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Characterization and Epitope Mapping of KI-41, a Murine Monoclonal Antibody Specific for the gp41 Envelope Protein of the Human Immunodeficiency Virus-1

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In this study, a mouse monoclonal antibody (mAb) against gp41(584-618), the immunodominant epitope of HIV-1 gp41 envelope protein, was generated. For this purpose, BALB/c mice were immunized with double branched multiple antigenic peptides derived from the HIV-1 gp41(584-618) sequence, and antibody-secreting hybridoma were produced by fusion of mice splenocytes with SP2/0 myeloma cells. One clone producing an antigen specific mAb, termed KI-41 (isotype IgG1) was identified, whose specific reactivity against gp41(584-618) could be confirmed by ELISA and Western blot analysis. Epitope mapping revealed the recognition site of the mAb KI-41 to be located around the sequence RILAVERYLKDQQLLG, which comprises the N-terminal region within the immunized gp41(584-618) peptide. Since this mAb recognizes this specific epitope within the HIV-1 gp41 without any cross-reactivity to other immunodominant regions in the HIV-2 gp36, KI-41 will provide some alternative possibilities in further applications such as the development of indirect or competitive ELISA for specific antibody detection in HIV-1 infection or for other basic researches regarding the role and function of HIV-1 gp41.

Keywords: HIV-1 gp41(584-599), Monoclonal antibody, Multiple antigenic peptide (MAP), Synthetic peptide.

Introduction

Human immunodeficiency virus (HIV) infection induces a strong antibody response to viral structural proteins encoded by viral genes such as the *env* (gp120 and gp41) and *gag* (p24) (Schupbach *et al.*, 1984; Chou *et al.*, 1988; Allain *et al.*, 1991). Among these antigenic proteins, gp41, a transmembrane envelope protein of HIV-1 has been successfully used as a coating antigen in enzyme-linked immunosorbent assays (ELISA) for detection of HIV-1-specific antibodies in AIDS diagnosis (Burke *et al.*, 1987; Schulz *et al.*, 1987; Filice *et al.*, 1991). In order to increase the sensitivity and specificity in the detection of anti-HIV antibodies from human serum, an alternative to native or recombinant viral proteins — synthetic peptides derived from immunodominant epitopes of the HIV-1 gp41 protein — have been successfully applied as coating antigens in ELISA (Khaotov *et al.*, 1990; Andreev *et al.*, 1991; Petrov *et al.*, 1991). For example, as reported previously, double branched multiple antigenic peptides (MAP) of the gp41(584-618) sequence which correspond to the most immunodominant epitope of the HIV-1 gp41 protein showed high sensitivity and specificity in detecting anti-HIV-1 antibodies from HIV-1 positive sera (Shin *et al.*, 1997).

Optimal blocking of non-specific bindings as well as the selection of the proper dilution factor of the sample solution are the prerequisites for setting up an ELISA system in direct detection of anti-HIV antibodies without false positive results derived from nonspecific reaction of human serum. But despite these efforts, false positive data induced by nonspecific bindings often appeared. Therefore, in the detection of anti-HIV-1 antibodies, an inhibitory ELISA using a mAb against the gp41 protein or to one of its immunodominant epitope sequence could be the solution for eliminating false positive results. Thus, in the present study, a mouse mAb termed KI-41 was generated, which shows high reactivity and specificity against HIV-1 gp41(584-618), containing the immunodominant region of gp41. The binding epitope of the mAb KI-41 was mapped using a set of overlapping

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synthetic peptides derived from the whole amino acid sequence of gp41(584-618). Also to determine other potential uses of this mAb, such as the discrimination of HIV-1 gp41 antigens from HIV-2 gp36 envelope proteins, or the selective diagnostics of HIV-1 antisera, the cross-reactivity of mAb KI-41 to the gp36(574-602) sequence, which is the structural counterpart region of the HIV-1 gp41(584-618) envelope glycoprotein (Charneau *et al.*, 1994), was evaluated by ELISA.

