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## Induction of a Protein-Targeted Catalytic Response in Autoimmune Prone Mice: Antibody-Mediated Cleavage of HIV-1 Glycoprotein GP120<sup>†</sup>

Natalia A. Ponomarenko,<sup>‡</sup> Ivan I. Vorobiev,<sup>‡</sup> Elena S. Alexandrova,<sup>‡,§</sup> Andrew V. Reshetnyak,<sup>||</sup> Georgy B. Telegin,<sup>⊥</sup> Sergey V. Khaidukov,<sup>‡</sup> Bérangère Avasse,<sup>#</sup> Alexander Karavanov,<sup>△,◇</sup> Herbert C. Morse III,<sup>○</sup> Daniel Thomas,<sup>#</sup> Alain Friboulet,<sup>#</sup> and Alexander G. Gabibov<sup>\*,‡,§,||</sup>

*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, RAS, 16/10, Miklukho-Maklaya str., Moscow 117871, Russia, Engelhardt Institute of Molecular Biology, RAS, 32, Vavilova str., Moscow 117984, Russia, Department of Chemistry, Lomonosov Moscow State University, Moscow 119899, Russia, Puschino Branch of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, RAS, Puschino 142290, Russia, CNRS UMR 6022, Compiègne Technological University, BP 20529, 60206 Compiègne Cedex, France, CIPHERGEN Biosystems, Inc., 6611 Dumbarton Circle, Fremont, California 94555, and Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5640 Fishers Lane, Rockville, Maryland 20852*

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**ABSTRACT:** We have induced a polyclonal IgG that degrades the HIV-1 surface antigen, glycoprotein gp120, by taking advantage of the susceptibility of SJL mice to a peptide-induced autoimmune disorder, experimental autoimmune encephalomyelitis (EAE). Specific pathogen-free SJL mice were immunized with structural fragments of gp120, fused in-frame with encephalitogenic peptide MBP<sub>85–101</sub>. It has resulted in a pronounced disease-associated immune response against antigens. A dramatic increase of gp120 degradation level by purified polyclonal IgG from immunized versus nonimmunized mice has been demonstrated by a newly developed fluorescence-based assay. This activity was inhibited by anti-mouse immunoglobulin antibodies as well as by Ser- and His-reactive covalent inhibitors. A dominant proteolysis site in recombinant gp120 incubated with purified polyclonal IgG from immunized mice was shown by SDS–PAGE. The SELDI-based mass spectrometry revealed that these antibodies exhibited significant specificity toward the Pro<sub>484</sub>–Leu<sub>485</sub> peptide bond. The sequence surrounding this site is present in nearly half of the HIV-I variants. This novel strategy can be generalized for creating a catalytic vaccine against viral pathogens.

Persistent viral infections such as HIV cause tremendous health and financial burden on society and threaten the human security. Vaccination is the most preferable approach to resist viral infection and is of proven efficacy in many viral diseases such as smallpox and poliomyelitis. However, current efforts to produce an efficient vaccine for HIV infection have been unsuccessful (1–3). The ability of HIV to utilize sophisticated mechanisms permitting to escape control by the host immune system complicates the develop-

ment of effective vaccines and antiviral therapeutics. The HIV virion surface antigen (Ag)<sup>1</sup> gp120 plays a significant, if not decisive, role in blocking the ability of the host immune system to resist pathogen penetration (4). Antibodies to the discontinuous or conformational epitopes of gp120 are prevalent in HIV-1-positive sera; thus antibodies to linear epitopes are less common, and most of them are directed against the hypervariable V3 region and highly specific to the HIV-1 variant (5). Direct virus elimination using gp120-specific antibody (Ab) has difficulty because a majority of neutralizing Ab fail to bind Ag organized in the form of a trimeric spike. Moreover, the viral surface proteins are coated with carbohydrates (sugars), which are made by human cells or otherwise they seem “self” and should not be recognized by antibodies (6, 7). Both the potential and the limits of humoral immunity in controlling HIV-1 infection were recently shown using the neutralizing monoclonal antibody

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\* To whom correspondence should be addressed at the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry. Tel: +7(095) 429-8269. Fax: +7(095) 330-7329. E-mail: gabibov@ibch.ru.

<sup>‡</sup> Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry.

<sup>§</sup> Engelhardt Institute of Molecular Biology.

<sup>||</sup> Lomonosov Moscow State University.

<sup>⊥</sup> Puschino Branch of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry.

<sup>#</sup> Compiègne Technological University.

<sup>△</sup> CIPHERGEN Biosystems, Inc.

<sup>◇</sup> Deceased.

<sup>○</sup> National Institutes of Health.

