A Broad-Spectrum Chemokine Antagonist Encoded by Kaposi's Sarcoma–Associated Herpesvirus

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Kaposi's sarcoma–associated herpesvirus encodes a chemokine called vMIP-II. This protein displayed a broader spectrum of receptor activities than any mammalian chemokine as it bound with high affinity to a number of both CC and CXC chemokine receptors. Binding of vMIP-II, however, was not associated with the normal, rapid mobilization of calcium from intracellular stores; instead, it blocked calcium mobilization induced by endogenous chemokines. In freshly isolated human monocytes the virally encoded vMIP-II acted as a potent and efficient antagonist of chemotaxis induced by chemokines. Because vMIP-II could inhibit cell entry of human immunodeficiency virus (HIV) mediated through CCR3 and CCR5 as well as CXCR4, this protein may serve as a lead for development of broad-spectrum anti-HIV agents.

Accumulating evidence indicates that human herpesvirus 8 (HHV-8) is the infectious agent responsible for Kaposi's sarcoma in patients with and without HIV infection (*1*). Several viral proteins with homologous human counterparts involved in cellular signaling are encoded by HHV-8 (*2*). Three open reading frames of the HHV-8 genome code for chemokine-like proteins with 28 to 56% amino acid identity to each other and about 40% identity to human CC chemokines (*3*) (Fig. 1A). We cloned the vMIP-II chemokine by polymerase chain reaction (PCR) amplification from a biopsy of a Kaposi's sarcoma lesion in an HIV patient and found the nucleotide sequence was identical to the one in GenBank (*4*). The vMIP-II gene was expressed in COS-7 cells, and we tested the secreted, recombinant protein in binding assays with various human chemokine receptors and with the human cytomegalovirus-encoded US28 receptor (*5*). Conditioned medium containing recombinant vMIP-II displaced ^{125}I-labeled MIP-1α (^{125}I–MIP-1α) from the human CC chemokine receptors CCR1 and CCR5 and from the US28 receptor as well as radiolabeled MCP-1 from the human CCR2 receptor (Fig. 2A). Surprisingly, vMIP-II also bound to CXCR4 in competition with the labeled CXC chemokine SDF-1. However, vMIP-II showed low activity on the CXCR2 [interleukin-8B (IL-8B) receptor]. It did not interfere with binding of labeled IL-8 to CXCR1 (IL-8A receptor) or binding of the unrelated peptide substance P to its 7TM receptor NK-1. Conditioned medium from COS-7 cells transfected with the empty expression vector did not displace any of the radioactively labeled chemokines from the indicated receptors (Fig. 2A).

The recombinant vMIP-II protein was purified by Bio-Gel P-30 gel filtration followed by reversed-phase high-pressure liquid chromatography (HPLC), and the chromatography was monitored by receptor analysis with human CCR5 stably transfected into HEK-293 cells (Fig. 1B) (*6*). NH_2-terminal sequence analysis of the first 10 residues of the recombinant vMIP-II demonstrated that a 23–amino acid signal peptide is removed from the precursor protein. Mass spectroscopy of the HPLC-purified vMIP-II gave a molecular mass of 7964, which indicates that a COOH-terminal Arg residue encoded by the open reading frame is also removed in the secretory pathway (theoretical molecular mass of the des-Arg protein is 7968). Thus, the vMIP-II sequence in Fig. 1A is likely to be the naturally occurring, secretory form of the viral protein (*7*).

In competition binding assays, HPLC-purified vMIP-II bound with high affinity to human CCR1 [median inhibitory concentration (IC_{50}) = 8.0 ± 3.6 nM], CCR5 (IC_{50} = 5.2 ± 1.9 nM), and CXCR4 (IC_{50} = 5.8 ± 2.8 nM) receptors and with very high affinity to human CCR2 (IC_{50} = 0.82 ± 0.16 nM) and the cytomegalovirus-encoded US28 receptor (IC_{50} = 0.63 ± 0.20 nM) (binding curves for CCR5, US28, and CXCR4 are shown in Fig. 2B). Chemically synthesized vMIP-II (*8*) gave very similar results (data not shown).

