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Short communication

Characterization of KI-24, a Novel Murine Monoclonal Antibody with Specific Reactivity for the Human Immunodeficiency Virus-1 p24 Protein

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The HIV-1 p24(202-221) sequence ETINNEEEWDRVHPV HAGP contains a B-cell epitope with the earliest immune response and the highest antibody titer against anti-mouse sera obtained by immunization with p24 antigens. A novel mouse monoclonal antibody (mAb) was generated against the immunodominant B-cell epitope of the HIV-1 p24 capsid protein, p24(202-221). BALB/c mice were immunized with the four branched multiple antigenic peptide (MAP) containing the HIV-1 p24(202-221) sequence, and antibody-secreting hybridoma were produced by fusion of mouse splenocytes with P3X63Ag8.653, mouse myeloma cells. One clone which produced the antigen-specific mAb named KI-24 (Isotype IgG1, light chain: κ) was identified. mAb KI-24 was highly specific for both the p24(202-221) and p24 proteins when analyzed by ELISA and Western blotting. Since p24(202-221) also contains a cytotoxic T-lymphocyte epitope, this specfic peptide epitope and the monoclonal antibody with specific reactivity against the p24 protein and p24(202-221) can be used in peptide vaccine development and p24 antigen detection from HIV patients.

Keywords: B-cell epitope, HIV-1 p24(202-221), HIV-1 p24 protein, Monoclonal antibody.

Introduction

Human immunodeficiency virus (HIV) infection induces a strong antibody response against viral structural proteins encoded by viral genes, such as *env* (gp120 and gp41) (Schupbach *et al.*, 1984; Chou *et al.*, 1988; Allain *et al.*, 1991). The HIV-1 gp41 protein and its derived synthetic peptides have been used as coating antigens in enzyme-linked immunosorbent assay (ELISA) for detection of HIV-1 specific antibodies in AIDS diagnosis (Schulz *et al.*, 1986; Burke *et al.*, 1987; Petrov *et al.*, 1990; Andreev, *et al.*,1991; Filice *et al.*, 1991; Shin *et al.*, 1997, 1998b). Since the titer of anti-HIV-1 gp41 antibodies in the early stage of HIV-1 infection is low, early detection using antibodies is difficult. On the other hand, a relatively large amount of the major capsid protein p24 antigen is present within 2-3 months after intitial HIV-1 infection. Furthermore, p24 antigen diagnosis is a useful method for the early detection of HIV-1 infection in neonates (Kessler, *et al.*, 1987; Backer, *et al.*, 1988). In this context a potential monoclonal antibody with specific reactivity against the p24 protein can be used for detecting early HIV-1 infection.

In our previous study (Lee *et al.*, 1996) four peptides whose sequences corresponded to 164-182, 202-221, 217-236, and 232-256 of p24 were identified as putative epitopes against a goat anti-p24 antibody. Among them, p24(202-221) the peptide with the sequence corresponding to 202-221 [p24(202-221): ETINNEEEWDRVHPVHAGP], showed the earliest immune response and the highest antibody titer when mice were immunized with p24 (Shin *et al.*, 1998a).

A novel mouse mAb KI-24 was generated by immunization of p24(202-221) and was characterized using ELISA and Western blotting. The antibody exhibited highly specific activity against p24(202-221) with a lower background than antibodies raised by immunization with p24 and, thus, may be useful for p24 detection.

Materials and Methods

Peptide synthesis. Peptides were synthesized by the solid phase method (Merrifield, 1986, Shin *et al.*, 1996) using the Fmoc-strategy. Fmoc-Lys(Fmoc)-OH was used in order to introduce the multiple antigenic peptide (MAP) structure. Fig. 1 shows the MAP with four branches for p24(202-221), named MAP-4. For the elongation of peptide chains, DCC (dicyclohexylcarbodiimide) and HOBt (N-hydroxybenzotriazole) were used as coupling agents. Protected peptide-Wang resins were

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washed with dichloromethane (DCM) and dried with N_2 gas. Thereafter, peptides were cleaved from the resins and deprotected using reagent K (King *et al.*, 1990; Choi and Aldrich, 1993; Shin *et al.* 1996) containing 3% triisopropylsilane. The crude peptides were purified by reversed-phase HPLC using a preparative C18 column (Deltapak, 15- μm, 19×30 cm).