Materials and Methods

Peptide synthesis The double-branched multiple antigenic peptide of HIV-1 gp41(584-618), termed MAP-1, and the other peptides, gp41(584-599), gp41(590-612), gp41(602-618), as well as the gp36(574-602) of HIV-2 were synthesized by the solid phase method (Merrifield, 1986) using Fmoc-chemistry. Fmoc-Lys(Fmoc)-OH was used for introducing the two branches of MAP-1. For the elongation of peptide chains, DCC (dicyclohexylcarbodiimide) and HOBt (N-hydroxybenzotriazole) were used as coupling agents. The protected peptide-Wang resins were washed with dichloromethane (DCM) and dried with N_2 gas. Thereafter, peptides were cleaved from the resins and deprotected with reagent K (King *et al.*, 1990; Choi and Aldrich, 1993; Shin *et al.* 1996) containing 3% triisopropylsilane. Crude peptides were purified by reversed-phase HPLC using a preparative C18 column (Deltapak, 15μ, 19 × 30 cm).

Gel electrophoretic analysis of MAP-1 Successful synthesis of MAP-1 was confirmed by tricine-SDS polyacrylamide gel electrophoresis (Schagger and von Jagow, 1987). The synthesized peptide (5 μg) was boiled in gel loading buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 12% glycerol, 2% mercaptoethanol, and 5% Brilliant Blue G) for 3 min, and the samples electrophoretically separated in a 33% tricine-SDS gel. After gel running, samples were stained in 10% acetic acid containing 0.025% Brilliant Blue G.

Generation of monoclonal antibody producing hybridoma Female 8-wk-old BALB/c mice were immunized 3 times with 100 μg of MAP-1 at 2-wk intervals. For the first immunization, MAP-1 was emulsified in complete Freund's adjuvant (Sigma, St. Louis, USA), and the emulsion injected abdominally. The second and third injection were performed under the same condition except using incomplete Freund's adjuvant for generating the emulsion. Three days prior to fusion, mice were boosted with 100 μg of MAP-1 in PBS by intravenous injection into the tail vein. Spleen cells were fused with SP2/0 mouse myeloma cells and selected in HAT media. Hybridoma clones specific for HIV-1 gp41(584-618) were identified by screening of the media supernatant. Positive clones were subjected to limiting dilution. The resulting monoclonal hybridoma, producing the gp41(584-618) specific mAb KI-41, was deposited at the Korean Culture Type Collection (KCTC), Taejon, Korea, under the deposit number KCTC 0332 Bp. This hybridoma clone as well as the produced monoclonal antibodies that are produced and any usage of them in generating diagnostic or research orientated applications are pending a patent (Korean Patent Agency, Pat. Pend. Number: 1997-32455)

Indirect enzyme linked immunosorbent assay (ELISA) For indirect ELISA, 96-well Maxisorb plates (Nunc, Roskilde, Denmark) were coated for 1 h at 37°C with 200 ng of MAP-1 per well in coating solution (0.1 M sodium carbonate, pH 9.5) and blocked for 1 h with 100 μl of TBS containing 2.7% casein, and then washed out with TBS (100 mM Tris-HCl, 1.5 M NaCl, pH 7.4) containing 0.05% Tween-20. The coated wells were incubated with 100 μl of hybridoma culture supernatant for 1 h at 37°C, and then washed 3 times with TBS containing 0.05% Tween-20. Specifically bound antibodies were detected by addition of horseradish peroxidase conjugated goat anti-mouse IgG (Sigma) as secondary antibody. After incubation for 1 h at 37°C, excessive antibodies were washed out for 3 times with TBS containing 0.05% Tween-20, and then 100 μl of the substrate solution (0.05 M sodium phosphate-citrate buffer, pH 5.0, containing o-phenylenediamine: 0.4 mg/ml and 37% H_2O_2: 0.4 mg/ml) was added to each well. The reaction was stopped by adding of the same volume of 2.5 M sulfuric acid. The plates were read at 492 nm in a *E.max* microplate reader (Molecular Devices, Sunnyvale, USA).

Immunoblotting Each 2.5 μg of HIV-1 gp160 and alcohol dehydrogenase were loaded, separated in a 12.5% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, USA). The membrane was blocked with TBS containing 2.7% casein for 1 h at 37°C and washed with TBS containing 0.05% Tween-20. Ten ml of a dense grown hybridoma culture supernatant were added and the membrane incubated for 1 h at 37°C. Excessive antibodies were washed out with TBS containing 0.05% Tween-20. Then, membrane was incubated for 30 min at 37°C with horseradish peroxidase conjugated goat anti-mouse IgG antibodies (Sigma), diluted 1 : 5,000 in TBS containing 0.3% casein. After antibody binding, membrane was washed with TBS containing 0.05% Tween-20. Finally, the bound conjugate was identified by incubation of the membrane in substrate buffer (0.5 mg/ml 4-chloro-1-naphthol in 1 : 5 v/v methanol/TBS and 0.015% H_2O_2 for 5 min at room temperature.

