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19. Cataplexy-off cells were defined as those having a ≥50% decrease in their discharge rate during cataplexy relative to that during the active waking period immediately before cataplexy. Cataplexy-on cells were identified by the same 50% criterion. Cataplexy onset was defined by the abrupt loss of muscle tone recorded bilaterally in the nuchal muscles. Cataplexy durations ranged from 6 to 43 s (mean, 18.4 ± 10.5 s).
20. A two-way analysis of variance of state (quiet waking, active waking, cataplexy, REM sleep, non-REM sleep) by type (cataplexy-off, cataplexy-on, other) showed a significant state effect ($P < 0.01$) and a significant interaction effect ($P < 0.05$).
21. The ratio of quiet waking rates to active waking rates was significantly higher in cataplexy-on cells than in cataplexy-off cells ($P < 0.001$, two-tailed t test).
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30. Supported by the Medical Research Service of the Veterans Administration; Public Health Service grants NS14610, HL41370, and NS23724; and the American Narcolepsy Association. We thank S. Goodman for advice on neurosurgical procedures.

24 September 1990; accepted 29 March 1991

Cloning, Expression, and Gene Structure of a G Protein–Coupled Glutamate Receptor from Rat Brain

KHALED M. HOUAMED,* JOSEPH L. KUIJPER, TERESA L. GILBERT, BETTY A. HALDEMAN, PATRICK J. O'HARA, EILEEN R. MULVIHILL,† WOLFHARD ALMERS, FREDERICK S. HAGEN

A complementary DNA encoding a G protein–coupled glutamate receptor from rat brain, Glu_GR, was cloned by functional expression in *Xenopus* oocytes. The complementary DNA encodes a protein of 1199 amino acids containing a seven-transmembrane motif, flanked by large amino- and carboxyl-terminal domains. This receptor lacks any amino acid sequence similarity with other G protein–coupled receptors, suggesting that it may be a member of a new subfamily. The presence of two introns flanking the central core suggests that Glu_GR may have evolved by exon shuffling. Expressed in oocytes, Glu_GR is activated by quisqualate > glutamate > ibotenate > *trans*-1-aminocyclopentyl-1,3-dicarboxylate, and it is inhibited by 2-amino-3-phosphonopropionate. Activation is blocked by *Bordella pertussis* toxin. These properties are typical of some metabotropic glutamate receptors.

L-GLUTAMATE (GLU) AND ITS ANALOGS are the predominant excitatory neurotransmitters in the central nervous system (*1*). Fast transmission is mediated by ionotropic glutamate receptors (GluRs) functioning as Glu-gated cation channels (*2*), whereas metabotropic GluRs act through second messenger systems (*3, 4*). The quisqualate (Quis) metabotropic receptor activates phospholipase C (PLC), which, in turn, generates inositol-1,4,5-trisphosphate (IP_3) (*5, 6*), leading to the liberation of intracellular Ca^{2+} (7). Quis also inhibits some neuronal voltage-gated cation channels (*8, 9*). Some of the effects of metabotropic GluR activation are mediated by G proteins because the effects can be inhibited by *Bordella pertussis* toxin (PTX) or mimicked by the intracellular application of guanosine triphosphate (GTP) and its analogs (*8, 10*). Here, we report the isolation of a cDNA clone encoding a G protien–coupled GluR (Glu_GR) that has the properties of the metabotropic Quis receptor (*4*).

Activation of the metabotropic Quis receptor, expressed in *Xenopus* oocytes injected with polyadenylated [poly(A)$^+$] whole brain RNA (Fig. 1A) or cerebellum RNA (Fig. 1B), evokes transient and often oscillatory inward currents (*3*). By a PTX-sensitive mechanism, this GluR activates the PLC-IP_3 cascade, leading to the generation of an oscillatory Ca^{2+}-activated Cl^- current [$I_{Cl(Ca)}$] (*11*). The $I_{Cl(Ca)}$ provided an assay for the functional expression of the metabotropic GluR. We constructed a cDNA expression library from rat cerebellum (*12*). RNA transcribed from pools of 100,000 clones (*13*) was injected into oocytes and assayed for $I_{Cl(Ca)}$ (*14*). A positive pool (Fig. 1C) was further subdivided until a single positive clone (45-A) was identified (Fig. 1D).

