The Fc and Not CD4 Receptor Mediates Antibody Enhancement of HIV Infection in Human Cells

JACQUES HOMSY, MIA MEYER, MASATOSHI TATENO,*
SARAH CLARKSON, JAY A. LEVY

Antibodies that enhance human immunodeficiency virus (HIV) infectivity have been found in the blood of infected individuals and in infected or immunized animals. These findings raise serious concern for the development of a safe vaccine against acquired immunodeficiency syndrome. To address the in vivo relevance and mechanism of this phenomenon, antibody-dependent enhancement of HIV infectivity in peripheral blood macrophages, lymphocytes, and human fibroblastoid cells was studied. Neither Leu3a, a monoclonal antibody directed against the CD4 receptor, nor soluble recombinant CD4 even at high concentrations prevented this enhancement. The addition of monoclonal antibody to the Fc receptor III (anti-FcRIII), but not of antibodies that react with FcRI or FcRII, inhibited HIV type 1 and HIV type 2 enhancement in peripheral blood macrophages. Although enhancement of HIV infection in CD4$^+$ lymphocytes could not be blocked by anti-FcRIII, it was inhibited by the addition of human immunoglobulin G aggregates. The results indicate that the FcRIII receptor on human macrophages and possibly another Fc receptor on human CD4$^+$ lymphocytes mediate antibody-dependent enhancement of HIV infectivity and that this phenomenon proceeds through a mechanism independent of the CD4 protein.

THE HUMORAL IMMUNE RESPONSE to HIV includes the formation of a heterogenous population of antibodies that can have inhibiting, promoting, or no effect on the virus pathogenicity in vitro (*1–5*). Neutralizing antibodies and antibodies mediating cellular cytotoxicity (ADCC) to HIV (*1, 2*) represent important responses sought for in an effective HIV vaccine. Antibodies that enhance HIV infectivity in vitro have been shown to occur in the serum or plasma of infected individuals and infected and immunized animals (*3–5*). These antibodies have been invoked as a possible explanation for the apparent lack of protection of neutralizing or cytotoxic antibodies against HIV pathogenesis in vivo (*3*). Enhancing antibodies are a particular concern for the development of a safe and effective HIV vaccine because any immunogen that would elicit their formation could pose serious risks to the immunized population.

It has been reported that a large proportion of neutralizing and nonneutralizing sera obtained from seropositive individuals could enhance HIV infectivity and mask neutralizing activity in an established T cell line in the presence of fresh complement (*3*). This observation suggested that the complement receptor could mediate antibody-dependent enhancement (ADE) of HIV infectivity. We and others have found that decomplemented sera from both immunized and infected subjects can also have enhancing properties not only in established T cell lines, but also in CD4$^+$ lymphocytes and fully differentiated macrophages derived from the peripheral blood (*4, 5*). Moreover, nonmacrophagetropic strains of either HIV-1 or HIV-2 subtype can productively infect these cells in the presence of enhancing sera (Table 1). Finally, decomplemented antisera lose their HIV-enhancing activity when the Fc portion of immunoglobulins (Ig) is removed and Ig aggregates compete with this activity (*5*). These observations have pointed to the Fc receptor as a likely mediator of ADE of HIV.

The aim of the present study was to identify further the receptor or receptors mediating this HIV enhancement in various cell types. Therefore, we performed blocking experiments on potential ADE receptors. To facilitate the interpretation of results, we used sera with high titers of en-

J. Homsy, M. Meyer, M. Tateno, J. A. Levy, Department of Medicine and Cancer Research Institute, School of Medicine, University of California, San Francisco, CA 94143.
S. Clarkson, Rosalind Russell Rheumatology Laboratory, San Francisco General Hospital, San Francisco, CA 94103.

*Present address: Department of Pathology, Sapporo City General Hospital, Chuoku Sapporo, 060 Japan.

