects that corticosteroids exert on the immune system. While searching for an endogenous antagonist of glucocorticoids, Bernhagen *et al.* (3) identified a 12.5-kDa peptide, secreted by the anterior pituitary cells in response to endotoxin stimulation, which reversed the suppressive effects of cortisol on cytokine production. Unexpectedly, this protein was found to be the previously described macrophage migration inhibitory factor (MIF) (3).

MIF was identified nearly 40 yr ago as a product of activated T lymphocytes that mediated the localization of macrophages to sites of delayed-type hypersensitivity reactions (4–7). MIF has emerged as a crucial mediator of the biological response to septic and toxic shock by strongly promoting the expression of proinflammatory mediators by macrophages and activating T cells (3, 8, 9). The paradoxical finding that MIF secretion is induced by glucocorticoids led Calandra *et al.* (10) to postulate that MIF might act to counterregulate the antiinflammatory activities of glucocorticoids. Thus, MIF and glucocorticoids appear to function as a physiological

Abbreviations: CD, Cushing's disease; HPA, hypothalamo-pituitary-adrenal; IPS, inferior petrosal sinuses; LDDST, low-dose dexamethasone suppression test; LPS, lipopolysaccharide; MIF, macrophage migration inhibitory factor.

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counterregulatory dyad that controls host immune responses to maintain homeostasis during severe inflammatory stress.

It was also reported that activation of the hypothalamo-pituitary-adrenal (HPA) axis in rats in response to endotoxin was associated with a concomitant rise in circulating MIF levels (3, 7, 10). This MIF appeared to be the same as that initially isolated from T lymphocytes. Subsequent immunocytochemical studies showed that pituitary MIF colocalizes within the granules present in ACTH and TSH cells of the normal human pituitary as well as in corticotroph adenomas (11, 12). However, data on circulating MIF in the human have been lacking. The aim of the present study was therefore to evaluate *in vivo* in humans whether alterations of MIF levels follow changes in ACTH levels after stimulation and suppression of the HPA axis in normal subjects, and whether MIF is cosecreted from the pituitary in patients with established pituitary ACTH hypersecretion [Cushing's disease (CD)].

Subjects and Methods

Subjects

Serum MIF was measured in normal subjects who underwent modulation of the HPA axis with an insulin tolerance test (n = 8), a CRH-stimulation test (n = 5), a short synacthen test (n = 5), and a low-dose dexamethasone suppression test (LDDST) (n = 6). Petrosal sinus samples were also studied in four patients with CD who underwent successful inferior petrosal sinus catheterization.

The normal subjects were healthy volunteers who provided informed consent (8 females, 10 males, mean age 37 ± 3 yr). For all subjects major diseases and endocrine abnormalities were excluded on clinical and biochemical grounds. After an overnight fast, an indwelling forearm cannula was inserted at 0830 h; the subject remained supine for the remainder of that study. For the CRH study, at 0900 h (0 min), a 100-μg bolus of human sequence CRH (Ferring Pharmaceuticals Ltd., Malmo, Sweden) was injected iv over a period of 15 sec. Blood was taken for estimation of plasma ACTH and cortisol at −15, 0, 15, 30, 45, 60, 90, and 120 min. Alternatively, for the insulin tolerance test, 0.15 U/kg insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) was given as an iv bolus, and blood was taken for estimation of plasma cortisol at 0, 30, 45, 60, 90, and 120 min. For the short synacthen test, 250 μg synacthen (Novartis, Berne, Switzerland) was administered by im injection, and blood was taken for measurement of plasma cortisol and MIF at −30, 0, 30, and 60 min. The LDDST was performed according to our standardized departmental protocol (13), with samples collected at 0900 h before and after 2 mg of dexamethasone administered as 0.5-mg tablets every 6 h for 48 h.

