Article

Preparation and Characterization of Three Monoclonal Antibodies against HIV-1 p24 Capsid Protein

Guangjie Liu[1], Jianping Wang[1], Jianchun Xiao[1, 2], Zhiwei Zhao[2] and Yongtang Zheng[1, 3]

HIV-1 p24 detection provides a means to aid the early diagnosis of HIV-1 infection, track the progression of disease and assess the efficacy of antiretroviral therapy. In the present study, three monoclonal antibodies (mAbs) p3JB9, p5F1 and p6F4 against HIV-1 p24 were generated. All mAbs could detect p24 of HIV-1_{IIIB}, HIV-1_{Ada-M}, HIV-1_{74V} mAbs p5F1 and p6F4 could detect HIV-1_{KM018}, while p3JB9 could not. Three mAbs did not react with HIV-2_{ROD}, HIV-2_{CBL-20} and SIVagm$_{TYO-1}$. The recognized epitope of p5F1 was located on the Gag amino acid region DCKTILKALGPAATLEEMMTAC. The p5F1 was used to establish a modified sandwich ELISA with rabbit anti-p24 serum and showed good specificity and high sensitivity, which has been used to measure HIV-1 p24 antigen levels in research. *Cellular & Molecular Immunology*. 2007;4(3):203-208.

Key Words: monoclonal antibody, HIV-1, p24, epitope, ELISA

Introduction

The p24 capsid protein is one of the main structural proteins of HIV-1. There are about 1,500 p24 molecules composing virus capsid in a mature virion (1, 2), therefore p24 protein becomes the most abundant protein produced during virus replication and can be detected in the very early stage of asymptom phase after HIV-1 infection. In the late stage of AIDS, p24 protein can be detected again because of rapid replication of viruses and decline of HIV-1 specific antibody production. Hence HIV-1 p24 detection provides a means to aid the early diagnosis of HIV-1 infection, track the progression of disease and assess the efficacy of antiretroviral therapy. Quantitative analysis of p24 protein has become an accepted way to measure HIV-1 production and infectivity in a wide variety of clinical and research settings (3). Monoclonal antibodies against p24 are key components of an HIV-1 p24 antigen diagnostic kit. In present study, three anti-HIV-1 p24 mAbs producing hybridomas, p3JB9, p5F1 and p6F4, were raised and characterized and an HIV-1 p24 antigen detection assay based on a modified sandwich ELISA was established.

Materials and Methods

Cell culture

NS-1 cells and P388D1 cells were maintained in RPMI-1640 (Gibco) with 10% heat-inactivated newborn calf serum (Gibco) in a humidified chamber with 5% CO_2 at 37°C. Conditioned medium was prepared by culturing 10^5 cells/ml of P388D1 for 72 h in complete IMDM with 10 μg/ml of *Escherichia coli* 055:B5 lipopolysaccharide (4).

Viruses and recombinant HIV-1 p24

The laboratory adapted virus HIV-1_{IIIB}, monocyte-tropic virus HIV-1_{Ada-M}, resistant to reverse transcriptase inhibitor virus HIV-1_{74V}, HIV-2_{ROD} and HIV-2_{CBL-20} were kindly donated by MRC AIDS Research Project or NIH AIDS Research and Reference Reagent Program. SIVagm$_{TYO-1}$ and SRV-1 were kindly donated by Dr. Preston Marx of the California Primate Research Center. The clinically isolated HIV-1_{KM018} was isolated by our laboratory from an HIV-1 infected individual

[1]Laboratory of Molecular Immunopharmacology, Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China;

[2]Key Laboratory of Conservation and Utilization for Bio-resources, Yunnan University, Kunming, Yunnan 650091, China;

[3]Corresponding to: Dr. Yongtang Zheng, Laboratory of Molecular Immunopharmacology, Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China. Tel & Fax: +86-871-519-5684, E-mail: zhengyt@mail.kiz.ac.cn