Generation of monoclonal antibody production Eight week old female BALB/c mice were immunized 3 times by the intraperitonical injection of 100 μg of MAP-4 mixed with Freund's adjuvant at 2 week intervals. Complete Freund's adjuvant was used for primary immunization and incomplete Freund's adjuvant was used for boosting. Three days prior to fusion, mice were intraveneously injected with 100 μg of MAP-4 in PBS. Spleen cells of immunized mice were fused with mouse myeloma cells, P3X63Ag8.653, using PEG-1500 and grown in HAT medium. Hybridoma clones specific to HIV-1 p24(202-221) were identified by ELISA against the culture supernatant. The monoclonal hybridoma was selected by limiting dilution and the mAb was named KI-24. This mAb was deposited in the Korean Culture Type Collection (KCTC), Yusong, Taejon, under deposit number KCTC 0358 BP (Patent pending, Korean Patent Agency, Number: 1997-52194).

Indirect enzyme-linked immunosorbent assay (ELISA) for anti-p24-antibody detection The synthetic MAP-4 antigen (200 ng per well) was absorbed on flat-bottom microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C. The plates were blocked for 1 h with 100 μl of the blocking solution (0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and 2.7 % casein), then washed in a washing solution of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and 0.05 % Tween-20. One hundred μl of hybridoma culture supernatant was added to each well. The plates were incubated for 1 h at 37°C and washed 3 times with the washing solution. Specifically bound antibodies were detected by addition of 100 μl (dilution 1/10,000) of horseradish peroxidase conjugated goat anti-mouse IgG (Sigma, A-9044) as a secondary antibody. After incubation for 1 h at 37°C the plates were washed and the reaction was developed with *o*-phenylenediamine [0.4 mg ml^{-1} in 0.05 M sodium phosphate-citrate buffer, pH 5.0, containing 0.03% (vol/vol) H_2O_2]. The reaction was stopped by adding 2.5 M sulfuric acid (100 μl). Absorbance at 490 nm was measured using an *E. max* microplate reader (Molecular Devices, Sunnyvale, USA).

Immunoblotting Each 2.5 μg of HIV-1 p24 and trypsinogen were loaded, separated in 12.5% SDS polyacrylamide gel, and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was placed in blocking solution for 1 h at 37°C then washed in washing solution. Ten ml of a dense, brown hybridoma culture supernatant was added and the membrane was incubated for 1 h at 37°C. Excessive antibody was then washed out with the washing solution. Thereafter, the membrane was incubated for 30 min at 37°C with horseradish peroxidase conjugated goat anti-mouse IgG antibodies (Sigma, A9044) diluted to 1:5,000 in the antibody dilution solution (0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and 0.3% casein). After antibody binding the 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-24 mAb was purified from the hybridoma culture supernatant by the method originally described by Ey *et. al.* (1978). For one preparation, 500 ml of culture supernatant was harvested by centrifugation and the antibody fraction was precipitated by addition of one volume of saturated ammonium sulfate. The pellet was 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. The next day the antibody solution was passed through a 3 ml protein A-sepharose column (Pharmacia, Uppsala, Sweden) preequilibrated with the column binding buffer. Non-specifically bound proteins were removed by washing the column successively with a 10 times column volume of 3.0 M NaCl/50 mM sodium borate and a 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 eluent was immediately neutralized with a 1/10 volume of 1.0 M Tris-HCl. Antibody purity was determined by gel electrophoresis and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, USA) using immunoglobulin as a standard.