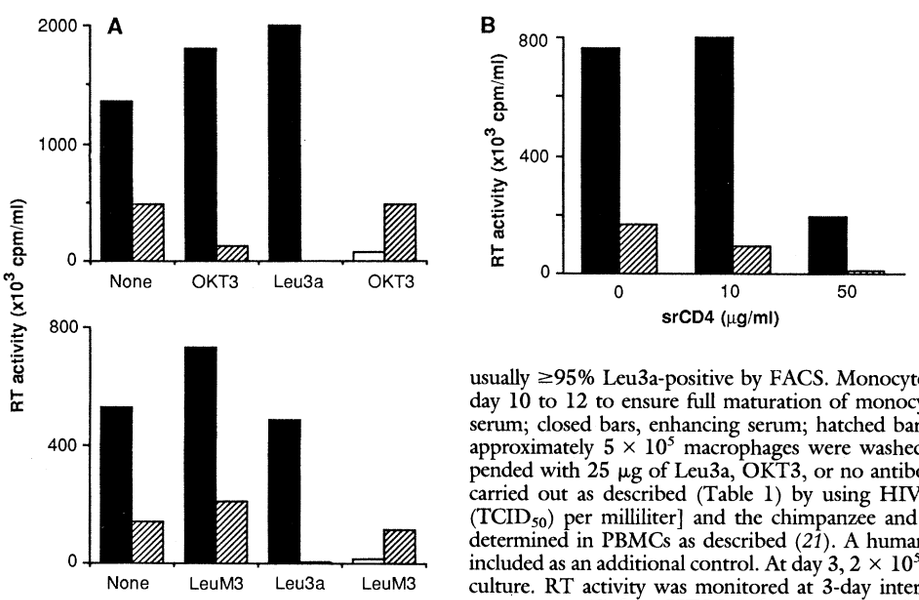

Fig. 1. The effect of blocking the CD4 protein on ADE of HIV infection of lymphocytes and macrophages. This figure is representative of at least three different experiments. PBMC were isolated from fresh blood obtained from HIV seronegative individuals by Ficoll-Hypaque separation (*7*). CD4$^+$ cells were separated from PBMCs by immunoadsorption on a polystyrene plate by using Leu3a (Becton Dickinson) according to the method of Wysocki and Sato (*20*). After separation, these CD4$^+$ cells were treated for 45 min with leucine methyl ester (Sigma) to lyse monocytes, and they were stimulated with phytohemagglutinin (PHA) (3 μg/ml) for 3 days before use (*7*). The final cell population obtained in this way was usually ≥95% Leu3a-positive by FACS. Monocytes were isolated as described (Table 1) and used by day 10 to 12 to ensure full maturation of monocytes into macrophages (*4*). Open bars, neutralizing serum; closed bars, enhancing serum; hatched bars, control serum. (**A**) CD4$^+$ lymphocytes (10^5) or approximately 5×10^5 macrophages were washed with phosphate-buffered saline (PBS) and resuspended with 25 μg of Leu3a, OKT3, or no antibody for 30 min at room temperature. Infection was carried out as described (Table 1) by using HIV_{SF128A} [100 median tissue culture infectious dose ($TCID_{50}$) per milliliter] and the chimpanzee and control sera (at 1:20 dilution). The $TCID_{50}$ was determined in PBMCs as described (*21*). A human neutralizing serum used at the same dilution was included as an additional control. At day 3, 2×10^5 CD4$^+$ lymphocytes were added to each lymphocyte culture. RT activity was monitored at 3-day intervals (*9*). Results represent the peak RT activity of supernatants (*8*) from HIV-1–infected cultures after 6 to 9 days for lymphocytes and 12 to 15 days for macrophages. Similar results were obtained with the guinea pig serum (*10*). (**B**) PHA-stimulated PBMCs (10^6) were infected as described above with $HIV\text{-}1_{SF128A}$ that had been incubated 1:1 (vol/vol) for 30 min with srCD4 (10 or 50 μg/ml), then with guinea pig enhancing or control serum diluted 1:50. Results are shown after 3 days of culture. Similar results were obtained when the sera were incubated with srCD4 before mixing with HIV.

hancing activity and no neutralizing activity. The three sera selected (*4*) came from a guinea pig (gp) hyperimmunized with HIV-1_{SF128A}, from an HIV-1_{SF2}–infected chimpanzee (ch), and from a patient (h) with acquired immunodeficiency syndrome (AIDS). The enhancing titers of these sera ranged from 1:250 (ch and h) to 1:1250 (gp). Controls included normal sera (ch and h) or homologous serum obtained before immunization (gp). Different HIV strains isolated in our laboratory, including macrophage-tropic strains of HIV-1 and HIV-2 (*7*), were used in the enhancement assays (*8–10*).