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Abbreviations: ELISA, enzyme linked immunosorbent assay; HIV-1, human immunodeficiency virus type 1; HAT, hypoxantine, aminopterin, thymidine; HLDH5, human lactate dehydrogenase isoenzyme 5; HRP, horseradish peroxidase; mAb, monoclonal antibody; rp24, recombinant HIV-1 p24; OD, optical density; PBS-T, PBS containing 0.05% Tween 20; SIV, simian immunodeficiency virus; SRV-1, simian type D retrovirus serotype 1.

in Yunnan Province of China as described (5). All virus stocks were stored in aliquots at -70°C. Different viruses were lysed and inactivated by addition tenth volume of 5% Triton X-100 to supernatants. In some experiments, HIV-1_{IIIB} virions were concentrated by PEG (5) and viral pellets were lysed in PBS supplemented with 0.5% Triton X-100.

The plasmid pGEX-6p-3/p24 subtype B was kindly donated from Dr. Kazuyoshi Ikuta of Research Institute for Microbial Diseases, Osaka University, Japan. Recombinant HIV-1 p24 (rp24) was produced as GST-p24 fusion protein in *E. coli* transformed with pGEX-6p-3/p24 subtype B. According to manufacturer's instruction, the fusion protein was purified by Glutathione Sepharose 4B (Amersham Pharmacia) affinity purification, and then p24 was cleaved from the fusion protein by PreScission protease (Amersham Pharmacia). The purities of the eluted solutions were estimated by Coomassie brilliant blue staining after sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE).

Peptides

Five peptides within HIV-1_{HXB2} (HIV-1_{HXB2} is a molecular clone derived from HIV-1_{IIIB} isolate) Gag p24 (GA-12, GQMREPRGSDIA; NI-15, NPPIPVGEIYKRWII; DR-16, DIRQGPKEPFRDYVDR; DC-22, DCKTILKALGPAATLE EMMTAC; PS-18, PGHKARVLAEAMSQVTNS) and one within HIV-2_{ROD} Gag (AG-23, AEWDVQHPIPGPLPAGQL REPRG) was synthesized according to Merrifield's solid-phase synthesis method by Professor Shan-Wei Jin of Shanghai Institute of Organic Chemistry, CAS. The position in Gag of peptides were shown in previous paper (6); a cysteine residue was added to each peptide.

Immunization

BALB/c mice (purchased from the Shanghai Laboratory animal Center, CAS) were immunized with 50 μg of purified rp24 which had been mixed and emulsified 1:1 with complete Freund's complete adjuvant (Sigma) for the initial injection and with Freund's incomplete adjuvant (Sigma) for subsequent boost injections *via* intraperitoneal injection at intervals of 30 days. Blood samples were collected from each mouse *via* a tail vein bleed and the immunological response was tested with ELISA. Three days before cell fusion, the mouse was given an intraperitoneal injection of 50 μg rp24 in PBS, at least 45 days after the last booster.

Cell fusion

Hybridomas were produced by fusing spleen cells from the immunized BALB/c mouse with myeloma cell NS-1 at a ratio of 5:1 in polyethylene glycol 1500 (Fluka) (5). They were selected in RPMI-1640 medium (Invitrogen) supplemented with 20% (v/v) newborn calf serum and 10% P388D1 cell Conditioned Medium (7), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM hypoxantine, 0.4 μM aminopterin, 16 μM thymidine (HAT) (Sigma). After 10-15 days, supernatants of the growing hybridomas were screened by ELISA for p24 specific antibody-secreting clones. The hybridoma cells of positive wells were subcloned three times by limiting dilution in aminopterin-free selection medium.

