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Separation and characterization of sperm antigens using two-dimensional gel electrophoresis and immunoblotting

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Separation and Characterization of Sperm Antigens Using Two-dimensional Gel Electrophoresis and Immunoblotting

by

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Abstract:

In order to help better separate and identify sperm antigens that may be involved in the fertilization process, a two-dimensional gel electrophoresis system was developed. This system separated proteins according to their isoelectric points on the first dimension and their molecular weight on the second dimension. More than 40 sperm protein spots can be identified using this system.

Those separated sperm proteins, after transferred onto PVDF membranes, were further identified using either a serum that came from an immunoinfertility patient or a monoclonal antibody that was previously developed by our lab. One sperm antigen which is about 13 Kd and pI about 4.5 was identified using the serum. This antigen is similar to the SAGA-1 antigen identified by Herr's lab. Another two sperm antigens have identical molecular weight (34 Kd) but different pIs (6.7 and 7.6 respectively) were identified using a monoclonal antibody that against sperm surface antigens.

Introduction:

Reproduction starts with the unison of gametes contributed by the male and female partners. Spermatozoa are unusual cells in many respects, such as their highly condensed nuclei, their unusual cytoplasmic compartments (such as acrosome) and their unique cell shape with the long flagella. (Kruger et al, 1986). In our lab, our researches focus on the sperm antigens. We hope by studying the sperm antigens, we can improve our understanding of the molecular events involved in the fertilization process, the complex processes of sperm differentiation and maturation, the mechanism of immunoinfertility and then, based on those information, we can contribute to the development of an effective immunocontraceptive vaccine.

Spermatozoa have some antigens that are unique, tissue-specific, immunogenic and can be accessed by antibodies easily. Binding of the antibodies to these antigens can affect many aspects of fertilization, such as reduce sperm motility, resulting in suppression of sperm travelling through the female reproductive tract (Mahony et al, 1991.) or reduction of sperm function at the stage of fertilization, such as antibody to FA-1 antigen can block sperm binding to the zona pellucida (Naz et al, 1986). Antibodies also can stop the embryo development by blocking cell cleavage signals (Naz et al, 1996). Up to 70% of vasectomized men form antisperm antibodies and up to 30% cases of involuntary infertility are associated with the presence of antisperm antibody in the male or female partner of the infertile couple. Those studies further confirmed

that antisperm antibodies not only associated with infertility but appear to be the cause of infertility (Bronson et al, 1984; Shulman, 1986). Examination of those immunoinfertility patients often shows no evidence of abnormality besides the fact that they are infertile. Based on those observations, people believe that at least some antigens derived from sperm surface are specific to the sperm cell and involved in fertilization, and thus constitute interesting molecules for the development of an antisperm contraceptive vaccine.

But the whole sperm *per se* can not be employed for the development of a immunocontraceptive vaccine due to the fact that some antigens present on the sperm cell are likely to be shared with various somatic cells (Naz et al, 1990). Also some clinical study found that antibody titer does not necessarily correlate with inhibition of sperm-oocyte fusion, sperm motility or fertilization. Sperm antibodies sometime can be detected in partners from a fertile couple and more interestingly, some antibodies can increase instead of inhibit sperm motility or stimulate sperm-egg interaction (Snow et al, 1992). All those observations suggest that only a limited number of sperm antigens may be associated with immunoinfertility. So careful distinction among and separation of sperm antigens is an important stage of immunocontraceptive vaccine development.

Based on their highly specificity, monoclonal antibodies have found several applications as probes for use in the understanding of reproductive mechanisms. In recent years, monoclonal antibodies have been generated against sperm antigens in

several species such as rabbit (M.G. O'Rand et al, 1984), guinea pig (P.Primakoff et al, 1988), mouse (Schmell et al, 1986), bull (Ambrosia et al, 1996) and human (Naz et al, 1996). Those monoclonal antibodies are valuable tools for identification, isolation and characterization of sperm antigens.

Since the 1970s (O'Farrell et al, 1977), two dimensional gel electrophoresis has become a powerful technique for separating proteins from complex mixtures. There are several different kinds of two dimensional gel systems, but the most commonly used system is called ISO-DALT system (Anderson et al, 1978). This system separates proteins on the basis of two independent properties. In the first dimension, proteins are separated based on their different isoelectric points, then on the second dimension, the proteins are further separated based on their mobility in SDS-polyacrylamide gels (SDS-PAGE). Since the isoelectric point of a protein can provide valuable information about its likely amino acid composition and the mobility of proteins in SDS-polyacrylamide gels is primarily a function of their molecular weight, two dimensional gels not only can achieve better separations but also can be used to characterize proteins. Because of those reasons, the ISO-DALT system has become pre-eminent both in terms of its range of applications and efforts of refinements. (O'Farrell et al, 1977; Hochstrasser et al, 1988)

In the first dimension of ISO-DALT system, a pH gradient can be generated and maintained by applying an electric field to a gel which containing a mixture of

amphoteric components called ampholytes (chemically, oligoamino, oligocarboxylic acids) which have closely spaced pIs, encompassing a given pH range. Electrophoretic transport causes the carrier ampholytes to stack according to their pIs, and a pH gradient, increasing from anode to cathode, is established. At the beginning of the run, the medium has a uniform pH equal to the average pI of the carrier ampholytes, thus most ampholytes have a net charge, so they move in the electric field. The most acidic ampholyte moves toward the anode where it concentrates in a zone whose pH equals to its pI, while the more basic ampholytes are driven toward the cathode. A less acidic ampholyte migrates adjacent and just cathodal to the previous one and so on, until all the ampholytes of the system reach a steady-state. Polypeptides move more slowly than carrier ampholytes and usually find themselves in an environment where they have a net negative or positive charge and then they migrate through the gel until they reach a position where they have no charge. Although the size of those polypeptides may affect the rate at which polypeptides migrate through the gel, their final position is determined solely by their isoelectric point (Hames et al, 1990).

The second dimension of ISO-DALT system is a typical SDS-PAGE. The proteins applied to this gel have been pre-incubated with SDS and a thiol reagent (usually 2-mercaptoethanol). Since most proteins bind SDS in a constant weight ratio (1.4g of SDS per gram of polypeptide), so they have essentially identical charge densities and migrate in polyacrylamide gels according to their molecular weights. The standard

proteins with the known molecular weight can be run together with the unknown proteins, then the molecular weights of unknown proteins can be calculated by comparison with those protein standards.