Results and Discussion

Recently, four putative B-cell epitopes of the HIV-1 p24 protein against anti-p24 goat antibody were determined (Lee *et al.*, 1996). Among these, the HIV-1 p24(202-221) was found to evoke the earliest immune response and the highest antibody titer when BALB/c mice were immunized with p24 (Shin *et al.*, 1998a). Therefore, HIV-1 p24(202-221) exhibits immuno-crossreactivity with the p24 protein and can be used for p24 detection in human HIV infected sera. In order to increase the immune response of p24(202-221), the four-branched multiple antigenic peptide MAP-4 (Fig. 1) of HIV-1 p24(202-221) was synthesized by introduction of two lysyl residues, then was used as the immunogen (Tam, 1989). To obtain a mouse monoclonal antibody against HIV-1 p24(202-221), spleen cells of mice immunized with MAP-4 were fused with P3X63Ag8.653 mouse myeloma cells and hybridoma were selected in HAT medium. Hybridoma producing

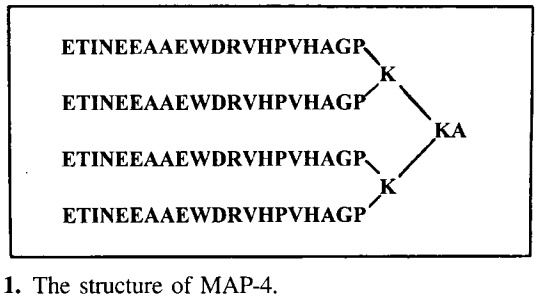

Fig. 1. The structure of MAP-4.

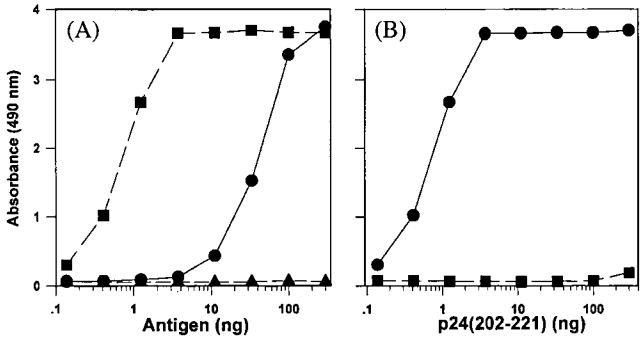

Fig. 2. Determination of antigen specificity of mAb KI-24. (A) Microtiter plates were coated with serially diluted HIV-1 p24 protein (●), HIV-1 p24(202-221) (■), and HIV-1 gp41(584-618) (▲). After blocking and washing, 100 μl of KI-24 culture supernatant was added to each well. (B) Microtiter plates were coated with serially diluted HIV-1 p24(202-221). After blocking and washing, 100 μl of KI-24 culture supernatant (●) and L243 (■) were added to each well.

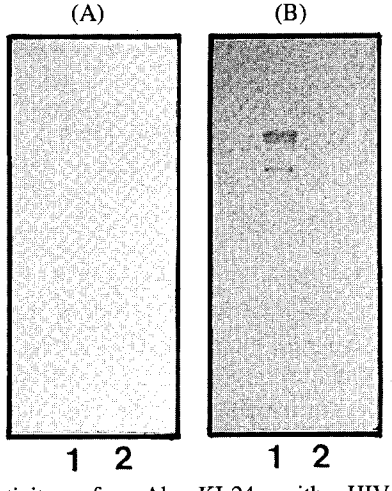

Fig. 3. Reactivity of mAb KI-24 with HIV-1 p24 as determined by Western blot analysis (Panel B). Panel A shows the Comassie brilliant blue R250 staining pattern. Lanes 1 and 2 of each panel show the HIV-1 p24 protein and trypsinogen, respectively.

antibodies reactive to the HIV-1 p24(202-221) antigen were subsequently generated. After a limiting dilution, the single clone KI-24 was obtained. The immunoglobulin isotype of mAb KI-24 was determined to be IgG1 (light chain: κ) using a commercially available isotyping kit (Mab-based Mouse Ig Isotyping Kit, PharMingen, San Diego, USA).