Direct ELISA

Polystyrene plates were coated with 5 μg/ml rp24 in 0.05 M biocarbonate buffer pH 9.6 overnight at 4°C, and were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Unbinding sites were blocked with 200 μl of 5% milk, 0.02% NaN_3 in PBS-T for 1 h at 37°C. Wells were washed, 100 μl of hybridoma supernatants were added and incubated for 1 h at 37°C. After washing, 100 μl of 1:5,000 diluted horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Sigma) and incubated for 1 h at 37°C. Substrate o-Phenylenediamine solution was added sequentially after washing. The colorimetric reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 after 10 min at room temperature (RT). Optical density absorbance (OD) at 490 nm (630 nm as reference) was determined using microplate reader ELx800 (Bio-Tek). The OD value of blank control was subtracted from OD values of each sample. The cut-off absorbance was designated as 0.2 OD. In later stage of screening, lysate of concentrated HIV-1_{IIIB} was used instead of rp24.

Modified sandwich ELISA

Polystyrene plates were coated with 1 μg/ml anti-mouse lgG Fc specific antibody in 0.05 M bicarbonate buffer pH 9.6 overnight at 4°C, and were washed three times with PBS-T. Unbinding sites were blocked with 200 μl of 5% milk, 0.02% NaN_3 in PBS-T for 1 h at 37°C. Hybridoma supernatants of 100 μl were added and incubated for 2 h at 37°C. After washing three times, 100 μl of HIV-1_{IIIB} lysate was added, and plates were incubated for 2 h at 37°C. One hundred microliter of 1:4,000 diluted rabbit anti-p24 serum was added after three washes and plate was incubated for 1 h at 37°C. After washing, 100 μl of 1:2,000 diluted HRP-labeled goat anti-rabbit IgG and incubated for 1 h at 37°C. Substrate o-Phenylenediamine solution was added sequentially after washing. The colorimetric reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 after 10 min at RT. Optical density absorbance at 490 nm (630 nm as reference) was determined using microplate reader ELx800 (Bio-Tek). The OD value of blank control was subtracted from OD values of each sample.

The cut-off absorbance was designated as 0.2. In specificity assay, different virus lysate were added instead of HIV-1_{IIIB} lysate and lysate of H9 culture supernatant was used as control and an anti-HIV-1 p24 mAb (kindly provided by Dr. Hiroo Hoshino of Gunma University School of Medicine, Japan) was used as positive control. In sensitivity assay, different dilution of rp24, HIV-1 lysate and a p24 standard of commercial p24 detection kit (RETRO-TEK HIV-1 p24 Antigen ELISA) were used.

Isotyping of mAb

The isotypes of mAbs was determined by antigen-mediated ELISA analysis using commercial mouse monoclonal antibody isotyping reagents according to the supplier's

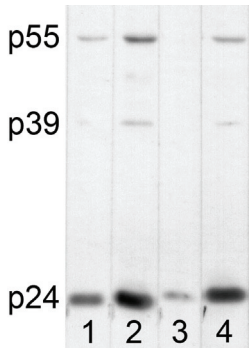

Figure 1. Western blotting analysis of the reactivity of three mAbs with the HIV-1 p24 protein. HIV-1_{IIIB} lysates transferred to nitrocellulose were detected with three mAbs or rabbit anti-rp24 serum by Western blotting. Lane 1, p3JB9; Lane 2, p5F1; Lane 3, p6F4; Lane 4, rabbit anti-p24 serum. The 24-kDa, 39-kDa and 55-kDa protein were indicated.

instructions (Sigma).

Western blotting analysis

Lysates of concentrated HIV-1 were lysed in SDS buffer (4% SDS, 20% glycerol, 0.12 M Tris pH 6.8, 1% bromophenol blue and 1% 2-mercaptoethanol). Lysates were immediately boiled for 10 min and equal amounts of proteins were loaded to 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-T for 1 h at RT and rinsed twice with PBS-T. Then the membrane was incubated with hybridoma supernatant and rabbit anti-recombinant p24 at RT for 1 h. After washing three times with TBS-T, HRP-labeled goat anti-mouse IgG was reacted with the membrane for 1 h at RT. Reaction product was visualized with diaminobenzidine (Sigma) for 5-10 min.