Antibody purification KI-41 mAbs were purified from hybridoma media culture supernatant by the method as originally described by Ey *et al.* (1978). Briefly, for one preparation, 500 ml of cell culture supernatant was harvested by centrifugation, and the antibody fraction was precipitated by addition of one volume of saturated ammonium sulfate. Pellet was collected and resuspended in column binding buffer (3.3 M NaCl, 100 mM borate buffer, pH 8.9) and dialyzed overnight at 4°C against the same buffer to remove excess sulfate salts. Next day, antibody solution was flown through a 3 ml protein A sepharose column (Pharmacia, Uppsala, Sweden) preequilibrated in column binding buffer. Non-specifically bound proteins were removed by washing the column successively with 10 times column volume of 3.0 M NaCl, 50 mM sodium borate, and 10 times column volume of 3.0 M NaCl, 10 mM sodium borate. Antibodies were eluted from the column with 100 mM glycine (pH 2.8), and the eluate was immediately neutralized with a 1/10 volume of 1.0 M Tris-HCl. Purity of eluted antibodies was determined by gel electrophoresis, and the concentration determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, USA).

Results and Discussion

To elicit maximal immune response from synthetic peptides with low molecular weight, in general, peptides coupled to carrier proteins such as bovine serum albumin, ovalbumin or keyhole limpet hemocyanin are used as immunogen (Malley *et al.*, 1965; Nizbet *et al.*, 1981). However, this method can alter the structure/conformation of the coupled antigen. The strategy of using multiple antigenic peptide with branched lysyl residues can avoid this problem while retaining strong immunogenic activities (Tam, 1989). Therefore, in this study, a double-branched MAP (MAP-1) of the HIV-1 gp41(584-618) was used as the antigen for the immunization of mice to obtain antibodies with a high reactivity against the immunized peptide. The structure of MAP-1 is shown in Fig. 1. The successfully branched structure of the synthesized MAP-1 was confirmed by its highly shifted molecular weight (MW) as determined by tricine gel electrophoresis. The observed MW value of MAP-1 was in agreement with its calculated value, 7661.7 Da (Fig. 2).

To obtain a mouse monoclonal antibody against the HIV-1 gp41(584-618), spleen cells of mice immunized with MAP-1 were fused with SP2/0 mouse myeloma cells, and hybridoma were selected in HAT media. Hybridoma producing antibodies reactive with the HIV-1 gp41(584-618) antigen was successfully generated. The immunoglobulin isotype of the mAb KI-41 was determined to be of IgG1 using a commercially available isotyping kit (ISO-1, Sigma).

MAb KI-41 showed high reactivity with HIV-1 gp41(584-618), but did not react with HIV-1 p24(164-182) which was used as the negative control in the coating of peptide antigens (Figs. 3 and 4). Also, the isotype matched control antibody, W6/32 (Brodsky and Parham, 1982), specific for a non-polymorphic determinant on human

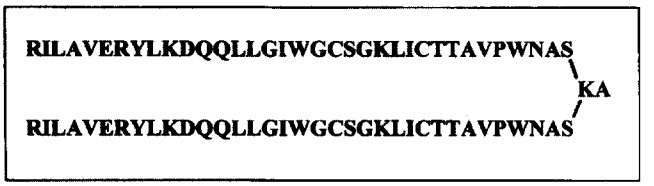

Fig. 1. The structure of MAP-1.

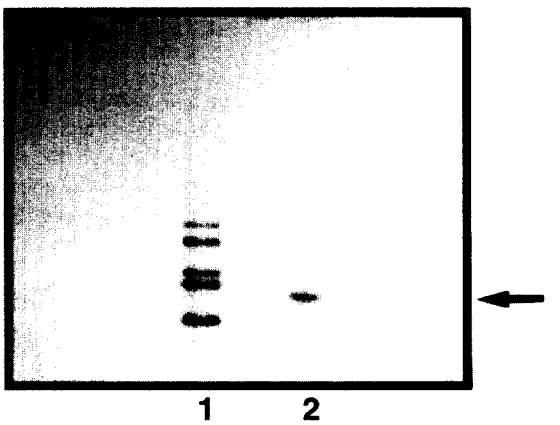

Fig. 2. Tricine gel electrophoresis of MAP-1. Lane 1: molecular weight markers (16,950, 14,440, 10600, 8,160, 6,120); Lane 2: MAP-1.