Receptor binding of endogenous chemokines is normally associated with a rapid, pertussis toxin–sensitive calcium response. Instead, HPLC-purified recombinant vMIP-II induced a slow, prolonged

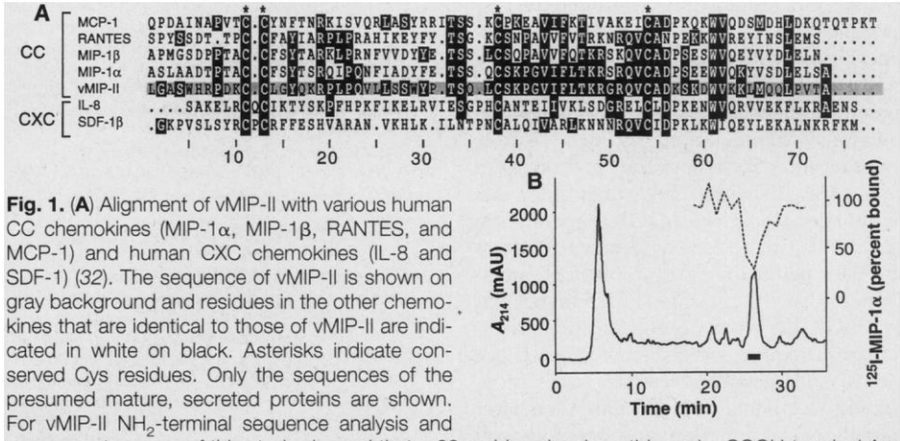

Fig. 1. (**A**) Alignment of vMIP-II with various human CC chemokines (MIP-1α, MIP-1β, RANTES, and MCP-1) and human CXC chemokines (IL-8 and SDF-1) (*32*). The sequence of vMIP-II is shown on gray background and residues in the other chemokines that are identical to those of vMIP-II are indicated in white on black. Asterisks indicate conserved Cys residues. Only the sequences of the presumed mature, secreted proteins are shown. For vMIP-II NH_2-terminal sequence analysis and mass spectroscopy of this study showed that a 23-residue signal peptide and a COOH-terminal Arg residue are removed from the virally encoded protein during synthesis and secretion in mammalian cells. (**B**) HPLC purification of recombinant vMIP-II expressed in COS-7 cells (solid line) monitored by testing aliquots for interference with ^{125}I–MIP-1α binding to CCR5 expressed stably in HEK-293 cells (dashed line). A_{214}, absorbance at 214 nm; AU, absorbance units.

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calcium response in CHO (Fig. 3A, upper left) but not HEK-293 cells stably transfected with human chemokine receptors (data not shown) (9). In contrast to the endogenous chemokines, vMIP-II was unable to cause rapid mobilization of calcium from intracellular stores—that is, increase intracellular calcium in the presence of EDTA (Fig. 3A). Both the HPLC-purified recombinant peptide and vMIP-II prepared by chemical synthesis in a dose-dependent manner blocked the calcium mobilization elicited by the relevant human chemokines through CCR1, CCR2, CCR3, CCR5, and CXCR4 (Fig. 3A). In contrast, vMIP-II had no effect on the calcium response induced by IL-8 through CXCR2 (data not shown). Thus, in some cellular contexts vMIP-II appears as an agonist—that is, it elicits slow, prolonged leakage of extracellular calcium into the cell (*10*). But with respect to mobilization of intracellular calcium, vMIP-II is a pure antagonist against the action of endogenous chemokines. Activation of two distinct calcium signaling pathways through the same receptor has recently been shown for the mammalian chemokines IL-8 and GRO-α acting through CXCR2 (*11*). It has been demonstrated that the primary biological effect of chemokine chemotaxis is associated with the rapid, pertussis toxin–sensitive calcium response, in contrast to the slow, prolonged calcium response (*12*).