The effect of Glu on oocytes expressing Glu_GR was dose-dependent, and the concentration of Glu producing a half-maximal effect (EC_{50}) was 12 μM (Fig. 1E). Three other Glu analogs were found to be effective agonists (Quis, EC_{50} = 0.7 μM; ibotenate, EC_{50} = 32 μM; and *trans*-1-aminocyclopentyl-1,3-dicarboxylate, EC_{50} = 0.38 mM) (*15*). In contrast, the ionotropic receptor agonists aspartate (1 mM), kainate (1 mM), *N*-methyl-D-aspartate (100 μM plus 10 μM Gly), 2-amino-4-phosphonobutyrate (APB) (100 μM) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) (100 μM) evoked no measurable response (n = 5). The putative Quis metabotropic receptor antagonist 2-amino-3-phosphonoproprion-

K. M. Houamed and W. Almers, Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, WA 98195.
J. L. Kuijper, T. L. Gilbert, B. A. Haldeman, P. J. O'Hara, E. R. Mulvihill, F. S. Hagen, Departments of Molecular and Cellular Biology and DNA Chemistry, ZymoGenetics, 4225 Roosevelt Way NE, Seattle, WA 98105.

*Present address: Department of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195.
†To whom correspondence should be addressed.

ate (1 mM) reduced Glu_GR currents evoked by 10 μM Glu to 56 ± 7% (SEM) of control (n = 4). Glu_GR currents were also significantly inhibited by PTX, indicating that a G protein lies in the signaling pathway of the receptor. After incubation of oocytes with PTX (*16*), peak currents were 58 ± 19 nA (n = 9), compared to 264 ± 73 nA (n = 6) in control oocytes from the same batch (P < 0.01, Student's t test). These results are consistent with those obtained for the metabotropic Quis receptor in brain and expressed in oocytes (*3, 4, 17–19*).

Clone 45-A contains an open reading frame of 3597 base pairs, corresponding to a 133-kD protein of 1199 amino acids (Fig. 2A). Glu_GR showed no sequence similarities to other G protein–coupled receptors (*20, 21*), suggesting that it represents a new subfamily. The receptor contains three structural domains: a 593–amino acid NH_2-terminal domain and a 367–amino acid COOH-terminal domain, separated by a central core of 239 amino acids containing the seven transmembrane domains (7TMD) characteristic of G protein–coupled receptors (Fig. 2B). The 7TMD region is distinguished by an unusually short loop between domains V and VI. This putatively cytoplasmic loop has been implicated in the coupling to the G proteins. The NH_2-terminal region of Glu_GR, which is unusually large for a G protein–coupled receptor, contains additional hydrophobic segments, which may interact with the membrane, including one at the NH_2-terminus. However, the absence of a signal cleavage site (*22*) suggests that the mature Glu_GR may start at the initiation methionine. The NH_2-terminal domain of Glu_GR has sequence similarity to that of the membrane form of the sea urchin guanylate cyclase (SUGC), a peptide receptor with a single transmembrane domain and an intracellular guanylate cyclase COOH-domain (*23*). In the region of highest similarity (a 104 stretch of amino acids starting at Lys^{153}), there was a 29% identity between the two proteins (Fig. 2C). The large COOH-domain of Glu_GR contains poly-(Gln), poly(Pro), and poly(Glu) repeats normally found in the third cytoplasmic loop of some other G protein–coupled receptors (*21*).

Northern analysis of brain and cerebellum poly(A)$^+$ RNA, probed with a fragment from clone 45-A (Fig. 3C), revealed two transcripts: strong hybridization was observed at ~7 kb, and a weaker signal appeared at ~4 kb. Two findings suggest that the larger mRNA encodes Glu_GR. First, size fractionation of brain poly(A)$^+$ RNA suggests that the metabotropic GluR is translated from a 6.5- to 7.5-kb mRNA (*24*). Moreover, two clones were isolated that

