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Isolation of human single chain variable fragment antibodies against specific sperm antigens for immunocontraceptive development

A.S. Samuel and R.K. Naz[1]

Reproductive Immunology and Molecular Biology Laboratories, Department of Obstetrics and Gynecology, West Virginia University, School of Medicine, Morgantown, WV, USA

[1]Correspondence address. Health Sciences Center North, Room 2084, West Virginia University, School of Medicine, Morgantown, WV 26506-9186, USA. Tel: +1-304-293-2554; Fax: +1-304-293-5757; E-mail: rnaz@hsc.wvu.edu

BACKGROUND: Contraceptive vaccines can provide valuable alternatives to current methods of contraception. We describe here the development of sperm-reactive human single chain variable fragment (scFv) antibodies of defined sperm specificity for immunocontraception. METHODS: Peripheral blood leukocytes (PBL) from antisperm antibody-positive immunoinfertile and vasectomized men were activated with human sperm antigens *in vitro*, and the complementary DNA prepared and PCR-amplified using primers based on all the variable regions of heavy and light chains of immunoglobulins. The scFv repertoire was cloned into pCANTAB5E vector to create a human scFv antibody library. RESULTS: Panning of the library against specific sperm antigens yielded several clones, and the four strongest reactive were selected for further analysis. These clones had novel sequences with unique complementarity-determining regions. ScFv antibodies were expressed, purified and analyzed for human sperm reactivity and effect on human sperm function. AFA-1 and FAB-7 scFv antibodies both reacted with fertilization antigen-1 antigen, but against different epitopes. YLP20 antibody reacted with the expected human sperm protein of 48 $\pm$ 5 kDa. The fourth antibody, AS16, reacted with an 18 kDa sperm protein and seems to be a human homologue of the mouse monoclonal recombinant antisperm antibody that causes sperm agglutination. All these antibodies inhibited human sperm function. CONCLUSIONS: This is the first study to report the use of phage display technology to obtain antisperm scFv antibodies of defined antigen specificity. These antibodies will find clinical applications in the development of novel immunocontraceptives, and specific diagnostics for immunoinfertility.

Keywords: single chain variable fragment antibodies; sperm antigens; antisperm antibodies; infertility; immunocontraception

Introduction

The world population has exceeded 6.65 billion and is increasing by 1 billion every 12 years at the present rate (World POP-Clock Projection, 2008). Ninety-five percentage of this growth is in developing countries. Besides population explosion, unintended pregnancies are a major public health issue. It is estimated annually in the USA that half of all pregnancies are unintended, resulting in over 1 million elective abortions (Henshaw, 1998). In over half of these pregnancies, the women were using some type of contraception. Thus, it is imperative to develop better methods of contraception that are acceptable, both in the developing and developed countries.

Contraceptive vaccines (CVs) have been proposed as a valuable alternative. CVs may be more acceptable than the currently available methods due to high specificity, limited or no side effects, low cost and infrequent administration. Various targets are being explored in several laboratories. These include targeting gamete production (anti-GnRH), targeting gamete function [antisperm and anti-oocyte zona pellucida (ZP)], and targeting gamete outcome (anti-HCG) (Talwar, 1999; Naz *et al*., 2005). Of all these, CVs targeting sperm are especially interesting since the presence of antisperm antibodies (ASA) have been shown to be associated with involuntary infertility in humans, which provides a strong rationale and model for the antisperm CV development. Sperm have both auto- and isoantigenic potentials and can produce ASA in both men and women. Up to 70% of vasectomized men produce ASA, and 2–30% of cases of infertility may be associated with the presence of ASA in the male and/or female partner of an infertile couple (Clayton and Moore, 2001; Ohl and Naz, 1995; Pillai *et al*., 1996; Bohring and Krause, 2003). Several sperm antigens have been delineated and their complementary DNAs (cDNAs) have been cloned and

sequenced. Active immunization with a few of them has been shown to cause a contraceptive effect.

Our laboratory has delineated two antigens, namely fertilization antigen-1 (FA-1) and YLP_{12}, present on human sperm. FA-1 antigen is a glycoprotein of 50 ± 4 kDa that undergoes phosphorylation at tyrosine, serine and threonine residues during sperm capacitation and/or acrosome reaction (Naz and Zhu, 1997, 2002). The monoclonal and polyclonal antibodies to FA-1 antigen inhibit human sperm capacitation/acrosome reaction and human sperm–ZP binding. Vaccination of female mice with murine recombinant FA-1 antigen and FA-1 cDNA cause a long-term reversible contraceptive effect (Naz and Zhu, 1998). FA-1 antigen is involved in human involuntary immunoinfertility (Naz *et al*., 1993). The immunoinfertile men and women, and vasectomized men have antibodies reactive with the FA-1 antigen. YLP_{12} is a dodecamer peptide sequence, YLPVGGLRRIGG, present on human and murine sperm (Naz *et al*., 2000). This sequence is involved in binding to ZP protein, ZP3, of oocytes. Antibodies to synthetic YLP_{12} peptide inhibit sperm–oocyte binding in both species. The peptide and DNA vaccines based on YLP_{12} sequence cause immunocontraceptive effects in female mice (Naz and Chauhan, 2002). Immunoinfertile men have antibodies to YLP_{12} (Naz and Chauhan, 2001).

The progress in CV development has been hindered due to the variability of the immune response observed in individuals after vaccination (Talwar, 1999; Naz *et al*., 2005). It is envisaged that this concern may be obliterated by the passive immunization approach using the preformed antibodies. The antibody therapies have been tried and found to be successful against various infectious diseases, both in animals and humans. Some of the antibodies have become treatment modalities in the clinics (Riethmüller *et al*., 1993; Casadevall, 1999; Dunman and Nesin, 2003). Phage display technology (Smith, 1985) has been widely used to obtain a variety of engineered antibodies, including single chain variable fragment (scFv) antibodies against several antigens (Rader and Barbas, 1997; Ye *et al*., 2002; Park *et al*., 2007; Zhang *et al*., 2007; Zhou *et al*., 2007). ScFv is an antibody fragment that plays a major role in the antigen-binding activity, and is composed of variable heavy (VH) and variable light (VL) chains connected by a peptide linker. The most widely used peptide linker is a repeat of a 15-residue sequence of glycine and serine (Gly4Ser)3. This linker provides a flexibility to move $\sim 35^{\circ}$A to 40°A between the carboxyl terminal of VH and the amino terminus of VL chains for efficient antibody binding. Also, the linker length promotes more intra-domain than inter-domain disulfide bonding between VH and VL chains. The affinity and stability of scFv antibodies produced in bacteria are comparable with those of the native antibodies and are maintained by a strong disulfide bond. ScFv antibodies can be produced on a large scale using specially modified bacterial hosts and have an advantage over the whole immunoglobulin (Ig) molecule. ScFv antibodies lack the Fc portion that obliterates unwanted secondary effects associated with Fc, and due to its small size can be easily absorbed into tissues and gene manipulated (Yokota *et al*., 1992). The mouse monoclonal antibody can elicit strong anti-mouse antibody reactions, and the chimeric antibody can cause anti-chimeric response against murine antibody variable regions when injected into humans (Koren *et al*., 2002; Mirick *et al*., 2004; Sidhu and Fellouse, 2006). The xenogenic complementarity-determining regions (CDRs) of humanized antibodies can also evoke an anti-idiotypic response. In order to overcome these problems, antibodies have to be of human origin if to be used in humans. The potential poor immunogenicity and toxicity of an antigen, and ethical issues, limit immunizing humans to obtain human antibodies. However, the phage display technology can be employed to obtain these antibodies against target antigens if they exist involuntarily in humans, such as ASA in immunoinfertile men and women, and vasectomized men. This manuscript describes the application of phage display technology to isolate, produce and characterize fully functional human scFv antibodies against two well-characterized sperm antigens (FA-1 and YLP_{12}) from immunoinfertile and vasectomized men, and examine their role in human sperm function. This is the first study to report the use the phage display technology to obtain antisperm scFv antibodies of defined antigen specificities from immunoinfertile/vasectomized men. The long-term objective of this study is to develop novel antisperm immunocontraceptives for humans.

Materials and Methods

Patient population and RNA isolation

Peripheral blood leukocytes (PBL) were isolated from heparinized blood of ASA—positive immunoinfertile and vasectomized men ($n = 6$, aged 27–37 years), and ASA-negative fertile ($n = 4$, aged 32–39 years) men by Ficoll-PaqueTM Plus gradient centrifugation (Amersham Biosciences AB, Uppsala, Sweden) following the manufacturer's protocol. The immunoinfertile and vasectomized men had ASA in their sera as revealed by the sperm immobilization technique (SIT), tray agglutination technique (TAT), and immunobead binding technique (IBT) (Isojima *et al*., 1987; Naz *et al*., 1993). All these sera demonstrated >20% sperm binding in IBT. The sera also demonstrated antibodies (IgG and IgA classes) to the purified cognate human sperm FA-1 antigen and synthetic YLP_{12} peptide in the enzyme linked immunosorbent assay (ELISA). The sera from fertile control men were negative for ASA when tested by SIT, TAT or IBT, and did not have antibodies to FA-1 antigen and YLP_{12} peptide. FA-1 antigen was purified from lithium diiodosalicylate (LIS)-solubilized human sperm extract (HSE) by using monoclonal immunoaffinity chromatography as described above. The purified FA-1 human sperm antigen showed a single band of 50 ± 4 kDa in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after staining with silver nitrate. The YLP_{12} peptide was synthesized by solid-phase synthesis using Fmoc chemistry (Biosynthesis Inc., Lewisville, TX, USA) and had >90% purity. The study was approved by the Institutional Review Board (IRB) for human studies and the appropriate consent to participate in this study was obtained. PBL was washed ($2\times$) in RPMI 1640 medium and 1×10^6 cells were then incubated (37°C, 5% CO2) with 8 μg each of FA-1 antigen, YLP_{12} peptide and HSE in a final volume of 200 μl RPMI 1640 medium containing 25 mmol/l Hepes buffer and L-glutamine, 10% fetal bovine serum, penicillin (100 U/ml) streptomycin (100 μg/ml) and kanamycin (100 μg/ml). After 5 days, the medium was changed to fresh medium of the same composition and incubated for an additional 2 days. The cells were then isolated, washed with

RPMI 1640 medium and used for RNA isolation. RNA was isolated using guanidium thiocyanate-phenol RNA-STAT-60 (TEL-TEST Inc., Friendswood, TX, USA) following the manufacturer's protocol and was used for cDNA synthesis.

