Antibody-Mediated In Vitro Neutralization of Human Immunodeficiency Virus Type 1 Abolishes Infectivity for Chimpanzees

EMILIO A. EMINI,^{1*} PETER L. NARA,² WILLIAM A. SCHLEIF,¹ JOHN A. LEWIS,¹ JOSEPH P. DAVIDE,¹
D. RICK LEE,³ JOSEPH KESSLER,² SHAWN CONLEY,⁴ SHUZO MATSUSHITA,⁵ SCOTT D. PUTNEY,⁶
ROBERT J. GERETY,^{1†} AND JORG W. EICHBERG³

Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania 19486¹; Frederick Cancer Research Facility, National Cancer Institute, Frederick, Maryland 21701²; Southwest Foundation for Biomedical Research, San Antonio, Texas 78284³; Program Resources, Inc., Frederick Cancer Research Facility, Frederick, Maryland 21701⁴; Kumamoto University Medical School, Kumamoto 860, Japan⁵; and Repligen Corporation, One Kendall Square, Cambridge, Massachusetts 02139⁶

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This study was undertaken to establish whether antibody directed against the human immunodeficiency virus type 1 (HIV-1) principal gp120 type-specific neutralization determinant can abolish the infectivity of HIV-1 in chimpanzees. Challenge inocula of the IIIb virus isolate were mixed in vitro with either immunoglobulin G (IgG) from an uninfected chimpanzee, nonneutralizing IgG from an HIV-seropositive human, a virus-neutralizing murine monoclonal antibody directed against the HIV-1 IIIb isolate, or virus-neutralizing IgG from a chimpanzee infected with the IIIb isolate. Both neutralizing antibodies were directed against the principal neutralization determinant of the challenge isolate. Establishment of infection following inoculation of each virus-antibody mixture into chimpanzees was assessed by virus-specific antibody development and by virus isolation. No protective effect was noted either with the control IgG or with the nonneutralizing anti-HIV IgG. By contrast, the polyclonal chimpanzee virus-neutralizing IgG prevented HIV-1 in vivo infection, while the neutralizing monoclonal antibody notably decreased the infectivity of the challenge virus. Hence, antibody to the gp120 principal neutralization determinant is able both to prevent HIV-1 infection in vitro and to inhibit infection in vivo.

The human immunodeficiency virus type 1 (HIV-1), a member of the *Lentivirinae* subfamily of retroviruses, is the causative agent of the acquired immunodeficiency syndrome. The virion surface is composed largely of a 120-kilodalton (kDa) glycoprotein, gp120, which is noncovalently bound to a 41-kDa anchor glycoprotein. Both glycoproteins derive by cleavage from a 160-kDa precursor. Previous studies have demonstrated that gp120-related immunogens, whether purified from virus-infected cells or expressed by several recombinant systems, commonly elicit a type-specific in vitro virus-neutralizing antibody response in various test animals (2, 3, 8, 11, 15, 18, 21, 23, 25). The structural determinant to which this virus-neutralizing antibody binds encompasses amino acid residues 296 to 331 of gp120 (IIIb isolate) and is termed the principal neutralization determinant (PND) (5-7, 9, 10, 24). This region represents the glycoprotein's third hypervariable domain (V3). Synthetic peptide fragments of this domain induce virus-neutralizing antibody (7, 10, 19), and a number of monoclonal antibodies have been derived which bind to this site and effectively neutralize virus infectivity in vitro (4, 14, 27, 28).

This study was undertaken to establish whether antibody directed against the PND can abolish the infectivity of HIV-1 for chimpanzees. Previously reported studies of actively or passively immunized chimpanzees challenged with HIV-1 have failed to address this issue directly. None of the animals in the active immunization experiments reported to date had circulating virus-neutralizing antibody titers at the

time of challenge (1, 3, 8), and while the passive immunization study reported by Prince et al. (20) did establish significant levels of circulating neutralizing antibody before virus challenge, the passively administered HIV-1 immune globulin was uncharacterized with respect to anti-PND antibody specific for the challenge virus isolate.