Because of its high resolution, sensitivity and reproducibility, this ISO-DALT system has been widely used in the molecular biology and biochemistry. For example, to distinguish five isoforms of actin (Ochs et al, 1985), to monitor the changes in protein expression that occurred during neural development (Geschwind et al, 1996), etc. By combining this technique with the computer analysis, scientists have assembled databases for proteins for different kinds of cells in different species, recently emerging as online database for adult drosophila (Encsson et al, 1997), database for heart proteins (Evans et al, 1997), database for human serum proteins (Bini et al, 1996), etc. In andrology, the two dimensional gel electrophoresis has been used to study the protein composition of the whole sperm cell (Naaby-Hansen et al, 1997), semen, prostatic fluid and seminal vesicle fluid (Lee et al, 1989). Sperm membrane antigens have been analyzed for boar (Russell et al, 1983; Peterson et al, 1991), bull (Noland et al, 1984), rat (Hall et al, 1989), mouse (Kramer et al, 1982), and man (Mack et al 1987; Naaby-Hansen, 1990).

For the present report, I have adopted an ISO-DALT system for our lab and used this system to separate sperm proteins. I used an antisperm-directed polyclonal antibody from an infertile patient and one monoclonal antibody to probe and characterize

interesting sperm antigens using western blotting.

Materials and Methods:

Preparation of spermatozoa

Semen specimens were obtained by masturbation from six healthy adult men after 24-48 hours of sexual abstinence. Specimens were allowed to liquefy at room temperature for 1 to 2 hours before use. Semen analysis was performed according to World Health Organization guidelines (1992). Normal semen analysis was defined as sperm concentration > 20 million sperm per ml, motility >60%, and morphology >50% normal forms. Direct immunobead tests were also performed on those specimens according to the procedure described below. Only specimens that tested negative in direct immunobead test and had normal semen parameters were used in the two dimensional gel electrophoresis analysis. Sperm were washed free of seminal plasma by mixing in 10 ml phosphate buffered saline at pH 7.4 following by centrifugation at 800× g for 10 minutes. After a second wash, the sperm pellets were stored at -20 °C.

Detection of antisperm antibodies by immunobead test (IBT)

The principles for immunobead test have been presented in several papers (Phillips et al, 1987; Bronson et al, 1982). Basically there are two types of immunobead tests, one is direct immunobead test (direct IBT) which is used to detect the human immunoglobulin (Ig) that bound on the sperm surface. The other type is indirect immunobead test (indirect IBT) which is used to detect antisperm antibodies that present in the human serum or in the human reproductive tract secretion. The isotypes

of the immunoglobulins can be determined by using isotype specific anti-immunoglobulin antibodies. The detailed procedure is described as below.

Immunobead Preparation:

Polyacrylamide beads with the diameter of 3-10 um were obtained from Bio-Rad. Those beads were conjugated by polyclonal rabbit-anit-human immunoglobulin IgA, IgG or IgM. To prepare the immunobead solutions, 6.25 mg of each kind of lyophilized beads was weighed out and washed twice by centrifugation at 800× g for 10 minutes with Phosphate Buffered Saline (PBS) solution to remove sodium azide and then resuspended in 1 ml of Phosphate Buffered Saline solution containing 10 mg of bovine serum albumin (BSA) to a final concentration of 6.25 mg bead/ml. Bead solutions were examined by light microscopy to confirm dispersion and disaggregation. Bead suspension can be stored at 4 °C for one month.

Direct Immunobead Test:

Direct IBT was performed on donors' sperm following IBT protocol for anitsperm antibody detection in WHO manual (1992). Briefly, donor sperm were washed twice in PBS solution which contains 5 mg/ml BSA at 800× g for 10 minutes and resuspended in the same solution to a final concentration of 20 million motile sperm/ml. 5 microliter of this sperm solution was mixed with 50 microliter of pre-prepared bead solution, and incubated at room temperature for 10 minutes. This suspension was votexed right before detection. 15-20 ul of sperm-bead mixture was added to a glass slide and covered by a coverslip, and scored by phase contrast microscopy at 200X. At least 200 motile sperm were scored for each immunoglobulin

class. The percentage of sperm with bead binding on their surface together with the locations of bead binding were recorded.

Indirect Immunobead Test:

The indirect immunobead test was performed on a donor's serum following IBT WHO protocol (1992). Briefly, the sperm which were within the normal ranges for morphology, motility and numbers based on WHO criteria and were negative for direct immunobead test for all three isotypes of immunoglobulin were used for indirect IBT. First, those sperm were washed twice at 800× g for 10 minutes with PBS containing 0.5 mg/ml BSA and resuspended to a concentration of 20 million motile sperm/ml. Then 25 ul of washed sperm were incubated with 275 ul of PBS with 0.5 mg/ml of BSA and 100 ul of human serum (previously inactivated at 56 degree for 30 minutes) for one hour at 37 °C. After incubation, the sperm were washed twice in PBS containing 0.5 mg/ml BSA at 800× g for 10 minutes and resuspended into the same solution. The subsequent steps were as above for direct IBT. The percentage of sperm with beads bound and the location of beads were carefully recorded for each of the immunoglobulin types.

Sperm membrane isolation

Sonication:

Sonication was used to disrupt the sperm cell membrane. Briefly, the stored sperm pellets (prepared as described above) were removed from -20 °C and warmed up to room temperature. Those pellets were washed once again in the 10 ml of PBS (pH

7.4) followed by centrifugation at 800× g for 10 minutes. Each pellet was resuspended in 1 ml cold PBS (pH7.4) which contained 1 mM of protease inhibitor PMSF (5 ul of 200 mM stock PMSF solution in 1 ml of PBS. Sigma), 1 mM of EDTA (5 ul of 200 mM stock EDTA solution in 1 ml of PBS. Sigma) and 2 ug/ml of Apotinin (Sigma). Sonication was carried out on ice for each sperm suspension separately while observing the sperm morphology closely under light microscope every minute during sonication. (Branson Scientific Cell Disrupter. 20k Hz, 100% duty cycle.) At about 7 minutes, almost all the sperm tails were detached. At about 12 minutes, some sperm heads region started to show irregular shape. After 25 minutes, almost all the sperm heads became irregular and unsmooth in the shape and tails were broken into several fragments, but the sperm nucleus' shape remained unchanged. At the same time, some round shape debris showed up in the solution surrounding the sperm. Sonication was stopped at this time. The entire sonication process was carried out on ice to inhibit the activity of protease. Sonicated sperm suspensions were centrifuged at 450× g for 5 minutes, the supernatant which contained the sperm membrane was kept. The pellet which contained the sperm nuclei, large sperm tail fragments and some unbroken sperm was removed. The supernatant was centrifuged again at 2000× g for 10 minutes at 4°C. The protein concentration of the resulting supernatant was measured following the procedure described in the next section.