The chemotactic property of vMIP-II has been probed in freshly prepared human monocytes (*13*). As expected from its lack of ability to cause rapid mobilization of intracellular calcium and in contrast to the endogenous chemokine RANTES, vMIP-II when given alone did not stimulate chemotaxis in the monocytes (Fig. 3B, upper). However, vMIP-II was an efficient and potent inhibitor of the chemotactic response to RANTES, MIP-1α, or MIP-1β in these cells (Fig. 3B, lower). Thus, it can be envisioned that vMIP-II is used by HHV-8 to block recruitment of leukocytes as part of the viral defense mechanism against the immune system of its host. Recently, it was suggested but not demonstrated that two other putative chemokines discovered as open reading frames in the genome of the human molluscum contagiosum poxvirus and a murine cytomegalovirus may act as anti-inflammatory agents by blocking chemokine receptors (*14*).

Although chemokines are often able to bind with high affinity to more than one receptor subtype, no known human chemokine has as broad a spectrum of activities as vMIP-II (*15*). RANTES and MCP-3, which bind to multiple CC chemokine receptors, are probably the most promiscuous of the human chemokines. But these CC chemokines do not bind with high affinity to any CXC chemokine receptor. Conversely, CXC chemokines such as IL-8 have negligible affinity for CC chemokine receptors. Nevertheless, despite relatively low levels of primary sequence identity, the monomeric forms of CC and CXC chemokines have very similar three-dimensional structures (*16*). Mutation and chemical modification of a single amino acid residue in IL-8 can introduce high affinity for this CXC chemokine on the CCR1 receptor where the affinity on its natural CXCR1 and -2 receptors is decreased two orders of magnitude (*17*). In the case of vMIP-II, this peptide appears to have been optimized by the virus to bind to and block multiple human chemokine receptor subtypes. HHV-8 also encodes a chemokine receptor called ORF74 (*18*). Although ORF74 binds IL-8 with high affinity, this virally encoded recep-

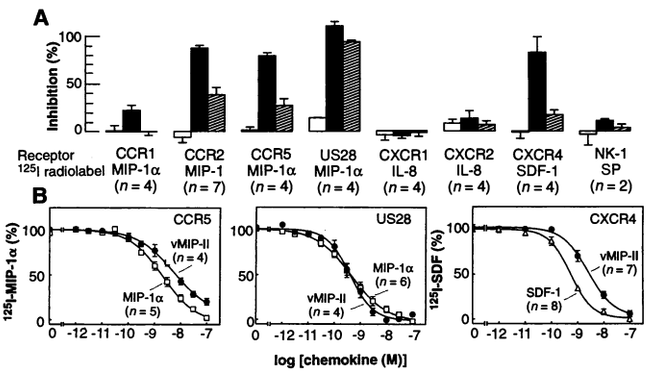

Fig. 2. Competition binding experiments with recombinant vMIP-II in various chemokine receptors. (**A**) Whole-cell binding experiments were performed with recombinant vMIP-II in conditioned medium from transiently transfected COS-7 cells diluted 1:10 (closed bars) and 1:50 (hatched bars) in transiently transfected COS-7 cells expressing CCR1, CCR2, US28, or CXCR4; stably transfected HEK-293 cells expressing CCR5; or CHO cells stably expressing either CXCR1, CXCR2, or the 7TM receptor for the nonchemokine neuropeptide substance P (NK-1). Conditioned medium from mock-transfected COS-7 cells was used as a control (open bars). Below the name of each of the receptors the chemokine peptide used as radioactively labeled ligand is indicated. Inhibition of 100% was defined by homologous displacement. (**B**) Competition binding experiments with HPLC-purified vMIP-II on CCR5 and CXCR4 expressed stably in HEK-293 cells and on US28 expressed transiently in COS-7 cells. Values on ordinate represent percent of maximum bound.