The design of the present study was experimentally straightforward. Virus and appropriately specific anti-PND antibody were mixed in vitro under conditions leading to complete in vitro neutralization of the virus. The virus-antibody mixture was then inoculated into a susceptible chimpanzee. Including controls, four purified immunoglobulin G (IgG) samples were tested: (i) nonneutralizing control chimpanzee IgG, (ii) nonneutralizing anti-HIV-1 polyclonal human IgG, (iii) a neutralizing murine monoclonal antibody against the PND, and (iv) neutralizing polyclonal chimpanzee IgG directed to the PND.

MATERIALS AND METHODS

Animals, virus, and antibodies. The study used four adult chimpanzees (*Pan troglodytes*). The animals were maintained under conditions exceeding the requirements specified by the National Institutes of Health Guide for the Care of Laboratory Animals. Inoculations and bleedings were performed after tranquilization with ketamine hydrochloride at 15.0 mg/kg.

The challenge virus stock of HIV-1 (IIIb isolate) was generously provided by Larry Arthur and Peter Fischinger (National Cancer Institute). This stock has been titrated previously in chimpanzees (1).

The HIV-seropositive human and the two chimpanzee IgG

* Corresponding author.

† Present address: Biogen Corporation, Cambridge, MA 02139.

TABLE 1. IgG preparation characteristics

Preparation	IgG sample	Anti-HIV-1 reactivity ^a	Neutralizing activity ^b	Binding to PND ^c
I	Seronegative chimpanzee x1	—	<1:10	—
II	Seropositive human (sample 498)	+	<1:10	—
III	0.5 β monoclonal antibody	NT ^d	1:5,120	+
IV	Seropositive chimpanzee x118	+	1:640	+

^a Anti-HIV-1 activity was measured by commercial Western blot (Du Pont, Wilmington, Del.). Symbols: +, positive binding to all virus-specific protein bands; —, no binding to any of the virus-specific proteins.

^b Neutralizing activity was measured against the IIIb isolate of HIV-1 as described in Materials and Methods. The values represent the highest dilution of the purified antibody preparation which completely neutralized the infecting virus. The quantity of virus used in this assay was identical to the quantity used for chimpanzee challenge (see text).

^c Binding was measured by ELISA with a synthetic peptide fragment (RP135 [24]) of the IIIb isolate PND as the substrate. Symbols: +, positive ELISA signal (≥ 10 -fold above background) with an undiluted sample of the antibody preparation; —, background ELISA reading with an undiluted sample.

^d NT, Not tested.

samples were prepared from plasma by protein A-affinity chromatography. The monoclonal antibody was purified from mouse ascites fluid by the same method.

In vitro neutralization and chimpanzee infection. The challenge virus was diluted 1:25 in physiologic saline prior to use. Cell culture titration of the virus at the time of use yielded an infectious-dose value equivalent to approximately 140 chimpanzee 50% infectious doses per ml as defined by Arthur et al. (1). Each purified antibody preparation (1 ml) was added to one of four identical 1.0-ml portions of virus. The two virus-neutralizing antibody preparations were mixed with the virus inoculum well in excess of the minimum levels needed for complete virus neutralization (Table 1). The antibody-virus mixtures were incubated at room temperature (19 to 25°C) for 90 min. Each mixture was then inoculated intravenously into one of four HIV-seronegative chimpanzees. The animals were tested for HIV-1 infection by virus isolation. Isolation results were confirmed by polymerase chain reaction (PCR)-mediated amplification of virus-specific DNA sequences from peripheral blood mononuclear cells (PBMC). The development of specific antibody responses was also assessed.