<sup>1</sup> Abbreviations: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; SELDI, surface-enhanced laser desorption–ionization; Ag, antigen; TSA, transition state analogues; PLP, proteolipid protein; SPF, specific pathogen-free; IPTG, isopropyl β-D-thiogalactopyranoside; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HRP, horseradish peroxidase; CHCA, α-cyano-4-hydroxycinnamic acid; PE, phycoerythrin; Trx, thioredoxin 1; AEBSEF, 4-(2-aminoethyl)benzenesulfonyl fluoride; TPCK, N<sup>α</sup>-tosyl-L-phenylalanyl chloromethyl ketone; LDI, laser desorption–ionization.

cocktail (2G12, 2F5, and 4E10) against gp120 and gp41 epitopes (8). Thus, the induction of specific molecular recognition of gp120 by Ab is one of the key problems of the modern life science.

A general approach to avoid virus invasion and proliferation is to disrupt the interaction of the viral particle with CD4 (4). It has been shown previously that gp120 (Supporting Information, Figure 1) cleaved by trypsin at residue 432 or by protease V8 at residue 269 is unable to interact with CD4 (6). These data suggest that specific cleavage of gp120 can be used to destroy gp120/CD4 interactions. At the same time, the cleavage of gp120 can expose masked parts of the surface protein to the immune system eliciting new constant Ag determinants. This could result in more efficient neutralization of the virus and/or elimination of virus-infected cells. One of the possibilities in the present case is the creation of a tailor-made proteolytic enzyme able to cleave gp120. Unfortunately, most probably this protease will be low specific to antigen and immunogenic. The obtaining of catalytic antibodies (abzymes) specifically cleaving gp120 would be the alternative. Abzymes, being immunoglobulin molecules, are expected to retain antigen specificity and remain nonantigenic. These features make it possible to use abzymes in humans (9). Moreover, the relatively low catalytic efficiency and high antigen-binding capacity of Ab catalysts can provide tighter binding of the abzyme to a target Ag and decrease the rate of nonspecific proteolysis which could be harmful to the organism.

In the present approach, we propose to apply recently found links between abzyme induction and autoimmunity (10, 11) for development of catalytic Ab toward gp120.

Natural catalytic Ab toward specific autoAg have been identified in sera of patients with systemic lupus erythematosus (SLE), Hashimoto's disease, asthma, multiple sclerosis (MS), and other human pathologies as well as in sera of animal models of autoimmune disorders (10–16). It has been found that the induction of abzyme-producing hybridomas by immunization with transition state analogues (TSA) is 100-fold more effective in SJL than in Balb/c mice (12). The SJL strain is known to be highly susceptible to EAE, a CD4<sup>+</sup> T cell-mediated acute autoimmune disease. EAE can be induced by immunization with PLP, MBP, or peptides corresponding to the immunodominant epitopes of these proteins (14). Recent data show that B-cell activation and anti-myelin autoAb response can play a significant role in the development of MS and EAE (17). Our previously published data showed the age-dependent appearance of a specific catalytic response toward MBP and some other autoAg in SJL mice (10). The peculiarities of SJL mice mentioned above can provide a unique setting for raising a specific catalytic response against proteins.

## MATERIALS AND METHODS

**Synthesis, Cloning, Expression, and Purification of Antigens.** A DNA fragment corresponding to bovine MBP<sub>85–101</sub> peptide (VVHFFKNIVTPRTPPPS) was synthesized using two overlapping oligonucleotides. The product was cloned into pET32b+ plasmid (Novagen), utilizing *NotI* and *XhoI* restriction sites according to ref 18. The expression product of this plasmid was Trx fused to MBP<sub>85–101</sub> peptide (Trx-mbp). The coding sequence of the c-myc epitope was added

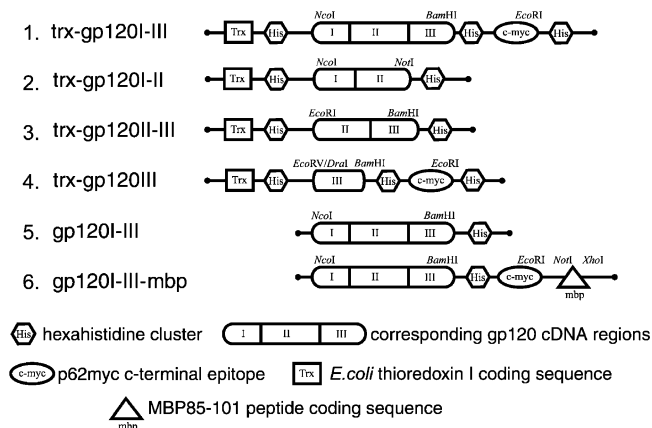


FIGURE 1: Scheme of expressed recombinant proteins. Recombinant protein abbreviations are in bold; restriction sites used are in italics.