Construction of human antibody phage display library

The first strand cDNA was synthesized by mixing 10 μg of total RNA from each PBL culture (1×10^6) with 1 μg of oligo (dT) 15 primer (500 μg/ml, Promega, Madison, WI, USA) in a 50 μl reaction volume. The contents were incubated at 70°C for 3 min and then chilled on ice. To this mixture, 10 μl of 5× first-strand buffer, 2.5 μl of 10 mM dNTP and 2.5 μl of moloney murine leukemia virus reverse transcriptase (200 units/μl, Invitrogen, Carlsbad, CA, USA) were added and mixed. The reaction was carried out at 37°C for 1 h and stopped by incubation at 70°C for 10 min. The mixture was stored at −20°C until further use.

The human antibody phage display library was constructed using the standard PCR procedure. The nucleotide sequences of all human antibody classes/subclasses (http://www.kabatdatabase.com) were collected and degenerate PCR primers were designed for amplification of VH and VL chains (Kabat *et al*., 1991). Briefly, PCR reactions were set to amplify 16 different VL and 8 different VH chains. PCR was performed using 3 μl of cDNA template, 1 U *Taq* polymerase, 10 mM dNTP, 25 mM MgCl2 and 50 pmol each of forward and reverse primers in a 50 μl reaction volume. After initial denaturation at 94°C for 2 min, PCR was performed for 35 cycles with denaturation at 94°C for 20 s, annealing at 55°C for 10 s and extension at 68°C for 30 s. The 330 bp PCR products from each reaction were mixed to form separate VH and VL pools, and the products were purified from the gel using QIATM quick gel extraction kit (QIAGEN Sciences, MD, USA). The scFv assembly of VH and VL chains, with a (G4S)3 linker between the chains, was performed using the splicing by overlapping extension PCR (SOE-PCR) procedure. The primers used in SOE-PCR contained overlapping linker sequences to allow the two genes to be spliced by an overlap extension. For SOE-PCR procedure, 20 ng of respective purified VH and VL products (mixed in equimolar ratio) were used as template for 25 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 50 s. A final PCR was performed to incorporate SfiI and NotI restriction sites to 5′ and 3′ ends of the assembled scFv, respectively. PCR was for 30 cycles at 94°C for 1 min, 55°C for 30 s and 72°C for 1 min. The final assembled PCR product (760 bp) was purified and then digested with restriction enzymes SfiI and NotI. One microgram of the purified digested PCR insert was ligated with 1.5 μg of SfiI— and NotI pre-digested and dephosphorylated pCANTAB5E (Amersham Biosciences AB), in a reaction volume of 50 μl containing 5 μl of l0× ligase buffer and 5 U of T4 DNA ligase (Fisher Scientific, Pittsburg, PA, USA). The ligation was performed overnight at 16°C, the residual ligase in the sample was inactivated (70°C, 10 min) and the reaction mixture was stored at −20°C. The ligated product was then transformed into electrocompetent TG1 cells (Stratagene, Cedar Creek, TX, USA) by electroporation programmed to give one pulse of 25 μF, 2.5 KV at 200 ohm. The transformed cells were suspended in 1 ml of 2× YT medium containing 2% glucose and incubated at 37°C for 1 h with shaking at 200 rpm, and then plated onto plates coated with SOB medium containing 100 μg/ml ampicillin and 2% glucose. Transformants were scraped using 2× YT medium containing 100 μg/ml ampicillin and 2% glucose (YT-AG), and stored as 1 ml aliquots in 30% glycerol at −80°C. For examining the transformation efficiency, 10 clones were randomly selected from the library and analyzed by PCR for the presence of scFv insert.

Screening of library and selection of specific clones

Phages displaying scFv antibodies were prepared from the primary library, and the phage rescue was performed using M13KO7 helper phage (Smith, 1985; Rader and Barbas, 1997). The phage titer was determined by infecting appropriate dilution of phages in exponentially growing *Escherichia coli* TG1 cells and plated in 2×YT-AG culture plates.

Phage panning was performed in 96-well microtiter plates following the standard protocol. Briefly, Nunc Maxisorb TM (Nunc Maxisorb, Roskilde, Denmark) plates were coated (10 μg /well) overnight at 4°C individually with each of purified cognate human sperm FA-1 antigen, synthetic YLP_{12} peptide or HSE diluted in 200 μl of 0.1 M carbonate buffer (pH 9.6). The wells were then washed (5×) with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T). To block the non-specific binding sites, the wells were incubated (37°C, 1 h) with PBS-T containing 1% bovine serum albumin (BSA) and then washed (5×) with PBS-T. The plates were incubated (37°C, 2 h) with 200 μl of the culture supernatant containing 1×10^{10} of the primary library phages. The wells were washed (3×) with PBS-T and then with PBS (3×). The bound scFv clones were eluted using three different procedures namely acidic, basic and antigen excess elutions. For acidic elution, glycine–HCl buffer (0.01 M, pH 2.2) was used, the basic elution was performed using Tris–HCl buffer (0.01 M, pH 9.0) and for the antigen excess elution 10× concentration of the respective antigen was used for elution. After three procedures, the eluted phages were immediately neutralized with 3 M Tris–HCl, (pH 8.0). The phage titer was determined by infecting *E. coli* TG1 cells as described before. For subsequent rounds of panning, the eluted phages were incubated with the coated wells as before, and the procedure was repeated twice, keeping all other conditions identical as before. The eluates from the third round of panning were pooled (all eluted using acidic, basic and antigen excess procedures) for each antigen (FA-1/YLP_{12}/HSE) and used to infect *E. coli* TG1 cells to isolate the individual clones. The single clones picked from the third round of panning were phage rescued using M13KO7 helper phage as mentioned before, and resulting recombinant phages were screened using ELISA.

For ELISA, each well was coated overnight with the antigen (cognate FA-1 antigen/synthetic YLP_{12} peptide/HSE) (Naz and Chauhan, 2001). After blocking with PBS-T containing 1% BSA, the wells were incubated (37°C, 2 h) with the recombinant phages, washed and then incubated (37°C, 1.5 h) with horseradish-peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (Amersham Biosciences AB) diluted 1:1000 in PBS-T containing 0.1% BSA. The wells were washed with PBS-T, and incubated (37°C, 20 min) with substrate solution (0.2 mg/ml, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) diluted in citric acid buffer (0.05 M, pH 4.0) containing 30% H_2O_2. The reaction was stopped using 10% SDS, and the ELISA plate was read at 405 nm.

The clones showing stronger reactivity [>2 optical density (OD) of the control wells] to the respective antigen were selected and PCR amplified. The ~850 bp PCR product was excised and the DNA was purified using QIA quick gel extraction kit. The eluted DNA was quantified and sequenced using ABI automated DNA sequencer (Biotech Core, Sunnyvale, CA, USA). The sequence analysis was done using IMGT®, the international ImMunoGeneTics information system (http://imgt.cines.fr) and gene tool software (www.biotools.com).

scFv antibody expression and purification

The individual colonies that showed the strongest reactivity with the sperm antigens (FA-1/YLP_{12}/HSE) were selected, grown and the

scFv antibodies were purified using RPASTM purification system with HiTrapTM anti-E-Tag Column (Amersham Biosciences AB) following the manufacturer's protocol. The protein concentration was determined using QuantiPro BCA kit (Sigma Chemical Co., Saint Louis, MO, USA), and the purity and authenticity of the antibodies were examined by using SDS–PAGE (Laemmli, 1970) and western blot procedures (Towbin *et al*., 1979). For SDS–PAGE, various concentrations (5–25 μg/lane) of the purified antibodies were run in 12% gel and the gel was stained with silver nitrate (Bio-Rad Labs, Hercules, CA, USA). For western blot procedure, the scFv antibodies were resolved in SDS–PAGE, transferred overnight to nitrocellulose membrane, the membranes were blocked with PBS containing 0.1% BSA, and then probed with anti-E-Tag mouse monoclonal antibody (1:2000 dilution), followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (H- and L-specific) (1:2000 dilution) (Southern Biotechnology Associates, Birmingham, AL, USA). The blot was washed and incubated with nitroblue tetrazolium–bromochloroindolyl phosphate (NBT–BICP) substrate solution to visualize the reactive protein bands. The blot treated similarly but not incubated with anti-E-Tag antibody, or the blot treated with a control scFv antibody (CAB-3) that did not react with any antigen (FA-1/YLP_{12}/HSE) in the panning/ELISA procedure, served as controls.

The purified antibodies were concentrated by lyophilization and examined again for reactivity in ELISA against the respective antigen. Only the purified antibodies showing the expected band(s) and having high titers (>1:2056 ELISA titers) were used for further analysis.

