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Ronald Q. Warren[a,b]
Hans Wolf[a]
Gregory W. Stunz[a,b]
Ronald C. Kennedy[a,b]

[a] Department of Virology and Immunology and
[b] Center for AIDS Research, Southwest Foundation for Biomedical Research, San Antonio, Tex., USA

Antibody Reactivity to Synthetic Peptides Representing the Principal Neutralizing Determinant of HIV-1 in Mouse Strains following Repeated Immunization with Recombinant gp160

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Abstract

The third variable region (V3) of the HIV-1 gp120 envelope molecule appears to represent a target for naturally occurring neutralizing antibodies in HIV-1-infected individuals. In this report, we examined the extent of antibody cross-reactivity to a panel of V3-based synthetic peptides in six inbred strains of mice following repeated immunization with a baculovirus-derived recombinant gp160 (rgp160) preparation formulated with alum. The amino acid sequence of the rgp160 used in these immunizations was based upon the HIV-1 IIIB (LAI) isolate. Following five injections with rgp160, all six strains developed antibodies to the homologous IIIB-based V3 peptides, designated 304–321 and RP135. However, antibody cross-reactivity to the other nonhomologous V3 peptides was either undetectable or limited among the strains of mice examined. No in vitro neutralizing activity against HIV-1 was observed in sera from any of the six inbred strains of mice that were examined. These results suggest that repeated immunization of mouse strains with a rgp160/alum formulation leads to nonneutralizing antibodies directed against the V3 region which remain predominantly type specific.

Introduction

The principal neutralizing determinant (PND) of HIV-1 is located in the V3 region of the envelope glycoprotein gp120. The V3 region is thought to consist of a disulfide linked loop structure spanning amino acids 303–338 of gp160 (IIIB). Comparison of V3 sequences from the United States and Europe have indicated that several conserved regions exist

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Ronald C. Kennedy, PhD
Center for AIDS Research
Southwest Foundation for
Biomedical Research, PO Box 28147
San Antonio, TX 78228-0147 (USA)

within this loop structure [1]. For example, conserved regions among isolates from the United States are present at the tip of the loop (defined by amino acid residues GPGRA) as well as at the base of the loop flanking the cysteine residues (amino acid residues CTRPNNNTR and GDIRXQAHC). These regions of conserved amino acids are interspersed between regions of high amino acid variability.

We have previously observed that sera from asymptomatic HIV-1-infected individuals were more likely to contain antibodies reactive with multiple V3 peptides composed of diverse sequences than were sera from symptomatic individuals [2]. This suggested that antibodies which recognized conserved regions within the V3 loop (and therefore cross-reacted with V3 peptides of diverse HIV-1 isolates) may have a possible beneficial effect in vivo.

In this study, we examined whether we could induce formation of neutralizing antibodies reactive with the more conserved regions of the V3 loop by repeatedly immunizing several groups of mice with a baculovirus-derived recombinant gp160 (rgp160). To more closely mimic the possible vaccine formulation used in humans, these immunizations were carried out using alum. This adjuvant has been approved for use in humans and the rgp160/alum formulation is presently being examined in human clinical trials. The induction of antibodies reactive with conserved regions within the V3 loop may potentially lead to an improved clinical outcome among HIV-1-infected individuals. Our results indicate, however, that the majority of mice repeatedly immunized with rgp160/alum continue to produce anti-V3 antibodies which remain type specific. We could detect no in vitro neutralizing activity in any of the sera that were examined. These results suggest that the variable regions within the V3 loop are immunodominant over the more conserved regions.

Materials and Methods

Animals

The following inbred strains of female mice were obtained from Jackson Laboratories (Bar Harbor, Me., USA): BALB/cByJ, A/J, CBA/CaJ, C57BL/10SnJ, DBA/1J, and SJL/J. Their ages at the time of the first immunization ranged from 6 to 8 weeks. Female New Zealand White rabbits were obtained from Hazelton Laboratories, Denver, Pa., USA.