Chromosomal analysis

The culture was treated by adding colcemid stock to give a final concentration of 0.1 μg/ml and incubated in the 37°C CO_2 incubator for 1 h. Centrifuge the cells and swell the cells with pre-warmed (37°C) 0.075 M KCl (hypotonic solution) for 10 min at 37°C. The cells were fixed with 3:1 methanol/ acetic acid fixative. After centrifugation, discard fixative and resuspend pellet by gentle flicking of the bottom of the tube. Add sufficient fixative such that the suspension appears opaque. Add 1 to 2 drops of the suspension onto a clean slide and allow the slide to dry. Examine by phase-contrast microscopy.

Dot blot

Ten microliter (1 mg/ml) of five HIV-1 p24 peptides, one HIV-2 gag peptide and rp24 were applied onto a nitrocellulose membrane; sites were shown in Figure 3. After air-dried, the membrane was blocked with 5% skim milk in PBS-T for 1 h at 37°C and rinsed twice with PBS-T. Then the membrane was incubated with hybridoma supernatant or

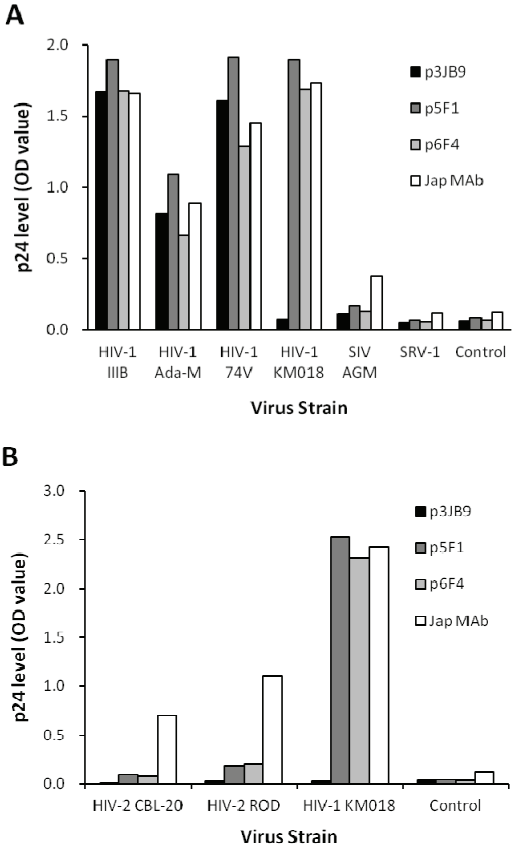

Figure 2. Specificity of mAbs evaluated by modified sandwich ELISA. Lysates of HIV-1_{IIIB}, HIV-1_{Ada-M}, HIV-1_{74V}, HIV-1_{KM018}, SIVagm$_{TYO-1}$ and SRV-1 (A) and lysates of HIV-1_{KM018}, HIV-2_{ROD} and HIV-2_{CBL-20} (B) were detected; H9 culture supernatant was used as control.

rabbit anti-rp24 serum at 37°C for 1 h. After washing three times with TBS-T, HRP-labeled goat anti-mouse IgG conjugate or HRP-labeled goat anti-rabbit IgG conjugate was incubated with the membrane for 1 h at 37°C. Reaction product was visualized with diaminobenzidine for 5-10 min.

Results

Generation of HIV-1 p24 mAbs

Following immunization with HIV-1 p24 protein, spleen cells from seropositive mice were fused with NS-1 cells. The fused cells were selected in HAT medium and screened by direct ELISA by coating plate with rp24 protein. Forty-one hybridomas were selected, among which only 3 hybridomas had ability to detect p24 in HIV-1 culture lysate. These 3 hybridomas were cloned three times by limiting dilution method and designated as p3JB9, p5F1 and p6F4. Chromosomal analysis showed that p3JB9, p5F1 and p6F4 possessed 89.7 ± 5.5, 91.0 ± 1.5 and 83.2 ± 4.6 (n = 20) chromosomes respectively. Isotypes of the mAbs p3JB9, p5F1 and p6F4 were found to be IgG1 determined by antigen-mediated ELISA analysis.