MHC class I, was shown not to interact with the HIV-1 gp41(584-618) peptide (Fig. 3), confirming again the specificity of this assay. To show that the specific binding activity of KI-41 is not only restricted against this free peptide epitope but also recognizes this sequence in context of its natural structural protein, — the HIV-1 gp41, — Western blot analysis was performed with the HIV-1 gp160 envelope protein which represents the

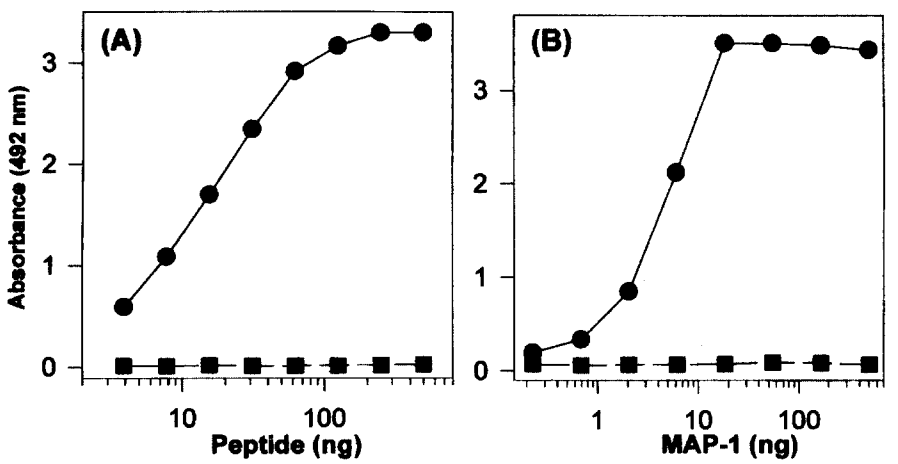

Fig. 3. Determination of antigen specificity of the mAb KI-41. (A) Microtiter plates were coated with serially diluted HIV-1 gp41(584-618) (●), and HIV-1 p24(164-182) (■). After blocking and washing, 100 μl of culture supernatant of KI-41 was added. (B) Microtiter plates were coated with serially diluted HIV-1 gp41(584-618). After blocking and washing, 100 μl of culture supernatant of KI-41 (●) and W6/32 (■) was added to each well, respectively.

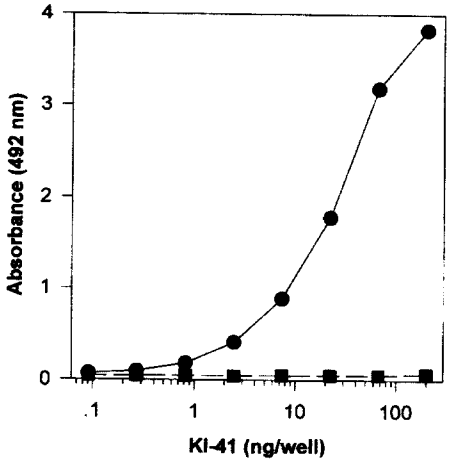

Fig. 4. Determination of antigen specificity of the mAb KI-41. Microtiter plates were coated with 1 mg/well of HIV-1 gp41(584-618) (●), and 1mg/well of HIV-1 p24(164-182) (■). After blocking and washing, the serially diluted KI-41 was added to each well.

precursor form of the gp41 (Decroly *et al.*, 1996), and therefore contains the KI-41 epitope. Indeed, as shown in Fig. 5, the specific reaction of the mAb KI-41 against the HIV-1 gp160 but not to an unrelated protein with similar molecular weight, the alcohol dehydrogenase (141 kDa), was confirmed.