Virus neutralization assay. The assay was performed as described by Robertson et al. (22). All test sera were heat-inactivated at 56°C for 60 min before use. Briefly, a titrated amount of HIV-1 stock was added to 1:2 serial dilutions of the test serum. Following a 60-min incubation at room temperature, 10⁵ MT-4 human lymphoid cells were added to each virus-serum sample. The cells then were incubated at 37°C for 7 days. Virus-mediated cell killing was assessed at this time by staining of the cell cultures with a tetrazolium dye. Virus neutralization was manifested by the prevention of virus-induced cell death. Results were confirmed by observation of the MT-4 cells with virus-specific immunofluorescence.

Detection of virus-specific DNA sequences. PBMC were obtained from the test chimpanzees by Ficoll gradient fractionation of heparinized blood and were processed in parallel with PBMC from three uninfected chimpanzees. High-molecular-weight DNA was isolated from the cells by sodium dodecyl sulfate-proteinase K digestion and phenol-chloroform extraction as described before (13). DNA samples were

digested with RNase A, reextracted, and concentrated by ethanol precipitation and centrifugation. The samples were then suspended in a buffer containing 10 mM Tris (pH 8.0), 25 mM NaCl, and 0.1 mM EDTA and adjusted to a concentration of 35 μ g/ml. PCR amplification was performed with the DNA thermal cycler and the GeneAmp kit of Perkin-Elmer Cetus (Norwalk, Conn.). Two sets of oligonucleotide primers flanking the HIV-1 PND (set 1 at positions 7065 to 7088 and 7366 to 7391 and set 2, nested within primer set 1, at positions 7096 to 7141 and 7282 to 7312 of the HXB2 genome; GenBank accession no. K03455) were prepared by the Synthecell Corporation (Rockville, Md.). Thirty PCR cycles, consisting of denaturation at 94°C for 1.5 min, hybridization at 55°C for 1.5 min, and polymerization at 72°C for 1.5 min were used to amplify 1.0 μ g of DNA with 100 pmol of the set 1 primers. Then, 5 μ l of this reaction was reamplified under the same PCR conditions with 100 pmol of the set 2 primers.

RESULTS

Four purified IgG preparations were tested for their ability both to neutralize HIV-1 in vitro and to abolish viral infectivity in vivo. The characteristics of each preparation are summarized in Table 1.

The preparation I sample was a control IgG purified from the plasma of an uninfected chimpanzee (x1).

Preparation II was IgG purified from the plasma of an HIV-1-seropositive human (sample 498). At the dilution used in this study, the purified IgG had no neutralizing activity directed against the challenge IIIb isolate and no binding activity directed against the isolate's PND. However, the IgG was positive for activity directed against all of the viral proteins as determined by commercial Western immunoblot with a viral lysate. This IgG was included in the study to establish whether nonneutralizing anti-HIV-1 antibody exhibits any in vivo protective effect.

Preparation III was a purified murine monoclonal antibody, 0.5 β . This antibody was elicited by the viral gp120 glycoprotein, is IIIb isolate specific in its neutralizing activity, and is directed against the IIIb isolate's PND (14).

Preparation IV was a polyclonal IIIb isolate-specific neutralizing IgG which had been purified from the plasma of a previously infected chimpanzee (x118). This animal had

TABLE 2. Absorption of preparation IV IgG virus-neutralizing activity by a PND-specific peptide^a

Sample	Column substrate ^b	RP135 ELISA titer ^c	Neutralizing antibody titer ^d
Preabsorption	RP135	1:5,120	1:640
Postabsorption	RP135	<1:10	<1:10
Preabsorption	Mock	1:5,120	1:640
Postabsorption	Mock	1:5,120	1:640

^a Absorption was performed with an affinity column of synthetic peptide covalently linked to Sepharose CL-4B. The antibody was loaded onto the column in phosphate-buffered saline, and the flowthrough was tested for anti-peptide and virus-neutralizing activities.

^b RP135 is a synthetic peptide representative of the IIIb isolate PND (24). The mock column contained unconjugated resin.

^c Anti-RP135 titers were determined by using a standard ELISA. Free RP135 peptide was used as the substrate. The values represent the highest dilution of column flowthrough which yielded an ELISA signal 2.1-fold above the control background.

