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Monoclonal antibodies to human sperm antigens – II

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As part of our continuous effort to elucidate the biochemical and immunological nature of human sperm surface antigens, monoclonal antibodies to human spermatozoa were generated by improved hybridoma techniques. Following immunizations with the membrane fraction of human spermatozoa and cell fusions, hybrid cells were cultured in a semi-solid HAT-selection medium to maximize the number of monoclones recovered. Subcultures were made in liquid phase 7 to 10 days after cell fusions by removing colonies from the initial medium. Based on the results of screening by microplate enzyme-linked immunoassay, 143 of 552 initial clones were found to secrete antibodies to human sperm antigens. More than one-hundred independently derived hybrid cell lines were established. Using indirect immunofluorescent procedures, 62 cell lines were shown to produce antibodies to surface antigens of human spermatozoa. Unique sperm antigens that react with monoclonal antibodies were identified by the SDS gel/protein blot radioimmunobinding method. Sperm agglutinating and immobilizing antibodies were exhibited by 4 and 15 hybrid cell lines, respectively. Fourteen of the monoclonal antibodies also exhibited cross-reactivity with methanol-fixed sperm cells of the rabbit or mouse or both whereas a reaction was not seen with viable sperm of these species. Generation of monoclonal antibodies against a wide spectrum of human sperm antigens should facilitate future investigations regarding immunologic-associated human infertility and fertility control.

Key words: *monoclonals, antibodies, antisperm, spermatozoa, antigens*

Introduction

Autoimmmune or isoimmune responses to human sperm antigens have been associated with human infertility (Shulman, 1971; Menge, 1980). The presence of antisperm antibodies can cause agglutination, complement-dependent immobilization, inhibition of cervical mucus penetration, and interference with ovum interaction of spermatozoa in the reproductive tract, resulting in a reduction of their fertilizing capacity (Rumke and Hellinga, 1959; Menge, 1980).

To elucidate these infertility-associated immunological factors, it is essential to understand the molecular nature and the immunogenicity of sperm antigens. Mono-

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clonal antibodies to surface antigens of human spermatozoa, especially those clinically related sperm auto- and iso-antigens are important tools for such investigations (Lee, et al., 1982a; Bellve' and Moss, 1983).

In a previous communication, we reported our initial attempt to generate hybrid cell lines that secrete monoclonal antibodies to human sperm antigens (Lee et al., 1982a). Out of six hybrid cell lines that were generated, three were shown to secrete antibodies that reacted with the same sperm antigen(s) having a molecular weight of 10,000. However, using the same cloning procedures (Kohler, 1978), subsequent attempts to generate more hybrid cell lines that produced antibodies to other sperm antigens were unsuccessful. When mixtures of sperm antigens are used as immunogens, it is likely that clones secreting antibodies to antigens of high immunogenicity are often recovered by using the early established hybridoma techniques (Kohler, 1978; David, 1982). The drawback of this procedure is that in culture slow-growing antibody-secreting clones may be taken over by fast-growing nonantibody-producing cells resulting in a loss of potentially valuable hybridomas.

This study was undertaken to establish a large number of hybridoma clones reactive with a wide spectrum of human sperm antigens by utilizing a modified cloning procedure and to characterize the resultant monoclonal antibodies by methods of agglutination, cytotoxicity, indirect immunofluorescence and SDS-gel/protein blot.

Materials and Methods

Animal and human semen

Male mice of BALB/c strain (6–10 wk old) were purchased from Jackson Laboratory, Bar Harbor, ME. Pooled human semen samples were obtained from healthy volunteer donors and rabbit sperm from male rabbits collected by use of an artificial vagina.

Chemicals

The analytical grade reagents for acrylamide gel electrophoresis were from Bio-Rad Laboratories, nitrocellulose filter paper from Millipore Corp. (0.45 μm pore size) and iodine-125 (50 mCi/mmole) from Amersham. The following chemicals were purchased from Sigma Chemical Company, St. Louis, MO: chloramine T, sodium bisulfite, Nonidet P-40 (NP-40), methylcellulose, and lipopolysaccharide (*Escherichia coli* 0111B4). Polyethylene glycol (PEG) 1500 was obtained from the British Drug House.