to the initial constructs using *BamHI* and *EcoRI* restriction sites for unambiguous identification of recombinant proteins by monoclonal anti-c-myc Ab. Resulting constructs were named pET32CH and pET32CHmbp. Three DNA fragments (named I, II, and III) corresponding to conservative regions of gp120 (Supporting Information, Figure 1) were amplified by PCR with specific primers and combined in-frame using a “splicing by overlap extension” technique. Cloned HXB2-env gene served as the initial template (19). The resulting fragment I–III and its I–II, II–III, and III parts were cloned into expression vectors pET32b+, pET32CH, and pET32CHmbp utilizing appropriate restriction sites (Figure 1, constructs 1–4). Recombinant proteins coded by these constructs that contained Trx fused to the gp120 fragments were used for immune response monitoring and cleavage analysis. I–III *NcoI*–*BamHI* and I–III *NcoI*–*XhoI* inserts of construct 1 were finally recloned into pET28aa vector (Novagen) by corresponding restriction sites (Figure 1, constructs 5 and 6) for expression in *Escherichia coli*. The recombinant proteins produced consisted of gp120 fragments I, II, and III alone or fused to MBP peptide and named gp120I–III and gp120I–III-mbp, respectively, and were used for immunization and cleavage analysis. A DNA fragment corresponding to the gp120-coding region of the HXB2-env gene with a deleted C-terminal  $\alpha 6$  helix part was amplified by PCR using specific primers and cloned into PMelBacB (Invitrogen) for production of a glycosylated form of gp120 in a baculovirus expression system.

Expression of recombinant proteins in *E. coli* was done according to vector manufacturer instructions. Insoluble recombinant proteins were purified according to (20), solubilized by 7 M urea and purified with a Talon SuperFlow (BD Biosciences) resin according to manufacturer's instructions. Purified denatured proteins were precipitated by dialysis against water and used for immunization as a corpusculate Ag or resolubilized with 0.02% SDS, 5 mM DTT and used in immune assays.

Expression of recombinant Trx-gp120I–III protein in soluble form in *E. coli* was carried out in Origami B(DE3) cells (Novagen) according to the manufacturer's instructions. The soluble form of the target protein was extracted and purified by metal chelating chromatography with a Talon Superflow resin as above. Eluted recombinant protein was desalted, applied onto a Mono Q column (Amersham Biosciences), and eluted by a linear gradient of NaCl

concentrations ranging from 0 to 500 mM at pH 9.0. Eluted target protein was concentrated and buffer-exchanged to TBS by ultrafiltration.

Transfection, screening, purification of virus particles, and expression of recombinant gp120 in the baculovirus expression system was performed according to the kit manufacturer's instructions (Invitrogen). Proteins of interest were collected from culture medium and purified by ammonium sulfate precipitation and affinity chromatography using immobilized lentil lectin (21).

**Synthetic Antigens.** Bovine MBP<sub>85–97</sub> peptide (VVH-FFKNIVTPRT) was synthesized using the Fmoc solid-phase peptide technique. Ag for ELISA was prepared by conjugating peptide and BSA by EDAC (Sigma) according to the EDAC manufacturer's protocol.

**Immunization.** Mice were obtained from Harlan Labs and maintained in SPF conditions. Female SJL and Balb/c mice, 6–8 weeks old, were immunized twice with an interval of 7 days according to ref 22. Mice were monitored every other day for the development of clinical symptoms. After 17 days, a boost injection of 50  $\mu$ g of antigen in PBS was given. Four days later, mice were euthanized. Blood sera, spleens, and lymph nodes were used for experiments. The following antigens were used in the same conditions as described above. gp120I–III-mbp was injected at 150 and 300  $\mu$ g (3.5 and 7 nmol) per mouse for groups SJL-3 and SJL-4. gp120I–III was injected at 150 and 300  $\mu$ g (3.5 and 7 nmol) per mouse for groups SJL-5 and SJL-6. Peptide MBP<sub>85–97</sub> was injected at 170  $\mu$ g (100 nmol) per mouse (group SJL-2). Nonimmunized SJL mice were analyzed as controls (group SJL-1).

**Analysis of Ag Specificity of Polyclonal Ab by ELISA.** All Ag were used at 500 ng per well; coating was performed overnight at 4 °C. Ag binding on the microplate surface was monitored by anti-c-myc monoclonal and anti-Trx polyclonal mouse Ab interaction. Dilution of sera was 1:10 for anti-MBP Ab detection and 1:50 or 1:200 for gp120-containing Ag's. Bound Ab were detected by horseradish peroxidase conjugated to goat anti-mouse IgG (Fc-specific) Ab and revealed with *o*-phenylenediamine substrate at 492 nm according to ref 20.