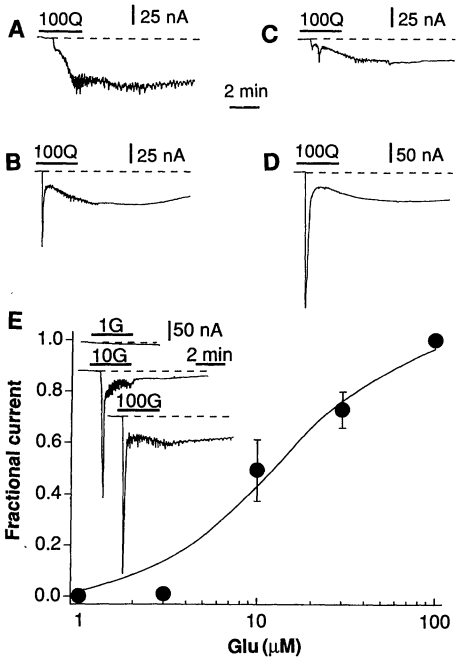

Fig. 1. Activation of the metabotropic GluR in oocytes injected with poly(A)$^+$ RNA from (**A**) whole brain (35 ng per cell), (**B**) cerebellum (60 ng per cell), (**C**) cRNA transcribed from a sublibrary of 100,000 clones (100 ng per cell), or (**D**) a single clone (45-A) coding for Glu_GR (5 pg per cell). Quis was used as an agonist to minimize activation of ionotropic glutamate receptors in (A) and (B). Dashed lines, resting membrane current. All recordings from oocytes of a single donor. Quis (100 μM) was applied at the bars marked 100Q. (**E**) Dose-dependent activation of Glu_GR by Glu. Peak current as a function of Glu concentration. Each dose of Glu was tested on four to five oocytes (mean ± SEM). In each oocyte, a final application of 100 μM Glu was used to normalize all other measurements. The curve is a nonlinear least squares fit to the following equation: fractional current = $(dose)^n/[(dose)^n + (EC_{50})^n]$, where n = 1 to 41. (**Inset**) Currents evoked by increasing concentrations of Glu (1 μM, 10 μM, or 100 μM Glu was applied at the bars marked 1G, 10G, and 100G, respectively) in an oocyte injected with Glu_GR cRNA (5 pg). More than 30 min elapsed between consecutive Glu applications to allow recovery from desensitization.