Initially we investigated whether the CD4 receptor, which has been considered a primary site of HIV entry into the cell, played a role in enhancement. We used Leu3a, a monoclonal antibody (MAb) against CD4a (anti-CD4a), and soluble recombinant CD4 (srCD4), which effectively inhibit HIV infection by preventing virus attachment to the cell surface (*11*). Controls included the anti-CD3 MAb OKT3 and the anti-macrophage MAb LeuM3. The presence of Leu3a prevented the infection of both purified $CD4^+$ lymphocytes and macrophages in the presence of control sera (Fig. 1A). It had no effect, however, when the virus had been incubated with enhancing sera before infection, even when up to 50 μg per 10^5 cells was used. As expected, treatment of cells with OKT3 or LeuM3 did not have any significant effect on infection in the presence of either the enhancing or a nonenhancing neutralizing serum (Fig. 1A). Furthermore, the addition of srCD4 at up to 50 μg per 10^6 peripheral blood mononuclear cells (PBMC) was not able to inhibit serum enhancement of HIV-1 infection (Fig. 1B), even though this concentration completely abolished HIV infectivity in the presence of control serum. The reduced level of reverse transcriptase (RT) activity observed at day 3 after infection with 50 μg of srCD4 (Fig. 1B) represents a delay in virus production and probably reflects the effect of srCD4 on residual free virus that is not bound to enhancing antibodies. Three days later, this culture reached high levels of RT activity, whereas the culture-receiving virus that was incubated with control serum remained negative. Similar results were obtained with two different strains of HIV-1 as well as HIV-2 in both types of blocking experiments (Leu3a and srCD4) (*10*). Finally, we tested $CD4^-$ human fibroblastoid cells for their ability to support enhancement of HIV infection. These cells do not express CD4 on their surface and do not contain a detectable amount of CD4 mRNA (*12*). However, after cocultivation with highly susceptible lymphoblastoid cells, both viral antigen production (>0.5 ng/ml) and cytopathology occurred only in cultures that had been infected in the presence of enhancing sera (*13*). All of these results indicate that the CD4 protein does not mediate ADE of HIV infectivity.

In studying further the role of the Fc receptors (FcRs) in this process, we attempted to block ADE of HIV infection of macrophages by treating these cells with antibodies known to bind and block the function of each human FcR. Three FcRs (FcRI, FcRII, and FcRIII) have been identified on human leukocytes (*14*). They are glycoproteins of 72,000, 40,000, and 50,000 to 70,000 M_r, respectively, and they can all bind IgG immune complexes or aggregates. However, only FcRI can bind monomeric IgG. FcRI is found on monocytes and macrophages and has been shown to be involved in ADCC, possibly in connection with interferon gamma (*14*). We used RPC-5, the product of a murine IgG2a myeloma, to bind and block the function of FcRI. When used at high concentrations, RPC-5 serves as an appropriate ligand to saturate the binding sites of FcRI (*6*). FcRII is a polymorphic receptor found on monocytes, neutrophils, eosinophils, B cells, and platelets. FcRII mediates the monocyte-dependent mitogenic response of T cells and may serve as a target for natural killer (NK) cell activity (*14*). These properties can be blocked by MAb IV.3 of the IgG2b subclass. Finally, FcRIII, also termed CD16, has been identified on neutrophils, eosinophils, NK cells, and differentiated macrophages and appears to be mainly responsible for the clearance of immune complexes through phagocytosis (*6*). MAb 3G8, an IgG1, binds human FcRIII and inhibits attachment and phagocytosis of IgG-coated red blood cells by macrophages (*6, 14*). In addition to these antibodies, we used MOPC-21, a murine IgG1 myeloma anti-