Immunoreactivity of scFv antibodies with human sperm and respective sperm antigens

The immunoreactivity of scFv antibodies with human sperm and purified human FA-1 antigen was examined using western blot procedure, immunoprecipitation procedure and indirect immunofluorescence technique (IFT). For western blot procedure, LIS-solubilized HSE, methanol-chloroform (MC)-solubilized human sperm preparation or purified human FA-1 antigen were run by SDS–PAGE (10–20 μg/lane) on a 12% gel, and transferred overnight to nitrocelluose membrane. The membranes were blocked with PBS containing 0.1% BSA, probed with scFv antibody (5 μg/100 ml) and then with anti-E-Tag mouse monoclonal antibody (1:2000 dilution). This was followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (H- and L-specific) (1:2000 dilution). The blot was washed and incubated with substrate (NBT–BICP) solution to visualize the reactive protein bands. The blot treated with the control scFv antibody (CAB-3) served as control. The LIS-solubilized HSE (Naz and Zhu, 1997, 1998, 2002) and MC-solubilized human sperm preparation was prepared as described elsewhere (Svennerholm and Fredman, 1980). MC solubilized sperm preparation was used only for testing AS16 antibody, since the sperm agglutination antigen-1 (SAGA-1) antigen is present on the inner acrosomal membrane and MC may increase the solubilization of the inner acrosomal membrane proteins.

For immunoprecipitation procedure, the scFv antibodies were conjugated to cyanogen bromide-activated Sepharose 4B and the conjugated beads were incubated with HSE overnight. To remove the unbound proteins, the beads were washed (5×) with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS), and the beads were then boiled in Laemmli buffer, centrifuged and the supernatant was run in SDS–PAGE. The indirect IFT was used to localize the scFv antibody immunoreactive subcellular sites on human sperm. IFT was performed on both live and methanol-fixed human sperm. Semen was collected from fertile, healthy men by masturbation, following institutional IRB-approved protocol. Motile sperm cells, collected by use of the swim-up procedure, were washed (3×) with PBS (800*g*, 10 min), air-dried (2×10^4 sperm/well) on 10-well slide Teflon-coated slides (Polysciences, Inc., Warrington, PA, USA) at room temperature, fixed in methanol for 10 min and air-dried again. The slides were then rinsed with PBS, blocked with PBS containing 3% BSA for 45 min and then incubated with 15 μl of purified scFv antibodies. The slides were washed and incubated for 1.5 h with anti-E-Tag monoclonal antibody diluted (1:2000) in PBS. After thorough washing in PBS, the slides were incubated for 1.5 h with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (H- and L-chain specific) (1:40 dilution, Southern Biotechnology Associates). The slides were washed in PBS, mounted in PBS containing 90% glycerol and 1,4- diazabicyclo-[2,2,2]-octane (10 mg/ml), and examined using Zeiss confocal microscopy axiovert 100 M under FITC filter, and microphotographed using laser scanning microscope (LSM 510) (Version 3.2) at our microscope imaging facility.

For IFT using live sperm, the motile human sperm (1×10^6 sperm/ml) in Ham's F-10 medium containing 0.1% BSA were incubated (4°C, 1.5 h) with scFv antibodies (5–10 μg/ml), washed (3×) with PBS and air-dried on Teflon-coated slides. The rest of the procedure was same as described above for the methanol-fixed sperm.

Human sperm acrosome reaction

The effect of scFv antibodies on human sperm function (capacitation/acrosome reaction) was performed as described elsewhere (Naz *et al*., 2000). Briefly, the swim-up sperm were capacitated at 37°C for 6–8 h in 5% CO_2 in air with 45 or 80 μg/ml of scFv antibody. The sperm were then washed to remove scFv antibody and incubated (37°C, 30 min) with calcium ionophore A23187 (Sigma) (5 μM final concentration) (Byrd *et al*., 1989). The sperm were washed (2×), methanol-fixed on the slides and the acrosome-reacted sperm were evaluated by using FITC-conjugated *Pisum sativum* agglutinin (ICN Biomedical, Inc., Aurora, OH, USA). Approximately 200–300 sperm were counted in each slide and each treatment was tested in 3–5 independent experiments on different days using sperm from three different men. Significance of differences was analyzed by using paired and unpaired Student's *t*-test. A *P*-value of <0.05 was considered significant.

Epitope analysis of scFv antibodies

Several peptides based upon immunodominant regions of published human (Naz and Zhu, 2002) and murine (Naz and Zhu, 1997) FA-1 amino acid sequences were synthesized by solid-phase synthesis using Fmoc chemistry (Biosynthesis Inc. or GenScript Corp., Piscataway, NJ, USA). The immunodominant regions were determined by using the online Invitrogen program (http://peptideselect.invitrogen.com/peptide/). The synthetic peptides were coated (10 μg/well) overnight in 96-well ELISA plate in 0.1 M carbonate buffer (pH 9.6). Non-specific binding sites were blocked using PBS-T containing 0.1% BSA. The wells were washed (3×) and incubated (37°C, 2 h) with the purified ScFv antibodies (5–20 μg/ml). The wells were washed (3×) again and incubated (37°C, 90 min) with HRP-conjugated anti-E-Tag antibody diluted 1:2000 in PBS-T containing 0.1% BSA. The wells were then incubated (37°C, 15 min) with substrate solution (0.2 mg/ml ABTS diluted in 0.05 M citric acid buffer, pH 4.0, containing 30% H_2O_2). The reaction was stopped using 10% SDS and read at 405 nm.

The absorbance reading of the control wells was subtracted from the absorbance reading of the peptide-coated wells, and the mean of the subtracted values was recorded. The absorbance readings were converted to standard deviation (SD) units by the formula: SD units =

mean (test) − mean (control)/SD of control group. The test samples with >2 SD units were considered as positive.

Results

Preparation of scFv antibody library

The cDNA prepared from RNA of activated PBL was used as a template for PCR amplification. A collection of 66 human Vκ chain sequences, 20 Vλ chain sequences, and 144 VH chain sequences from the Kabat database (Kabat *et al*., 1991) were used to design the consensus PCR primers. The human Vκ are classified into four, Vλ into five and the VH into three subgroups. For amplification of VL chains, 11 different amplification reactions were performed using 11 forward primers and an equal mixture of two reverse primers. Similarly, five different PCRs were performed for amplification of VK chains using five different forward primers and an equal mixture of two reverse primers. The VH sequences were similarly amplified in eight different PCR reactions using eight forward primers and an equal mixture of two reverse primers. Each primer set produced a distinct band of predicted size of ∼330–350 bp. The Vκ and Vλ sequence products obtained using all the primer pairs were pooled to form a VL pool. Similarly, products of all eight reactions performed to amplify VH were pooled to form a VH pool. Fifty nanogram DNA of each VL and VH pools were mixed to perform SOE-PCR. Ten nanogram of assembled scFv product were purified and used for construction of the library. The SfiI- and NotI-digested scFv repertoire was cloned into predigested SfiI- and NotI-digested and dephosphorylated vector, pCANTAB5E and transformed into *E. coli* TG1 cells to create the human scFv antibody library. The created library consisted of 3.8×10^5 primary transformants. PCR analysis of the individual clones ($n = 10$) randomly screened from the library showed 80% to be the expected recombinants, having a specific PCR product of 850 bp.

Panning of the library using sperm proteins

Rescue of phagemids encoding scFv fusion protein using helper phage (M13KO7) yielded phage particles displaying scFv. Selection of phages displaying scFv was carried out by performing three rounds of selection process comprising incubation of phages with sperm antigen (FA-1/YLP_{12}/HSE)-coated wells, washing unbound non-specific phages and then elution of the bound specific phages. For the first round of

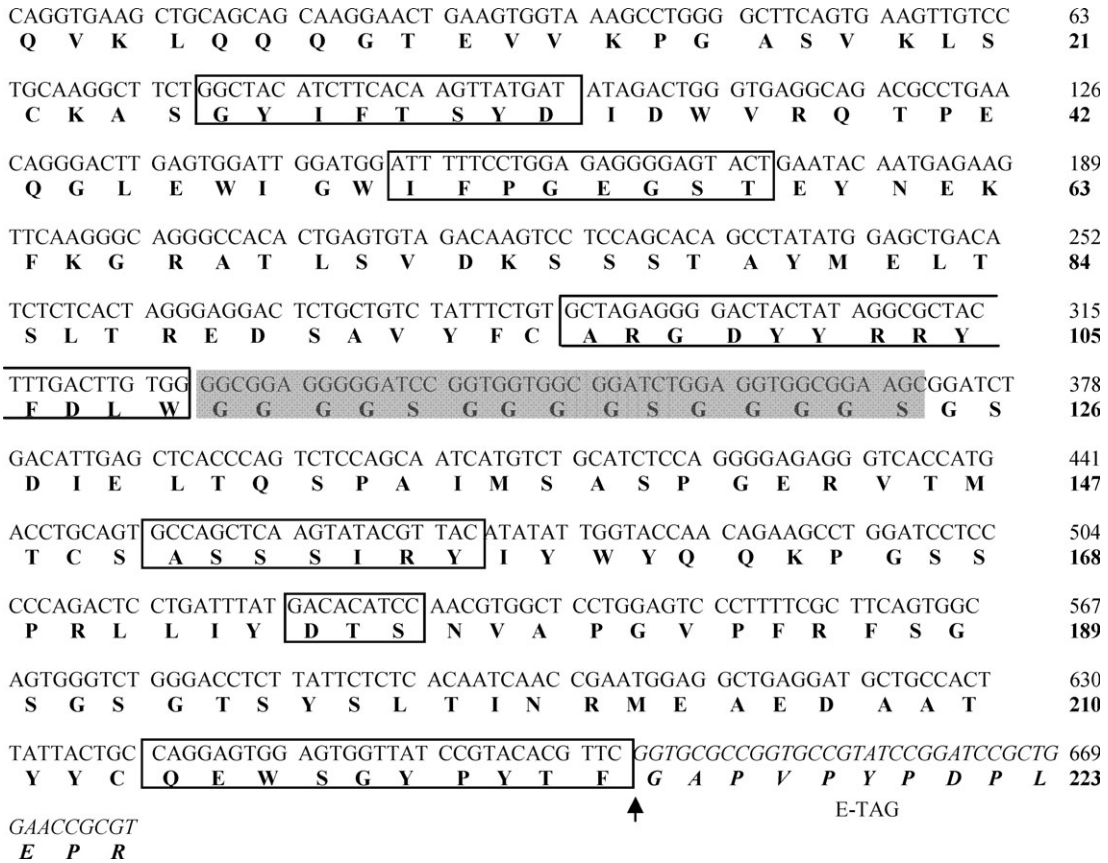

Figure 1: The cDNA sequence and the corresponding amino acid (aa) sequence of single chain variable fragment (scFv) clone (AFA-1) reactive with FA-1 antigen