Immunizations

Each strain of inbred mice (5 mice/group) received a total of six intraperitoneal injections of alum-precipitated rgp160 in 200 µl of borate-buffered saline (5 µg/injection; kindly supplied by Gale Smith, MicroGeneSys, Inc., New Haven, Conn., USA). The amino acid sequence of the rgp160 was based upon the HIV-1 IIIB isolate [3]. The first three injections were performed biweekly, with subsequent injections at monthly intervals. Sera were collected from the tail vein 2–4 weeks after each injection. Preimmune sera from each strain were used as controls.

V3 Region Peptides

Peptide 304–321, which corresponds to the amino terminus of the IIIB V3 region, was synthesized as previously described [4]. The remaining V3 peptides (RP135, RP139, RP142, and RP145) correspond to the central region of the V3 epitope of isolates IIIB, RF, MN, and the composite HAN/SC, respectively, and were kindly supplied by Scott Putney (Repligen Corp., Cambridge, Mass., USA). The amino acid sequences of the synthetic V3 peptides used in this study are shown in table 1.

ELISA

Mouse sera were tested for antibody reactivity to the panel of synthetic peptides, as well as rgp160, by ELISA, as previously described [5]. Briefly, microtiter wells were coated with synthetic peptides (250 ng/well), dissolved in bicarbonate buffer, pH 9.6, by overnight incubation at 4°C. Wells were blocked with 10% normal goat serum, followed by incubation with mouse sera for 1 h at 37°C. To detect antibody reactivity to the peptides, horseradish peroxidase-labeled anti-mouse IgG (Cappel, Westchester, Pa., USA) was added to the wells for 1 h at 37°C. Finally, substrate

Table 1. Amino acid sequences of the V3 peptides examined in this study

Peptide	HIV-1 isolate	Sequence
304-321	IIIB	(CGY)TRPNNNTRKSIRIQRGPG[1]
RP135	IIIB	NNTRKSIRIQRGPGRAFVTIGKIG(C)
RP139	RF	NNTRKSITKGPGRVIYATGQIIG(C)
RP142	MN	YNKRKRIHIGPGRAFYTTKNIIG(C)
RP145	HAN/SC	NNTRKGIHIGPGRA/FYATGDIIG(C)

[1] Amino acids in parentheses were added to facilitate coupling to carrier proteins.

consisting of 0.22 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma Chemical Co., St. Louis, Mo., USA), in 0.05 *M* citric acid, pH 4, and 0.015% hydrogen peroxide was added. Binding was quantitated by determining the optical density (OD) of the wells at 410 nm. The cutoff for a positive reaction was determined as being three times the mean OD value of the preimmune sera. Peptide Hep122, corresponding to amino acids 122–137 of the S region of hepatitis B surface antigen, was used as a control peptide [6].

Antibody reactivity to rgp160 was measured by ELISA as described above except that the microtiter wells were coated with 20 ng of rgp160 overnight at 4°C.

Neutralization Assay

These assays have been described in detail elsewhere [2]. Briefly, mouse sera were obtained 2 weeks following the sixth immunization and tested for neutralizing activity against HIV-1 in vitro. All sera were tested at a final dilution of 1/100 with preimmune sera serving as a control for each individual serum being examined. A cytotoxicity assay with a dye uptake step to determine cell viability was used to assess the neutralizing activity of sera against the IIIB isolate (100 50% tissue culture infective doses). Sera were incubated with HIV-1 for 1 h at room temperature, then added to SupT1 cells and cultured for 8 days at 37°C. On day 8, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue; 0.012 *M*; Sigma, St. Louis, Mo., USA) was added to the wells and incubated for 4 h at 37°C. The cultures were solubilized by the addition of 0.04 *N* HCl in isopropanol, and the OD was determined at 570 nm. Sera were considered to neutralize HIV-1 IIIB when more than 50% of the SupT1 cells remained viable when compared to wells containing either preimmune sera or no virus. A p24 antigen capture ELISA was also used to examine the neutralizing activity of sera against the MN isolate (Coulter Corp., Hialeah, Fla., USA). The HIV-1 MN isolate was not cytopathic for the target human T cell line (SupT1) which precluded the use of the dye binding cytotoxicity assay. For the p24 assay, we followed the manufacturer's recommended procedure. Again, sera were considered to neutralize the MN isolate when p24 levels were reduced by 50% when compared to preimmune sera.