Table 1. ADE of HIV-1 and HIV-2 infectivity in macrophages. Monocytes were isolated from PBMC by adherence onto a polystyrene plate and incubated in RPMI 1640 medium containing 10% fetal calf serum and 5% blood group–matched, heat-inactivated human serum. Adherent monocytes were kept in culture for 10 to 12 days before infection to ensure full differentiation into macrophages (*4*). Cells isolated in this way were ≥ 95% positive for LeuM3 staining. After washing and trypsinization, 5 × 10^5 macrophages were infected with 100 μl of HIV-1 or HIV-2 culture supernatants (RT activity was ≈ 10^6 cpm/ml) that had been incubated 1:1 (vol/vol) with enhancing or control serum (1:20) for 1 hour at room temperature (*4*). The gp serum came from an animal hyperimmunized with HIV-1_{SF2} (formerly ARV-2), the ch serum from an animal infected with HIV-1_{SF2}, and the h serum from a patient with AIDS. These sera had no neutralizing activity. Cultures were incubated for 2 hours at 37°C, washed with Hanks buffer, and resuspended in appropriate medium. HIV production was monitored by measurement of RT activity at 3-day intervals (*9*). Results represent the peak RT activity measured 12 to 15 days after infection (*8*). In the presence of normal serum, RT activity never increased over the levels shown here for HIV-1_{SF2c} (molecular clone of HIV-1_{SF2}) (*18*), HIV-1_{SF33}, HIV-2_{UC2}, or HIV-2_{UC3} (*7*). A representative of two experiments giving similar results is shown.

Virus strain	RT activity (×10^3 cpm/ml)		Source
	Control serum	Enhancing serum	
HIV-1			
SF2c	9.4	111.6	gp
SF33	4.3	164.4	ch
SF128A	426.8	1465.5	gp
HIV-2			
UC1	293.1	2131.4*	gp
UC2	3.6	298.3	h
UC3	102.8	420.2	ch

*HIV-2_{UC1}, a macrophage-tropic isolate (*19*), reached high levels of RT activity only 12 days after infection in the presence of enhancing serum.

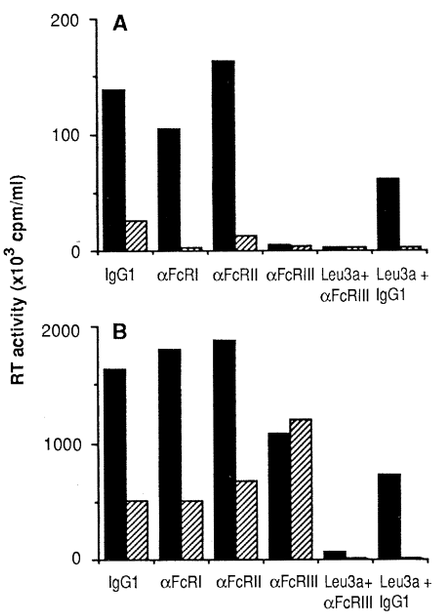

Fig. 2. The effect of FcR blockage on ADE of HIV-1 and HIV-2 infection of macrophages. Isolation of macrophages and enhancement experiments were as described in legends to Table 1 and Fig. 1A, except that anti-FcR, control antibodies, and Leu3a were used at a concentration of 5 μg per 5 × 10^5 cells. (**A**) HIV-1_{SF128A} (100 $TCID_{50}$ per milliliter) or (**B**) HIV-2_{UC1} fluid (5 × 10^5 cpm/ml) were used in these experiments with the chimpanzee and the human serum, respectively (diluted 1:20), and the normal control sera (1:20). Results represent peak RT activity of culture supernatants (*8, 9*) measured between day 15 and 18 (A) or day 12 (B) after infection. RPC-5 (Litton Bionetics) was used to block FcRI, anti-FcRII MAb was MAb IV.3 (*14*), anti-FcRIII MAb was MAb 3G8, and IgG1 was MOPC-21 (*6*). The RPC-5 antibody used at the concentrations given saturates the binding sites of FcRI. A representative experiment of two different experiments is shown. Closed bars, enhancing serum; hatched bars, control serum.

body, to control for nonspecific binding of MAb 3G8.