The mAb KI-24 showed high reactivity with both the HIV-1 p24 protein and the HIV-1 p24(202-221) peptide, but had no reactivity with the HIV-1 gp41(584-618) peptide which was used as a negative control (Fig. 2-A). Also, the isotype matched control antibody L243, which is specific for a non-polymorphic determinant on human MHC class II molecules, did not interact with the HIV-1 p24(202-221) peptide (Fig. 2-B).

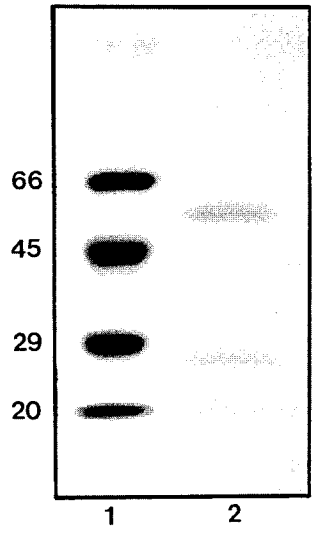

Fig. 4. Gel electrophoresis of purified mAb KI-24. Lane 1: molecular weight markers (66 KDa, 45 KDa, 29 KDa, 20 KDa); Lane 2: prurified mAb, KI-24.

Western blot analysis was performed with the HIV-1 p24 protein to determine if the specific binding activity of KI-24 is restricted to this free peptide epitope. As shown in Fig. 3, the specific reaction of mAb KI-24 against HIV-1 p24, but not to trypsinogen (24 kDa, an unrelated protein with similar molecular weight) indicates that KI-24 recognizes this sequence in the context of its natural structural protein HIV-1 p24. The purity of KI-24 was confirmed by 10% SDS gel electrophoresis. As shown in Fig. 4, purified KI-24 treated with β-mercaptoethanol displayed a single heavy chain and a single light chain on a 10% SDS gel.

The HIV-1 p24 antigen is an important prognostic marker of HIV-1 infection (Alter *et al.*, 1989). As the p24 antigen can be detected in the initial period between infection and seroconversion, p24 antigen testing is useful for early detection of HIV-1 infection in neonates (Kessler *et al.*, 1987; Backer *et al.*, 1988; Nishanian *et al.*, 1990). A specimen is interpreted as HIV-1 positive when two of three possible bands (p24, gp41, gp120/160) appear in a Western blot (Centers for Disease Control, 1989). The p24 band is observed in the Western blot of the indeterminate period of HIV infection. Also, indeterminate Western blot patterns occur frequently in persons who are not infected by HIV (Schochetman *et al.*, 1989). This may be due to immuno-crossreactivity between the HIV-1 p24 protein and the core proteins of other retroviruses, such as human T-cell leukemia viruses (Schochetman *et al.*, 1989). Therefore, a highly specific mAb against the p24 peptide which has low sequence homology with the core proteins of other reteroviruses is necessary for the detection of p24 antigen. Since the HIV-1 p24(202-221) peptide used as the immunogen in this study has low sequence homology with the core proteins of other reteroviruses, and KI-24 has a highly specific reactivity against the HIV-1 p24 protein, KI-24 can be useful for specific

detection of the HIV-1 p24 antigen.

In recent years the polyclonal T cell lines established by *in vitro* antigen-specific stimulation of peripheral blood mononuclear cells (PBMC) from long-term asymptomatic (LTA) individuals have been tested for cytotoxic activity against a set of 48 partially overlapping peptides spanning the entire *Gag*. The p24(202-221) sequence ETINEEAAEW stimulates a cytotoxic T lymphocyte (CTL) (van Baalen *et al.*, 1996). Since this specific sequence is involved in the p24(202-221) used in this study, this specific epitope will be applied in HIV vaccine development.

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