All patients with CD (three females and one male, age range 20–50 yr) were clinically and biochemically hypercortisolemic, the diagnosis being subsequently confirmed by the presence of positive immunostaining for ACTH in the pituitary adenoma. The catheterization involved the placement of venous sampling catheters in the inferior petrosal sinuses (IPS; these drain the pituitary venous effluent), with simultaneous peripheral and bilateral IPS sampling before and after a CRH test (14). Blood samples for evaluation of plasma ACTH and serum MIF were taken at 0, 3–5, 8–10, and 13–15 min after CRH injection. Informed written consent for the use of spare plasma for research samples was obtained from every patient.

Immunohistochemistry

Paraffin-embedded sections (3 μm) of human pituitary corticotroph adenomas collected at *trans*-sphenoidal surgery from three of the patients with CD were routinely prepared for immunohistochemistry using the standard avidin biotin complex method (Vectastain, Peterborough, UK). Mouse monoclonal anti-MIF antibody or rabbit polyclonal anti-MIF antiserum were applied on serial sections (respective dilutions 1:1000 and 1:100) and incubated for 40 min at room temperature with antigen localization by activated 3′, 3′-diaminobenzidene-tetrahydrochloride solution (Kentec DAB tablets 4170, Biostat, Stockport, UK). Controls included negative staining of other tissue, blocking with human recombinant MIF, and replacement of antibody with nonimmune bovine serum.

Assays

Serum MIF levels were measured using a human MIF ELISA (9), identical to that used for monocyte/lymphocyte studies. The sensitivity of the assay is 200 pg/ml; however, because ceratin samples required dilution, the working sensitivity is in the range of 600–800 pg/ml. The ELISA showed no significant cross-reactivity with other proinflammatory cytokines, including TNF-α, the interferons, and IL-1 and IL-6. The separated serum was stored at −20 C until assay; any sample showing evidence of hemolysis was discarded. Plasma cortisol was measured by an in-house unextracted nonchromatographic RIA, plasma ACTH by our routine in-house Vycor (Societe-A.T.A., Geneva, Switzerland) glass-extracted RIA (14).

Statistical analysis

The criteria applied to the IPS for the diagnosis of CD, calculated as central:peripheral plasma ACTH gradients, were either a basal ratio of 2.0 or greater or a CRH-stimulated ratio of 3.0 or greater. The peripheral response to the CRH test was calculated as the percent rise in the mean circulating plasma ACTH values at 15 and 30 min and circulating plasma cortisol values at 30 and 45 min above the mean basal values at −15 and 0 min. The integrated response of serum MIF to dynamic testing was measured as area under the curve calculated by the trapezoid method on the series of blood samples. The nonparametric Friedman two-way ANOVA and the Wilcoxon tests for related variables were used to evaluate responsiveness. Possible correlations between changes in serum MIF and cortisol and ACTH values were investigated by Spearman's coefficient of correlation. Data are given as mean plus or minus SE unless otherwise stated. Results were considered statistically significant if the P value was less than 0.05.

Results

Normal subjects

The response of serum MIF to stimulation of the HPA axis during the insulin stimulation test is shown in Fig. 1 (*solid line*). No significant change was seen in spite of a significant rise in serum cortisol (mean peak values 665 ± 41 mmol/liter, $P < 0.01$; Fig. 1, *dashed line*) and ACTH (mean peak values 114 ± 33 ng/ml, $P < 0.01$) occurring in all subjects.

The fluctuations of serum MIF levels before and after the CRH stimulation test are shown in Fig. 2, plotted as the mean plus or minus SE of the study population (*solid line*). When applying to serum MIF the same criteria used to assess the ACTH response (*i.e.* the percent rise in the mean values at 15 and 30 min above the mean basal values at −15 and 0 min), serum MIF levels increased by a mean of 28% plus or minus 14% from baseline. Nonparametric two-way ANOVA showed that the time trend of serum MIF during the CRH test just reached statistical significance ($P < 0.05$); however, as shown in Fig. 2, the fluctuations of serum MIF resembled that of a pulsatile hormone, with no major change in the amplitude of pulses after stimulation with CRH. In all patients, significant rises in serum cortisol and plasma ACTH were observed, which were not associated with any change in serum MIF.