MOPC-21, RPC-5, and MAb IV.3 at up to 25 μg per culture (~5 × 10^5 cells) were not able to block enhancement of HIV in macrophages (Fig. 2). The addition of Leu3a MAb did not change these results (*10*). In contrast, treatment with anti-FcRIII MAb 3G8 efficiently blocked the enhancement of both HIV-1 and HIV-2 infection of macrophages (Fig. 2). Because the infectious dose of HIV-2 was greater than that of HIV-1, some HIV-2 replication but no enhancement occurred in the presence of the anti-FcRIII MAb alone. Nevertheless, the addition of Leu3a MAb to the anti-FcRIII MAb completely eliminated HIV-1 and HIV-2 replication in the presence of either enhancing or control serum. Because the presence of Leu3a alone did not reduce enhancement, this observation supports the conclusion that free virus as well as antibody-complexed virus account for infectivity in the presence of enhancing antibodies. Finally, the addition of the control IgG1 MOPC-21 to Leu3a had no effect on enhancement. This finding excludes the possibility that MAb 3G8 blocked ADE of HIV by attachment of its own Fc portion to the Fc receptor. The fluctuating level of virus replication observed in the control cultures treated with FcR MAbs (Fig. 2A) showed no reproducible pattern in repeat experiments. This variation—as well as the increased level of HIV-2 replication in anti-FcRIII–treated cells compared to the other treatment groups (Fig. 2B)—may represent different activating effects of the antibodies on the cells. In any case, the difference in virus enhancement observed between treatment groups was consistent in repeat experiments, independent of the level of virus replication. These results strongly implicate this epitope of FcRIII/CD16 as critical for HIV enhancement.

The presence of FcRs on T cells has been reported (*15*), but it is not clear which receptor is present and which cell subset expresses it. When we examined monocyte-depleted $CD4^+$ cells for expression of FcRIII/CD16 by fluorescence-activated cell sorting (FACS), we found that less than 5% of them were positive with either Leu11 (an NK cell marker) or 3G8 MAb. When the experiment in Fig. 1 was repeated with this cell population, no blocking of HIV-1 enhancement was observed with any of the antibodies to FcR, including anti-FcRIII MAb 3G8 (Fig. 3). Nevertheless, normal human immunoglobulin aggregates, but not free IgG, inhibited this enhancement. This block was most effective in the presence of Leu3a (Fig. 3). The Leu3a was used to prevent infection by free HIV. This observation suggests that HIV

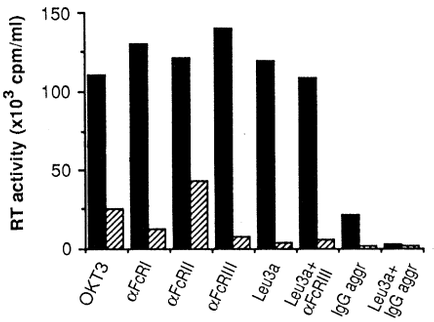

Fig. 3. The effect of FcR antibodies, IgG, and IgG aggregates on ADE of HIV infection of $CD4^+$ lymphocytes. Isolation, treatment, and infection of monocyte-depleted $CD4^+$ cells were conducted as described in Fig. 1 and as described for macrophages in Fig. 2. IgG aggregates were prepared from concentrated HIV-negative human IgG (Gamastan, Cutter Labs, Berkeley, California) by heating at 60°C for 10 min. Large aggregates were removed by centrifugation at 10,000*g* for 10 min. IgG aggregates were used at 25 μg per 10^5 cells. Antibody concentrations were 5 μg per 10^5 cells, and no difference in results was observed at 25 μg per 10^5 cells. The SF128A strain of HIV-1 and the human enhancing serum were used in these experiments. Results represent RT activity of culture supernatants at day 9 after infection (*8, 9*). A representative of two different experiments is shown. Closed bars, enhancing serum; hatched bars, control serum.

enhancement in $CD4^+$ lymphocytes involves a receptor that binds immune complexes. Because the biochemical structure of FcRIII may vary substantially on different cells that express this receptor (*6*), conceivably MAbs 3G8 and Leu11 could not bind or block this receptor as expressed on $CD4^+$ lymphocytes. This possibility applies to other FcRs and their epitopes as well, but these results do not exclude that another unidentified FcR could also be responsible for ADE of HIV in these cells.

Our findings establish that in the presence of enhancing antibodies, HIV can efficiently infect a cell independently of the CD4 protein. We have shown that the FcRIII receptor mediates uptake of HIV-enhancing antibody complexes into human macrophages; this result was not seen with $CD4^+$ lymphocytes. Since Ig aggregates were effective in blocking enhancement in lymphocytes, it is thus likely that either a different epitope of FcRIII or another FcR mediates HIV enhancement in these cells. Finally, the receptor responsible for ADE of human fibroblastoid cell infection remains to be identified.