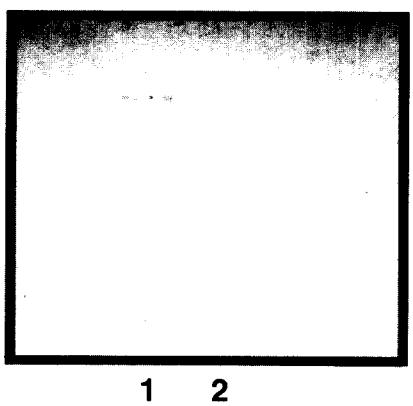

Fig. 5. Reactivity of the mAb KI-41 with HIV-1 gp160 as determined by Western blot analysis.
Lane 1: gp160;
Lane 2: alcohol dehydrogenease.

The binding epitope of the mAb KI-41 was more precisely mapped using a set of synthetic peptides which covers the whole region of MAP-1. The sequence of these peptides are as listed in the following: HIV-1 gp41(584-599), HIV-1 gp41(590-612), and HIV-1 gp41(602-618). The amino acid sequences of each of these peptides are shown in Fig. 6. Since monoclonal antibodies specific for linear epitopes recognize only a short stretch of peptides within a given polypeptide sequence, — usually ranging from 8 to 15 amino acids (Worthington and Morgan, 1994), — it was expected that KI-41 would also have a shorter binding site on the gp41(584-618) than recognizing the whole molecule. As shown in Fig. 7, KI-41 displayed higher reactivity to HIV-1 gp41(584-599) than gp41(590-612), but showed no binding at all to the HIV-1 gp41(602-618). In competitive ELISA, gp41(584-599) inhibited more effectively the bindings of the KI-41 and gp41(584-618) than gp41(590-612) and gp41(602-618) (Fig. 8). These results indicated that the recognition site of KI-41 is located around the sequence, 584-599, RILAVERYLKDQQLLG, which comprises the N-terminal region of gp41(584-618). Actually, due to the structural composition and the extramembraneous localization, this specific gp41 epitope had long been a subject of extensive research in HIV-1 diagnosis, and according to this great interest, a large array of poly- and monoclonal antibodies had been generated. These monoclonal antibodies had been reported to be specific for the 26-meric sequence RILAVERYLKDQQLLGIWGCSGKLIC (Mani *et al.*, 1994), the 23-meric sequence RILAVERYLKDQQLL-GIWGCSGK (Poumbourios *et al.*, 1992), the 21-meric sequence RILAVERYLKDQQLLGIWGCS (Benjouad *et al.*, 1993), and so on. But when compared to all of the antibodies raised against this region of the HIV-1 gp41, the

gp41(584-599):	**RILAVERYLKDQQLLG**
gp41(590-612):	**RYLKDQQLLGIWGCSGKLICTT**
gp41(602-618):	**GCSGKLICTTAVPWNAS**

Fig. 6. The amino acid sequences of gp41(584-599), gp41(590-612), and gp41(602-618).

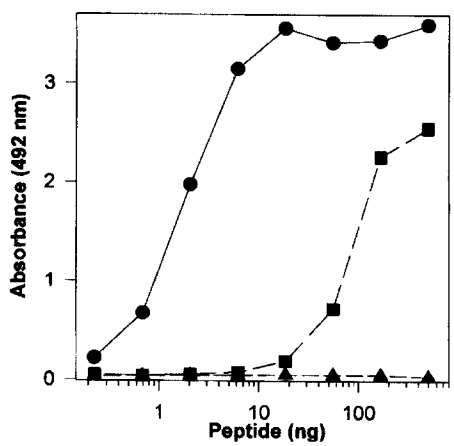

Fig. 7. Reactivity of the mAb KI-41 with gp41(584-599) (●), gp41(590-612) (■) and gp41(602-618) (▲). Microtiter plates were coated with serially diluted gp41(584-599), gp41(590-612) and gp41(602-618), respectively. After blocking and washing, 100 μl of culture supernatant of the hybridoma producing mAb KI-41 was added to each well.