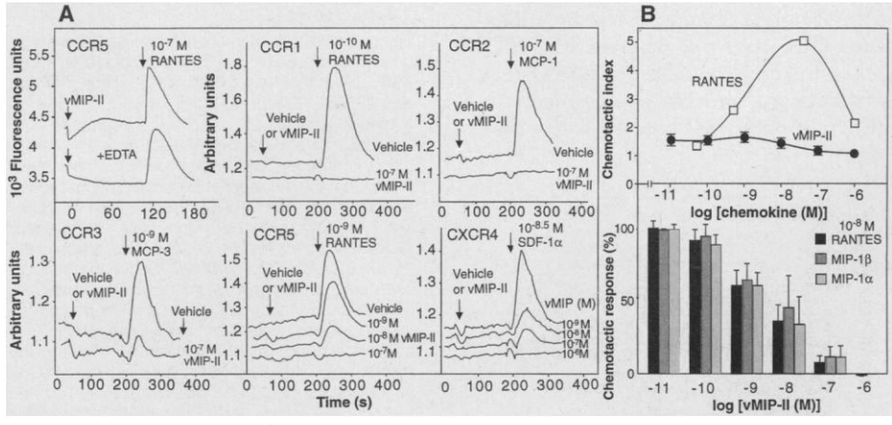

Fig. 3. Effect of vMIP-II on $[Ca^{2+}]_i$ in transfected cells and on chemotactic activity of human monocytes. (**A**) Traces of fluctuations in $[Ca^{2+}]_i$ measured by Fura-2 fluorescence in CHO cells stably transfected with various chemokine receptors. All experiments except the one shown in the upper left were done with 10 mM EDTA in the extracellular medium to study calcium mobilization only from intracellular stores. (Upper left) Upper trace shows effect of 10^{-9} M vMIP-II followed by 10^{-7} M RANTES in cells expressing CCR5; lower trace shows an experiment performed under the same conditions but with 10 mM EDTA added to the medium. All other panels show the effect of pretreatment with vMIP-II (either a single dose of 10^{-7} M or a full dose-response series on the calcium mobilization induced by a submaximal dose of an appropriate endogenous human chemokine in cells expressing various chemokine receptors: CCR1, 10^{-10} M RANTES; CCR2, 10^{-7} M MCP-1; CCR3, 10^{-9} M MCP-3; CCR5, 10^{-9} M RANTES; CXCR4, $10^{-8.5}$ M SDF-1α). HPLC-purified recombinant vMIP-II and synthetic vMIP-II gave similar results; the experiments with CCR5 were done with recombinant material, and the rest were done with synthetic peptide. (**B**) Chemotaxis of freshly isolated human monocytes. (Upper) Dose-response curve for vMIP-II (n = 8) given alone. A single dose-response curve for RANTES is shown for comparison. (Lower) Dose-response curves for inhibition by vMIP-II of chemotaxis induced by 10^{-8} M RANTES (IC_{50} for vMIP-II = 3.1 nM; n = 5), MIP-1α (IC_{50} = 2.7 nM; n = 2), or MIP-1β (IC_{50} = 6.2 nM; n = 2).

tor is in fact also surprisingly promiscuous, as it binds both CXC and CC chemokines (*19*)—just as vMIP-II acts as a ligand for both CC and CXC chemokine receptors.

Although CCR5 and CXCR4 are the main co-receptors for HIV cell entry (*20*), CCR3 also appears to be important for HIV infection of microglia (*21*). Because vMIP-II binds to all these receptors (*22*), its ability to block HIV cell entry was tested (*23*). The 89.6 strain of HIV-1 was used as a convenient probe as it is able to exploit multiple chemokine receptors for cell entry (*24*). In U87/CD4 cells, vMIP-II could block infection of strain 89.6 mediated through CCR3, CCR5, or CXCR4 (Fig. 4). On CCR5 and CXCR4, vMIP-II was less potent but equally efficient (at higher doses) compared with RANTES and SDF-1, respectively, whereas on CCR3, vMIP-II was highly potent and even more efficient than RANTES in blocking infection with strain 89.6 (Fig. 4). Against the SL-2 strain of HIV-1, which selectively uses CCR5 as co-receptor (*25*), vMIP-II and especially RANTES appeared less potent, whereas the semisynthetic analog AOP-RANTES as previously reported blocked infection very potently (*26*) (Fig. 4). Similar results were obtained with SF-162, a second NSI strain that uses CCR5 exclusively as co-receptor (data not shown). In peripheral blood mononuclear cells stimulated with phytohemagglutinin (PHA) and IL-2, vMIP-II at 200 nM reduced infectivity of SL-2 and SF-162 by a maximum of 50 to 75%, whereas RANTES blocked infectivity >98%. In this system, no significant reduction in infectivity with strain 89.6 was observed with either vMIP-II or RANTES (data not shown). Recently it was shown by cotransfection with CCR5 in $CD4^+$ cells that one of the other HHV-8–encoded chemokines, vMIP-I, can also inhibit transmission of CCR5-dependent HIV-1 strains (*2*). The binding profile of at least vMIP-II, including several co-receptors for HIV cell entry, could make this protein a possible lead for development of broad-spectrum therapeutic agents against HIV infection.