Protein concentration measurement:

Protein concentration was measured using Bradford method. Briefly, protein standard was made by dissolving 10 mg of BSA in 1 ml of distilled H_2O (DH_2O) to make final

concentration of 10 mg/ml of protein. This protein standard solution was further diluted with DH_2O to 1 mg/ml. Five identical test tubes were prepared. Each contained either 5 ul, 10 ul, 15 ul, 20 ul or 25 ul of the diluted protein standard solution, DH_2O was used to bring the volume upto 800 ul. 200 ul of Bradford reagent (Bio-Rad) was added into each tube. So the final volume in every tube was 1 ml but the concentrations were different, from 5 ug/ml to 25 ug/ml depended on the volume of protein standard solution that added into each tube. Absorbance was measured at 595 nm in a spectrophotometer, 800 ul of DH_2O plus 200 ul of Bradford reagent was used as a blank. A standard curve for BSA was generated based on the readings. The concentration of the isolated sperm proteins was measured and then calculated using this standard curve.

Concentration of the sperm antigen preparation:

Since the protein concentration from the original isolation solution measured was 0.592 mg/ml, concentration procedure was carried out using contriprep -10 concentrator. (Amicon Inc, MA) This procedure was carried out at 4 °C to avoid the damage to sperm proteins. This type concentrator allows molecules whose molecular weight below 10K to pass the membrane, since most sperm proteins size are much larger than 10K, this step would not leading to loss any interesting proteins. First the outer chamber of the concentrator was rinsed with DH_2O, then 8 ml sperm antigen solution was put into that chamber. After centrifugation at 3000× g for 30 minutes at 4°C , sperm antigen solution was removed from the outer chamber and protein concentration was measured. The protein concentration was about 0.832 mg/ml. Since

it was still not high enough, this concentrate step was repeated again and the protein concentration was measured right after that. The resulting protein concentration was about 1.27 mg/ml which would be high enough for the two-dimensional gel electrophoresis assay. The final solution left in the outer chamber was taken out from the concentrator and transferred into microcentrifuge tubes and stored in -20 °C.

Two dimensional gel electrophoresis analysis

IEF gel preparation

IEF gel contained 3.85 g of urea, (Sigma) 1.16 ml of 30% acrylamide/ Bis acrylamide solution, (29:1, 3.3%C, Bio-Rad) 1.4 ml of 10% NP-40 stock solution and 350 ul of ampholines solution (pH 3-10, 40% stock, Pharmacia) in a volume of 7 mls. This solution was incubated at 37 °C to help urea dissolve. After urea completely dissolved and before pouring the gel, 3.5 ul of TEMED (Bio-Rad) and 35 ul of Ammonium Persulfate (APS) stock solution (0.1 g of APS in 1 ml of DH_2O, fresh-made, Fisher Scientific) were added. After mixing thoroughly, this gel solution was poured into the slab gel apparatus (12 cm × 8.3 cm × 0.75 mm). The gel was allowed to polymerize for one hour. After the gel completely polymerized, the comb was taken out and the wells were rinsed with DH_2O to remove precipitated UREA. IEF gels were prepared fresh for each experiment.

Sample preparation:

Sample buffer (1 ml) was made containing 9.5 M urea (0.57g in 1 ml, Sigma), 2% NP-40 (200 ul of 10% stock, Sigma), 5% 2-mercaptoethanol (Bio-Rad), 2%

ampholines (pH 3-10, 40% stock, Pharmacia) and 0.5 ul of 200 mM PMSF stock solution. After mixing, DH_2O was added to bring up the volume. The tube was incubated at 37 °C to help urea dissolve. The sample buffer was butched and stored at -20 °C. Once thawed, it must be used and can not be re-frozen.

This sample buffer can be either used to dissolve power sample to the desired concentration or mixed with liquid sample using 1:1 ratio. Concentrated sperm antigen solution was mixed with an equal volume of this sample buffer and incubated at room temperature for half hour. Timing in this step is very critical. Too short incubation permits incomplete dissolving of proteins, too long incubation allows protein modification by the high concentration of urea. It is also very important to keep incubation time constant for each experiment to avoid unnecessary variation. The two dimensional gel standards (Sigma) were incubated in parallel with isolated sperm antigens. Samples were loaded onto the wells in the slab IEF gel after incubation and covered with overlay buffer to protect those proteins from base at the cathode. Overlay buffer was prepared by mixing equal amount of sample buffer with DH_2O.

IEF separation:

The slab gel was then attached to the Mighty Small II Electrophoresis Cell (SE-250, Hoefer Scientific Instruments, San Francisco). The upper cathodic chamber was filled with 25 mM NaOH. The lower anodic chamber was filled with 10 mM H_3PO_4. The IEF gel was run at constant current of 2.75 mA for 16 hours using Model 1000/500 power supply (Bio-Rad). The running was carried out at 4 °C in a cold room to avoid the heat generated during the running damaging proteins inside the gel. When the

focusing finished, the gel was taken out and cut into ten lanes according to the positions of the wells. One gel lane without antigens containing 15 ul of standard methyl red plus 15 ul of sample buffer was cut into small pieces, 0.5 cm per piece. Those pieces were incubated with DH_2O, 1 ml DH_2O each for at least 2 hours at room temperature. pH were measured for those resulting solutions to see whether there was a pH gradient built up. Other gel lanes were stored in -20 °C. They can be saved for up to 3 months.

Preparation of SDS-PAGE gels:

Mini discontinuous SDS-PAGE gel was used as the second dimensional gel. The separating gel was made by mixing 2.5 mls of acrylamide/ bisacrylamide solution (29:1, 3.3%C, Bio-Rad), 1 ml of 10× separating gel buffer (0.4538 g of Tris-base and 50 ul of 12N HCl, pH 8.8), 0.1 ml of 10% SDS (W/V), 6.35 ml of DH_2O, 50 ul of APS stock solution (0.1g/ml, fresh made) and 5 ul of TEMED (Bio-Rad). After mixing, this solution was poured into the gel apparatus (12 cm x 8.3 cm x 1.5 mm) and covered with DH_2O. The gel was allowed to polymerize for one hour at room temperature. After the gel completely polymerized, DH_2O was poured out and filter paper was used to remove remaining water. The stacking gel solution was prepared by mixing 0.2 ml of acrylamide/ bisacrylamide solution (29:1, 3.3%C, Bio-Rad), 0.2 ml of 10x stacking gel buffer (1.512 mg of Tris-base, 1 ul of 12N HCl, pH 6.8), 20 ul of 10% SDS stock solution (W/V), 1.56 ml of DH_2O, 17 ul of APS (0.1g/ml, fresh made) and 1 ul of TEMED (Bio-Rad). This solution was poured onto the top of the separating gel and the comb was put in. This gel was allowed to polymerize at room

temperature for at least half hour. Then the comb was removed and wells were rinsed with DH_2O. Those SDS-polyacrylamide gels can be stored in 4 °C by covering with plastic wrap (Handiwrap) for up to one week.