The aa sequence contains heavy chain (1–109 aa), linker (Gly4Ser)3 (110–124 aa) shown in shaded grey box, light chain (125–223 aa) and E-Tag (224–236 aa) sequence (shown after ↑ in italics). The aa sequence of immunoglobulin (Ig)G1 heavy chain includes framework region 1 (1–25 aa), framework region 2 (34–50 aa), framework region 3 (59–96 aa), and their corresponding CDR-1 (26–33 aa), CDR-2 (51–58 aa) and CDR-3 (97–109 aa) regions. Similarly, the aa sequence of Igκ3 light chain includes framework region 1 (125–150 aa), framework region 2 (158–174 aa), framework region 3 (178–213 aa), and their corresponding CDR-1 (151–157 aa), CDR-2 (175–177 aa) and CDR-3 (214–223 aa) regions. All CDRs regions are enclosed in rectangular boxes.

panning, 1×10^{10} phages were added to each FA-1/YLP_{12}/HSE-coated well. The titers of the eluted phages were 3.6×10^6 colony forming units (CFU)/ml for FA-1-coated wells, 2.4×10^6 CFU/ml for YLP_{12}-coated wells and 1×10^7 CFU/ml for HSE-coated wells after the first round of panning. For the second round, the input phages from the first round of panning were used for incubation with the respective antigen-coated wells. The eluted phages had titers of 3×10^3, 3.6×10^6 and 9×10^6 CFU/ml for the FA-1, YLP_{12} and HSE, respectively. After the third round, using the eluate from the second round of panning, the titers were 1.3×10^2, 1.5×10^3 and 6×10^3 CFU/ml for FA-1, YLP_{12} and HSE, respectively. For each panning procedure, the specific phages were eluted using three strategies, namely low pH, high pH and antigen excess. The eluates after the third panning obtained from all the three elution procedures were pooled for each antigen, and the mixture was infected into *E. coli* TG1 cells to isolate individual colonies. Individual clones were grown and re-examined for their immunoreactivity with the respective sperm antigen. After these procedures, 9 clones were found to react with FA-1 antigen, 7 clones with YLP_{12} and 14 with HSE.

Five clones reacting the strongest with each antigen were selected, and their phagemids isolated and analyzed for nucleotide sequence. Analysis of these clones in immunogenetics database revealed them to be Ig sequence of human origin belonging to IgG class with κ chain as their light chain.

Sequence analysis of selective recombinant scFv antibodies

Four of these clones, two reactive with FA-1 (AFA-1 and FAB-7), and one with YLP_{12} (YLP20) clones were selected because they reacted strongest with the FA-1 antigen and YLP_{12} peptide, respectively. The fourth clone, AS16, reactive with HSE was selected because it showed homology with a mouse antisperm monoclonal antibody (RASA) developed against a defined sperm antigen discussed below. AFA-1, FAB-7 and AS16 had IgG1, and the YLP_{12} scFv clone had IgG3 subclass heavy chain sequence. AFA-1, FAB-7 and YLP20 had light chain belonging to Igκ3 subclass, and the AS16 clone had light chain belonging to Igκ2 subclass. These scFv clones were further analyzed to determine homology with the consensus CDRs of human antibody sequences in the database. The scFv clone AFA-1 had 82% homology in CDR-1 region and 0% homology in CDR-2 and CDR-3 regions

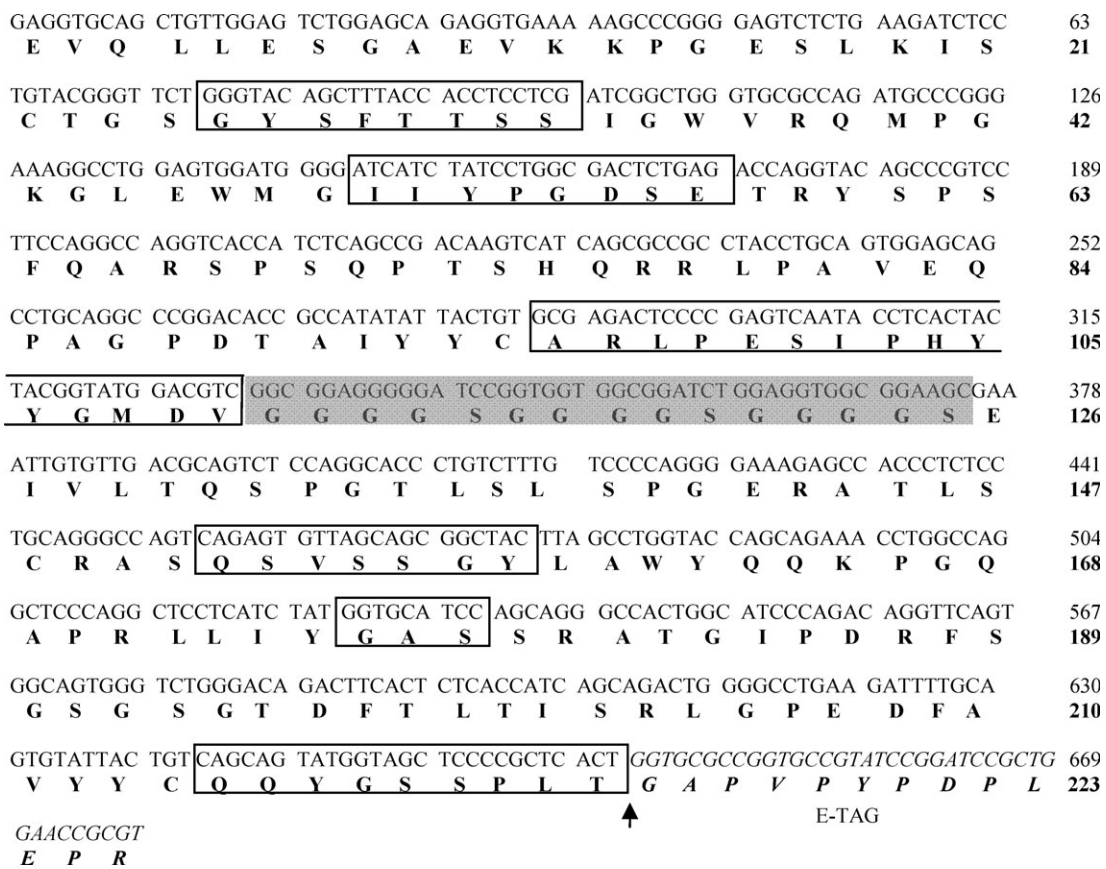

Figure 2: The cDNA sequence and the corresponding amino acid (aa) sequence of scFv clone (FAB-7) reactive with FA1 antigen
The aa sequence contains heavy chain (1–110 aa), linker (Gly4Ser)3 (111–125 aa) shown in shaded grey box, light chain (126–223 aa) and E-Tag (224–236 aa) sequence (shown after ↑ in italics). The aa sequence of IgG1 heavy chain includes framework region 1 (1–25 aa), framework region 2 (34–50 aa) and framework region 3 (58–95 aa), and their corresponding CDR-1 (26–33 aa), CDR-2 (51–57 aa) and CDR-3 (96–110 aa) regions. Similarly, the aa sequence of Igκ3 light chain includes framework region 1 (126–151 aa), framework region 2 (159–175 aa) and framework region 3 (179–214 aa), and their corresponding CDR-1 (152–158 aa), CDR-2 (176–178 aa) and CDR-3 (215–223 aa) regions. All CDRs regions are enclosed in rectangular boxes.

in VH region of human antibody sequence (accession no. M99637). It had 43%, 67% and 20% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VL region of human antibody sequence (accession no. L19272) (Fig. 1). The scFv clone FAB-7 had 63%, 88% and 20% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VH region of human antibody sequence (accession no. M99686). It had 100% homology in CDR-1 and CDR-2 regions and 90% homology in CDR-3 region in VL region of human antibody sequence (accession no. X12686) (Fig. 2). The scFv clone YLP20 had 75%, 13% and 23% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VH region of human antibody sequence (accession no. M99679). It had 58%, 33% and 67% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VL region of human antibody sequence (accession no. X12686) (Fig. 3). The scFv clone AS16 had 75%, 75% and 0% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VH region of human antibody sequence (accession no. M62106). It had 82%, 100% and 45% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VL region of human antibody sequence (accession no. X62106) (Fig. 4). The amino acid homology of clone AS16 was also compared with mouse antisperm scFv antibody RASA. The scFv clone AS16 had 88%, 75% and 0% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VH region of this mouse antibody sequence (accession no. AF276797). It had 73%, 67% and 67% homology in CDR-1, CDR-2 and CDR-3 regions, respectively, in VL region of this mouse antibody sequence (accession no. AF276797).