The FcR MAbs used in this study may not represent all functional epitopes on these receptors, as more than one FcR on macrophages or other cells could be involved in ADE of HIV infectivity. Indeed, FcRIII is not present on the U937 monocytic cell line that has been shown to support ADE of HIV infectivity (*3, 5*). This fact suggests that alternative Fc receptors may be used by viral:immune complexes, depending on the type of cells being infected by HIV. However, the relevance to the in vivo situation of enhancing experiments performed on transformed cell lines remains to be established.

Enhancing antibodies are likely to be found in many individuals as part of the immune response to HIV infection, even though such antibodies may be masked by the presence of neutralizing antibodies. Albeit less frequently, we have identified sera with only enhancing activity (*4, 16*), and previously we had found that immune complexes precipitated from the serum of individuals with AIDS or AIDS-related complex were infectious in culture (*17*). These observations, and the fact that HIV enhancement represents an alternative infectious pathway independent of CD4, suggest that, from a therapeutic perspective, blocking of the CD4 binding sites on HIV may not suffice to reduce or stop virus spread. Blocking of relevant Fc receptors might also be required to prevent uptake of viral: immune complexes by macrophages and other cells in vivo. In this regard, soluble FcRIII/CD16 might represent a therapeutic approach worth evaluating as a complement to soluble CD4 treatment. In any case, our results imply that enhancing antibodies can substantially contribute to the "underground" spread of the virus in vivo, can increase the tropism of some viral strains (Table 1), and might enhance an individual's susceptibility to HIV infection. The viral epitope or epitopes involved in this reaction appear to be conserved since the same sera enhanced several strains of both HIV-1 and HIV-2 (Table 1). Therefore, the identification of the viral antigen or antigens eliciting the formation of such antibodies is critical for vaccine development.

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8. In these studies, enhancement was measured primarily by the first peak of RT activity of culture supernatants (*9*). For all experiments, peak RT activity was always from the same time points for enhanced versus control samples and was always

reached in the cultures receiving enhancing serum before or at the same time as the peak RT activity of control infections. Moreover, we have shown in other experiments that RT activity mirrors viral antigen (p25) as well as the amount of infectious virus production (*10*). Thus, HIV enhancement is characterized not only by accelerated kinetics of viral replication but also by increased virus progeny.

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13. Human osteosarcoma (HOS) cells were infected with HIV-1_{SF33} (*12*) in the presence or absence of enhancing sera (1:20 dilution). In brief, DEAE-dextran (25 μg/ml)–treated HOS cells were infected with 0.1 ml of HIV_{SF33}–containing fluid (RT activity, 10^6 cpm/ml) that had been incubated with an equal volume of test (immunized guinea pig or infected chimpanzee) and control serum (1:10) for 1 hour at 37°C. After overnight incubation at 37°C, the cells were trypsinized three times (500 μg/ml) during 9 days, washed in Hanks buffer, and cocultivated with MT-2 cells for 3 days. MT-2 cells were then removed and cultured alone. HIV production was monitored by measuring p25 antigen levels (Dupont HIVp24 kit) [J. Goudsmit *et al.*, *Lancet* **ii**, 177 (1986)] in the MT-2 cell supernatants. Cytopathic effect (CPE) was assessed by the presence of multinucleated giant cells and balloon formation in MT-2 cells. The p25 and CPE were detected only in the presence of enhancing serum and as early as day 3 and 7, respectively, after initiation of cocultures.
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Pathological Changes Induced in Cerebrocortical Neurons by Phencyclidine and Related Drugs

JOHN W. OLNEY, JOANN LABRUYERE, MADELON T. PRICE

Phencyclidine (PCP), a dissociative anesthetic and widely abused psychotomimetic drug, and MK-801, a potent PCP receptor ligand, have neuroprotective properties stemming from their ability to antagonize the excitotoxic actions of endogenous excitatory amino acids such as glutamate and aspartate. There is growing interest in the potential application of these compounds in the treatment of neurological disorders. However, there is an apparent neurotoxic effect of PCP and related agents (MK-801, tiletamine, and ketamine), which has heretofore been overlooked: these drugs induce acute pathomorphological changes in specific populations of brain neurons when administered subcutaneously to adult rats in relatively low doses. These findings raise new questions regarding the safety of these agents in the clinical management of neurodegenerative diseases and reinforce concerns about the potential risks associated with illicit use of PCP.