Sample preparation for SDS-PAGE:

One IEF sample lane was removed from -20 °C and warmed to room temperature. Equilibration buffer was prepared by mixing 600 ul of 10% SDS (W/V), 300 ul of 2-mercaptoethanol, a trace of bromophenol blue in 6 ml of DH_2O. The thawed IEF sample lane was incubated in this equilibration buffer for 15 to 20 minutes at room temperature. The purpose of this step is to increase the SDS concentration in the IEF sample lane to help protein migration in the SDS-PAGE gel. Too short incubation may not allow the SDS penetrate the IEF gel completely, too long incubation may cause protein loss from the IEF gel. So timing is critical in this step. Once the most suitable incubation time was found, it was kept constant to avoid unnecessary variation between experiments.

Loading the SDS-PAGE gel:

The IEF sample lane, after incubation with equilibration buffer for 20 minutes, was loaded on top of the SDS-PAGE gel. Care was taken to avoid breaking the IEF gel. The IEF gel was sealed in position with 0.5% agarose solution (W/V). At the same time, the protein molecular weight standards (Bio-Rad) were prepared by mixing 1 ul of this molecular weight standard with 20 ul of SDS sample buffer (5 ml of 10X stacking gel buffer, 1 g of SDS, 10 mls of Glycerol and 50 mg of bromophenol blue in 100 ml DH_2O) and heated at 100 °C for three minutes. This standards were loaded

into an SDS-PAGE gel standard well.

SDS-PAGE:

After loading with the IEF gel and protein molecular weight standards, the slab gel was attached to the Mighty Small II Electrophoresis Cell (SE-250, Hoefer Scientific Instruments, San Francisco). The upper chamber and lower chamber were filled with the same electrophoresis buffer (2.4g of Tris-base, 11.52g of Glycine, 4 mls of 10% SDS in 800 mls of DH_2O, pH 8.3). This SDS-PAGE gel was run at constant voltage at 85V using Model 1000/500 power supply (Bio-Rad). Stop running until the bromophenol blue line reached the end of the gel which was about 2 hours. Then the gel was either subject to silver staining or Western transfer and immunoblotting according to the procedures described below.

Silver staining:

Silver staining was carried out according to Morrisey (1981) with a little modification. It is very important to use the best quality water that available to do the silver staining in order to avoid the high background. In this project, the minipore water was used. First the gel was put into the 50% Methanol- 50% DH_2O plus 0.1 ml/100 ml formaldehyde (37% stock) for overnight to remove the SDS in the gel. The next day, the gel was transferred into 100 ml of DH_2O plus 25 ul of 100 mM DTT stock solution and incubated for 2 hours. The gel was then put into 100 mls of DH_2O plus 0.1 g of $AgNO_3$ and incubated for one half hour. During this time, developer solution can be prepared by mixing 3g of Na_2CO_3 and 50 ul of formaldehyde (37% stock) in 100 ml DH_2O. After incubating with $AgNO_3$, the gel was rinsed once with DH_2O and

twice with a small amount of developer solution to reduce the background. After this, the gel was left in the developer solution and observed closely. When the staining intensity reached the desired level, the reaction was stopped with 10 mls of citric acid. The amount of citric acid used is very important, since too little can not stop the reaction completely and too much will bleach the protein dots on the gel. Citric acid was allowed to incubate with gel for 10 minutes, then the gel was washed several times with DH_2O. After this step, the gel can be either scanned using Collage image analysis software (FotoDyne Incorporated) or dried using a gel drying apparatus.

Western Transfer, immunoblotting and visualization:

The western transfer was done using a wet transfer apparatus called Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The transfer buffer contains 3g of Tris-base, 14.4g of Glycine and 200 ml of 100% methanol in a final volume of 1000 mls.

The proteins were transferred onto the polyvinyldifluoride (PVDF) membrane (Hybond-P, Amersham Life science, England) using constant voltage at 100 V for 4 hours. The ice bucket in the transfer cell was changed every 2 hours.

After the transfer, the PVDF membrane was put into STBST solution contains 5% non-fat dry milk (The STBST solution containing 9 g of NaCl, 2.42 g of Tris-base and 2 mls of Tween 20 in 1000 mls of DH_2O. Adjusted to pH 7.4) overnight to block the unspecific binding sites on the membrane.

On the next day, the membrane was incubated with either hybridoma culture supernatant (undiluted) or 1:50 diluted human serum (diluted with 1% milk in

STBST) for 11/2 hours. Then the membrane was rinsed with STBST once for 20 minutes and three more times with STBST for five minutes each time. Then the membrane was incubated with an appropriate biotin-conjugated secondary antibody for one hour. When the primary antibody was diluted human serum, the secondary antibody was Biotin conjugated goat-anti-human IgG and IgA mixture (Vector Laboratories, 1:1000 dilution in 1% milk-STBST). When the primary antibody was monoclonal antibody supernatant, then the secondary antibody was biotinylated goat anti-mouse IgG (Vector Laboratories, 50 ul in 10 ml of 1% milk-STBST). After incubating with secondary antibody for one hour, the membrane was washed once with STBST for 20 minutes, following three washes with STBST for 5 minutes each time. The membrane was incubated with Avidin-biotin-peroxidase complex for half hour (50 ul of avidin and 50 ul of biotin-peroxidase, Vector Laboratories, in 5 ml of 1% milk-STBST) to amplify the signal. After incubation, the membrane was washed once for 20 minutes in STBST and three times with STBST for 5 minutes each time. Then the membrane was incubated with ECL reagent (Amersham International , England) for exact one minute and allowed to expose to the X-ray film (sigma). Then the film was developed and fixed following standard procedure. The film can be scanned and analyzed using Collage image Analysis software (FotoDyne Incorporated).

Results:

Immunobead test :

The direct immunobead test was used to detect immunoglobulins that bound on the sperm surface. The results are presented in table 1. These results confirmed that none of the donor sperm that were used to isolate the sperm antigens contained immunoglobulins on their surface. The indirect immunobead test for the human serum 05 is shown in table 2. The indirect immunobead test result for this donor was positive, this confirmed that this donor serum contains antisperm antibodies that can recognize the human sperm surface antigens.