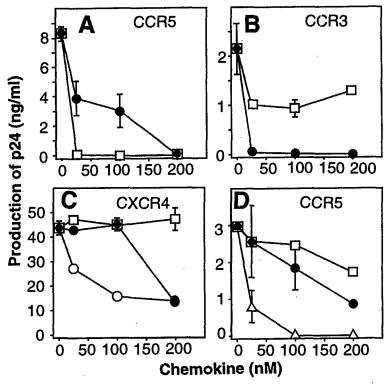

Fig. 4. Inhibition of HIV-1 infection of US87/CD4 cells stably expressing CCR5, CCR3, and CXCR4 with vMIP-II (●), RANTES (□), SDF-1α (○), and AOP-RANTES (△). The 89.6 strain of HIV-1 can use all three chemokine receptors as co-receptors for cell entry (**A** to **C**), whereas the SL-2 strain selectively uses CCR5 (**D**). Nanomolar concentrations of chemokines are indicated for direct comparison with receptor binding and signal transduction studies (100 nM ~ 800 ng/ml).

Conceivably, the original mammalian counterpart of the viral MIP was obtained by HHV-8 through an act of molecular piracy, as has been described for other herpesviruses (*27*). Genetic combinatorial chemistry combined with phenotypic selection has optimized the mammalian chemokines to benefit the virus—in analogy with methods used in pharmaceutical drug development. In the case of vMIP-II, it will be interesting to determine the structural basis for the promiscuous receptor recognition as well as the chemical modifications the virus has exploited to transform the protein into an antagonist.