Figure 3 shows the mean variations of serum MIF levels (*open squares*) during a formal LDDST along with changes in

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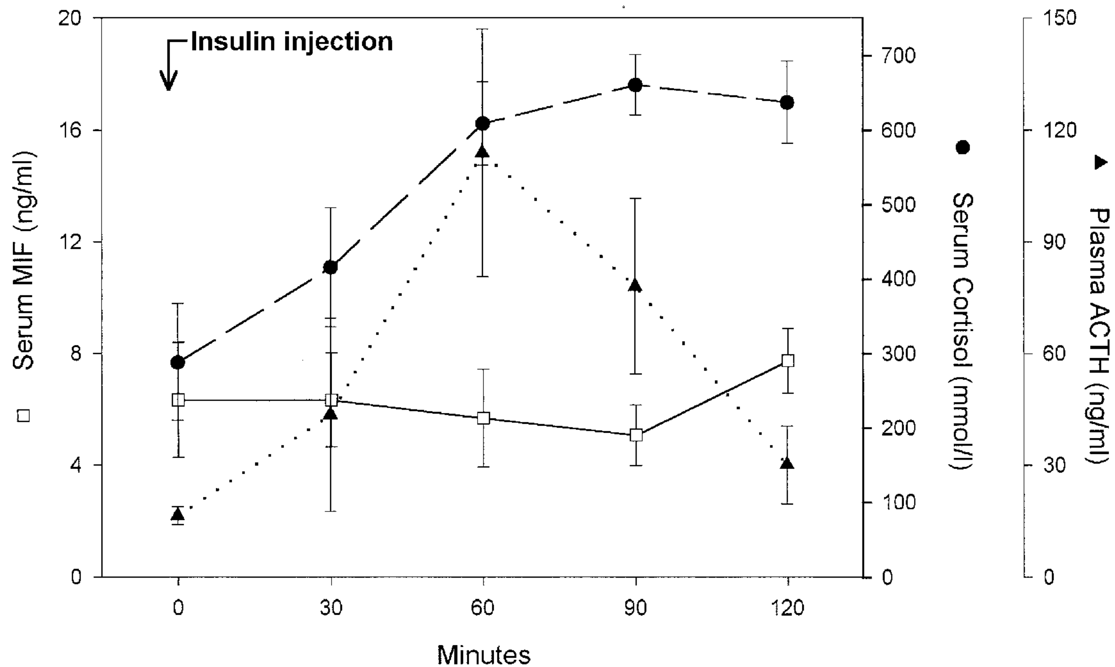

FIG. 1. Serum MIF (□, *solid line*), serum cortisol (●, *dashed line*), and plasma ACTH (▼, *dotted line*) levels during insulin-induced hypoglycemia in normal subjects (n = 8).