Results

Various inbred strains of mice were examined to determine whether repeated immunizations with a clinically approved formulation of rgp160/alum would broaden the fine specificity of antibody reactivity to the V3 region of gp120. The testing of six strains of mice, each differing in H-2 haplotype, allowed us to further investigate whether differences in the major histocompatibility complex (MHC) might influence antibody fine specificity to the PND.

An analysis of sera obtained after the fifth immunization with rgp160 indicated that antibody titers to rgp160 ranged from 1,600 in sera from DBA/1J mice ($H\text{-}2^q$) to greater than 12,800 with A/J mice ($H\text{-}2^a$) (table 2). The relatively low titers in most strains of mice following five immunizations indicated that the rgp160/alum formulation used in this study was not highly immunogenic.

Table 2. Endpoint titers of mouse sera to rgp160 following the fifth immunization

Strain	H-2 haplotype	Endpoint titers[1]					
		gp160	V3 peptides				
			304–321	RP 135	RP 139	RP 142	RP 145[2]
BALB/c	d	12,800	400	100	–	–	–
A/J	a	>12,800	800	800	400	200	–
C57BL	b	12,800	1,600	200	–	–	–
CBA	k	12,800	1,600	800	–	–	–
DBA/1J	q	1,600	200	100	–	200	–
SJL	s	12,800	400	200	–	–	–

[1] Endpoint titers were calculated as the reciprocal of the highest dilution that resulted in an OD greater than three times the OD of the preimmune sera.

[2] Five mice of each of the inbred strains received 5 injections of 5 μg rgp160. Sera were pooled for each strain and titrated against each of the V3 peptides. Sera with titers less than 1:50 were considered negative. All sera tested were unreactive with a control peptide.

Antibody endpoint titers to each of the V3 peptides were also determined for these sera (table 2). We were interested in examining whether elevated titers to the IIIB based V3 peptides would correlate with cross-reactivity to the other peptides. However, no correlation between anti-V3 antibody titers and cross-reactivity to other V3 peptides was observed. For example, sera from strains C57BL and CBA, which exhibited the highest titers to the IIIB derived peptide 304-321, contained no detectable antibody cross-reactivity to the non-IIIB based peptides examined in this study. In addition, the relatively weak anti-gp160 titers in these mice appeared to contribute to low antibody titers against the V3 peptides.

We then examined whether repeated immunizations of mice with rgp160 would broaden the specificity of V3-reactive antibodies to include reactivity with conserved regions within the V3 loop. After each immunization, sera were pooled and tested for reactivity to a panel of V3-based synthetic peptides. As seen in table 3, immunization with rgp160 (IIIB) elicited antibodies reactive with the IIIB-based peptide 304-321 in all strains by the fourth immunization. Antibodies to the other IIIB-based peptide, RP135, appeared later in sera from strains BALB/c, CBA, and SJL than was observed with peptide 304-321. V3 peptides whose amino acid sequences were based upon other HIV-1 isolates were less reactive with the mouse antisera. For example, peptides RP139 and RP145, which correspond to V3 sequences from the RF and a composite of the HAN/SC isolates, respectively, were reactive only with sera from A/J mice, and then only after the fifth or sixth immunization, respectively. Sera from two strains of mice (A/J and DBA/1J) contained antibodies which cross-reacted with the MN-based peptide RP142. These results indicate that immunization with the rgp160/alum preparation did not result in a strong antibody response to the V3 region of gp120 in the various inbred stains of mice tested. In addition, we observed that