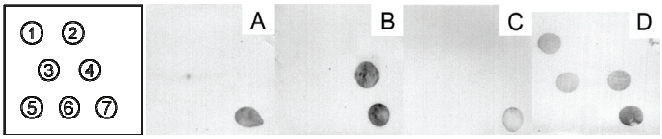

Figure 3. Epitope analysis by dot blot. Diagram showed sites that each peptide or rp24 was applied to. Dot 1-7 represent GA-12, NI-15, DR-16, DC-22, PS-18, AG-23 and rp24 respectively. Membranes were reacted with mAbs p3JB9 (A), p5F1 (B), p6F4 (C) and rabbit anti-rp24 serum (D), respectively. MAb p5F1 could recognize HIV-1 peptide DC-22 (Gag aa329-350, DCKTILKAL GPAATLEEMMTAC).

Western blot analysis showed that a 24 kDa band was detected by three hibridomas and two additional bands, a 39 kDa and a 55 kDa, were detected by p3JB9, p5F1. The 3 bands were recognized by rabbit anti-rp24 serum (Figure 1). Twenty-four kDa band was HIV-1 p24 capsid protein, 55 kDa band was Gag protein, precursor of p24; and 39 kDa band was one of cleavage intermediates of Gag and was precursor of p17 and p24 (8).

Specificity by modified sandwich ELISA

The specificity of mAbs p3JB9, p5F1 and p6F4 was evaluated with ELISA respectively, in which lysates of HIV-1_{Ada-M}, HIV-1_{74V}, HIV-1_{KM018}, HIV-2_{ROD}, HIV-2_{CBL-20}, $SIVagm_{TYO-1}$ and SRV-1 were added instead of HIV-1_{IIIB} lysate. Three mAbs could react with p24 of all tested HIV-1 strain except p3JB9 which could not recognize p24 of HIV-1_{KM018} (Figure 2A), which was a primary isolated from an HIV-1 positive individual in Kunming. Three mAbs could not react with two HIV-2 strains, CBL-20 and ROD and $SIVagm_{TYO-1}$ (Figures 2A and 2B).

Epitope determination by dot blot

Reactivity of three mAbs to five HIV-1 p24 peptides and one HIV-2 gag peptide adsorbed onto a nitrocellulose membrane was evaluated by dot blot. Dot 7 was rp24 and served as positive control; three mAbs recognized rp24 dot (Figure 3). Dot 6 (a HIV-2 peptide, AG-23) was not recognized by any of three mAbs and by rabbit anti-rp24 serum (Figure 3). Rabbit anti-rp24 serum reacted with three peptide dots 1 (peptide GA-12), 3 (peptide DR-16), 4 (peptide DC-22) and rp24 dot, but did not recognize dots 2 (peptide NI-15) and 5 (peptide PS-18) (Figure 3D). The p3JB9 and p6F4 did not recognize any peptide dot (Figures 3A and 3C). The p5F1 specifically recognized peptide DC-22, an HIV-1 peptide on Gag aa329-350, which demonstrated that epitope of p5F1 was DCKTILKALGPAATLEEMMTAC (Figure 3B).