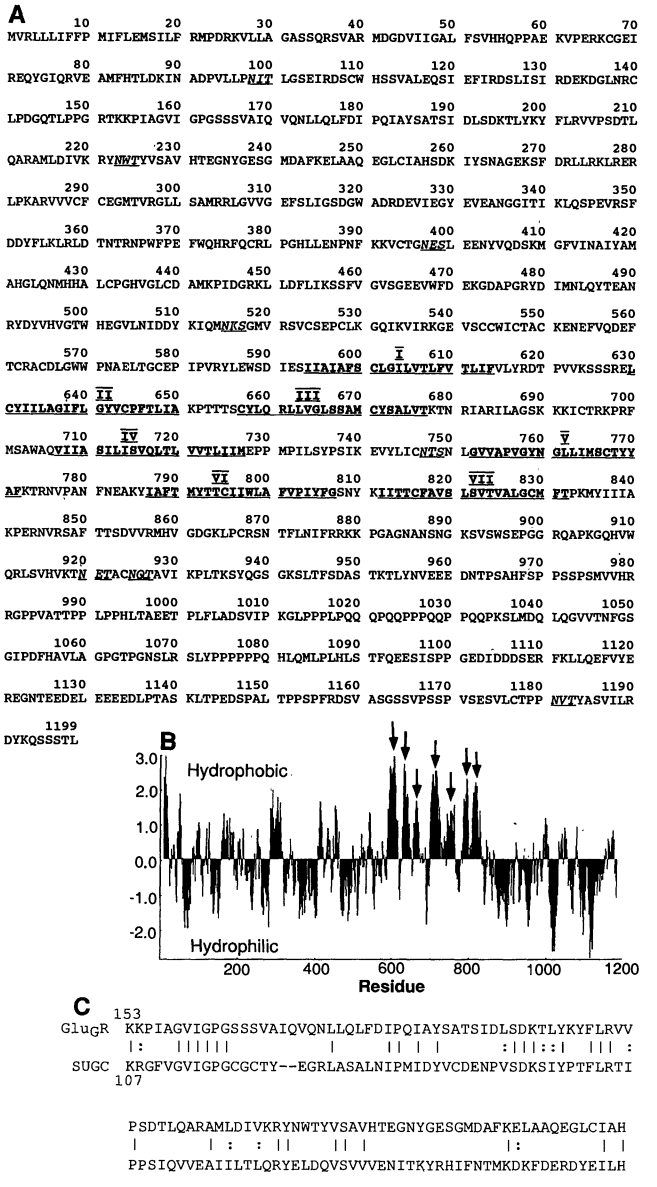

Fig. 2. Sequence analysis of Glu_GR cDNA. (**A**) Both strands of clone 45-A were sequenced with a modified chain termination method, with Sequenase (U.S. Biochemical, Cleveland, Ohio) (*27*). The amino acid sequence of the Glu_GR predicted from clone 45-A is shown in one-letter code (A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; and V, Val). The 7TMD are underlined and numbered with Roman numerals. Possible N-linked glycosylation sites are underlined and italicized. The nucleotide sequence has been deposited in GenBank (accession number M61099). (**B**) Hydrophilicity profile of the amino acid sequence of Glu_GR (*28*) showing the seven hydrophobic peaks (arrows). (**C**) Alignment of amino acids 153 to 257 of Glu_GR with amino acids 107 to 209 of SUGC. Vertical bars connect identical amino acids, colons connect conservative substitutions.

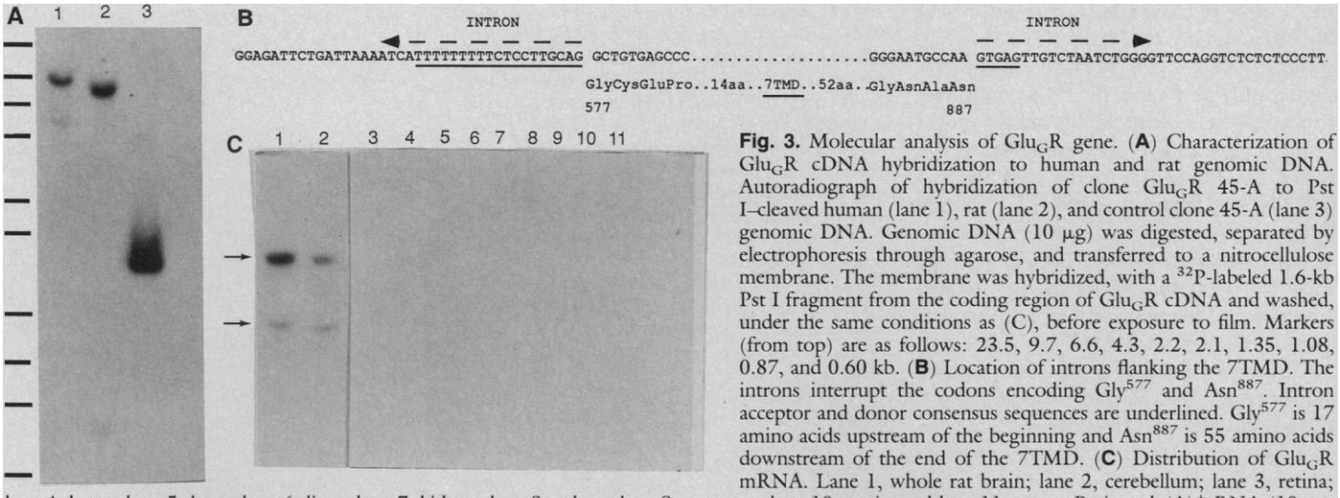

Fig. 3. Molecular analysis of Glu_GR gene. (**A**) Characterization of Glu_GR cDNA hybridization to human and rat genomic DNA. Autoradiograph of hybridization of clone Glu_GR 45-A to Pst I–cleaved human (lane 1), rat (lane 2), and control clone 45-A (lane 3) genomic DNA. Genomic DNA (10 μg) was digested, separated by electrophoresis through agarose, and transferred to a nitrocellulose membrane. The membrane was hybridized, with a ^{32}P-labeled 1.6-kb Pst I fragment from the coding region of Glu_GR cDNA and washed, under the same conditions as (C), before exposure to film. Markers (from top) are as follows: 23.5, 9.7, 6.6, 4.3, 2.2, 2.1, 1.35, 1.08, 0.87, and 0.60 kb. (**B**) Location of introns flanking the 7TMD. The introns interrupt the codons encoding Gly^{577} and Asn^{887}. Intron acceptor and donor consensus sequences are underlined. Gly^{577} is 17 amino acids upstream of the beginning and Asn^{887} is 55 amino acids downstream of the end of the 7TMD. (**C**) Distribution of Glu_GR mRNA. Lane 1, whole rat brain; lane 2, cerebellum; lane 3, retina; lane 4, heart; lane 5, lung; lane 6, liver; lane 7, kidney; lane 8, spleen; lane 9, pancreas; lane 10, testis; and lane 11, ovary. Brain poly(A)$^+$ RNA (10 μg, lanes 1 and 2) was obtained as in (*12*); poly(A)$^+$ RNA from other tissues (5 μg) was purchased from Clontech Labs. Integrity of the RNAs was confirmed by reverse transcription and polymerase chain reaction amplification of a 381-bp fragment from the 5′ end of the housekeeping gene glucose-6-phosphate dehydrogenase. RNA was denatured in glyoxal, separated by electrophoresis through agarose, transferred to a nitrocellulose membrane, probed with a ^{32}P-labeled 3474-bp Eco RI to Xba I fragment of Glu_GR cDNA [50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 50 mM NaH_2PO_4, 5 mM EDTA, salmon sperm DNA (0.1 mg/ml), 5× Denhardt's solution at 42°C], and washed at high stringency (30 mM NaCl, 3 mM sodium citrate, 0.1% SDS at 65°C). The size of the two bands (arrows) was determined with the use of glyoxalated DNA markers.