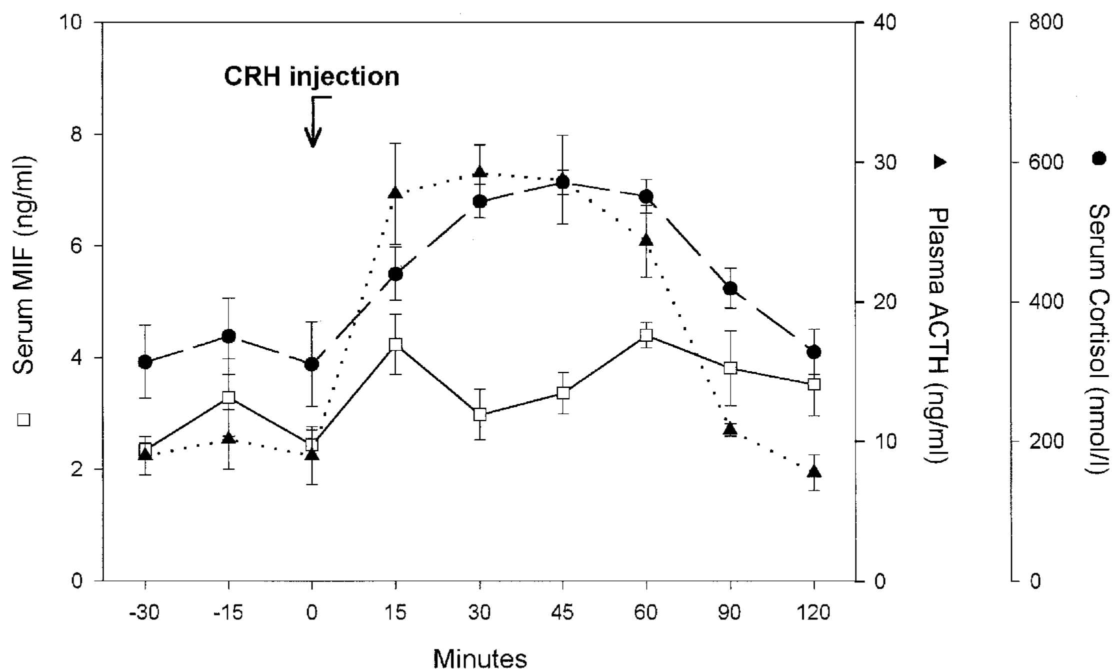

FIG. 2. Serum MIF (□, *solid line*), serum cortisol (●, *dashed line*), and plasma ACTH (▼, *dotted line*) levels during the human CRH stimulation test in normal subjects (n = 5).

serum cortisol (*filled circles*). No significant change was observed in serum MIF levels during the test, in spite of the fact that serum cortisol became undetectable (<50 nmol/liter) in all patients.

Figure 4 shows the response of serum cortisol (*filled circles*) and serum MIF levels (*open squares*) during a short synacthen test. Serum cortisol showed the expected rise, unassociated with any change in plasma MIF.

Patients with CD

Results of the simultaneous peripheral (F), right (○), and left (▽) IPS catheterizations are reported for each patient in Fig. 5. All four patients showed clear gradients in ACTH after CRH stimulation, three on the right and one in the left IPS (Fig. 5). Before CRH administration, in patients A and B, a gradient of 1.6 and 3.2, respectively, was observed in serum

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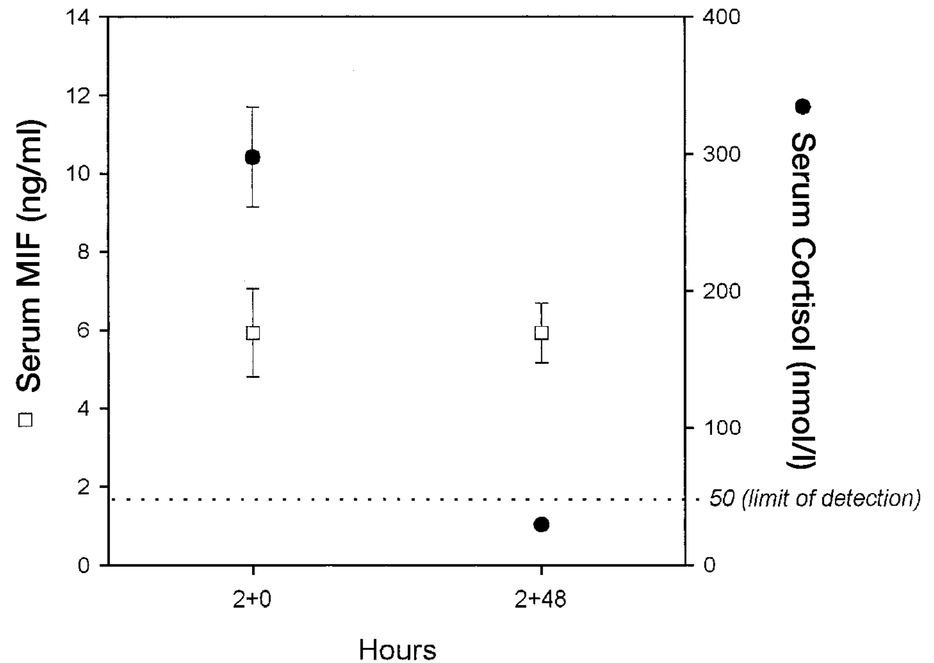

FIG. 3. Serum MIF levels during an LDDST (2 mg) in normal subjects. 2+0: baseline levels; 2+48: serum levels after 48 h of 6-h 0.5 mg dexamethasone tablets (n = 6) (□, serum MIF; ●, serum cortisol).