Table 3. Antibody reactivity of sera from rgp160 immunized mice to V3 peptides[1]

Peptide	Strain	Number of immunizations					
		1	2	3	4	5	6
304-321	BALB/c	–	–	+	+	+	+
	A/J	–	–	+	+	+	+
	C57BL	–	–	+	+	+	+
	CBA	–	–	+	+	+	+
	DBA/1J	–	–	–	+	+	+
	SJL	–	–	+	+	+	+
RP135	BALB/c	–	–	–	–	+	+
	A/J	–	–	+	+	+	+
	C57BL	–	–	+	+	+	+
	CBA	–	–	–	+	+	+
	DBA/1J	–	–	+	+	+	+
	SJL	–	–	–	–	+	+
RP139	BALB/c	–	–	–	–	–	–
	A/J	–	–	–	–	+	+
	C57BL	–	–	–	–	–	–
	CBA	–	–	–	–	–	–
	DBA/1J	–	–	–	–	–	–
	SJL	–	–	–	–	–	–
RP142	BALB/c	–	–	–	–	–	–
	A/J	–	–	+	+	+	+
	C57BL	–	–	–	–	–	–
	CBA	–	–	–	–	–	–
	DBA/1J	–	–	–	+	+	+
	SJL	–	–	–	–	–	–
RP145	BALB/c	–	–	–	–	–	–
	A/J	–	–	–	–	–	+
	C57BL	–	–	–	–	–	–
	CBA	–	–	–	–	–	–
	DBA/1J	–	–	–	–	–	–
	SJL	–	–	–	–	–	–

[1] Five mice of the indicated inbred strains received injections with 5 μg of rgp160. Sera were pooled from each strain and reacted against each of the peptides at a 1:50 dilution. Sera that resulted in OD values greater than 3 times that of the control sera were considered positive.

antibodies reactive to the V3 region remained type specific following multiple immunizations with rgp160 in the majority of mouse strains examined.

Since the V3 region has been designated as the PND of HIV-1 gp120, we also examined the ability of the anti-gp160 sera to neutralize HIV-1 in vitro following the sixth immunization with rgp160. Our results indicated that no detectable levels neutralizing activity (1/100 dilution of sera) were observed against HIV-1 isolates IIIB or MN in sera from any of the six inbred strains of mice (data not shown).

Discussion

As a retrovirus, HIV-1 lacks the enzymes necessary to correct errors which occur during DNA synthesis. This results in a high mutation rate, averaging about one error per 10,000 bases [7–10]. High levels of base pair substitutions can lead to the presence of quasi-species of HIV-1 within an individual and may represent a mechanism for the virus to evade neutralization in vivo [11–14]. An effective vaccine must therefore overcome this variability by eliciting an immune response which is directed primarily against conserved, neutralizing epitopes.

In the present study, we examined whether repeated immunizations of mice with a rgp160/alum formulation would lead to antibodies which recognized conserved regions within the PND of HIV-1. This rgp160/alum preparation was selected because it is presently undergoing human clinical trials as a potential vaccine [15]. Six inbred strains of mice were immunized to examine whether antibody fine specificity to the V3 region might be influenced by differences in H-2 haplotypes.

When immunized with rgp160 (IIIB), each of the strains of mice produced antibodies

reactive with the IIIB-based peptides, designated 304-321 and RP135. Antibody reactivity to the non-IIIB peptides, however, was not detected in sera from BALB/c, C57BL, CBA, or SJL mice. Since each of the V3 peptides examined share conserved amino acid sequences, antibodies reactive with these conserved regions were apparently not present in sera from these mice. Following immunization with rgp160, only sera from the A/J mice contained antibodies reactive with the entire panel of V3 based peptides. The presence of cross-reactive antibodies in A/J mice did not appear to correlate with high antibody titers to rgp160 since other strains with similar titers to rgp160 lacked these cross-reactive V3 antibodies. It therefore appears that the variable regions within the V3 loop are immunodominant over conserved sequences in the majority of mice strains immunized with rgp160/alum. Immunodominance of variable regions within the PND may pose a significant problem for a PND-based vaccine to elicit group specific antibodies.