Sensitivity of HIV-1 p24 capture ELISA

Serially diluted rp24, lysate of HIV-1_{IIIB} culture supernatant and p24 standard protein were added to evaluate detection limit of mAbs p3JB9, p5F1 and p6F4 by modified sandwich ELISA. Three mAbs reacted well with rp24 and native p24 in viral lysate. MAbs p6F4, p3JB9 and p5F1 could detect p24 in about 950, 5000 and 10000 times diluted lysate, respectively (Figure 4A). Detection limit for rp24 of mAbs p3JB9, p5F1 and p6F4 was about 15 ng/ml (Figure 4B). For p24 in standard, mAbs p3JB9 and p5F1 could detect as low as 40 pg/ml of p24 in standard of a commercial RETRO-TEK HIV-1 p24 Antigen ELISA kit, while p6F4 could not detect 125 pg/ml of p24 standard (Figure 4C). MAbs p3JB9 and p5F1 showed similar ability to detect p24, but p5F1 was better than p3JB9.

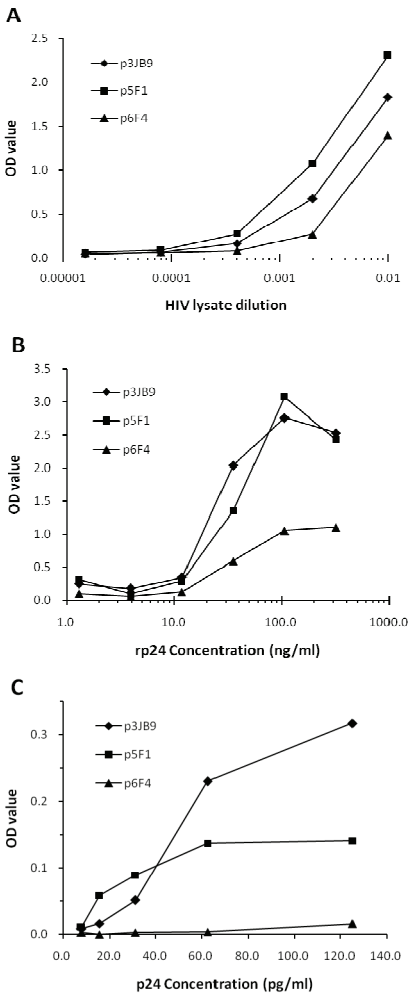

Figure 4. HIV-1 p24 detection limit by modified sandwich ELISA. (A) Five-fold diluted HIV-1_{IIIB} lysate, starting from 1:100. (B) Three-fold diluted rp24, starting from 318 ng/ml. (C) Two-fold diluted p24 standard protein (RETRO-TEK HIV-1 p24 Antigen ELISA kit), starting from 125 pg/ml.

Discussion

Two improvements were introduced into the procedure of anti-HIV-1 p24 mAbs production in the present study. One was that P388D1 culture conditioned medium (8) was employed instead of macrophage feeder cells to enhance growth of new hybridoma in HAT medium after fusion. This

modification reduced the labor intensiveness and accelerated the hybridoma screening process.

The other improvement was modification of ELISA screening method. The typical screening method was direct ELISA in which positive antibodies reacted to the antigen adsorbed onto a plate. Although 41 hybridomas were found to react to rp24 when antigen was adsorbed onto a polystyrene plate, only 3 of them had ability to detect p24 in HIV-1 lysate solution by the modified sandwich ELISA. It probably resulted from conformational change of p24. First, conformation of rp24 expressed in *E. coli* might be different from natural p24 present in solution. Second, adsorption onto a plate brought rp24 a conformational change which hid epitopes recognized by B cells of free rp24 immunized mice. It was reported that human lactate dehydrogenase isoenzyme 5 (HLDH5) was denatured and could be recognized by an mAb MF30 when the enzyme was adsorbed onto a plate but HLDH5 in solution could not be recognized by MF30 (9). Studies on mAbs against the β_2-subunit of *E. coli* tryptophan synthase also showed similar results. Antibodies which bound rapidly in solution to the native protein recognized epitopes present on the native protein, while those reacting very slowly in solution bound preferentially to the denatured form of the protein (10). This finding including ours demonstrated that ELISA commonly used for screening specific hybridomas may lead to retention of antibodies not specific for the native conformation of the antigen. Although purified antibodies adsorbed onto plate could react with native antigen in solution, it is impracticable to adsorb antibodies in hybridoma supernatant onto plate to screen mAbs. In our study, an additional mouse IgG Fc fragment specific antibody coating step was applied to immobilize antibodies probably secreted into supernatant by hybridomas. This improvement overcame all drawbacks mentioned above and offered additional advantages compared to another alternative-competitive ELISA. First, coated mouse IgG Fc fragment specific antibody can concentrate antibodies, maintain conformation of antibody and expose the antigen binding sites away from plate to solution phase when capturing antibodies that probably secreted into supernatant by hybridomas, which increased testing sensitivity. Second, it needs only HIV-1_{IIIB} culture supernatant that was inactivated by adding 0.5% of Triton X-100, which avoided dangerous concentration of HIV and complicated purification of p24 from HIV lysate and can screen mAbs specific for native p24.