Tissue culture media and supplies

Tissue culture media including Dulbecco's modified Eagle's medium (DMEM), Iscove's medium (IMDM), RPMI 1640 and the supplements; sodium pyruvate, glutamine, hypoxanthine (H), aminopterin (A), thymidine (T) and fetal calf serum were from GIBCO, Canada. The tissue culture plates and dishes were from FLOW Laboratories, and the microtiter plates were from Dynatech, Alexandria, VA.

Myeloma cells, NS-1 were routinely maintained in RPMI 1640 containing 10% fetal calf serum. After the fusion, all hybrid cell lines were grown in a specified culture medium containing 10–20% heat inactivated fetal calf serum, in a 5% CO_2 incubator at 37°C.

Immunizations, cell fusion and cloning

BALB/c mice were immunized with the membrane fraction of human spermatozoa as described previously (Lee et al., 1982). Three days after booster injections, the spleen cells of the immunized mice were fused with NS-1 myeloma cells at a 5 : 1 ratio in 50% PEG and diluted with serum free IMDM. After fusion, the cell mixtures were cultured in a semi-solid IMDM medium containing 1.25% (w/v) methylcellulose, 2% (v/v) fetal calf serum, 1% (v/v) HAT stock (100 × concentrate), 50 μg/ml lipopolysaccharide, 3×10^6/ml thymocytes, 2.5×10^6/ml spleen cells and 5.0×10^5 myeloma cells in Petri dishes. After 7–10 days in culture, the plates were examined for the presence of visible colonies (Davis et al., 1982). Colonies greater than 0.5 mm in diameter were removed by sterile pipette tips and cultured in 24-well culture plates with each well holding 1 ml of RPMI medium containing 15% fetal calf serum and 1% (v/v) HAT stock (100 ×) solution. When the suspension cultures had reached a density of about 1×10^6 cells/ml or higher, the supernatant was checked for the presence of antibodies reacting with human sperm antigens by the microplate enzyme-linked immunoassay (ELISA) described below. Hybrid cell lines were frozen in liquid nitrogen storage tanks in a 40% (v/v) fetal calf serum, 10% (v/v) DMSO and 50% RPMI 1640 mixture.

Microplate enzyme-linked immunoassay (ELISA)

This assay was performed according to the method of Voller et al. (1979). Briefly, sonicated washed sperm were pelleted by centrifugation and resuspended in the wells (1×10^6 cells/well) of microtiter plates with carbonate buffer containing 1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$, 0.2 g/l NaN_3 and 0.5 ml/l Tween-20. After overnight incubation at 4°C, the plates were washed three times with PBS–Tween (phosphate buffered saline, 0.2 g/l NaN_3 and 0.5 ml/l Tween-20). The coated sperm proteins were incubated with 0.5% glutaraldehyde for 1 h followed by three washes with PBS–Tween. The unbound sites in the wells were blocked with 0.5% bovine serum albumin in PBS for 1 h at 37°C. The sperm coated wells were then washed three times with PBS–Tween and finally left at 4°C in PBS.

The enzyme immunoassay was performed by first incubating 100 μl culture supernatant overnight at 4°C followed by three washes with PBS–Tween. Peroxidase labeled goat anti-mouse IgG in PBS containing 0.5% BSA was added to each well for 1 h incubation at 37°C. After three washes with PBS, 100 μl of substrate (containing 0.02% H_2O_2, 0.2% *O*-phenylenediamine (*O*PD) in 0.1 M citrate buffer at pH 5.5) was added to initiate the enzymatic reaction (Voller et al., 1979). After 20 min in the dark, the color reaction was terminated by the addition of 100 μl of 4.5 M sulfuric acid. The optical density of each well was read at 492 nm with an automatic microplate reader. Culture medium for NS-1 and mouse anti-human sperm sera were used as negative and positive controls, respectively.