We observed that each strain of mice required repeated immunizations with the rgp160/alum preparation to elicit antibody responses to the V3 loop region of gp120. Antibodies reactive with the V3 peptides were not detectable in any strain of mice until the third or fourth immunization. In addition, the rgp160/alum preparation led to relatively low antibody titers against both rgp160 and the V3 peptides. Low levels of immunogenicity of rgp120 and rgp160 have been reported previously in nonhuman primates and human volunteers [15–19]. In addition, we have reported that anti-gp160 titers among HIV-1-infected individuals range only between 7,000 and 12,000 [5]. Together these studies indicate that the envelope glycoproteins of HIV-1 represent relatively weak immunogens.

Following immunization with rgp160, mice of the H-2^d (BALB/c) and H-2^s (SJL) haplotypes responded similarly, with both possessing nearly identical antibody titers to peptides 304-321 and RP135. Also, sera from these two mouse strains did not contain antibodies reactive with any of the non-IIIB-based peptides. Of the remaining four strains examined, only mice with the H-2^a (A/J) and H-2^q (DBA/1J) haplotypes produced antibodies reactive with non-IIIB based peptides. Previous studies have suggested that the immune response to specific HIV-1 epitopes may be under genetic restriction. For example, MHC-class-I-restricted cytotoxic T lymphocyte (CTL) responses to gp160 epitopes have been reported by others [20–24]. In addition, following immunization with rgp160, mice with H-2^k or H-2^d haplotypes reportedly mounted a T-cell-proliferative response to gp160 peptide 324-338, while mice bearing other haplotypes failed to demonstrate this reactivity [25].

Nara et al. [26] have previously examined whether group-specific neutralizing antibodies could be generated in animals following repeated immunization with gp120. For these studies, gp120 was injected with either complete Freund's adjuvant (CFA) or RIBI. These studies demonstrated that repeated immunization of animals with gp120/CFA or gp120/RIBI resulted in neutralizing antibodies that remained type specific. In addition, Arthur et al. [27] reported that repeated immunization of chimpanzees with gp120/alum resulted in type-specific neutralizing antibodies. Unlike these previous authors, we failed to observe significant neutralizing activity to HIV-1 IIIB or MN in any of the anti-gp160 sera that were examined. This may reflect differences in the in vitro neutralizing assays that were used, the source of the rgp160 and the adjuvant formulations. We performed our studies to mimic the most likely rgp160 immunization protocol in human clinical trials. Indeed, increased anti-gp160 titers

and immunogenicity have been reported when formulating the gp160 with different adjuvants, such as ISCOMS [28]. However, since alum is presently the only approved adjuvant for use in the United States, we selected it as the adjuvant of choice for these studies. It was of interest that no HIV-1 IIIB-specific neutralizing antibodies were detected in mice immunized with rgp160 even in the presence of anti-V3 responses as detected by ELISA. Previously, we reported the lack of correlation between antibodies to V3 peptides, as assessed by ELISA, and in vitro neutralizing activity in sera from HIV-1-infected individuals from geographically diverse populations [29]. The results reported herein, using sera from mice immunized with rgp160, support our previous findings.

Our study of antibody fine specificity to the PND in rgp160 immunized mice provides additional information related to the clinical trials of rgp160, rgp120, and vaccinia-gp160 immunizations of humans [15, 19, 30, 31]. Early indications from the human trials suggest that rgp160 is safe when used to immunize uninfected volunteers and may potentially broaden the specificity of the humoral immune response to gp160 when used to immunize HIV-1-infected individuals. Our results indicate that vaccine strategies which utilize baculovirus-derived gp160 and alum may not induce antibodies that cross-react with V3 epitopes from divergent HIV-1 isolates.

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