Quantitation of HIV-1 p24 antigen has become an accepted way to measure HIV-1 production and infectivity in clinical and research settings. Production of mAbs specific to HIV-1 p24 antigen would be a valuable tool to establish an ELISA based p24 quantitation assay. In this study, we established three hybridomas which secreted mAbs specifically reacted to HIV-1 p24. MAbs p3JB9, p5F1, p6F4 reacted to neither HIV-2, including ROD and CBL-20 strain, nor SIVagm$_{TYO-1}$, but specifically to p24 of HIV-1 including HIV-1_{IIIB}, HIV-1_{Ada-M}, and HIV-1_{74V} (Figure 1). The mAbs p5F1 and p6F4 could detect a primary isolate HIV-1_{KM018} but p3JB9 not suggested that the epitope recognized by p3JB9 was different from those by p5F1 and p6F4. The subtype of primary isolate HIV-1_{KM018} has not been determined yet. But different sensitivity of three mAbs to HIV-1_{KM018} suggested that HIV-1_{KM018} maybe underwent a mutation in Gag which led to the amino acid residues changes. All three mAbs did not recognize SRV-1 as did control mAb.

As suggested in specificity experiments, epitope recognized by p3JB9 was different from those by p5F1 and p6F4. We confirmed that these three mAbs recognized distinct epitopes by an ELISA. By dot blot, the epitope of p5F1 was located between the Gag amino acid residues (aa) 329-350, DCKTILKALGPAATLEEMMTAC (DC-22). This region of p24 had defined as epitope by peptide blocking of binding to native protein (11, 12). Another murine mAb 13B5 recognized LGPAATLEEM which lays in this region (12, 13). The homologous sequences of DC-22 (HIV-1) had been compared among HIV-2, SIV and SRV. Corresponding sequence A-QAAIRPYRKKTD-TGYIRL- ("-" reprsents the same amino acid residue in DC-22) in SRV gag was found without similarity with DC-22, which explained why p5F1 did not recognize SRV. The homologous sequences of SIVagm$_{TYO-1}$ (---V---G--MHP-----L---) and HIV-2_{ROD} (---LV--G--MNP -----L---) had 6-7 amino acid residues different to DC-22, which might cause p5F1 to fail to react with SIV and HIV-2. Peptides NI-15, DR-16 and PS-18 used in present study covered the regions that had been defined as epitopes by mAbs, but the other two mAbs p3JB9 and p6F4 did not reacted with them. The epitopes of them might locate between the p24 aa1-26, aa44-60, aa69-90 and aa170-190 (14).

In conclusion, three mAbs p3JB9, p5F1 and p6F4 against HIV-1 p24 with high specificity and sensitivity were generated. The recognized epitopes of the mAbs are different and p5F1 recognized epitope is DCKTILKALGPAATLEE MMTAC. The p5F1 was used to establish a modified sandwich ELISA with rabbit anti-p24 serum with good specificity and high sensitivity, which has been used to measure HIV-1 p24 antigen levels in our research (15, 16). It could also promisingly become a commercial HIV-1 p24 quantitation assay.

Acknowledgements

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