Immunobiological characterization of scFv antibodies

E. coli containing recombinant phagemid of these four clones were grown, and the antibodies were expressed and purified using anti-E-Tag column. The purified scFv antibodies showed the expected single protein band of ~28 kDa in SDS–PAGE after staining with silver nitrate (Fig. 5, lane a). The antibodies tend to aggregate into dimeric and sometimes into trimeric forms during freezing. Anti-E-Tag antibody specifically recognized all these forms in the western blot procedure (Fig. 5, lane b). The purified antibodies showing only the specific bands were used for further studies.

AFA-1 scFv antibody specifically recognized a protein band of 50 ± 4 kDa corresponding to FA-1 antigen in western blot procedure involving LIS-solubilized HSE (Fig. 6A, lane b).

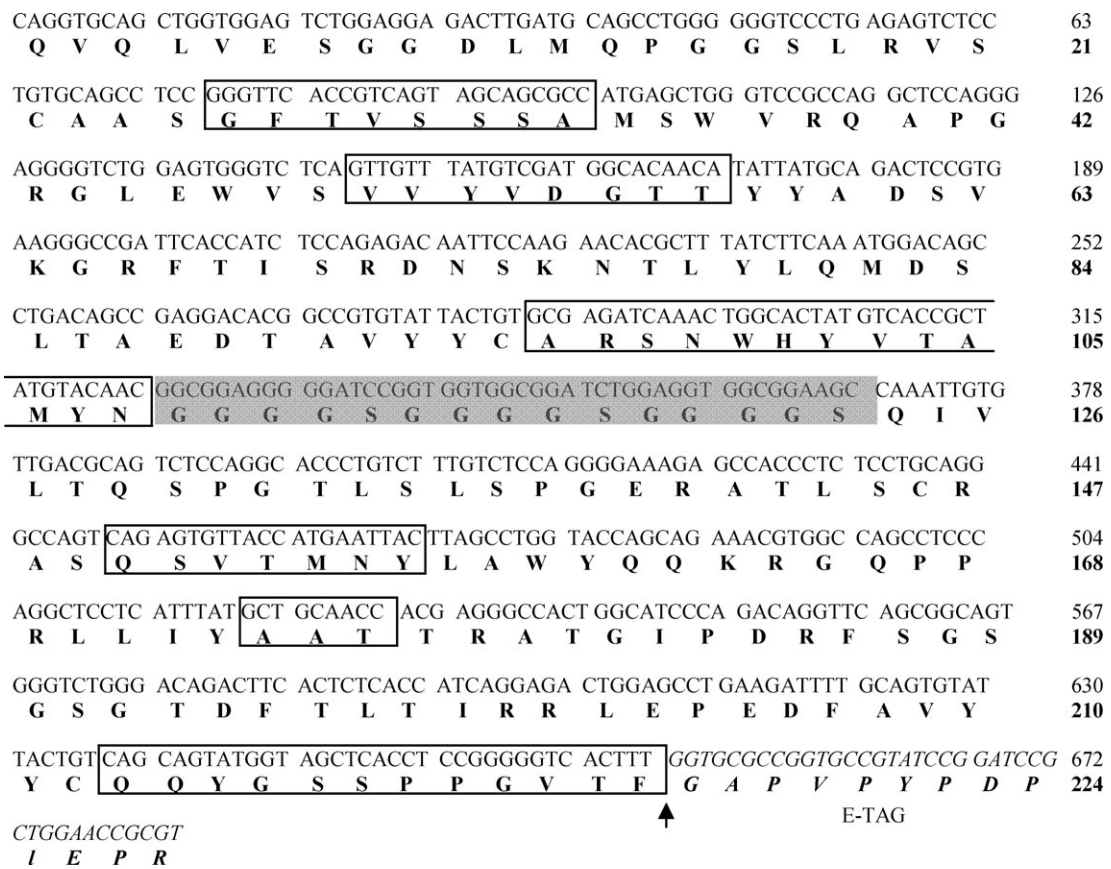

Figure 3: The cDNA sequence and the corresponding amino acid (aa) sequence of scFv clone (YLP20) reactive with YLP_{12} antigen
The aa sequence contains heavy chain (1–108 aa), linker (Gly4Ser)3 (109–123 aa) shown in shaded grey box, light chain (124–224 aa) and E-Tag (225–237 aa) sequence (shown after ↑ in italics). The aa sequence of IgG3 heavy chain includes framework region 1 (1–25 aa), framework region 2 (34–49 aa) and framework region 3 (58–95 aa), and their corresponding CDR-1 (26–33 aa), CDR-2 (50–57 aa) and CDR-3 (96–108 aa) regions. Similarly, the aa sequence of Igκ3 light chain includes framework region 1 (123–149 aa), framework region 2 (157–173 aa) and framework region 3 (177–212 aa), and their corresponding CDR-1 (150–156 aa), CDR-2 (174–176 aa) and CDR-3 (213–224 aa) regions. All CDRs regions are enclosed in rectangular boxes.

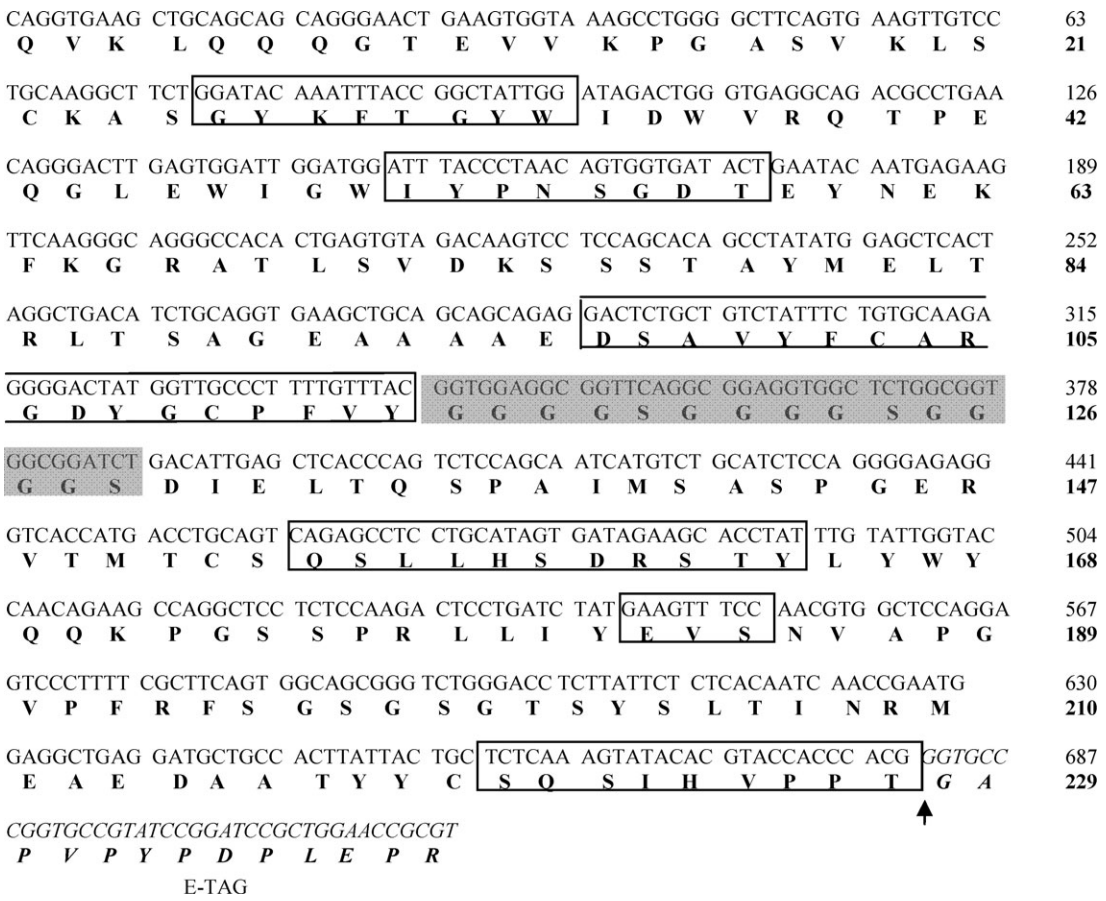

Figure 4: The cDNA sequence and the corresponding amino acid (aa) sequence of scFv clone (AS16) reactive with HSE
The aa sequence contains heavy chain (1–114 aa), linker (Gly4Ser)3 (115–129 aa) shown in shaded grey box, light chain (130–229 aa) and E-Tag (230–242 aa) sequence (shown after ↑ in italics). The aa sequence of IgG1 heavy chain includes framework region 1 (1–25 aa), framework region 2 (34–50 aa) and framework region 3 (59–96 aa), and their corresponding CDR-1 (26–33 aa), CDR-2 (51–58 aa) and CDR-3 (97–114 aa) regions. Similarly, the aa sequence of Igκ2 light chain includes framework region 1 (130–153 aa), framework region 2 (165–181 aa) and framework region 3 (185–220 aa), and their corresponding CDR-1 (154–164 aa), CDR-2 (182–184 aa) and CDR-3 (221–229 aa) regions. All CDRs regions are enclosed in rectangular boxes.