extend the sequence of Glu_GR ~2.5 kb beyond the 3′ end of clone 45-A (*25*). The extension is noncoding and terminates in a polyadenylation sequence.

In contrast to many G protein–coupled receptor genes, the coding region of Glu_GR contains at least two introns. We isolated three independent cDNA clones (*25*) that contained the 7TMD of clone 45-A flanked by noncoding sequences with intron donor and acceptor sites (*26*) (Fig. 3B). These clones probably originated from incompletely processed precursor mRNA. The region enclosed by these introns has the essential structural features of other (intronless) G protein–coupled receptor genes (7TMD flanked by short NH_2-end and COOH-end overhangs). It is therefore possible that the Glu_GR gene evolved by coupling of exons coding for large NH_2- and COOH-terminal extracellular portions to a primordial G protein–coupled receptor gene.

The presence of introns within the Glu_GR gene was confirmed by Southern (DNA) blot analysis. Rat and human genomic DNA, digested with Pst I, was probed with a 1.6-kb Pst I fragment coding for amino acids 562 to 1084 of Glu_GR (Fig. 3A). The absence of a 1.6-kb hybridizing species in the human and rat DNA suggests that there is at least one intron within the 1.6-kb Pst I fragment.

We have cloned a GluR from cerebellum with the same functional and pharmacological properties as the Quis metabotropic GluR. Athough this receptor bears little sequence similarity to any other known G protein–coupled receptor, there is little doubt that it acts through a G protein because it mediates IP_3 release, an effect normally regulated by a G protein, in a PTX-sensitive manner. The presence of the 7TMD structural motif also lends support to the hypothesis that this receptor is distantly related to the family of G protein–coupled receptors. The location of the introns flanking the central 7TMD suggests a possible mechanism of evolutionary relatedness by exon rearrangement.

Note added in proof: After this report was submitted, Masu *et al.* (*29*) reported the cloning of a GluR with identical amino acid sequence.

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13. Template DNA, prepared by alkaline lysis and CsCl centrifugation, was transcribed in vitro with T7 RNA polymerase (Pharmacia) in the presence of GpppG cap (Pharmacia) by standard protocols [D. A. Melton *et al.*, *Nucleic Acids Res.* **12**, 7035 (1984)], except that the plasmid was not linearized.
14. The methods of oocyte isolation, injection, maintenance, and the voltage clamp recording were as in previous work [N. Dascal, T. P. Snutch, H. Lubbert, N. Davidson, H. A. Lester, *Science* **231**, 1147 (1986); C. Methfessel *et al.*, *Pfluegers Arch.* **407**, 577 (1986)].
15. All drug responses were normalized with respect to a subsequent application of 100 μM Glu. The response to 100 μM Glu was the maximum recorded; 300 μM and 1 mM Glu produced smaller responses, presumably because of desensitization. All three agonists, at the maximum concentrations tested, evoked responses ≥80% of the response

evoked by 100 μM Glu, suggesting that all three are full agonists.
16. The oocytes were treated with PTX (4 μg/ml) in Barth's medium [88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 10 mM Hepes, pH 7.4] for 24 hours at 19°C [N. Dascal *et al.*, *Mol. Brain Res.* **1**, 201 (1986); T. M. Moriarty *et al.*, *J. Biol. Chem.* **264**, 13524 (1989)].
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13 February 1991; accepted 5 April 1991