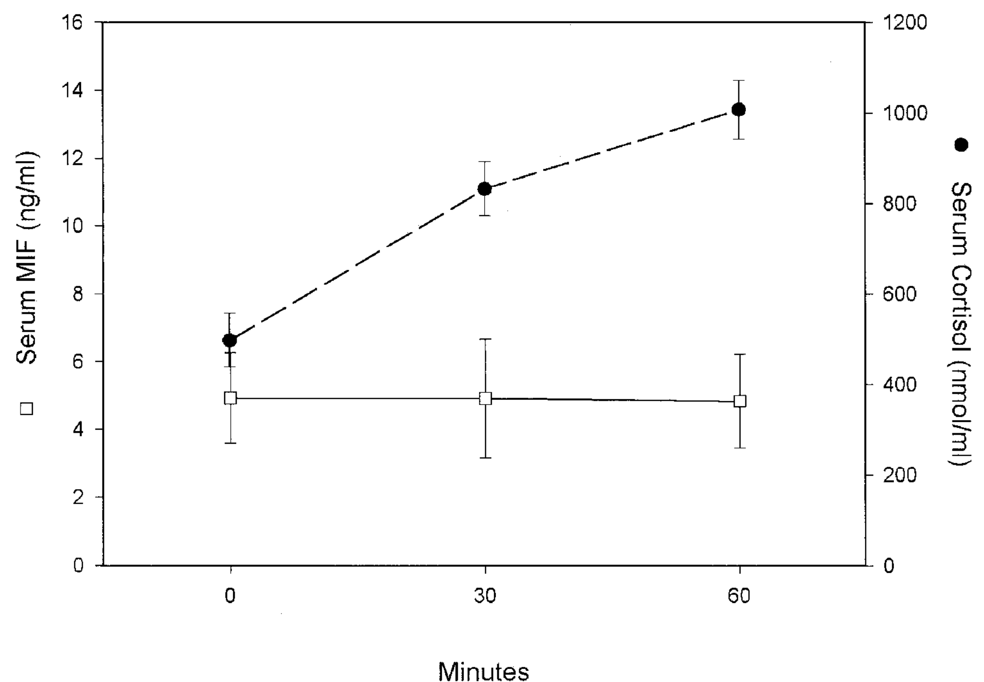

FIG. 4. Serum MIF and cortisol levels during a short synacthen test (250 μg) in normal subjects (n = 5) (□, serum MIF; ●, serum cortisol).

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MIF levels between the right petrosal sinus and the periphery, and in patients C and D, the central-to-peripheral ratio was less than 1.2. No significant trend of response in serum MIF levels was observed after CRH stimulation. No central-to-peripheral MIF gradient was observed following CRH stimulation. Basal and stimulated MIF levels during the IPS catheterizations were not significantly different from those observed in normal subjects. Serum MIF changes during the test did not parallel those observed in plasma ACTH.

Figure 6 shows the immunostaining for ACTH (A) and MIF (B and C) of the pituitary adenoma from patient D. Strong positive staining for MIF, using either a monoclonal antibody (B) or polyclonal antiserum (C), was obtained.

None of the reported changes in serum MIF levels following CRH or insulin-induced stimulation, dexamethasone suppression, or during the short synacthen test or in patients with CD significantly correlated with the observed changes in either ACTH or cortisol levels.