^d Neutralizing activity was measured against the IIIb isolate of HIV-1 as described in Materials and Methods. The values represent the highest dilution of the column flowthrough which completely neutralized the infecting virus.

TABLE 3. Chimpanzee p.i. serological and virus isolation profiles

Section (chimpanzee no.)	Inoculum	Time p.i. (wk)	HIV-1 ^a		Neutral- izing antibody ^b	Virus isola- tion ^c
			ELISA titer	Western blot		
A (x222)	Virus + prepn I	0	<1:20	—	<1:10	ND ^d
		2	<1:20	—	<1:10	ND
		4	<1:20	—	<1:10	ND
		6	1:40	+/-	<1:10	ND
		8	1:20	+/-	<1:10	ND
		10	1:640	+	1:20	ND
		12	1:1,280	+	1:160	+
		14	1:2,560	+	1:160	+
		16	1:5,120	+	1:320	ND
		17	1:5,120	+	1:320	+
		19	1:10,240	+	1:320	+
		21	1:10,240	+	1:320	+
		23	1:10,240	+	1:320	+
		25	1:5,120	+	1:320	ND
		27	1:5,120	+	1:320	+
		31	1:10,240	+	1:640	+
		36	1:5,120	+	1:640	ND
		42	1:5,120	+	1:640	+
		67	1:5,120	+	1:640	+
		76	1:5,120	+	1:1,280	+
B (x177) ^e	Virus + prepn II	0	<1:20	—	<1:10	ND
		2	<1:20	—	<1:10	ND
		4	1:20	—	<1:10	ND
		6	1:160	+/-	<1:10	ND
		8	1:320	+/-	<1:10	ND
		10	1:160	+	1:10	ND
		12	1:320	+	1:10	+
		14	1:320	+	1:40	+
		16	1:160	+	1:40	ND
		17	1:640	+	1:40	+
		19	1:640	+	1:40	+
		21	1:1,280	+	1:80	+
		23	1:640	+	1:80	+
		25	1:1,280	+	1:80	ND
		27	1:1,280	+	1:80	+
		31	1:5,120	+	1:160	+
		36	1:2,560	+	1:320	ND
		42	1:12,560	+	1:320	+
C (x)	Virus + prepn III	0	<1:20	—	<1:10	ND
		2	<1:20	—	ND	ND
		4	<1:20	—	ND	ND
		6	<1:20	—	ND	ND
		8	<1:20	—	ND	ND
		10	<1:20	—	ND	ND
		12	<1:20	+/-	ND	+
		14	1:20	+/-	ND	—
		16	1:20	+/-	ND	ND
		17	1:20	+/-	ND	—
		19	1:40	+/-	ND	—
		21	1:40	+/-	ND	+
		23	1:80	+/-	<1:10	—
		25	1:80	+/-	ND	ND
		27	1:80	+/-	<1:10	—
		31	1:80	+/-	<1:10	—
		36	1:160	+/-	<1:10	ND
		42	1:160	+	<1:10	—
		67	1:1,5120	+	1:160	+
		76	1:15,120	+	1:160	+

Continued

TABLE 3—Continued

Section (chimpanzee no.)	Inoculum	Time p.i. (wk)	HIV-1 ^a		Neutral- izing antibody ^b	Virus isola- tion ^c
			ELISA titer	Western blot		
D (x81)	Virus + prepn IV	0	<1:20	—	<1:10	ND
		2	ND	—	ND	ND
		4	ND	—	ND	ND
		6	ND	—	ND	ND
		8	ND	—	ND	ND
		10	ND	—	ND	ND
		12	ND	—	ND	—
		14	ND	—	ND	—
		16	ND	—	ND	ND
		17	ND	—	ND	—
		19	ND	—	ND	—
		21	ND	—	ND	—
		23	ND	—	<1:10	—
		25	ND	—	ND	ND
		27	<1:20	—	<1:10	ND
		31	ND	—	ND	—
		36	<1:20	—	ND	ND
		42	<1:20	—	<1:10	—
		67	<1:20	—	<1:10	—
		76	<1:20	—	<1:10	—