**IgG Purification.** All IgG preparations used were obtained according to ref 13. Briefly, polyclonal IgG antibodies were isolated by triple 50% ammonium sulfate precipitation, reconstituted by buffer TN (20 mM Tris-HCl, pH 7.6, and 50 mM NaCl), and applied onto a protein G–Sephadex fast-flow (Amersham) column. The column was extensively washed by 50 volumes of TN. IgG antibodies were eluted by 100 mM Gly-HCl, pH 2.6, solution. All solutions used in the chromatographic step were sterilized by autoclaving prior to use. Before each purification run, the column was washed with 15 volumes of 100 mM Gly-HCl, pH 2.6, solution and 2 M LiCl solution. The purity of the IgG obtained was checked by SDS–PAGE (Coomassie staining).

**Size Exclusion Chromatography.** Size exclusion chromatography in strong acidic solution was performed according to ref 13 with minor changes: a Superose 6 column (Amersham) was employed instead of a TSK gel-based column.

**Proteolytic Activity Detection by Fluorescent Assay.** Fluorescent substrate FITC–gp120I–III was synthesized and characterized according to ref 23 utilizing a 1:1 protein:FITC

weight ratio. The degree of FITC conjugation was found to be 6:1 mol/mol. Hydrolysis of substrate by an excess of trypsin gives a 7.0 times fluorescence increase for FITC–gp120I–III.

Sera from five mice in each group tested were pooled for antibody purification and analysis. Determination of proteolytic activity in Ab samples was done by mixing purified Ab and fluorescent substrate with final concentrations of 0.7 and 0.25  $\mu$ M in TN, supplemented with 0.02% NaN<sub>3</sub>. Three independent measurements of fluorescence intensity ( $\lambda_{\text{ex}}$  = 480 nm,  $\lambda_{\text{em}}$  = 530 nm) were performed at 0, 24, and 48 h.

**SDS–PAGE Analysis of the gp120 Degradation Pattern.** Biotinylated BSA was synthesized according to ref 20 and was used as a nonspecific control substrate. IgG isolated from groups SJL-1 (unimmunized control) and SJL-3 mice, at a concentration 0.3  $\mu$ M, was incubated with gp120I–III (0.25  $\mu$ M), glycosylated baculovirus-expressed gp120 (0.5  $\mu$ M), and biotinylated BSA (0.5  $\mu$ M) for 24 h at 37 °C. gp120-derived peptides were visualized by silver staining, using Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> activation according to ref 24 in the case of gp120I–III and the Bio-Rad silver stain kit (Bio-Rad) in the case of glycosylated gp120 and BSA. Biotinylated BSA was visualized by Western blot analysis using a neutravidin–HRP conjugate (Pierce). In the case of glycosylated gp120, the membrane was incubated with biotinylated polyclonal mouse anti-gp120I–III antibodies, with subsequent incubation with the neutravidin–HRP conjugate. Color was developed with 4-chloro-1-naphthol (Sigma).

**SELDI Determination of the gp120 Hydrolysis Pattern.** Purified IgG was incubated at 37 °C overnight at a 5:1 molar ratio with Trx–gp120I–III protein and baculovirus-derived glycosylated gp120, both taken in a concentration of 1  $\mu$ M. Following the incubation, samples were diluted 10-fold to a final volume of 60  $\mu$ L with the incubation buffer (100 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 0.1% Triton X-100). Samples were then incubated on a shaker with Protein Chip IMAC3 array (Ciphergen Biosystems, Inc.) loaded with Ni ions and washed twice with 170  $\mu$ L of the binding buffer for 15 min each. Peptides not containing His<sub>6</sub> clusters were washed out by 170  $\mu$ L of 20 mM Tris-HCl and 50 mM imidazole hydrochloride, pH 7.5, for 5 min. Imidazole was removed by a 1 min water wash. One microliter of 20% CHCA solution (Ciphergen Biosystems, Inc.) was added to each spot. Prepared chips were read at a laser intensity of 190 in the PBS II reader (Ciphergen Biosystems, Inc.).

**Flow Cytometry Analysis.** Spleens and lymph nodes from three randomly chosen mice from each group were pooled for cytometry analysis (25–27). Lymphocytes from mouse spleens and lymph nodes were purified by centrifugation in Ficoll plaque gradient, density 1.0077. Cells were fixed and double-stained with anti-CD4 Ab–PE and various anti-CD Ab–FITC conjugates (CALTAG Laboratories). Cells were counted by a Coulter Epics Elite flow cytometry apparatus.