Technical Comments

CD14 and Immune Response to Lipopolysaccharide

Lipopolysaccharide binding protein (LBP) is a normal serum component that can bind to soluble lipopolysaccharide (LPS) and to LPS that is expressed on bacterial surfaces (*1*). Binding of the LPS-LBP complex to macrophages induces production of tumor necrosis factor (TNF)(*2*), which is a primary mediator of endotoxic shock. Moreover, LBP concentration increases after induction of the acute phase response (*1*). These results suggest that LBP has a major regulatory role in Gram-negative bacterial clearance and in the destructive processes of LPS-induced schock. Wright *et al.* reported (*3*) that CD14, a 55-kD glycoprotein expressed by monocytes and macrophages, is a cell surface receptor for LBP-LPS. Induction of TNF release by LBP-LPS complexes was blocked by anti-CD14 monoclonal antibodies in a highly specific manner. Thus, CD14 expression represents a second mechanism by which the immune response to LPS might be regulated.

In 1985, Maliszewski *et al.* reported (*4*) on the purification and biochemical characterization of My23, a 55-kD protein on monocyte cell surfaces that was recognized by the monoclonal antibody AML-2-23. (The My23 antigen was later designated "CD14" by the International Workshops on Leukocyte Antigens.) We demonstrated that a soluble form of CD14 could be purified from myeloid cell culture supernatants and that the binding of AML-2-23 to myeloid cells was inhibited by soluble CD14 and by monoclonal antibodies to the soluble CD14 protein. These results suggested that a similar phenomenon might occur in vivo, a suggestion that was supported by the finding that soluble CD14 was present in normal human plasma and could be purified on AML-2-23 immunosorbent beads (*4*). A possible mechanism for the generation of a soluble CD14 peptide was subsequently provided by Haziot *et al.* (*5*), who demonstrated that CD14 is attached to the cell surface by a glycosylphosphatidyl-inositol linkage.

One hypothesis that emerges from these data is that soluble CD14 could be a natural inhibitor of the deleterious effects of endotoxin. LBP-LPS complexes could be neutralized by soluble CD14, which would prevent their interaction with macrophages and the induction of TNF release. This protective mechanism would be circumventedonce the levels of LBP-LPS exceeded the effective inhibitory concentration of soluble CD14. Thus, under normal conditions, soluble CD14 might neutralize the concentrations of LBP-LPS in a limited Gram-negative infection. Taken a step further, a process that triggers the release of CD14 from macrophages would enhance this protective effect in the face of a greater LBP-LPS load.

Clearly, additional experimentation is required to verify or disprove this hypothesis. Nevertheless, it suggests an obvious therapeutic application for recombinant soluble CD14 in Gram-negative sepsis.

CHARLES R. MALISZEWSKI
Department of Immunology, Immunex, Seattle, WA 98101

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5 November 1990; accepted 21 March 1991

Response: Maliszewski correctly points out that CD14 can be found not only on the cell surface but also in culture supernatants of CD14-bearing cells and in human plasma (*1*). This observation was first made in 1985 and has been confirmed and extended (*2*). Because cell surface CD14 binds LPS-LBP complexes and appears crucial for initiating responses to LPS (*3*), Maliszewski hypothesizes that soluble CD14 may "neutralize" LPS-LBP complexes and thereby prevent responses to LPS. While this hypothesis is attractive on theoretical grounds, other observations suggest that soluble CD14 competes inefficiently with cell-bound CD14.

Bazil *et al.* (*2*) estimated the concentration of CD14 in plasma from healthy adults to be 2 to 6 μg/ml. This concentration ($\sim 10^{-7}$ M) is over a thousand times greater than the peak concentration of LPS (2×10^{-11} M) observed in human serum during sepsis (*4*). Thus, humans can respond to LPS briskly, and fatal-

Table 1. Loss of CD14 from monocyte-derived macrophages during response to LPS. The indicated stimuli were added to Teflon beakers containing 4-day cultures of human monocytes (10^6 cells per milliliter in RPMI, 10% normal human serum). After 18 hours of culture, cells were washed, stained with monoclonal antibodies to CD14 (3C10), CD18 (IB4), or HLA (W6/32) and fluoresceinated $F(ab)_2$ antimurine immunoglobulin G, and analyzed by FACS. Data are presented as mean fluorescent intensity. The loss of cell surface CD14 induced by LPS is unlikely to be secondary to the secretion of TNFα as addition of TNF enhanced, not decreased, the expression of CD14.

Antigen	Stimulant		
	None	LPS (100 ng/ml)	TNFα (10^{-9} M)
CD14	579	26	1138
CD18	1700	1636	1788
HLA	2292	2472	2399
Control	23	20	24