SDS gel/protein blot radioimmunobinding method

Human sperm antigens that react with the mouse anti-human sperm monoclonal antibodies were identified by the SDS gel/protein blot radioimmunobinding method (Lee et al., 1982a, b). SDS acrylamide gel electrophoresis was performed according to the procedure of Laemmli (1970) using a 10% acrylamide gel. After blotting the protein in the gels to nitrocellulose strips and performing the radioimmunobinding procedure, the human sperm antigens bound specifically by monoclonal antibodies were visualized on the nitrocellulose strips by autoradiography using Kodak X-OMAT AR-2 film.

Indirect immunofluorescence

Immunofluorescent staining of human spermatozoa followed the procedure of Johnson and Menge (1975) using undiluted culture supernatants. Human spermatozoa were examined by three different procedures: fixation by methanol; pretreatment with Triton X-100 and dithiothreitol to induce swollen heads and exposure of nuclear proteins such as protamine (Zirkin and Chang, 1977); and in the viable state. For staining of viable spermatozoa, culture supernatants (25 μl) from different hybrid cell lines were incubated with human sperm samples (4×10^5 sperm) having motility greater than 60% for 1 h at room temperature followed by two washed with PBS. The sperm were finally resuspended in PBS and dried on slides. Appropriately diluted fluorescein-labeled goat anti-mouse IgG + IgM was then added as the second antibody to stain spermatozoa. In all experiments, PBS normal mouse serum, normal mouse ascites fluid and culture supernatant of NS-1 cells were used as controls. Methanol-fixed mouse and rabbit spermatozoa were also used to determine the cross-reactivity of the generated anti-human sperm antibodies with sperm of other mammalian species. Slides were observed under dark field illumination with a Zeiss photomicroscope equipped with a halogen-quartz light system and appropriate filters.

Sperm agglutination and immobilization tests with monoclonal antibodies

To determine the ability of antibodies secreted by hybrid cell lines to agglutinate or to immobilize human spermatozoa, the respective methods of Friberg (1974) and a microadaptation of Isojima et al. (1968) were followed.

Sperm specificity of monoclonal antibodies

Specificity of the supernatants toward sperm was examined using cytotoxicity and immunofluorescent methods on plasma mononuclear cells from ten individuals. The cells were isolated using the Ficoll–Hypaque technique and subjected to immunufluorescence as performed with viable sperm cells. Cytotoxicity was assayed in a 96-well microtiter tray using 50 μl rabbit complement (1 : 2 dilution). After incubation for 1 h at 37°C in 5% CO_2 in air trypan blue was added and incidence of viable cells was estimated. Negative controls as well as a positive control, mouse anti-human sperm serum (1 : 10 dilution) were included.

Results

Cell fusion and cloning

Five-hundred and fifty-two colonies cultured in a semi-solid methylcellulose medium were initially chosen for subculturing 7–10 days after the cell fusion. Among these, 143 clones were recovered that initially secreted anti-human sperm antibodies as judged by the ELISA. Finally, of these, 117 stable hybrid cell lines were established for further biochemical and immunological characterization.

SDS gel/protein blot radioimmunobinding method

The SDS gel/protein blot radioimmunobinding method demonstrated that the supernatants of some hybrid cell lines contained antibodies that reacted with unique human sperm antigens (Fig. 1). The R_f values of the detected protein bands and relative molecular weights are listed in Table 1. As shown in Fig. 1, the majority of these monoclonal antibodies reacted with more than one sperm protein band. Clones 14C2, 20B3 and 20A6 bound as many as six protein bands of different molecular weights, whereas, only one sperm antigen(s) was recognized by antibody secreted by clones 14D2 and 6D5. The monoclonal nature of these hybrid cell lines was tested by subcloning using the limiting dilution method (Kohler, 1978). The multiplicity of the detected sperm antigens on autoradiograms, however, did not change with the recloning.

Indirect immunofluorescent and sperm function assays

Distinct patterns of indirect immunofluorescence due to antibody binding were observed for human sperm prepared by the three different procedures. Out of 117 hybrid cell lines that were positive in the initial screening, 53 were shown to secrete antibodies that reacted with methanol-fixed human spermatozoa (Table 2 and Fig. 2). On the other hand, antibodies from 20 and 19 clones, respectively, were shown to bind the pre-treated sperm with swollen heads and the viable sperm.