## RESULTS

Here we present a novel approach aimed at inducing a protein-specific catalytic response in SJL mice under conditions of an ongoing autoimmune disorder stimulated by gp120 fragments fused with the encephalitogenic peptide MBP<sub>85–101</sub>. gp120-based Ag's for immunization of SJL mice



Table 1: Specific Immune Response of SJL Mice Measured by ELISA<sup>a</sup>

group of animals <sup>b</sup>	SJL-1	SJL-2	SJL-3	SJL-4	SJL-5	SJL-6	Balb-1	Balb-2
immunogen	—	MBP <sub>85–97</sub> peptide	gp120I–III-mbp	—	gp120I–III	—	—	gp120I–III-mbp
dose ( $\mu$ g)	—	170	150	300	150	300	—	300
Trx-gp120I–III	—	—	+	+	+	+	—	+
Trx-gp120I–II	—	—	+	+	+	+	—	+
Trx-gp120III	—	—	+	+	+	+	—	+
Trx-mbp <sub>85–101</sub>	—	+	+	+	—	—	—	+
MBP <sub>85–97</sub> –BSA	—	+	+	+	—	—	—	+
Trx	—	—	—	—	—	—	—	—

<sup>a</sup> The ELISA test was considered positive if the signal for all mice in the corresponding group was more than three times the background. Serum dilutions used varied for different antigens and are given in the Materials and Methods section. Levels of antibodies to different gp120 fragments for immunized SJL mice are presented in more detail in Supporting Information, Figure 2. <sup>b</sup> Five animals in each group.

were chosen on the basis of prior analysis of potentially immunogenic sites within gp120 (28–30). To produce Ag for gp120-targeted Ab induction, coding DNA sequences for the immunodominant, highly variable gp120 domains, V1, V2, and V3 (6), as well as three nonessential hydrophobic  $\alpha$  helices,  $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 6, with their flanking sequences were deleted. Three resulting DNA fragments (named I, II, and III) corresponding to the highly conserved regions of gp120 necessary for CD4 binding and structural consistency and essential for Ab-mediated protein cleavage were kept unmodified (Supporting Information, Figure 1). As a basic template for design of gp120-containing recombinant proteins, the HXB2-env gene, obtained from the AIDS Research and Reference Reagent Program (AIDS NIH Division), was used (19). gp120I–III-mbp fusion, gp120I–III recombinant proteins, and synthetic peptide MBP<sub>85–97</sub> were selected as Ag for this set of experiments. To obtain folded soluble recombinant Ag for screening purposes (both binding and catalysis), the designed proteins were fused to Trx (31) (Figure 1). A glycosylated form of gp120, corresponding to the whole HXB2-env gene part, coding gp120 with a deleted C-terminal  $\alpha$ 6 helix, was obtained in a baculovirus expression system (Invitrogen) and used in the cleavage analysis.

We followed the “short” immunization protocol (12) proposed for raising catalytic Ab-producing clones in autoimmune mice.

Ab capture ELISA analysis of the immune response at day 21 after immunization revealed the presence of Ab to all three gp120 fragments as well as to encephalitogenic MBP<sub>85–97</sub> peptide (Table 1, Supporting Information, Figure 2). The response to gp120I–III immunogen alone, without fused mbp<sub>85–101</sub> peptide, was comparatively low. No significant relationships between the levels of immune response toward discrete fragments I, II, and III of the gp120 molecule and immunization conditions in various groups were found due to high differences in the distribution pattern of specific Ab in individual mice inside groups.

Flow cytometry analyses of spleen and lymph node cells from immunized mice (Supporting Information, Figure 3) showed that CD4<sup>+</sup> T cells were activated following injection of MBP<sub>85–97</sub> peptide and gp120I–III-mbp fusion protein. In the case of immunization by gp120I–III alone the response of T cells was comparatively reduced or delayed.

The development of autoimmune and encephalomyelitis-like disorders in SJL mice (groups SJL-2–SJL-4, Table 1) was confirmed by histological analysis at 1 month after immunization. Examination of tissue sections demonstrated hypertrophy and hyperplasia of lymphoid follicles in spleen and lymph nodes together with dilation of their sinuses, all

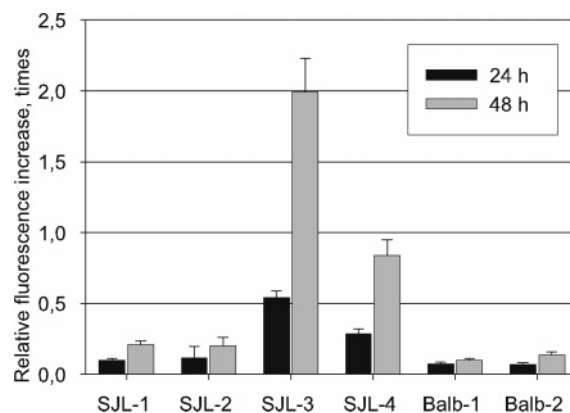


FIGURE 2: Induction of gp120-specific abzyme response in SJL mice. Relative fluorescence signal increase due to proteolytic degradation of gp120I–III substrate, heavily labeled by FITC. The relative fluorescence increase was calculated as the ratio of the difference of fluorescence intensities at time  $t$  ( $F_t$ ) and at initial time ( $F_0$ ) to fluorescence intensity at initial time ( $F_0$ ):  $A = (F_t - F_0)/F_0$ . Data are the average of three independent measurements; error bars represent standard deviation.