^a HIV-1 ELISA was performed with a commercial assay kit. Titers were calculated according to the manufacturer's instructions. HIV-1 viral lysate Western blot was performed with a commercial kit. The sera were tested at a 1:10 dilution. Symbols: +, reactivity noted with all of the virus-specific protein bands; +/-, reactivity noted with four or fewer virus proteins; —, no reactivity against any of the virus proteins.

^b Neutralizing antibody was determined against the IIIb isolate of HIV-1 as described in Materials and Methods. The values represent the highest serum dilution which completely neutralized the assay's infecting virus.

^c Virus isolation from peripheral blood mononuclear cells (PBMC) was performed by in vitro co-cultivation with uninfected human PBMC as described by Nara et al. (16).

^d ND, Not done.

^e Chimpanzee x177 died before the week 67 sample was obtained. Death was due to causes unrelated to this study.

been inoculated 1 year earlier with the HIV-1 IIIb isolate. Plasma from x118 was obtained with the kind permission of H. J. Alter (National Institutes of Health). Absorption studies with a synthetic peptide (RP135 [24]) representative of the IIIb isolate PND showed that the IgG's entire neutralizing capacity was directed against this determinant. Absorption onto an RP135-containing resin led to complete loss of in vitro neutralizing activity (Table 2).

Each IgG sample was mixed with a sample of the challenge virus and incubated at room temperature for 90 min. Then, 1/40th of each mixture was withdrawn and analyzed for its in vitro infectivity with the highly HIV-susceptible MT-4 lymphoid cell line. The virus inocula mixed with the nonneutralizing IgG preparations I and II both proved infectious, yielding virus-specific immunofluorescence several days after inoculation onto MT-4 cells. In contrast, no virus-specific infection was observed for 8 weeks following cell culture inoculation of virus mixed with neutralizing IgG preparations III and IV.

The remainder of each virus-antibody mixture was injected intravenously into one of four HIV-seronegative chimpanzees. Each animal was then assessed as described in Materials and Methods for the development of persistent infection. The results are presented in Table 3.

The animal inoculated with the virus-preparation I control IgG mixture (chimpanzee x222) exhibited initial immunological signs of HIV-1 infection by 6 weeks postinoculation

(p.i.). Neutralizing antibody to the challenge inoculum developed at 10 weeks p.i., and all virus isolation attempts from PBMC yielded virus. Virus-specific antibody titers remained high through 76 weeks (Table 3A). Similar observations were made with the animal (chimpanzee x177) which had received the virus-nonneutralizing preparation II IgG mixture (Table 3B).

In contrast, chimpanzee x30, inoculated with the virus-neutralizing monoclonal preparation III IgG mixture, exhibited a delayed serological response to the virus. Specific virus Western blot-reactive and virus enzyme-linked immunosorbent assay (ELISA)-reactive antibodies were first noted at 12 and 14 weeks p.i., respectively. The ELISA titers remained low through 31 weeks of observation, and the Western blot reactivity patterns were restricted to four or fewer viral proteins during this time. Neutralizing antibody to the challenge inoculum was not noted until the week 67 sample. The ability to isolate virus from the PBMC was sporadic (Table 3C).

Chimpanzee x81, inoculated with the virus-neutralizing preparation IV IgG sample, has not developed serological signs of HIV-1 infection through the 76-week observation period. Virus was not isolated at any time (Table 3D).

PBMC samples obtained from each of the animals at weeks 42, 67, and 76 were processed as described in Materials and Methods for PCR-mediated amplification of HIV-specific DNA sequences. In control studies, the PCR procedure was capable of detecting 1 infected cell in a population of 10^5 cells (data not shown). Unambiguously positive signals were obtained with samples from chimpanzees x222, x177, and x30. However, the signal intensity from animal x81 DNA was not reproducibly stronger than the background signal from uninfected chimpanzee DNA obtained and processed in parallel.