The sperm function assays revealed 15 and 4 monoclonal antibodies, respectively, exhibiting immobilizing and agglutinating activities against human spermatozoa (Table 2).

TABLE 1

R_f values of the protein bands that were recognized by monoclonal antibodies to human sperm antigens on nitrocellulose strips by SDS gel/protein blot radioimmunobinding method

Clone no.	R_f Values
A. 4D1, 21A6	0.52, 0.64, 0.75, 0.85
B. 8A2, 21C4, 23B1, 23C2	0.80, 0.85, 0.92
C. 14D2	0.31
D. 14C2, 16C4, 20A6, 20B3, 21D2	0.52, 0.64, 0.73, 0.76, 0.86, 0.92
E. 6D5, 11B1	0.40

The R_f values for the molecular weight standards, bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease are 0.39, 0.55, 0.80 and 0.95, respectively. They have the corresponding molecular weights of 68,000, 45,000, 25,000 and 13,000, respectively.

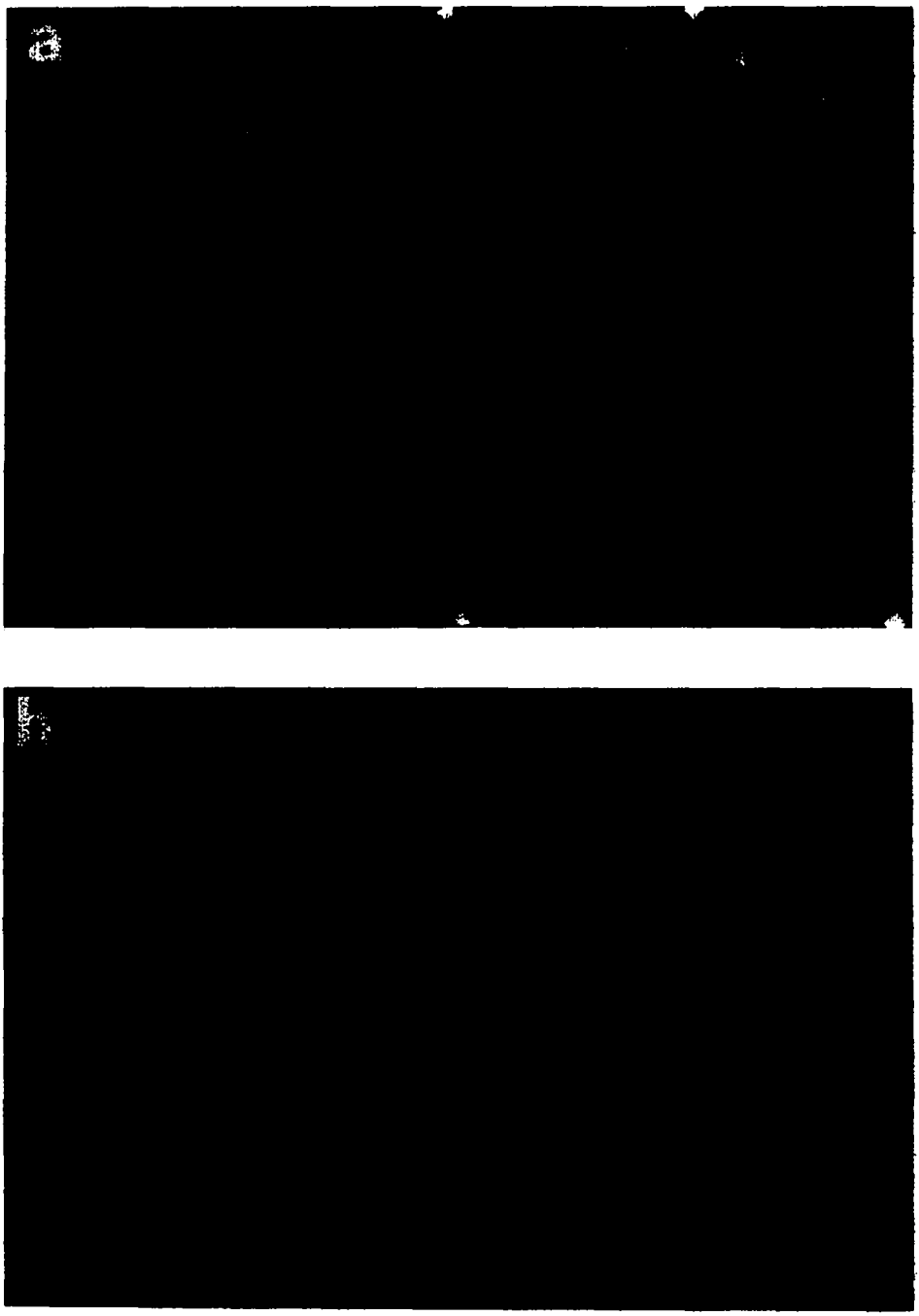
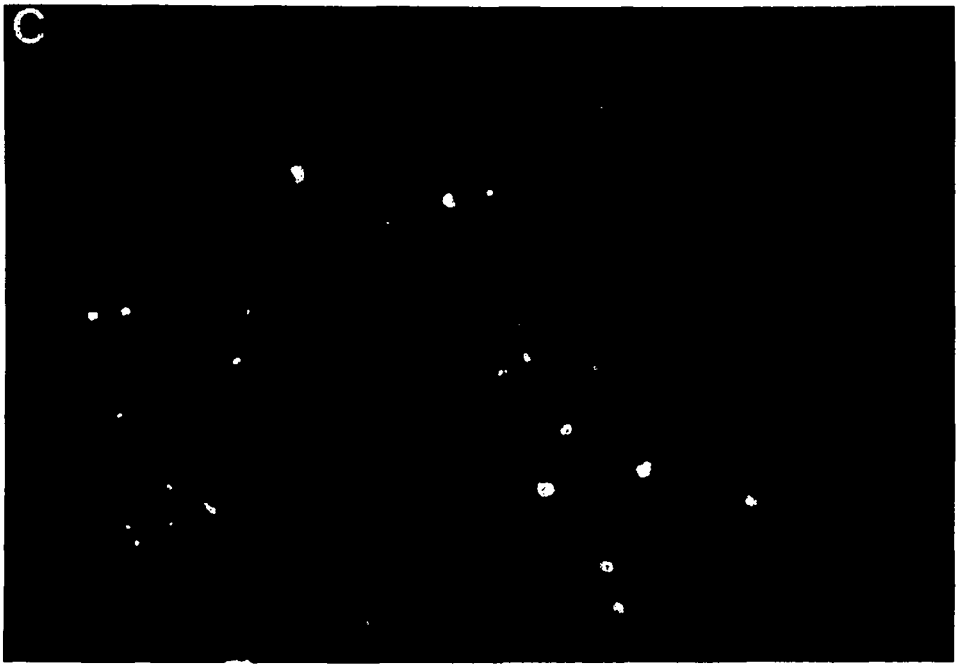