indicative of a pronounced immune response. Kidneys exhibited features characteristic of glomerulonephritis as part of a progressive autoimmune disorder (14) (data not shown). A diffuse lymphocytic infiltration of neural glia and degeneration of cortical neurons were also observed (Supporting Information, Figure 4).

These changes are characteristic of the developing encephalomyelitis-like disorder (14) and directly demonstrate that MBP<sub>85–101</sub> peptide fused in-frame with a protein of interest acts as an encephalitogenic agent.

To demonstrate the appearance of proteolytic activity, immunoglobulin fractions were isolated from sera of immunized SJL mice and subjected to a previously developed purification scheme for the study of natural abzymes (13).

The proteolytic activity of abzymes, contained in the purified IgG pool, was measured by a quantitative technique using polypeptide substrate heavily labeled with FITC (10, 32). This analysis revealed the appearance of time-dependent cleavage of FITC-gp120I–III (Figure 2). Compared to bovine trypsin, assayed in the same conditions, IgG from SJL-3 mice is only 3800-fold less active (data not shown). Kinetic data showed the induction of a catalytic response toward gp120, namely, a 9-fold increase of proteolytic activity when compared with nonimmunized SJL mice or mice immunized with MBP peptide, the level of proteolytic activity being higher at 150  $\mu$ g/mouse antigen dose than at 300  $\mu$ g/mouse (Figure 2). This inverse proportion can be attributed to the high titer of gp120-binding antibodies

Table 2: Inhibition Analysis of Abzyme Activity<sup>a</sup>

group of animals <sup>b</sup>	SJL-1	SJL-3	SJL-4
anti-mouse IgG <sup>c</sup>	0.18	0.15	0.25
AEBSF <sup>d</sup>	0.25	0.09	0.14
TPCK <sup>e</sup>	0.30	0.55	0.13

<sup>a</sup> The inhibition rate is shown as the ratio of activity in the presence of inhibitor to uninhibited activity. The reaction time for anti-mouse IgG was 48 h and for other inhibitors 24 h. All other assay conditions are the same as in Figure 2. <sup>b</sup> Five animals in each group. <sup>c</sup> Inhibition was performed by 1 h preincubation of immobilized goat anti-mouse IgG antibodies with the IgG under study. Control immobilized goat anti-mouse IgM antibodies and immobilized anti-human IgG antibodies, incubated with SJL-3 and SJL-4 mouse IgG preparations in same conditions, gave no detectable inhibition (data not shown). <sup>d</sup> Final concentration 3  $\mu$ M. IgG and AEBSF were incubated for 1 h before adding substrate. <sup>e</sup> Final concentration 3  $\mu$ M. IgG and TPCK were incubated for 1 h before adding substrate.

extensively masking antigen in the case of 300  $\mu$ g/mouse immunization (Supporting Information, Figure 2).

The background, but nonzero level of proteolytic activity of IgG from naive SJL mice is consistent with our previous findings (10). Utilizing nonspecific FITC-BSA substrate, we found that the general proteolytic activity of IgG from immunized and nonimmunized SJL mice did not change significantly (data not shown).

Special attention was paid to avoid possible contribution of usual enzyme contamination to the induced activity. The observed proteolytic activity was present only in 150 kDa IgG peak after gel filtration in acidic conditions (data not shown) and removed following passage over immobilized anti-mouse IgG antibodies (Table 2). At the same time, IgG from both nonimmunized and immunized Balb/c mice (groups Balb-1 and Balb-2), purified by the same scheme,

was found to be proteolytically inactive (Figure 2). All of these data are consistent with the activity being Ab-mediated. The observed activity was inhibited by covalent Ser- and His-reactive covalent inhibitors AEBSF and TPCK (Table 2).