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4. A biopsy was taken from a Kaposi's sarcoma skin lesion on an HIV-infected patient and a QIAamp blood kit (Qiagen) was used to extract total DNA. Based on the nucleotide sequence deposited in GenBank, the vMIP-II gene was amplified by PCR. The full-length coding sequence was inserted into the eukaryotic expression vector pTEJ8, which uses the ubiquitin UbC promoter (*28*). Sequence analysis was performed on an Alf Express sequencing system (Pharmacia Biotech). COS-7 cells were transiently transfected by a calcium phosphate precipitate method with addition of chloroquine. Serum-free medium was collected 24 to 48 and at 48 to 72 hours after transfection. COS cells were transfected in parallel with the empty pTEJ8 vector and serum-free medium was collected as a control.
5. Whole cell binding (1 to 3 × 10^5 cells per well) was performed at 4°C for 3 hours in 0.5 ml of 25 mM Hepes buffer containing 1 mM $CaCl_2$ and 5 mM $MgCl_2$ at pH 7.2, supplemented with 0.5% bovine serum albumin (BSA) on either transiently transfected COS-7 cells or stably transfected CHO or HEK-293 cells as indicated in the figure legends. The incubation was stopped by washing twice in 0.5 ml of ice-cold binding buffer except for CXCR4, where washing was performed four times with 0.5 ml of binding buffer made 0.5 M in NaCl. Cell-associated radioactivity was determined after extraction of the cells with 8 M urea in 3 M acetic acid supplemented with 1% NP-40. Nonspecific binding, determined in the presence of the relevant chemokine peptide (1 μM), was subtracted. The following radioactively labeled peptides (9 to 12 pM used for binding) were from Amersham: ^{125}I–MIP-1α (IM285), ^{125}I–MCP-1 (IM280), and ^{125}I–IL-8 (IM249). ^{125}I–SDF-1α was prepared in-house by oxidative iodination with Iodogen followed by HPLC purification to remove unlabeled from labeled compound. The chemokine peptides RANTES and IL-8 were expressed in *Escherichia coli* and purified in-house (Glaxo Biomedical Research Institute, Plan-les-ouates, Switzerland); MCP-1 and MIP-1α were obtained from Pepro Tech (London, UK) and SDF-1α was a generous gift from Michael Luther (Glaxo Wellcome, Research Triangle Park, NC).
6. Proteins were precipitated from culture medium with acetone (5:1) at room temperature in the presence of about 300 mg of BSA added to the medium, and the dried and reconstituted proteins were subjected to Bio-Gel P-30 gel filtration. This was followed by reversed-phase HPLC on a Vydac C8 column eluted with 0.1% trifluoroacetic acid (TFA) in water and a gradient of CH_3CN. Alternatively, conditioned medium was made 0.15% in TFA, filtered, and pumped directly onto the HPLC column. The elution position of the recombinant vMIP-II was monitored by receptor analysis of aliquots of fractions with ^{125}I–MIP-1α and HEK-293 cells stably expressing CCR5. Some batches of HPLC purified recombinant vMIP-II were contaminated with small amounts of ubiquitin as revealed by NH_2-terminal sequence analysis. NH_2-terminally deleted forms of vMIP-II, which otherwise could have acted as receptor antagonists, were not detected by NH_2-terminal sequence analysis or mass spectroscopy.
7. Conceivably, the carboxypeptidase B–like enzyme, which is found in the secretory pathway of most eukaryotic cells (*29*), is responsible for the COOH-terminal trimming of the vMIP-II protein by removal of the Arg residue. Nevertheless, the synthetic form of vMIP-II, which includes the COOH-terminal Arg residue [see (*9*)], and the recombinantly produced peptide had similar receptor binding and signal transduction profiles.
8. vMIP-II including the COOH-terminal Arg, which normally is removed in the secretory pathway, was synthesized with *t*-butoxycarbonyl chemistry and automated solid-phase methods as described elsewhere (*30*). The peptide was reconstituted in 1 M guanidinium hydrochloride and tris acetate at pH 8.5 and folded in 10% dimethyl sulfoxide in guanidine hydrochloride at pH 8.5, purified by reversed-phase HPLC, and analyzed by electrospray mass spectroscopy (*30*).
9. Alterations in intracellular calcium concentration ($[Ca^{2+}]$) in CHO and HEK-293 cells stably expressing various chemokine receptors were determined by loading with Fura-2 AM (Fluka), and fluorescence was monitored in a Jobin Yvon FluroMax-2 (results expressed as the ratio of 340 nm to 380 nm) or a JASCO PF777 spectrofluorometer. For studies without calcium in the extracellular medium (Krebs-Ringer buffer at pH 7.4) EDTA was added to a final concentration of 10 mM.
10. The possible biological consequences associated with the slow, prolonged calcium response to vMIP-II are unclear. However, a secondary, similarly slow and prolonged increase in intracellular calcium after stimulation with RANTES has been shown to be G-protein independent and possibly connected to activation of tyrosine kinases (*12*). Thus, agonistic effects of vMIP-II on cell differentiation or proliferation possibly related to the Kaposi's sarcoma lesion should be investigated.
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22. Actual binding of vMIP-II to CCR3 was not determined because of the lack of success in establishing a valid binding assay with radioactively labeled eotaxin or MCP-3. However, the observation that pretreatment with vMIP-II could inhibit the calcium mobilizing effect of MCP-3 in cells expressing CCR3 indicates that vMIP-II does bind to CCR3 (Fig. 3A).
23. U87/CD4 cells expressing CCR3, CCR5, and CXCR4 were kindly provided by Dan Littman. Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coat white blood cells from blood banks. After separation by density-gradient centrifugation on Ficoll-Paque, cells were cultured and stimulated with PHA. After 2 days IL-2 was added and after 3 to 5 days cells were infected. The HIV-1 strains have been described (*25, 26*). Virus stocks were prepared in PBMC cultures stimulated with PHA and IL-2. Although the same tissue culture ID_{50} (1900 for PBMC) of virus was used to challenge the cells, replication and infection were more efficient on the U87/CD4 cells expressing CXCR4 than on those expressing CCR3 or CCR5. However, lowering the amount of virus failed to substantially increase the inhibition by chemokines shown in Fig. 4 for the CXCR4-expressing cells. For determination of chemokine inhibition of infectivity, cells were seeded into 96-well dishes at 4×10^3 cells per well. On the following day chemokines were added and incubated for 30 min at 37°C before virus was added and incubated an additional 3 hours before washing three times to remove residual virus. The cells were then incubated for 5 days at 37°C before the medium was harvested and p24 concentrations were estimated (*31*). Initial time-course studies had determined that optimal production of p24 was obtained after 5 days with the virus strains that were used in the U87 cells before confluency was reached.
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Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress

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Variations in maternal care affect the development of individual differences in neuroendocrine responses to stress in rats. As adults, the offspring of mothers that exhibited more licking and grooming of pups during the first 10 days of life showed reduced plasma adrenocorticotropic hormone and corticosterone responses to acute stress, increased hippocampal glucocorticoid receptor messenger RNA expression, enhanced glucocorticoid feedback sensitivity, and decreased levels of hypothalamic corticotropin-releasing hormone messenger RNA. Each measure was significantly correlated with the frequency of maternal licking and grooming (all r's > -0.6). These findings suggest that maternal behavior serves to "program" hypothalamic-pituitary-adrenal responses to stress in the offspring.

Several years ago Levine, Denenberg, and others (*1*) showed that the development of hypothalamic-pituitary-adrenal (HPA) responses to stress is modified by early environmental events, including infantile stimulation [or handling (*2*)]. As adults, animals exposed to brief periods of handling daily for the first weeks of life show reduced pituitary adrenocorticotropic hormone (ACTH) and adrenal corticosterone (the principal glucocorticoid in the rat) responses to stress compared with nonhandled animals (*3*). These differences are apparent as late as 24 to 26 months of age (*4*), indicating that the handling effect on HPA function persists throughout life.

Glucocorticoids act at a number of neural sites to exert an inhibitory, negative-feedback effect over the synthesis of hypothalamic releasing-factors for ACTH, notably corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (*5*). Postnatally handled animals show enhanced glucocorticoid negative-feedback sensitivity compared with nonhandled rats (*6*) and therefore decreased hypothalamic CRH and AVP mRNA expression, as well as lower levels of both CRH and AVP immunoreactivity (*7*). The handling effect on feedback sensitivity is mediated by an increase in glucocorticoid receptor (GR) expression in the hippocampus (*8, 9*), a region that has been strongly implicated in glucocorticoid negative-feedback regulation (*10*). The increased hippocampal GR gene expression is therefore a central feature of the handling effect on HPA responsivity to stress, resulting in increased feedback inhibition of CRH and AVP synthesis and reduced pituitary ACTH release during stress.

A number of authors (*11*) have proposed that the effects of postnatal handling are mediated by changes in mother-pup interactions and that the handling manipulation itself might map onto naturally occurring individual differences in maternal care. Specifically, Levine proposed that handling of the pups altered the behavior of the mother and that these differences in mother-pup interactions then mediate the effect of handling on the development of endocrine and behavioral responses to stress. The question, then, is how this maternal mediation might occur and whether such factors might contribute to naturally occurring individual differences in HPA responses to stress.

In the Norway rat, mother-pup contact occurs primarily within the context of a nest-bout, which begins when the mother approaches the litter and gathers the pups under her; she then nurses her offspring, intermittently licking and grooming the pups (*12, 13*). Handling results in changes in mother-pup interactions (*14*). Mothers of handled pups spend the same amount of time with their litters as mothers of non-

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