This band was not recognized by the control scFv antibody (CAB-3) (lane b'). AFA-1 antibody conjugated to Sepharose 4B immunobeads also specifically immunoprecipitated a protein band of 50 ± 4 kDa, corresponding to FA-1 antigen, from HSE (lane c), that was not immunoprecipitated by the control antibody immunobeads (lane c'). The cognate FA-1 antigen that was purified from LIS-solubilized HSE by immunoaffinity column involving a mouse monoclonal antibody (MA-24) conjugated to Sepharose 4B immunobeads, and showed a single band of 50 ± 4 kDa in SDS–PAGE (lane d). This band was specifically recognized by AFA-1 scFv antibody (lane e) and not by the control antibody (e') in the western blot procedure.

FAB-7 scFv antibody, which was also identified using the FA-1 antigen in the panning procedure, showed a similar immunoreactive pattern with HSE and FA-1 antigen (Fig. 7) as observed with the AFA-1 antibody, described above (Fig. 6). FAB-7 scFv antibody specifically recognized a protein band of 50 ± 4 kDa, corresponding to FA-1 antigen, in the western blot of HSE (Fig. 6A, lane b). Control antibody (CAB-3) did not react with any specific band in the western blot procedure (lane b'). FAB-7 scFv antibody-conjugated to Sepharose 4B immunobeads immunoprecipitated a specific protein of 50 ± 4 kDa, corresponding to FA-1 antigen, from HSE (lane c). Control antibody conjugated immunobeads did not immunoprecipitate any specific protein from HSE (lane c'). The purified cognate FA-1 antigen was specifically recognized by FAB-7 antibody (lane e) and not by the control antibody (lane e') in the western blot procedure.

YLP20 antibody specifically reacted with a protein band of 48 ± 5 kDa in the western blot of HSE (Fig. 8A, lane b), which was not recognized by the control antibody (lane b'). YLP20 antibody-conjugated to Sepharose 4B immunobeads (lane c) and not the control antibody immunobeads (lane c'), immunoprecipitated a protein of 48 ± 5 kDa from HSE.

AS16 antibody recognized four protein bands, one major (18 kDa) and three minor (37, 55 and 110 kDa) on the western blot of LIS-solubilized HSE (Fig. 9B, lane b'). Using the western blot of MC-solubilized human sperm preparation, AS16 antibody reacted with a major (18 kDa) and a minor (100 kDa) protein band (lane c). These protein bands were not recognized by the control antibody (lanes b' and c').

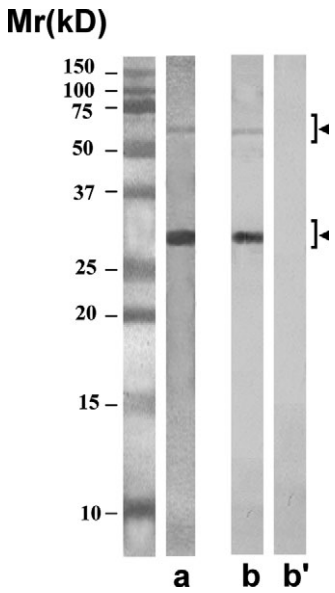

Figure 5: Purity of the scFv antibodies isolated by using anti-E-Tag antibody column

The purified scFv antibodies showed predominantly the expected single protein band of ~28 kDa in SDS–PAGE after staining with silver nitrate (lane a). On freezing, the antibodies had a tendency to polymerize into dimeric form of ~56 kDa, and sometimes into trimeric form of ~84 kDa (not shown). All these forms were specifically recognized by the anti-E-Tag monoclonal antibody (lane b) and not by the myeloma control monoclonal antibody (lane b') in the western blot procedure.

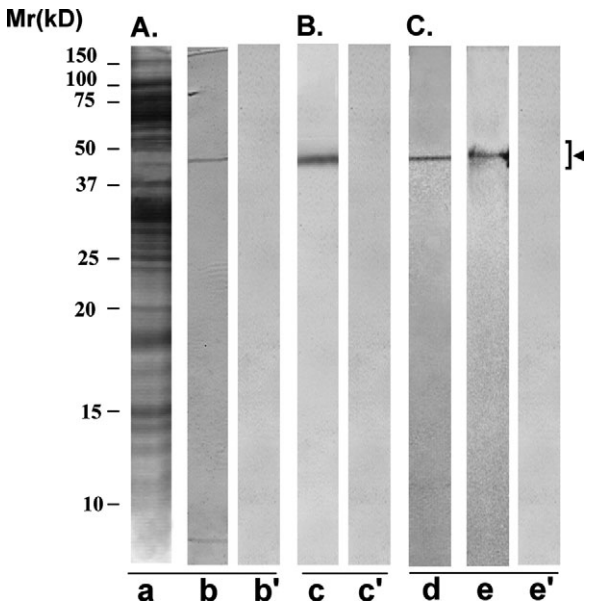

Figure 6: Immunoreactivity pattern of AFA-1 scFv antibody with HSE and FA-1 antigen

The purified scFv antibody was examined for its immunoreactivity with LIS-solubilized HSE (**A** and **B**) and purified cognate human sperm FA-1 antigen (**C**), using western blot (A and C) and immunoprecipitation (B) procedures. (A) SDS–PAGE of HSE revealed several protein bands of various molecular identities after silver staining (lane a). AFA-1 scFv antibody specifically recognized a protein band of 50 ± 4 kDa, corresponding to FA-1 antigen on western blot of HSE (lane b). Control scFv antibody did not react with any specific band on the western blot (lane b'). (B) AFA-1 scFv antibody Sepharose 4B immunobeads reacted with a specific protein in HSE that on elution with glycine–HCl (0.1 M, pH 2.8) showed a single band of 50 ± 4 kDa, corresponding to FA-1 antigen, in SDS–PAGE (lane c). Control scFv antibody Sepharose 4B immunobeads did not react with any protein in HSE (lane c'). (C) The cognate FA-1 antigen purified from HSE using immunoaffinity column involving mouse monoclonal antibody MA-24 showed a single band of 50 ± 4 kDa in SDS–PAGE (lane d) that was specifically recognized by AFA-1 scFv antibody (lane e) and not by the control scFv antibody (lane e') in the western blot procedure.

In the indirect IFT, all the three antibodies that were examined, namely AFA-1, FAB-7 and YLP20, reacted with both the methanol-fixed and unfixed live human sperm (Fig. 10). AFA-1 (a and a') and FAB-7 (b and b') antibodies predominantly reacted with post-acrosomal, midpiece and tail regions of methanol-fixed (a and b) and unfixed live human sperm (a' and b'). YLP20 antibody reacted with acrosomal, midpiece and tail regions of methanol-fixed (c) and unfixed live (c') human sperm.

Effect of antibodies on human sperm capacitation/acrosome reaction

To examine the effect of antibodies on human sperm function, the antibodies (AFA-1/FAB-7/YLP20) were incubated with sperm during capacitation, and the percentage of sperm undergoing acrosome reaction was determined (Table I). Following treatment with ionophore, an average of 74% sperm underwent acrosome-reaction when they were capacitated in the presence of 85 μg/ml of control antibody (CAB-3). Capacitation of sperm with the AFA-1/FAB-7/YLP20 antibody caused a concentration-dependent inhibition in percentage of acrosome-reacted sperm compared with control antibody (Table I). The highest inhibition in acrosome reaction was induced by AFA-1 antibody, especially at 80 μg/ml concentration. There was no effect of any of these antibodies on sperm motility or viability, and they did not cause agglutination of sperm. AS16 caused agglutination of sperm, thus was not tested for its effects on sperm capacitation/acrosome reaction.

Epitope analysis of FA-1 reactive antibodies

Both AFA-1 and FAB-7 antibodies demonstrated similar reaction patterns with HSE, FA-1 antigen and human sperm, but revealed different nucleotide and amino acid sequences in the CDRs. It was hypothesized that these antibodies may be directed against different epitopes of FA-1 antigen. To investigate, several peptides based on the immunodominant regions of mouse and human FA-1 sequences were synthesized and examined for their immunoreactivity with AFA-1 and FAB-7 antibodies. AFA-1 antibody showed a positive reaction (>2 SD units) only with peptides based on human $FA\text{-}1_{200\text{-}219aa}$ and mouse $FA\text{-}1_{117\text{-}136aa}$ sequences. This epitope was designated as FA-1a (Table II). FAB-7 antibody showed a positive reaction only with peptides based on human $FA\text{-}1_{82\text{-}97aa}$ and mouse $FA\text{-}1_{2\text{-}19aa}$ sequences. This epitope was designated as FA-1b. These antibodies did not react with other human and mouse peptide sequences (data not shown). YLP20 antibody did not react with these peptides, and AFA-1 and FAB-7 antibodies did not react with YLP_{12} peptide (Table II).