Purified IgG was examined for the presence of proteolytic activity by SDS-PAGE following incubation with corresponding substrates including BSA as a nonspecific control substrate. The results of these experiments are summarized in Figure 3. Panels A–C display the Ab-mediated cleavage of recombinant gp120I–III and glycosylated gp120. One distinct degradation product of gp120I–III was detected by SDS-PAGE in the case of immunized SJL mice (Figure 3A, marked by triangle). The band shift of the degraded protein was calculated and found to be about 3 kDa, corresponding to a cleavage site located either near the C-terminal His<sub>6</sub> cluster but still inside gp120 moiety or in the N-terminal part of gp120I–III. It was found that BSA is completely resistant to Ab-mediated hydrolysis, according to SDS-PAGE and Western blot (Figure 3D,E), as well as SELDI analysis (data not shown).

To determine the exact location of the cleavage site, the Trx-fused form of recombinant gp120, Trx-gp120I–III, was used for analysis (Figure 1). This protein, expressed in soluble monomeric form, possesses a His<sub>6</sub> cluster between the Trx and gp120 parts and two adjacent His<sub>6</sub> clusters, spaced by a c-myc epitope at the C-terminus. Thus, cleavage of this protein by catalytic Ab at the same site as in the gp120I–III protein and near the C-terminus of the gp120 part should produce a 2.2 kDa larger peptide (5–6 kDa) with two His<sub>6</sub> clusters, not bound to the rest of the protein molecule by disulfide bonds and presumably not recognizable

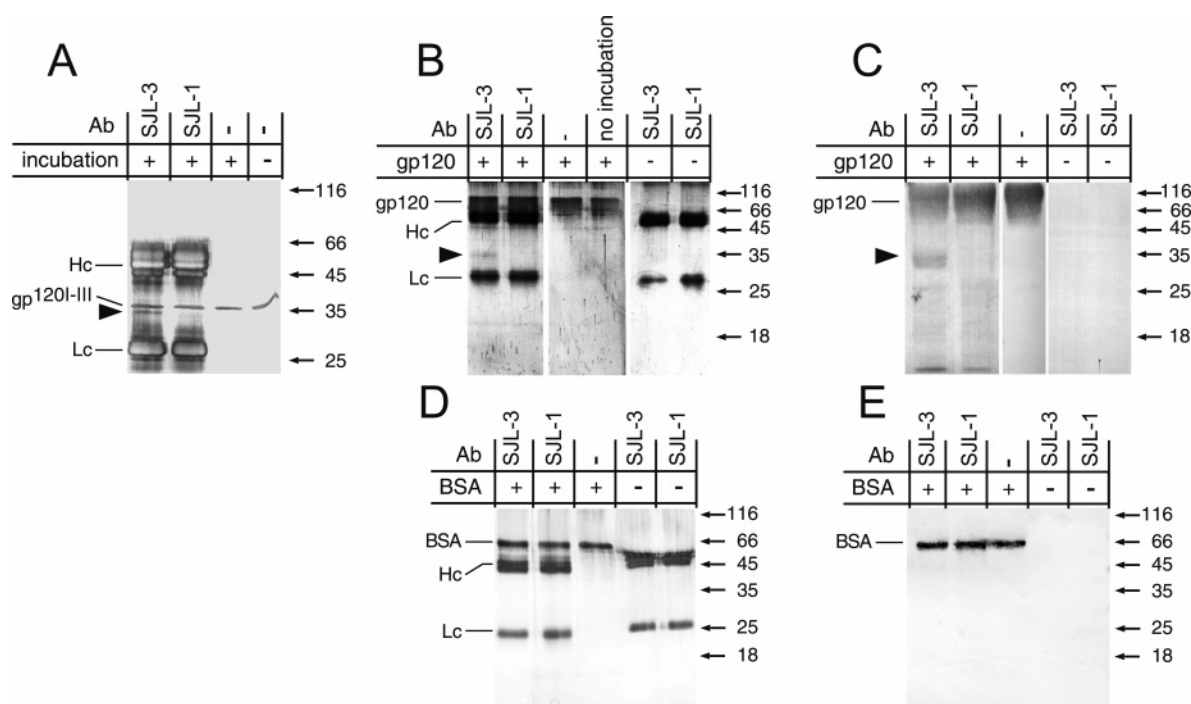


FIGURE 3: IgG-mediated proteolytic degradation pattern of (A) gp120I–III by SDS-PAGE, (B) glycosylated baculovirus-expressed gp120 by SDS-PAGE, and (C) Western blotting and absence of degradation of biotinylated BSA (D) by SDS-PAGE and (E) by Western blotting. Reaction conditions are described in the Materials and Methods section. 15  $\mu$ L aliquots were subjected to SDS-PAGE. Gel slabs were silver stained (A, B, D) or transferred to membrane for subsequent blotting. Molecular mass protein marker band positions are shown by arrows and given in kDa; IgG heavy chains, light chains, and degradation product positions are marked as Hc, Lc, and triangle sign, respectively.