Two dimensional gel electrophoresis separate the isolated sperm antigens :

Two dimensional gel electrophoresis was used to separate the sperm antigens. About 15 ul of sonication isolated antigens plus 15 ul of sample buffer was applied in to each IEF sample well. The first dimensional was an isoelectric focusing gel and the second dimensional was an SDS-PAGE gel. The gel was silver stained and scanned using the Collage Image Analysis Software. The actual pH gradient that built up was measured. The result of the separation is shown in figure 1. The pH gradients for most runs were from 4.5 to 8.0. From this figure we can see that the sperm antigens were separated successfully. More than forty protein dots can be identified from the gel following the detection by silver staining. If better computer image analysis system was available, more proteins dots are expected to be identified. Those proteins dots

were distributed across the whole gel area, but more concentrated in the range of pI's from 5.0 to 7.6. The molecular weights of those proteins were varied from very low molecular weight to very high molecular weight. The original protein concentration that was applied onto the two dimensional gel was about 1.27 mg/ ml. Even though this concentration is high enough for most one dimensional electrophoretic analysis, but it was still not high enough for two dimensional gel analysis. Not all types of proteins contributed equally to the total protein concentration. Some proteins have relatively high concentrations so they show very dark dots on the silver stained gel. In contrast, some protein concentrations were very low, so the dots barely showed up. Those low concentration proteins dots can be seen during and right after silver staining, but disappeared during the scanning process using computer. Other dots showed ample silver staining intensity and did not disappear even after being kept in DH_2O for a long time. These results suggest that if the original sperm antigen concentration could be further increased, additional stable dots could be resolved after silver staining.

Western Blotting Using A Human Serum :

After separating the sperm antigens on the gel using two dimensional electrophoresis, a wet transfer method was used to transfer separated antigens to the PVDF membrane in order to carry out the detection by immunoblotting. After blocking those unspecific binding sites on the membrane overnight in 5% milk-STBST, the membrane was incubated with human serum 05 (1:50 dilution in 1% milk-STBST) for one and one

half hour. Serum 05 came from a involuntary immunoinfertility patient. This patient has been infertile for more than two years and the serum does contain anti- human sperm surface antigen confirmed by indirect immunobead test. After incubation, four washes with STBST were carried out to wash away the unbound antibodies. Then the membrane was incubated with biotinylated goat-anti-human IgG and IgA mixture for one hour. After washes, the membrane was then incubated with avidin-biotin-peroxidase complex for one half hour. After washes the membrane was subjected to ECL detection. The detection result is shown in figure 2. From this figure we can see that the 05 antisperm positive serum can recognize a single sperm antigen. This antigen is a low molecular weight protein and its isoelectric point is low, too. This protein's molecular weight was estimated by comparison with Bio-Rad molecular weight markers. The isoelectric point of this antigen was calculated using Sigma isoelectric focusing gel standards. The sperm antigen targeted by human serum 05 has a molecular weight about 13 Kd and an isoelectric point of not more than pI= 4.5. The isoelectric point for this protein may be even lower than the number showed here if we use a mixture of different pH range ampholines instead one. (see discussion.)

Western Blotting Using A Monoclonal Antibody :

The western blotting using monoclonal antibody developed in our lab was carried out after wet transfer of the two dimensional gel electrophoresis separated sperm proteins from the polyacrylamide gel onto the PVDF membrane. The monoclonal antibody used was MAb 102 (Tang and Bean, 1997). This monoclonal antibody is one of the

monoclonal antibodies that developed in our lab that target sperm surface antigens. The affinity of this monoclonal antibody for the sperm surface has been separately characterized. And more interesting, this antibody blocks sperm penetration into the zona pellucida free hamster eggs (Tang and Bean, unpublished data). During the immunoblotting, the undiluted monoclonal antibody supernatant was incubated with the experimental membrane for one and one half hour, then after washes with STBST, the membrane was incubated with biotinylated goat anti-mouse IgG for one hour (since this monoclonal antibody was generated in mice so the secondary antibody used should be anti-mouse IgG). After four washes with STBST, the membrane was incubated with avidin-biotin-peroxidase complex for one half hour. The membrane was washed four times and subjected to the very sensitive ECL method. The control membrane was made by incubating with 1% milk STBST instead of monoclonal antibody as the primary antibody, then every other steps were identical as the experimental membrane. The result are shown in figure 3.

The control membrane showed the unspecific binding pattern for secondary antibody alone. The experimental membrane showed two clear spots that did not appear on the control membrane, suggesting that monoclonal antibody 102 can recognize two sperm antigens. Interestingly, even though they have different isoelectric points (which are about 6.7 and 7.6), but these two sperm antigens do have the identical molecular weight which are about 34 Kd. This result will be further discussed in the discussion section.

Discussion:

This project was designed to develop a two dimensional gel electrophoresis system for our lab, and to apply it in the characterization of important antigens of human sperm.

Several investigators who work on two dimensional gel electrophoresis for sperm are using whole sperm extract as the start material. This approach achieves high enough sperm protein concentration to carry out two dimensional gel electrophoresis easily. But the major disadvantage of this approach is that the whole cell protein extract may be contaminated with the nucleic acid which can interfere with the isoelectric focusing process. The other method people used to isolate sperm antigens is nitrogen cavitation followed by differential centrifugation. This method has been used successfully to obtain plasma membrane vesicles from sperm of boar (Gillis et al, 1978; Peterson et al, 1980), rat (Olson et al, 1987) and man (Mark et al, 1986; Chong Xu et al, 1994). In the two papers that addressed the use of nitrogen cavitation in man have shown the electron microscopy pictures that demonstrated clearly that the sperm membrane proteins can be separated successfully using this method.

It has been confirmed by scientists that not only sperm plasma membrane antigens that play important roles in fertilization, some antigens that located on the inner acrosomal membrane and inside acrosome (such as SP-10) also involved in critical fertilization steps. Sperm membrane particles may have lost some important antigens. So it is preferable to prepare a mixture of sperm antigens that contains sperm surface proteins

and acrosomal proteins, but does not contain sperm nucleic acids.

The modified nitrogen cavitation method was tried in our lab. Nitrogen cavitation was used to break cells without using differential centrifugation to get membrane vesicles. Such extracts are expected to contain a mixture of sperm proteins that from membrane and acrosome. But the major shortcoming of this method is that it is very hard to monitor the process since it is carried out in a closed chamber. Also it needs special instrument and nitrogen gas and it is very hard to release sample from the high pressure chamber without losing some of them.

Sonication was then tried to substitute the nitrogen cavitation based on the idea that both methods are dependent on the physical force to break down the cells. Compared to nitrogen cavitation, sonication is easier to conduct and monitor. So sonication was used combining with carefully monitoring using light microscopy during the whole process.