Discussion

Our data show that serum MIF levels in humans do not mirror the changes in ACTH/cortisol levels observed during

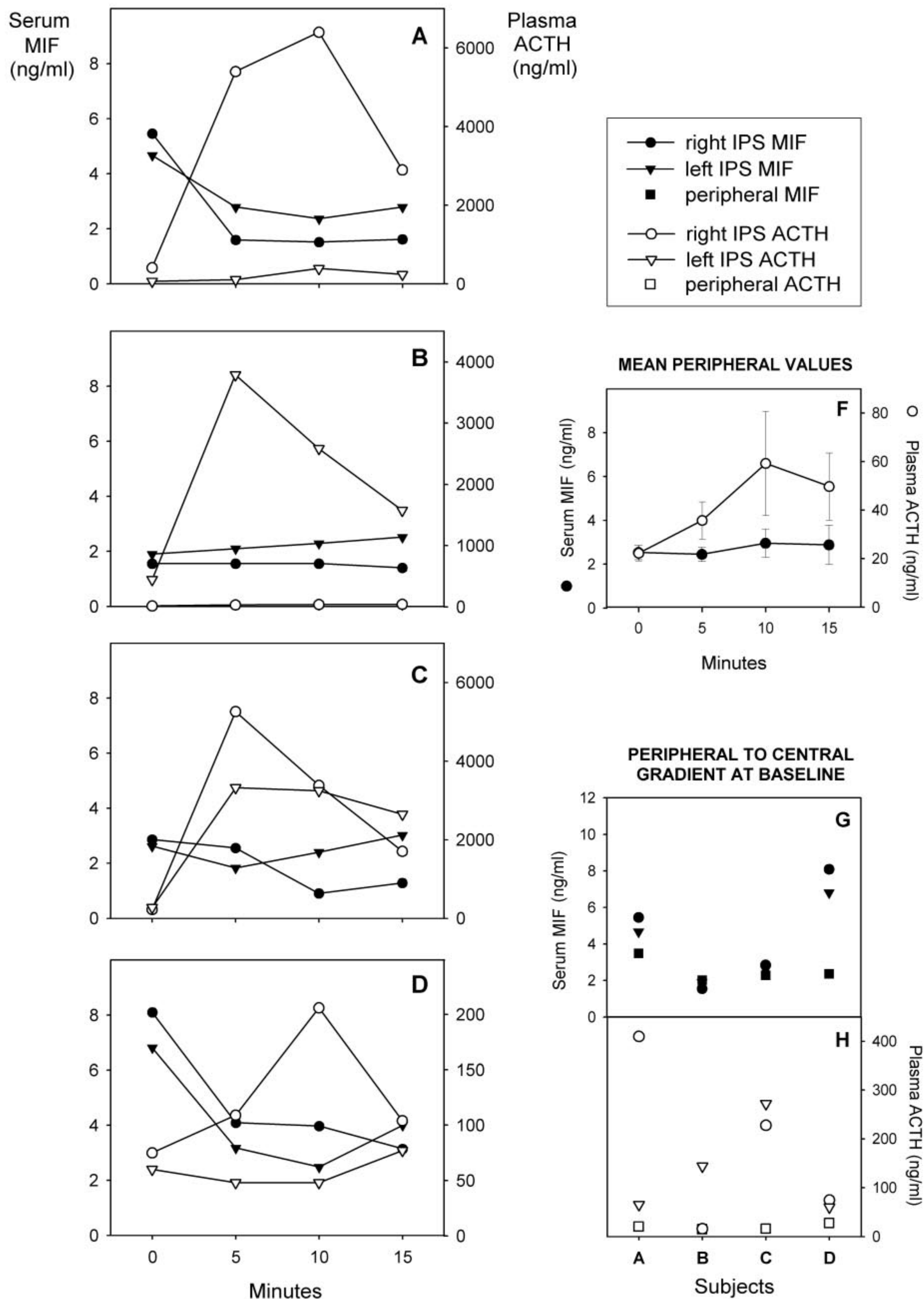

FIG. 5. Serum MIF levels (*solid*) and plasma ACTH levels (*blank*) during the simultaneous sampling from the R petrosal sinus (○) and L petrosal sinus (▽), before (time 0) and at 3, 8 and 13 min after the administration of human CRH in four patients (A, B, C, and D) with CD. Mean simultaneous peripheral MIF and ACTH levels are reported in *panel* F. Central to peripheral gradient for MIF and ACTH are shown in *panels* G and H, respectively.