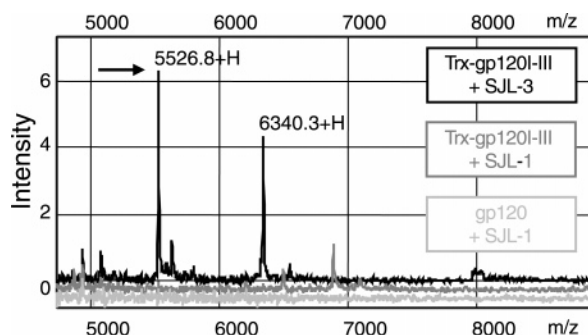


FIGURE 4: SELDI spectra of IgG-mediated cleavage of gp120. Mass spectra of His<sub>6</sub>-containing peptides, derived from the Trx-gp120I-III protein and glycosylated gp120, incubated overnight with IgG from groups SJL-3 and SJL-1 mice. The dominant 5526.8 Da peptide, marked by an arrow, was identified as the C-terminal fragment of the Trx-gp120I-III protein. Control spectra, indicated by black and gray colors, have been shifted down for clarity.

by the majority of gp120-binding Ab. Cleavage near the N-terminus of the gp120 moiety should give rise to two large molecules.

Mass spectrometry analysis was performed by SELDI technology, which provides a combination of chromatographically active surfaces with LDI-type mass detection. The presence of two His<sub>6</sub> clusters in the expected peptide provided us with the opportunity to use IMAC-3 surfaces (CiphaGen Biosystems, Inc.) loaded with Ni ions specifically to capture His<sub>6</sub> cluster-containing fragments of the protein. Figure 4 shows the results of such mapping analysis of recombinant Trx-gp120I-III subjected to abzyme-mediated proteolysis. SELDI spectra showed a predominant peak of 5526.8 Da. The structural analysis of the gp120 fragment cleaved by abzymes permitted us to locate the cleavage at the Pro<sub>493</sub>–Leu<sub>494</sub> bond (residue numbers according to the HXB2CG numbering system). This corresponds to positions 484–485 in a recombinant protein generating a fragment from the C-terminus of a recombinant gp120 with theoretical average mass 5528 Da.

Analysis of a second peak detected at 6340 Da gave no good agreement with theoretical masses of C-terminal peptides.

According to a database of HIV-I Env protein sequences (33), a 12-mer peptide surrounding the cleavage site is entirely present in 34% of the cases. Heptapeptide potential Ab epitopes which include the cleavage point are present in 48% of the Env protein sequences (average value for six possible epitopes). Hence, approximately half of the sequenced HIV-I variants may be susceptible to proteolysis by the catalytic Ab under study.

## DISCUSSION

The data obtained in this study support the idea that a protein-specific catalytic response can be elicited in the course of an inducible autoimmune disorder. In our approach, we intended to take advantage to induce autoimmune disorders for obtaining Ab that cleave specific sequences of constant regions of gp120. This approach is conceptually more akin to vaccination (Vaczyme) than to straightforward elimination of virus by means of drug therapy.

Classical vaccination designed to recruit a vigorous immune response is aimed at induction of sterilizing immunity rather than at direct virus elimination. This first

attempt to raise protein-specific abzymes using peptide-inducible autoimmune disorder may stimulate investigators to expand these assumptions to other proteins critical for specific viral and bacterial infections. The suggested approach may be more general and can be regarded as “passive vaccination”. It may help to obtain a catalytic vaccine to proteins of interest.

Abzymes are known to be relatively “slow” but highly specific biocatalysts (34). Their turnover properties against specific sequences of virus proteins may contribute to efficient virus neutralization and stimulate classical immune responses to viral particles. Even if low turnover properties are characteristic of abzymes in general (35), their specificity and stability in the bloodstream have been clearly demonstrated to be sufficient to inactivate target proteins (36). Moreover, it was shown recently that IgM antibodies from individuals not exposed to gp120 are able to cleave gp120 to some extent (37). The alternative, chemically assisted induction of anti-gp120 catalytic responses was described previously by the same group (38). Additionally, it was shown that another HIV-1 surface glycoprotein, gp41, can be cleaved by the free light chain of anti-gp41 monoclonal antibody (39).

The first remarkable attempts in the abzyme field (40–42) encouraged investigators to make valuable contributions in medicine. The induction of a protein-specific catalytic response together with newly described abzymological pathways of medical importance (36, 38, 39, 43–45) opens exciting perspectives for this field of research.

## SUPPORTING INFORMATION AVAILABLE

Figure 1, scheme of gp120 and sites of cleavage; Figure 2, distribution of immune response to gp120 in immunized SJL mice; Figure 3, T-lymphocytes phenotype changes during immunization; Figure 4, tissue stains of immunized mice. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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