For two dimensional gel electrophoresis, an ISO-DALT system was used. But instead of using a traditional tube gel to carry out isoelectric focusing, a slab gel was used. It was difficult to make identical mini- isoelectric focusing tube gels even if multiple gels were made at the same time. Sometimes the air bubbles were trapped at the end of the tubes and this could be found out only after the gels were polymerized. And because those tubes are so tiny which make it very hard to avoid trapping air bubbles during loading the samples. The slab gel was found easier to make, more uniform and easier to observe. The sample loading and gel apparatus cleaning are not problems any

more. Also one major problem of isoelectric focusing is the reproducibility of the system. Using a slab gel, all the samples are separated in the same gel under the identical conditions, thus giving more reproducible separations than can be obtained using tube gels. So the problem of reproducibility is solved easily. Since isoelectric focusing can be done once for ten gels altogether by using slab gel, time is saved.

The sperm antigens were separated using two dimensional gel electrophoresis as shown in the result section. By analyzing the silver stained gel, more than 40 protein dots can be identified. It has been reported that some labs can separate more than 60 sperm antigens after nitrogen cavitation, but they utilized several kinds of ampholines instead of one kind. In this project, only one broad range ampholines (pH 3.0-10.0) was used. During the present investigation, this ampholines alone can not build a broad pH gradient, say from pH 3 to pH 10. The best pH gradient obtained was pH 4.5- pH 8. In order to force separation at the very acidic and very basic range, it is necessary to use a mixture of different kinds of ampholines. For example, ampholines with pH range from 2 to 5 should enhance the pH gradient at the acidic pole, and ampholines with pH range from 8 to 10 can build a better pH gradient at the basic pole. So by using a mixture of different kinds of ampholines, proteins can be successfully separated in the broader pH range, and better resolution can be achieved. Also another way to improve the separation is to use a gradient gel instead of a discontinuous gel in the second dimension. Based on our lab's experience, using gradient gel does improve separation, but not a lot. Also based on some researches

that the final quality of the two dimensional gel separation is very dependent on the degree of resolution obtained in the first dimension. (Rickwood et al, 1990). So the discontinuous gel was kept using in the second dimension of the system.

Silver staining can detect proteins which concentration in the nanogram range. Although total amount of proteins applied into each well of 2D gel is about 2 ug, since the solution contains so many different kinds of proteins and each kind of protein has different concentration, it is highly possible that some protein concentrations below the limit of silver staining and could not showed up on the final stained gel. Increasing the concentration of proteins mixture always help improve the resolution of the system.

By comparison to similar systems, ie, using one kind of ampholines in the first dimension and discontinuous gel in the second dimension, eg. Naz et al, 1986, this resolution is not bad at all.

The sperm antigen array on the two dimensional gel system were targeted via western blotting using either sera that came from immunoinfertility patients or monoclonal antibodies that against sperm surface antigens that were developed in our lab. The serum 05, donated by an immunoinfertility patient recognizes a single protein spot on the two dimensional electrophoresis system. This interesting sperm protein has a molecular weight about 13 Kd and is acidic with an isoelectric point not more than 4.5. This is a very interesting discovery. A new report from John Herr's group (Diekman et al, 1997) also identified a sperm antigen SAGA-1 that can be recognized

by the monoclonal antibody S19. Monoclonal antibody S19 has been demonstrated to agglutinate human spermatozoa, inhibit sperm penetration of cervical mucus, and inhibit sperm-zona binding. The SAGA-1 antigen recognized by this antibody has important roles in the fertilization and remains a strong candidate for an immunocontraceptive vaccine. The SAGA-1 antigen turned out to be a small protein with molecular weight about 15 Kd and an isoelectric point of about 2.5-3.0. The protein that identified by serum 05 is very similar to SAGA-1 since they have similar molecular weight and they are both acidic proteins. The pI reported here is about 4.5, that is the lowest pI that I can determine in my system. In future experiments, by using a mixture of ampholines to enhance the resolution at acidic side, it is possible that the actual pI of this protein may be revealed to be lower than 4.5. If the protein identified by serum 05 turned out to be the same protein as SAGA-1, that would be further confirm its importance, especially since the naturally generated antibody that against this protein is tightly associated with immunoinfertility.

Other interesting sperm antigens were also identified using monoclonal antibodies that developed in our lab (Tang and Bean, 1997). The MAb used here is 102, which has shown positive for immunolocalization on the sperm surface and was found can efficiently block sperm penetration into the zona-free hamster eggs (Tang and Bean, unpublished data). The results of western blotting using this monoclonal antibody are very interesting. This monoclonal antibody recognizes two protein spots on the two dimensional gel. More interestingly, those two proteins have the identical molecular

weights which are about 34 Kd, but they have different isoelectric points. The pI for one protein is about 6.7 and pI for the other is about 7.6. On a one-dimensional SDS-PAGE gel, MAb 102 would identify a single protein band since these two proteins have identical molecular weight. This observation further demonstrates the importance of the two dimensional system for characterization of sperm antigens. The simplest explanation is that the two proteins identified by monoclonal antibody 102 are isoforms of the same protein. The different charges of proteins usually arise from their charged carbohydrate side chains, many sperm proteins have been reported to undergo modifications of their polysaccharide components, and such changes may occur during the final stages of sperm maturation and fertilization-related events. The idea that these isoforms differ in their carbohydrate portions can be tested experimentally. Further experiments can be carried out by modification or cleaving of those carbohydrate side chains following by two dimensional gel analysis and western blotting.
Alternatively, it remains possible, although less likely, that the two proteins identified by MAb 102 are fundamentally different. If so, they would be expected to share a common epitope that is recognized by MAb 102. In addition, their similarity in molecular weight would have to be considered a coincidence.

In conclusion, a two dimensional gel electrophoresis system developed for our lab and reasonable resolution was achieved. Initial analytic applications of this system are reported here. An interesting sperm antigen was identified by a serum that came from a immunoinfertility patient. This protein is similar to the antigen SAGA-1 recently

characterized by John Herr's lab. One monoclonal antibody was used as a probe, and it identified two protein spots that have identical molecular weight but different isoelectric points. These observations further demonstrate the importance of two dimensional gel systems for the analysis of sperm antigens.

	IgG			IgA			IgM		
	negative	Positive	percent of positive	negative	positive	percent of positive	negative	positive	percent of positive
TD	218	1 (head)	0	205	2 (head)	0	222	0	0
CB	241	0	0	267	0	0	221	0	0
KW	200	0	0	200	0	0	200	0	0
KO	204	0	0	200	0	0	208	0	0
LD	209	0	0	213	0	0	200	0	0
KN	240	0	0	209	0	0	225	0	0

Table1 The direct immunobead test results for the six donors sperm which were used to isolate the sperm antigens for two-dimensional gel analysis.