THE DRUG PCP, A DISSOCIATIVE ANesthetic, is best known as an abused street drug with potent psychotomimetic properties. PCP binds with high affinity and specificity to a unique class of membrane receptors in the mammalian central nervous system (CNS) (*1*), suggesting that the CNS may contain a yet to be identified PCP-like peptidergic neuromodulator and that dysfunction of this neuromodulatory system might underlie psychotic disorders such as schizophrenia (*2*). PCP receptors are colocalized with *N*-methyl-D-aspartate (NMDA) receptors (a subtype of glutamate receptor) (*3*), and PCP antagonizes NMDA receptor–mediated neuroexcitatory (*4*) and neurotoxic (*5*) phenomena. MK-801 is a PCP-like compound that displays even greater potency than PCP in binding to the PCP receptor and in antagonizing the excitatory (*6*) and toxic (*7*) actions of NMDA. MK-801 or PCP can protect CNS neurons against hypoxic-ischemic, hypoglycemic, or epilepsy-related brain damage (all of which are postulated to be NMDA receptor–mediated processes) (*8, 9*). Here we report that, in addition to their potent neuroprotective properties, these agents induce pathomorphological changes in certain CNS neuronal populations when administered subcutaneously to adult rats.

Treatment of rats with kainic acid causes persistent limbic seizures that result in a distinctive pattern of seizure-related brain damage (*10*). We have observed that PCP and MK-801 protect against this type of damage (*11*). However, the protected brains, although appearing normal in regions typically vulnerable to seizure-related brain damage, displayed neuropathological changes (vacuolization of neuronal cytoplasm) in the posterior cingulate and retrosplenial neocortices, changes that are subtly different from those typically associated with kainic acid treatment. Examination of the brains of control animals treated only with MK-801 or PCP revealed these same changes in cingulate and retrosplenial neurons, even though these animals had not been exposed to any convulsant and had not experienced any seizures. No such changes could be found in control animals that received no drug treatments. Because the vehicle was water (*12*), it could not have caused the changes. Therefore, we evaluated the possibility that PCP and MK-801 might have cytotoxic effects on CNS neurons.

Adult, female Sprague Dawley rats (300 g) were injected subcutaneously (sc) with an aqueous solution (*12*) of MK-801 (0.05 to 1.0 mg per kilogram of body weight, sc) or PCP (0.5 to 5.0 mg/kg sc) and were killed 4 hours later for histopathological evaluation of the brains by light and electron microscopy (*13*). Both compounds caused a dose-dependent vacuolar reaction detectable by light microscopy in cingulate and retrosplenial neurons, the ED_{50} (*14*) being 0.18 mg/kg (0.12 to 0.24) (n = 36) for MK-801 and 2.83 mg/kg (1.72 to 3.93) (n = 36) for PCP. Adult, male Sprague Dawley rats (450 g) were also susceptible but at a slightly higher dose, ED_{50} being 0.32 mg/kg (0.25 to 0.39) (n = 24) for MK-801 and 4.29 mg/kg (2.87 to 5.70) (n = 20) for PCP.

Electron microscopic evaluation of the affected neurons 2 to 4 hours after PCP or MK-801 treatment corroborated that the changes consisted of the formation of multiple vacuoles of heterogeneous size occupying the cytoplasmic compartment (Fig. 1, A and B). At 2 hours, when vacuoles first became evident, it appeared that they were forming from saccules of endoplasmic reticulum, that mitochondria or other cytoplasmic components were being incorporated within them, and that the incorporated structures were undergoing a process of dissolution. At 4 hours, the cytoplasmic compartment of an affected neuron appeared to be packed with vacuoles and devoid of mitochondria. The vacuoles varied in diameter from 3 to 15 μm. A time course study by light microscopy revealed that the vacuoles were detectable 2 hours after a

Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110.