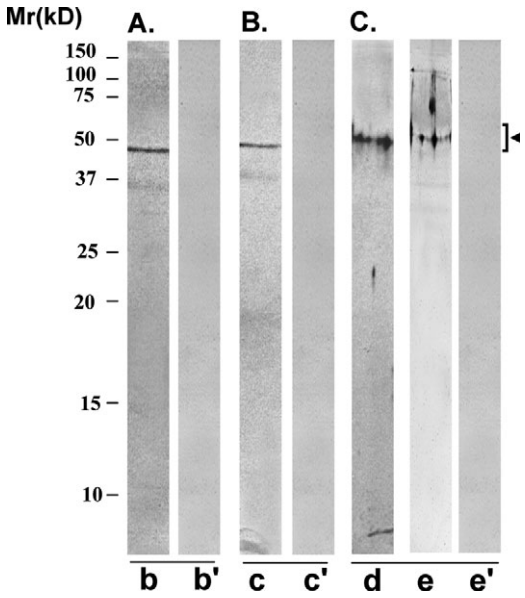

Figure 7: Immunoreactivity pattern of FAB-7 ScFv antibody with HSE and FA-1 antigen

The purified scFv was examined for its reactivity with LIS-HSE (**A** and **B**) and purified cognate human sperm FA-1 antigen (**C**), using western blot (A and C) and immunoprecipitation (B) procedures. (A) FAB-7 scFv antibody specifically recognized a protein band of 50 ± 4 kDa, corresponding to FA-1 antigen, on western blot of HSE (lane b). Control scFv antibody did not react with any specific band on the western blot (lane b'). (B) FAB-7 scFv antibody Sepharose 4B immunobeads reacted with a specific protein in HSE that on elution with glycine–HCl (0.1 M, pH 2.8) showed a single band of 50 ± 4 kDa, corresponding to FA-1 antigen, in SDS–PAGE (lane c). Control scFv antibody Sepharose 4B immunobeads did not react with any protein in HSE (lane c'). (C) The cognate FA-1 antigen purified from HSE using immunoaffinity column involving mouse monoclonal antibody MA-24 showed a single band of 50 ± 4 kDa in SDS–PAGE (lane d), that was specifically recognized by FAB-7 ScFv antibody (lane e) and not by the control scFv antibody (lane e') in the western blot procedure.

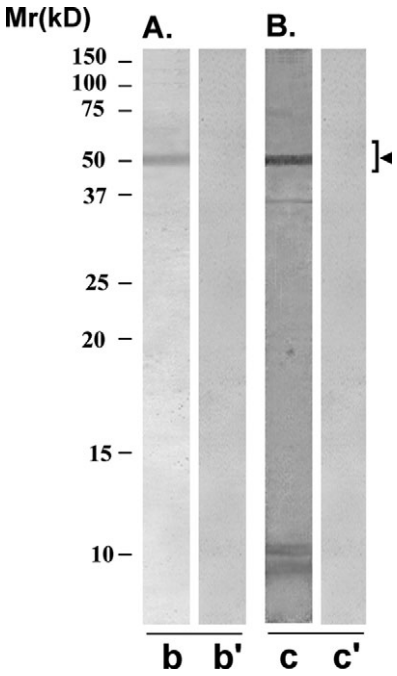

Figure 8: Immunoreactivity pattern of YLP20 scFv antibody with HSE

The purified scFv was examined for its reactivity with LIS-solubilized HSE using western blot (**A**) and immunoprecipitation (**B**) procedures. (A) YLP20 scFv antibody specifically recognized a protein band of 48 ± 5 kDa, corresponding to YLP_{12} antigen, on western blot of HSE (lane b). Control scFv antibody did not react with any specific band on the western blot (lane b'). (B) YLP20 scFv antibody Sepharose 4B immunobeads reacted with a specific protein in HSE that on elution with glycine–HCl (0.1 M, pH 2.8) showed a single band of 48 ± 5 kDa in SDS–PAGE (lane c). Control scFv antibody Sepharose 4B immunobeads did not react with any protein in HSE (lane c').

Discussion

Using phage display technology involving cDNA cloned from lymphocytes of immunoinfertile and vasectomized men, we have isolated, characterized and produced fully functional human scFv antibody fragments against two well-characterized sperm specific antigens, namely FA-1 and YLP_{12}. The role of FA-1 antigen and YLP_{12} sequence in human sperm function and human sperm–oocyte ZP binding is well documented (Naz and Zhu, 1997, 1998, 2002; Naz *et al*., 2000; Naz and Chauhan, 2002). The immunoinfertile men and women, and vasectomized men have circulating and testis antibodies to these antigens (Naz *et al*., 1993; Naz and Chauhan, 2001). Vaccination with these antigens causes a contraceptive effect and the combination vaccination results in an enhanced reduction in fertility (Naz and Zhu, 1998; Naz and Chauhan, 2002).

For construction of the antibody library, immunoinfertile/vasectomized men that had high titers of ASA, especially against FA-1 antigen and YLP_{12} peptide, were selected. PBL were activated with sperm antigens (FA-1/YLP_{12}/HSE) to enrich them with specific RNAs. PCR primers were constructed to amplify at least 90% of the human antibody sequences available in the Kabat database (Kabat *et al*., 1991). The phages displaying recombinant scFv antibodies were made from the primary library and used for panning against the sperm antigens (FA-1/YLP_{12}/HSE). The positive clones, after three rounds of panning, were selected, sequenced and analyzed using the immunogenetics database.

Of the 30 clones reacting positively with these antigens, four strongest reacting were selected for further analysis. Of these four, two were reactive with FA-1 antigen, one with YLP_{12} and one with HSE. The nucleotide and amino acid sequences of these clones did not show a complete homology with any of the existing sequences in the human database, indicating them to be novel. The antigen binding site is comprised of six CDRs, three each contributed by heavy and light chain, and confer specificity for binding to a specific epitope. All these antibody clones had a similarity in the framework region with other human antibody sequences in the database, but their CDR regions were novel, indicating that these were directed against different antigenic determinants.

Three antibodies (AFA-1/FAB-7/AS16) had heavy chain belonging to IgG3 and one (YLP20) had heavy chain belonging to IgG1 subclass. On the basis of the nucleic acid homology, the human Ig heavy chains consist of 50 functional gene segments belonging to seven gene families (VH1–VH7). The majority (86%) of the contribution is provided by three families

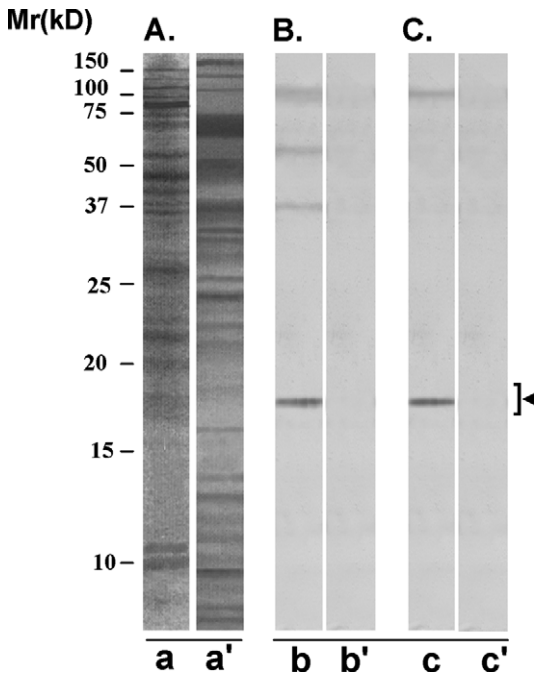

Figure 9: Immunoreactivity pattern of AS-16 scFv antibody with HSE

The purified scFv antibody was examined for its reactivity with LIS-solubilized HSE (**B**) and MC-solubilized human sperm preparation (**C**), in the western blot procedure. (**A**) SDS–PAGE of LIS-solubilized human sperm preparation (lane a) and MC-solubilized human sperm preparation (lane a') revealed several protein bands of various molecular identities after silver staining. (B) AS16 scFv antibody specifically recognized four protein bands of 18 (major band), 37, 55 and 100 kDa, respectively, on the western blot of LIS-solubilized human sperm preparation (lane b). Control scFv antibody did not react with any band on the western blot (lane b'). (C) AS-16 ScFv antibody recognized two protein bands of 18 (major band) and 100 kDa (minor band), in the western blot of MC-solubilized human sperm preparation (lane c). Control scFv antibody did not react with any specific band on the western blot (lane c'). The 18 kDa protein was the major band specifically recognized in both the LIS-solubilized human sperm preparation (lane a) and MC-solubilized human sperm preparation (lane a') and it corresponds to SAGA-1 sperm protein.

(VH1, VH3 and VH4) (Cook and Tomlinson, 1995) and our four clones fall in these families. Antibodies reactive with the FA-1/YLP_{12}/HSE in the sera of immunoinfertile and vasectomized men primarily belong to the IgG class, and we have examined these antibodies for subclass specificity. The light chain of all the four clones belonged to κ-chain family. The relative roles of heavy and light chains, and their classes and subclasses in antigen recognition and binding, as pertaining to sperm immunity, have not been determined.

These four clones were expressed to produce and purify larger quantity of antibodies for various immunobiological assays. Antibodies had an E-Tag sequence at the end that made it easy to purify them using an anti-E-Tag monoclonal antibody column. The purified antibodies showed the expected single band of 28 kDa in the silver-stained gel that was specifically recognized by the anti-E-Tag antibody. The antibodies had a tendency to polymerize (dimers and occasionally trimers) during freezing. This has been reported to occur with several other scFv antibodies (Arndt *et al*., 1998; Deng *et al*., 2003). The yield of antibodies using our vector system was low. Various methods such as high-level expression vectors, increase of linker length and reversing the domain orientation from VH–VL to VL–VH have been used to increase the expression level for several scFv antibodies (Nieba *et al*., 1997; Hamilton *et al*., 2001).

The authenticity of clones (AFA-1 and FAB-7) obtained by panning against cognate FA-1 antigen was confirmed by: (i) immunoreactivity of AFA-1/FAB-7 antibodies with the specific expected band of $\sim 50 \pm 4$ kDa, corresponding to FA-1 antigen, in the western blot and immunoprecipitation procedures; (ii) binding of these antibodies with the post-acrosomal, midpiece and tail regions of unfixed live and methanol-fixed human sperm, the subcellular sites of human sperm previously shown to have FA-1 antigen expression; and (iii) binding of these antibodies with the purified cognate human sperm FA-1 antigen. Both these antibodies, AFA-1 and FAB-7, showed a similar immunoreactivity pattern in all these assays.