	Negative cells	Positive cells	Percent of Positive
IgG	65	47(head, midpiece)	42%
IgA	164	14(tail)	7.8%
IgM	92	65	41.4%

Table2. The indirect immunobead test result for human serum 05. According to WHO criteria, percentage of cells bound by beads (positive cells) larger than 20% is considered as positive. So human serum 05 does contain anti-sperm surface antibodies. The antibodies isotyes are mainly IgG and IgM.

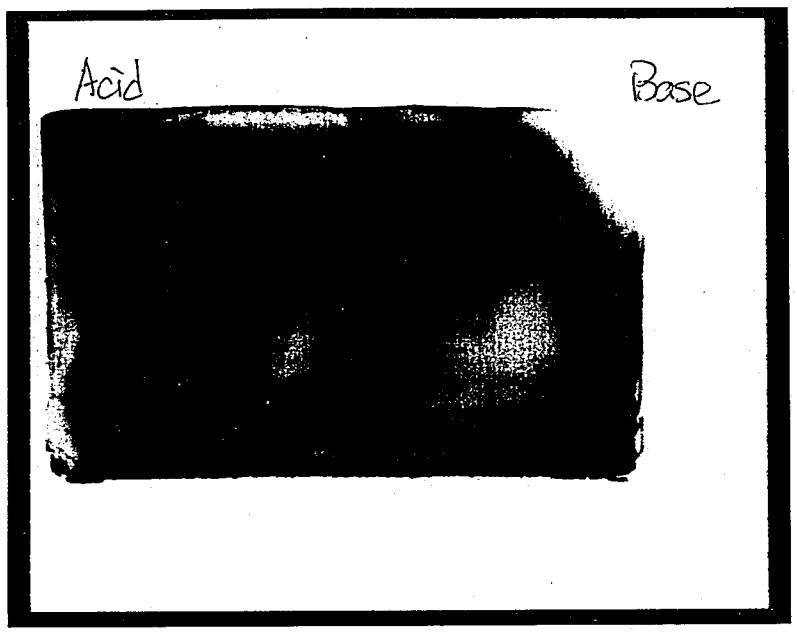

Fig 1. Sperm antigens separated by two-dimensional gel electrophoresis. Sperm antigens were isolated using sonication. The gel was silver stained and was scanned using collage image analysis software. More than forty protein spots can be identified.

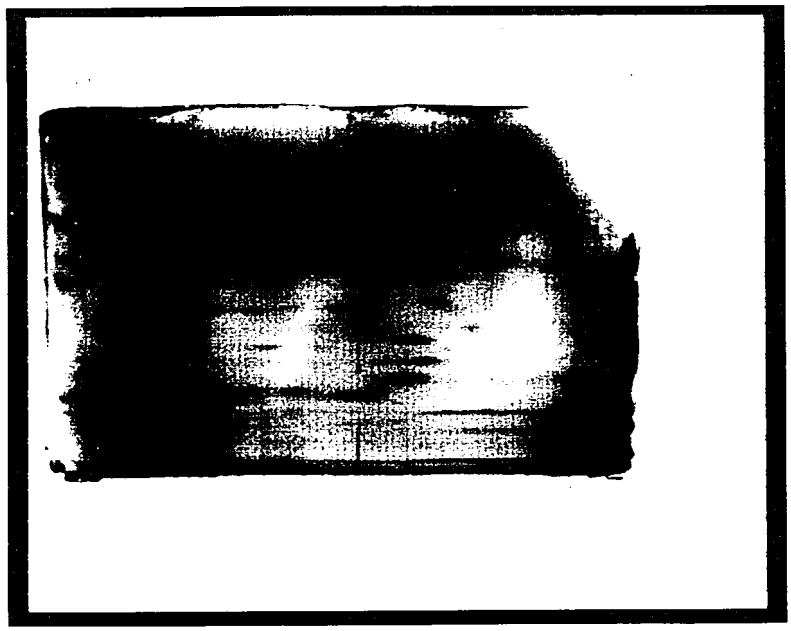

Fig 1. Sperm antigens separated by two-dimensional gel electrophoresis. Sperm antigens were isolated using sonication. The gel was silver stained and was scanned using collage image analysis software. More than forty protein spots can be identified.

Fig2. Sperm antigen identified using human serum 05. Sperm antigens were separated by two-dimensional gel electrophoresis and transferred onto PVDF membrane. Immunoblotting was carried out using human serum 05 and the result was detected using sensitive ECL method.

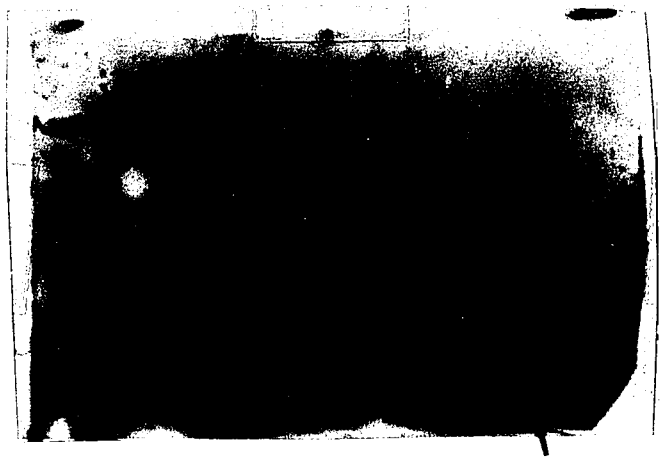

Fig2. Sperm antigen identified using human serum 05. Sperm antigens were separated by two-dimensional gel electrophoresis and transferred onto PVDF membrane. Immunoblotting was carried out using human serum 05 and the result was detected using sensitive ECL method.