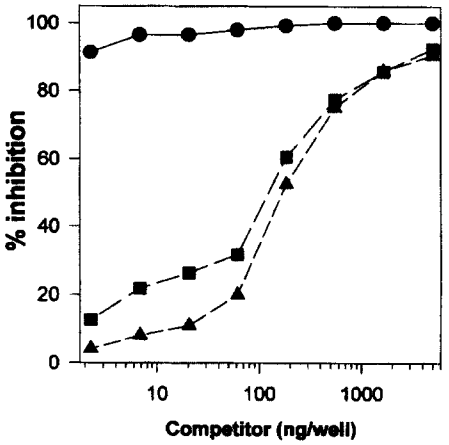

Fig. 8. Percent inhibition by gp41(584-599) (●), gp41(590-612) (■), and gp41(602-618) (▲) of the reaction of the KI-41 and the gp41(584-618). The microtiter plates were coated with 10 ng/well of MAP-1. After blocking and washing, the serially diluted competing peptides and KI-41 (20 ng/well) were added to each well.

mAb KI-41 generated in this study represents an antibody with the shortest binding epitope (16-mer) to this immunodominant region as reported so far. Having such a short binding domain will have further implications in the use of the mAb KI-41 in diagnostic assays as well as in the development of HIV research tools, since this short epitope will largely diminish non-specfic binding due to cross-reaction with other proteins.

Beside the aspect described above, KI-41 will also be of potential use in characterizing HIV subtypes, such as the discrimination of HIV-1 infection from HIV-2 infections, since the KI-41 binding epitope is a highly conserved region even in mutated HIV-1 and other well known HIV-1 subtypes. This point becomes even more clear, when one considers the fact that most of the ELISA for detection of anti-HIV-1 antibodies have been reported to show positive reactions with 60–90% of anti-HIV-2 antibody positive serum samples (George *et al.*, 1990). Since antigenic differences between HIV-1 and HIV-2 are mostly due to the amino acid sequence of their envelope glycoproteins such as the gp41 of HIV-1 and gp36 of HIV-2, synthetic peptides corresponding to the immunodominant epitope regions of gp41 of HIV-1 and gp36 of HIV-2, respectivley, have been conventionally used to discriminate HIV-1 and HIV-2 infections (Gnann *et al.*, 1987; Norrby *et al.*, 1989) Similarly, in our previous study, synthetic peptides of gp41(584-618) and gp36(574-602) had been used successfully as coating antigens in the detection of specific antibodies to HIV-1 and HIV-2, respectively (Shin *et al.*, 1997). To determine whether the mAb KI-41 could be used as a control antibody in a potential diagnostic ELISA assay for discriminating the antibody response to HIV-1 and HIV-2, the ELISA assay was performed with peptide antigens whose sequences were derived from the same ectotopic region of HIV-1 and HIV-2. In this assay, it was questioned, whether KI-41 might have recognized a possible structural conformation formed by the 16-meric peptide from gp41(584-618) which critical conformation would still be retained in a form as a coated antigen or in a membrane bound form (see Western blot analysis, Fig. 5), and not a wholly denatured linear sequence. In the former case, it was expected that there would be a cross-reaction to the HIV-2 derived pepide gp36(574-602), which represents exactly the same structural counterpart of the gp41(584-618), and therefore show the same conformational characteristics (Charneau *et al.*, 1994). As shown in Fig. 9, the mAb KI-41 showed no binding to the gp36(574-602), which confirms the linear binding epitope of KI-41, and also assures its highly specific reaction to HIV-1 without any cross-reaction to HIV-2.

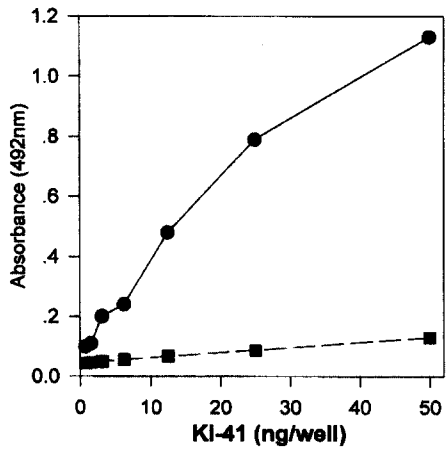

Fig. 9. Analysis of reactivity of the mAb KI-41 with gp36(574-602). Microtiter plates were coated with 100 ng/well of HIV-1 gp41(584-599) (●), and HIV-2 gp36(574-602) (■), AIEKYLKDQAQLNSWGCAFRQVCHTVPG. After blocking and washing, the serially diluted mAb KI-41 was added to each well.

In summary, the newly generated exclusively HIV-1 specific mAb KI-41 will provide some novel possibilities in further applications such as the development of indirect or competitive ELISA in the detection of HIV-1 infections or some other basic research tools regarding the role and function of HIV-1 gp41.

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