The authenticity of YLP20 clone obtained by panning against synthetic dodecamer YLP_{12} peptide was confirmed by: (i) immunoreactivity of YLP20 antibody with a specific protein band of $\sim 48 \pm 5$ kDa, corresponding to YLP_{12} antigen, in the western blot and immunoprecipitation procedures; and (ii) binding of the antibodies to the acrosomal, midpiece and tail regions of live and methanol-fixed human sperm, the subcellular sites of human sperm previously shown to have YLP_{12} expression. YLP_{12} dodecamer amino acid sequence is a part of a 72 ± 5 kDa protein that is synthesized during spermatogenesis in the testis and later gets modified/cleaved to form a 48 ± 5 kDa protein in the mature ejaculated sperm cell (Naz *et al*., 2000; Naz and Chauhan, 2001, 2002).

There were several clones reactive with HSE. The clone AS16 was selected for further analysis. This clone demonstrated 67–88% homology in CDRs of both heavy and light chains with a RASA. RASA is a mouse scFv monoclonal antibody directed against a tissue-specific epitope of human SAGA-1 that is a sperm glycoform of CD52 antigen (Diekman *et al*., 1997; Norton *et al*., 2001). Antibodies against the SAGA-1 cause agglutination of human sperm. As with FA-1 and YLP_{12}, SAGA-1 has been proposed as a candidate for immunocontraception. Besides having homology in CDRs with RASA, AS16 scFv antibodies reacted with the specific protein of ~ 18 kDa in HSE, corresponding to SAGA-1 and caused agglutination of human sperm. AS16 seems to be a human homolog of the mouse monoclonal scFv, RASA.

All the four antibodies (AFA-1/FAB-7/YLP20/AS16) inhibited human sperm function. AS16 caused an agglutination of sperm and the other three antibodies (AFA-1/FAB-7/YLP20) inhibited human sperm capacitation/acrosome reaction. The polymerized antibodies demonstrated more agglutination than the freshly isolated antibodies. In general, scFv fragments should not agglutinate sperm, as they are single chain Igs. It may be a tendency of the antibodies to aggregate into dimeric and trimeric forms that cause sperm

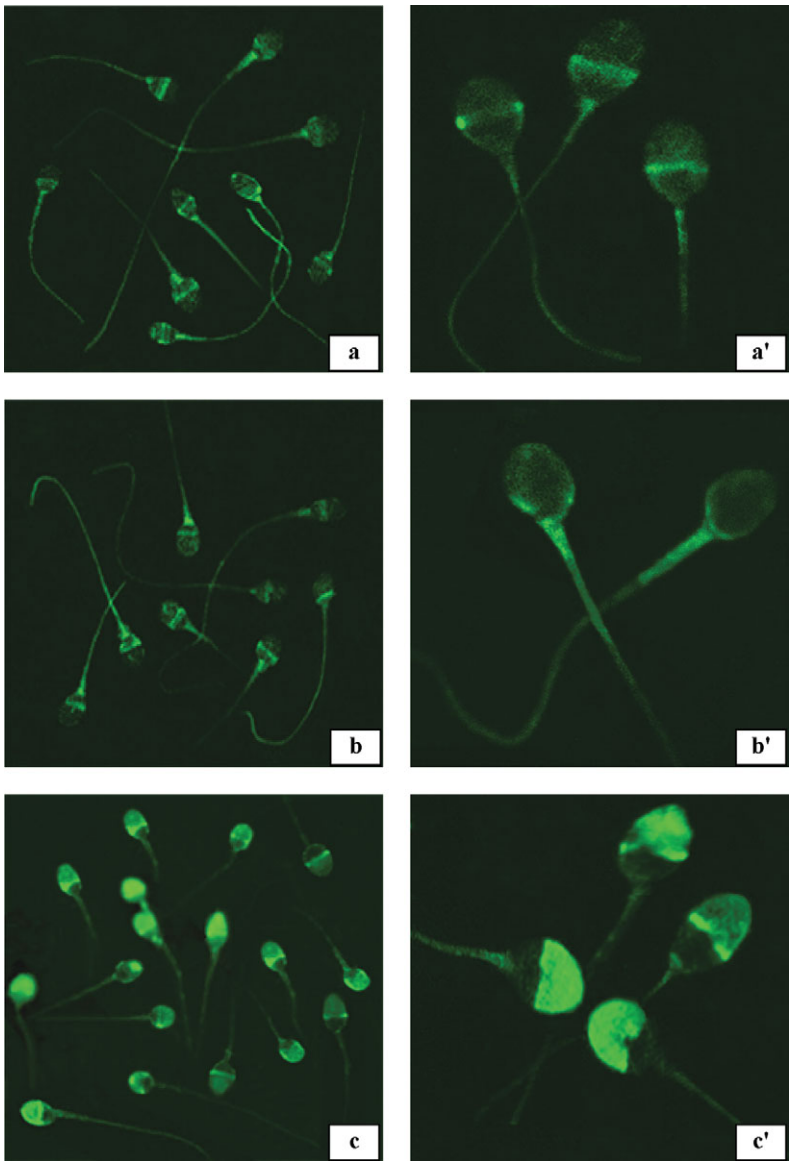

Figure 10: Epifluorescent photomicrographs indicating the indirect immunofluorescent reaction pattern of AFA-1, FAB-7 and YLP20 scFv antibodies with methanol-fixed (a, b and c) and unfixed live human sperm (a', b', and c')
AFA-1 (a and a') and FAB-7 (b and b') antibodies predominantly reacted with post-acrosomal, midpiece and tail regions of methanol-fixed (a and b) and unfixed live human sperm (a' and b'). YLP20 antibody reacted with acrosomal, midpiece and tail regions of methanol-fixed (c) and unfixed live (c') human sperm. Magnifications: a, b and c $\times 698$; a', b' and c' $\times 1924$.

agglutinating activity. Polyclonal antibodies raised in rabbits/mice against FA-1 antigen and YLP_{12} also inhibit human sperm capacitation/acrosome reaction. These three antibodies did not agglutinate or immobilize sperm when they were in monomeric form. Both AFA-1 and FAB-7 antibodies had different sequences in their CDRs. The data indicate that these two antibodies are directed against two different epitopes of FA-1 antigen. AFA-1 antibody is directed against an epitope, designated as FA-1a, which is present on human FA-$1_{200\text{-}219aa}$ and mouse FA-$1_{117\text{-}136aa}$ sequences. FAB-7 antibody is directed against an epitope, designated as FA-1b, which is present on human FA-$1_{82\text{-}97aa}$ and mouse FA-$1_{2\text{-}19aa}$ sequences. In these two epitope regions, the human and mouse FA-1 sequences have 60–61% homology. These data also indicate that these antibodies are directed against the polypeptide sequence and not against the carbohydrate moiety of the FA-1 glycoprotein. The findings may also indirectly suggest that these epitopes may be more immunogenic and produce antibodies in human immunoinfertility and after vasectomy. Indeed, in a recent study, we found that 24.6–41.8% of the sera from immunoinfertile women have antibodies reactive with synthetic peptides based on sequences of these two epitopes (Williams *et al*. in press).

In conclusion, using phage display technology involving cDNA isolated from lymphocytes of immunoinfertile and vasectomized men, we have isolated four novel fully functional scFv human antibodies reactive with well-characterized human sperm antigens (FA-1/YLP_{12}/SAGA-1). These antibodies inhibit human sperm function, thus may find applications in immunocontraceptive development and specific diagnosis of

Table I. Effect of various single chain variable fragment (ScFv) antibodies on human sperm acrosome reaction.

Antibody	Concentration (μg/ml)	Acrosome-reacted sperm (%)
1. AFA-1	45	50 ± 6^{a}
	80	36 ± 7^{b}
2. FAB-7	45	58 ± 8^{c}
	80	44 ± 6^{b}
3. YLP20	45	52 ± 7^{c}
	80	$42 \pm 3^{b,d}$
4. Bovine Serum Albumin/ Control antibody	85	74 ± 5^{e}

[a,b,c,d,e]Values with different superscripts are significantly different, $P < 0.01$ to $P < 0.001$.

Table II. Immunoreactivity of ScFv antibodies with various synthetic peptides.

Antibody	FA-1_a	FA-1_b	YLP_{12}	FA-1 antigen	Control
AFA-1	23.6–37.1[a]	1.4	0.1	91.0[a]	0.1
FAB-7	1.8	48.7–60.2[a]	1.1	89.5[a]	0.3
YLP20	0.1	0.2	27.2[a]	1.3	0.5
Control antibody	0.1	0.7	0.3	0.9	0.1

[a]Immunoreactivity is expressed as SD units. Values with superscript a are positive (>2 SD units), and the others are negative (<2 SD units). FA-1_a epitope refers to peptides based on human FA-$1_{200-219\ aa}$ and mouse FA-$1_{117-136\ aa}$ sequences, and FA-1_b epitope refers to peptides based on human FA-$1_{82-97\ aa}$ and mouse FA-$1_{2-19\ aa}$ sequences. Control peptide is based on human FA-$1_{220-240\ aa}$ sequence.

immunoinfertility. There are a few reports on the development of human hybridoma secreting ASA (Isojima *et al*., 1987) and antibody Fab fragments by combinatorial phage display reactive with human spermatozoa (Clayton *et al*., 1998). This is the first report on the development of human scFv ASA of defined specificity related to immunoinfertility/vasectomy. Their *in vivo* efficacy as passively administered immunocontraceptives is presently being explored. Various methods such as PEGylation (Chapman, 2002), fusion with serum albumin (Smith *et al*., 2001) and multimerization (Hudson and Kortt, 1999) are being examined to increase the efficacy and half-life of these genetically engineered recombinant antibodies.

Acknowledgements

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