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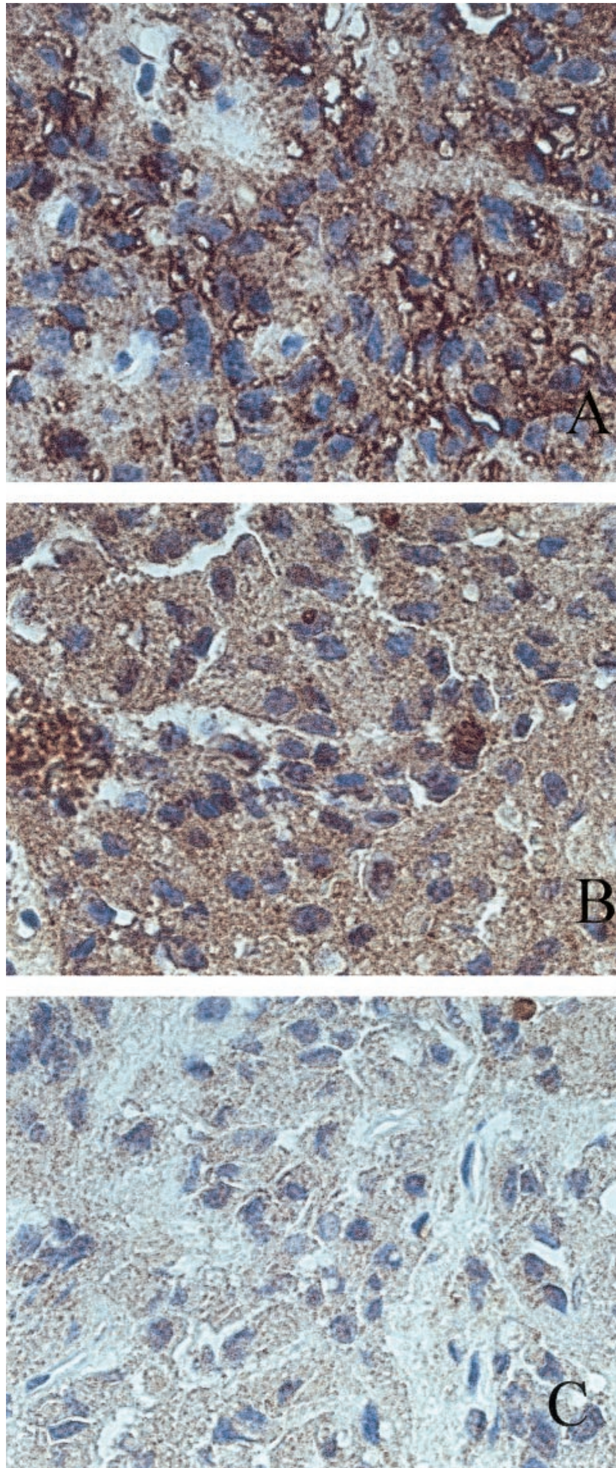

FIG. 6. Serial sections of pituitary corticotroph adenoma from patient D immunostained for ACTH (A) and monoclonal (B) or polyclonal (C) MIF antibodies show a colocalization for MIF, both polyclonal and monoclonal antibodies and ACTH as well. The staining is stronger, however, with MIF polyclonal antibody (three populations of cells observed: weak, moderate, and strong staining for both MIF antibodies).

stimulation or inhibition of the HPA axis in normal subjects or in patients with CD. By contrast to previous studies that investigated the modifications of serum MIF levels in response to endotoxemia or lipopolysaccharide (LPS) stimulation, the present study is the first report addressing the issue of the physiological modulation of MIF levels *in vivo* in humans. Our findings indicate that basal and stimulated MIF levels are not significantly different in patients with CD from controls, and that CRH stimulation of the HPA axis is not directly associated with an acute release of preformed MIF from the pituitary. In addition, we have shown that the lack of response of serum MIF levels was not because of the loss of expression of MIF in our patients with CD because immunohistochemistry confirmed that MIF was expressed by the ACTH-secreting adenomas.