DISCUSSION

The observations reported here constitute the first experimental evidence that anti-PND-dependent HIV-1 neutralization may have *in vivo* biological relevance. We are confident that the protection we observed in chimpanzee x81 resulted from virus neutralization mediated by the anti-PND antibody in preparation IV, since the preparation II antibody, which contained anti-HIV-1 Western blot reactivities identical to those of preparation IV but lacked anti-PND antibody for the inoculum virus, failed to neutralize virus infectivity for chimpanzee x177. Chimpanzee x81, which had received virus neutralized *in vitro* by the preparation IV IgG, has not developed signs of infection throughout the 76-week observation period, whether assessed by development of virus-specific antibody, virus isolation, or PCR-amplified detection of virus-specific nucleic acid.

Antibody to the PND also appears to have contributed to a significant delay in the HIV-1 infection of chimpanzee x30. This animal was inoculated with virus which had been completely neutralized for cell culture infectivity by the anti-PND monoclonal antibody, yet the virus inoculum retained infectivity for the chimpanzee, although the infection was notably delayed in its appearance. We can offer several explanations for this result. First, the IgG1 murine antibody-virus complexes may not have been efficiently cleared from the chimpanzee's circulation, thereby permitting escape of sufficient infectious particles to establish a delayed infection. Second, it may be that the restricted immunological specificity of the monoclonal antibody allowed the *in vivo* selection of HIV-1 variants from the

challenge inoculum which were capable of infecting a cell compartment not represented in the cell culture neutralization assay. Such variants, if present, would have been neutralized *in vivo* by the polyclonal anti-PND IgG, given the broad specificity of antibody elicited during an infection. Finally, the restricted specificity of the monoclonal antibody and the possible delayed clearance of circulating virus-antibody complexes together may have allowed the *in vivo* selection of a neutralization-escape variant which normally would not have been selected under the *in vitro* conditions used in this study. In this regard, preliminary characterizations of two viral isolates obtained from chimpanzee x30 showed that both are identical to the inoculum virus in the amino acid sequence of the antibody-binding determinant. However, both required approximately 30-fold higher concentrations of the antibody than did the parental virus to achieve the equivalent degree of *in vitro* neutralization (unpublished observations).

Further studies are planned to assess the usefulness of anti-PND antibodies as components of a vaccine-elicited protective immune response. Such studies will include a determination of whether passively administered circulating antibodies to the PND are capable of providing protection upon intravenous virus challenge. Both polyclonal chimpanzee and chimeric human monoclonal anti-PND antibodies will be used in these experiments. In addition, methods for actively eliciting high titers of anti-PND antibodies are under investigation in order that the immunogenic potential of the PND, in various antigenic presentation contexts, may be assessed. Finally, continued observations of chimpanzee x81 are planned so as to firmly establish the long-term protective effect of the anti-PND antibody. The observations reported in the present study do not exclude the possibility that a silent latent infection may have been established in a noncirculating cell compartment. Such a latent infection, if present, may ultimately manifest itself by the activation of viral replication. The animal will continue to be tested for the development of virus-specific antibodies and for the presence of virus at regular intervals.

These continuing studies are justified both by the conclusions reported here and by the previously established biological characteristics of the antibody to the PND. This antibody, in addition to its ability to neutralize free virus, also prevents the *in vitro* fusion of infected and uninfected lymphoid cells (26). The antibody retains full access to its determinant following the binding of gp120 to the CD4 cell receptor (12, 26), and the antibody neutralizes cell-adsorbed virus as efficiently as it neutralizes cell-free virus (16; unpublished observations). These *in vitro* properties established a strong interest in the PND as a potential HIV-1 vaccine immunogen. This interest is now increased by the observed *in vivo* virus-inhibitory effects of antibody against the determinant.

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