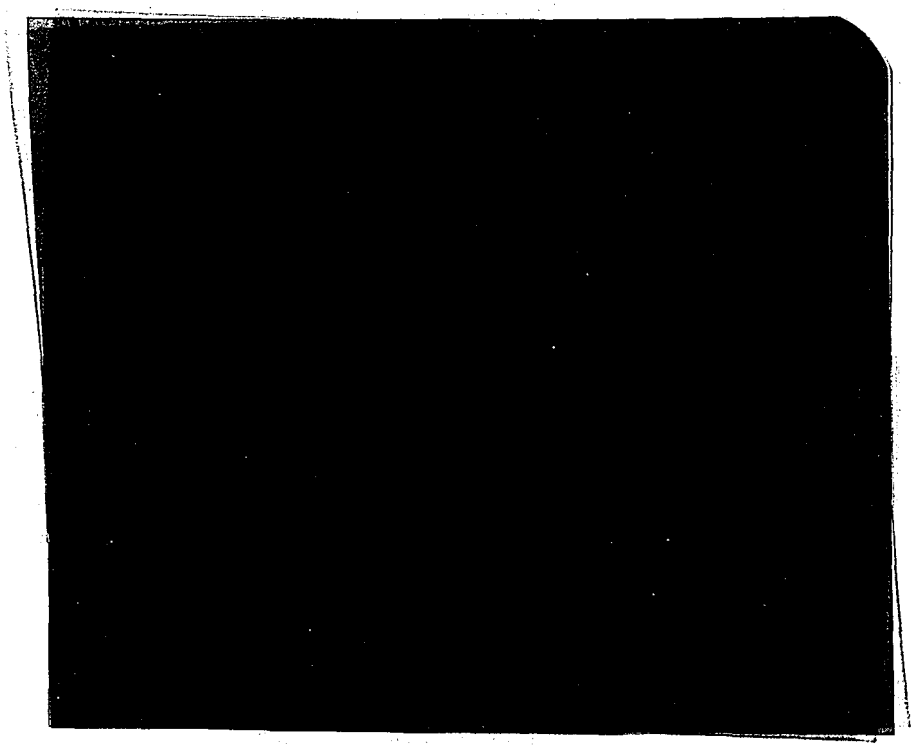

Fig3. Sperm antigen identified by MAb 102. Sperm antigens were isolated using sonication and separated by two-dimensional gel electrophoresis. Sperm antigens were transferred to PVDF membrane and immunoblotting was carried out using MAb 102. The result was detected using sensitive ECL method. The left-half film shows the control membrane without primary antibody. The right-half film shows the experimental membrane.

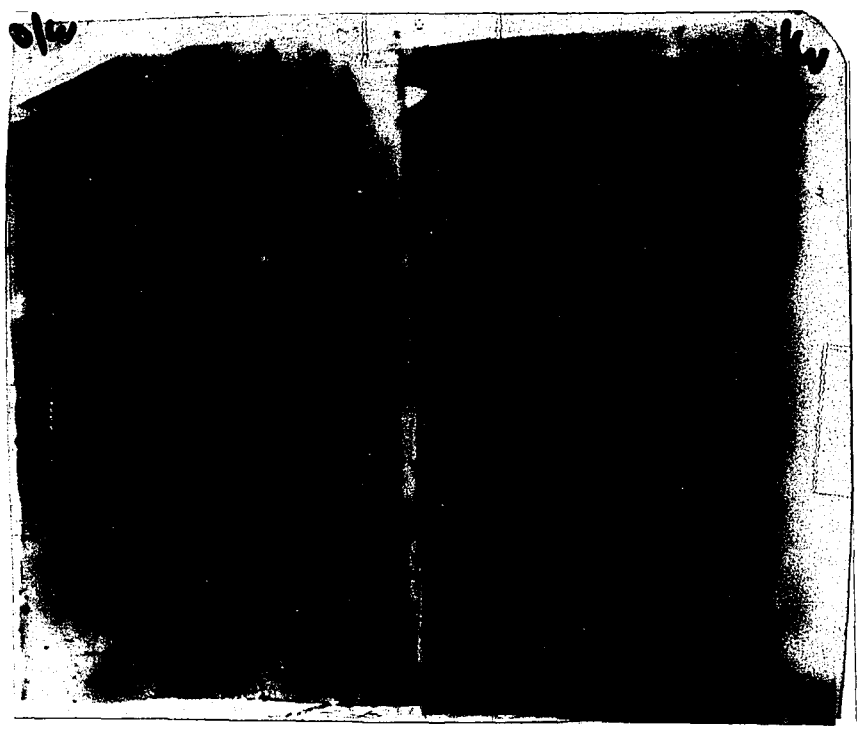

Fig3. Sperm antigen identified by MAb 102. Sperm antigens were isolated using sonication and separated by two-dimensional gel electrophoresis. Sperm antigens were transferred to PVDF membrane and immunoblotting was carried out using MAb 102. The result was detected using sensitive ECL method. The left-half film shows the control membrane without primary antibody. The right-half film shows the experimental membrane.

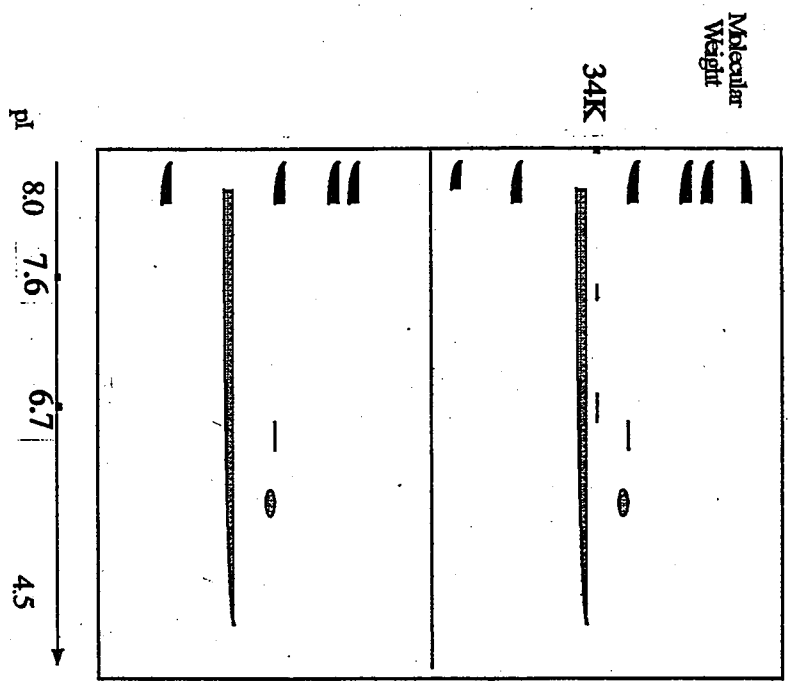

Fig4. A graph represent the film showed on the previous page. The left-half graph shows the control membrane without primary antibody. The right-half graph shows the experimental membrane which using MAb 102 as primary antibody.

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VITA

I was born in Beijing, People's Republic of China , on June 27, 1970. I am the child of Mr. Jierui Xu and Ms. Changqing Ji.

I received my high school education at the High School attached to Beijing University, where I developed my strong interests in biology and medicine. I entered the Beijing Medical University in September 1989 and received the degree of Bachelor of Medicine in July 1994. I worked in the Hematology department of The Third Hospital attached to Beijing Medical University for four months. During those years that I spent in the medical school and clinical hospitals, I found the key to treat all kinds of diseases is to understand the mechanisms of the diseases. In order to understand those mechanisms, more knowledge about the life unit- cells is needed. That was the reason why I attended the Biological Science Department at Lehigh University and started my master studies in August, 1995.

END OF TITLE