In contrast to other immune mediators, MIF exists preformed in a variety of tissues and can be released from intracellular pools in response to adequate stimuli (15). In rodent and human pituitary glands, MIF is a prominent constituent of the corticotroph cells: Its basal expression is high, and the overall MIF content within this gland has been estimated to be approximately 0.05% of total pituitary protein, compared with 0.2% of ACTH (3, 11). *In vivo* studies in mice showed that pituitary MIF mRNA levels increased after LPS challenge, and the pituitary content of stored MIF protein decreased to almost undetectable levels. The contribution of pituitary MIF to circulating MIF during endotoxemia, assessed in hypophysectomized mice, showed that pituitary-derived MIF significantly contributed to circulating MIF present in the postacute phase of endotoxemia (3, 7).

In vitro studies showed that MIF is stored in granules within the corticotroph and thyrotroph cells and is released in circumstances such as endotoxic shock (3, 11, 12), although contradictory results have been reported (11). Nishino *et al.* (11) also found that MIF exists in the pituitary cells packed into two distinct granular subtypes, one containing MIF alone and one containing both MIF and either ACTH or TSH. However, endotoxemia induced a significant decrease in MIF content by a selective reduction in the granule population that contained MIF alone, suggesting that these subsets are regulated independently and/or may represent advanced stages in the maturation of the secretory granules. Indeed, further *in vitro* studies using the AtT-20 corticotroph cell line suggested that CRH could act as an MIF secretagogue via the cyclic AMP/CRE-binding protein signaling system, although the pathway appeared to be distinct from that controlling ACTH release (11, 16). In addition, LPS in subnanogram concentrations can directly induce MIF release from primary pituitary cells or the AtT-20 corticotroph cell line *in vitro* (3). These findings suggest that the pathways of MIF and ACTH secretion are quite distinct, allowing MIF a more specific activity consistent with its pivotal role in the regulation of septic shock. Our results suggest that the regulation of circulating MIF in the human is different from that in rodents because stimuli to the HPA axis, such as CRH or hypoglycemia, were ineffective in stimulating circulating levels. The clear changes in serum cortisol were dissociated from MIF, indicating that either the majority of circulating MIF is not derived from the pituitary or it is regulated discordantly to ACTH. Indeed, a dominant source for circulating MIF outside the pituitary corticotroph is suggested by endotoxemia studies in the rat (15) and by our petrosal sinus catheterization study, in which no central-to-peripheral gra-

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dient for MIF was evident, either before or after CRH. The fact that the tumors obtained from patients in whom a significant central-to-peripheral gradient of ACTH was observed also showed positive immunostaining for MIF further supports independent regulation and location of the two peptides. However, we cannot entirely exclude a contribution to circulating MIF from the pituitary, but quantitatively such a contribution, if it exists, is likely to be small.

The dissociation of circulating MIF from ACTH and cortisol, along with the fact that MIF is not secreted at high levels into petrosal blood after hypoglycemic or CRH stimulation, suggests that pituitary MIF does not access the peripheral circulation to any major extent. Cytokines such as IL-6 have also been demonstrated within the anterior pituitary, particularly after inflammatory stress, and are thought to exert local autocrine and/or paracrine effects (17). Similarly, leptin has recently been located within, and secreted from, anterior hypophysial cells but does not appear to influence circulating levels of leptin (18). We propose that a network of cytokines and cytokine-related peptides is functioning within the pituitary gland, which, at least in the absence of systemic inflammatory stressors, remain confined to the pituitary. We further suggest that pituitary-derived MIF is unlikely to contribute to circulating levels to any major extent.

In conclusion, these data do not support the speculation that MIF and ACTH are cosecreted into the blood stream in response to physiological challenge of the HPA axis. We suggest that if pituitary MIF is released into the peripheral circulation and contributes significantly to circulating levels, it may do so only in response to a subset of more severe stressors, such as inflammation, endotoxemia, or tissue invasion. The increasingly broad spectrum of activities attributed to MIF (19–29), in particular the recently described tumor growth-promoting properties of MIF (20, 23, 26–29), suggest that pituitary-derived MIF is more likely to be involved in the regulation of pituitary growth and/or metabolism.

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