Fig. 1. See page 234 for figure legend.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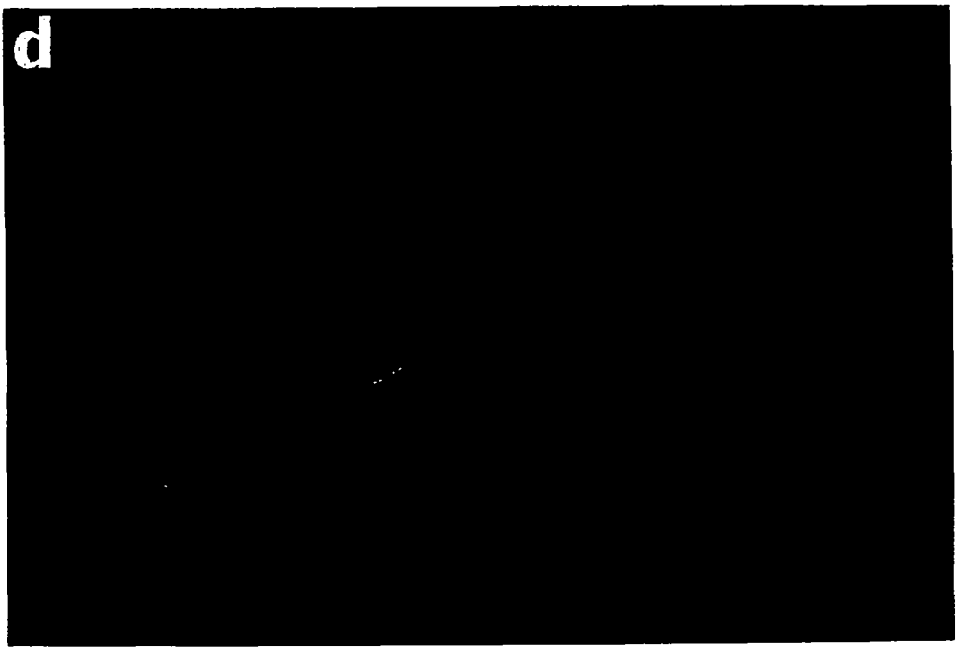
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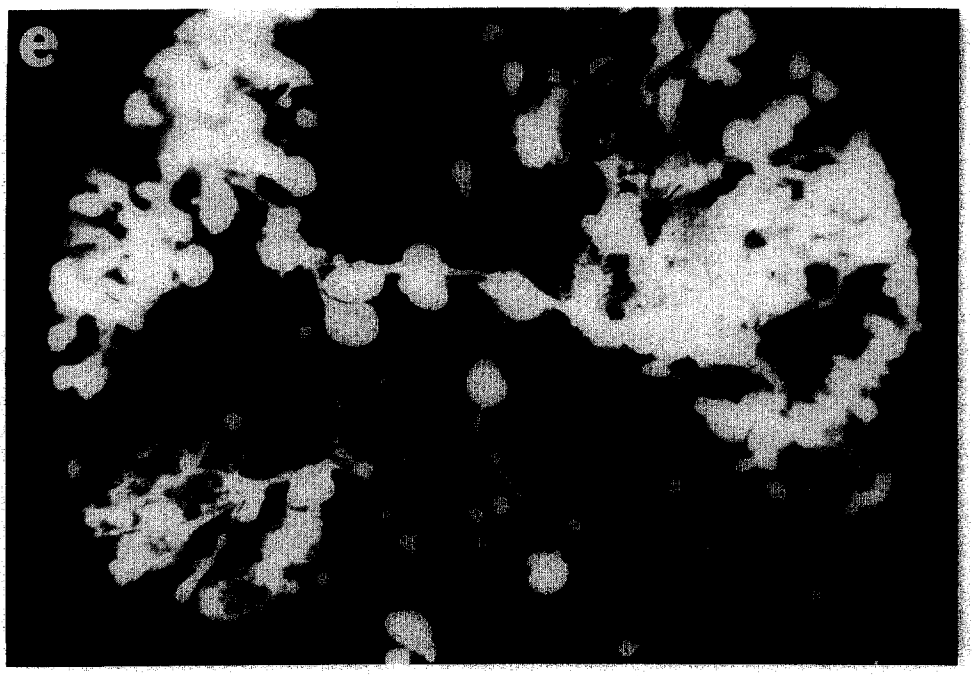

Fig. 1. SDS gel/protein blot radioimmunobinding method to reveal the binding of monoclonal antibodies derived from different hybrid cell lines to human sperm antigens; autoradiograms (24 h exposure) are (A) clone 21A6; (B) 8A2; (C) 14D2; (D) 20A6. (E) 11B1.

In order to determine the specificity of the generated monoclonal antibodies, cytotoxicity and indirect immunofluorescent assays with human lymphocytes were performed. As shown in Table 2, out of 70 monoclonal antibodies that reacted positively with human sperm, cross-reactions against lymphocytes were observed in nine by indirect immunofluorescence, three by cytotoxicity and two exhibited both

TABLE 2

Number of hybrid cell lines that secrete monoclonal antibodies reacting with human sperm and lymphocytes determined by different methods

Sperm Cells						Lymphocytes	
	Indirect immunofluorescence			Agglutination	Immobilization	Cytotoxicity	Immunofluorescence
	Methanol-fixed	Swollen sperm	Viable sperm				
Total	117	117	117	117	117	70	70
Positive	53	20	19	15	4	3	9
Sperm-specific positive	47	19	16	11	3	–	–

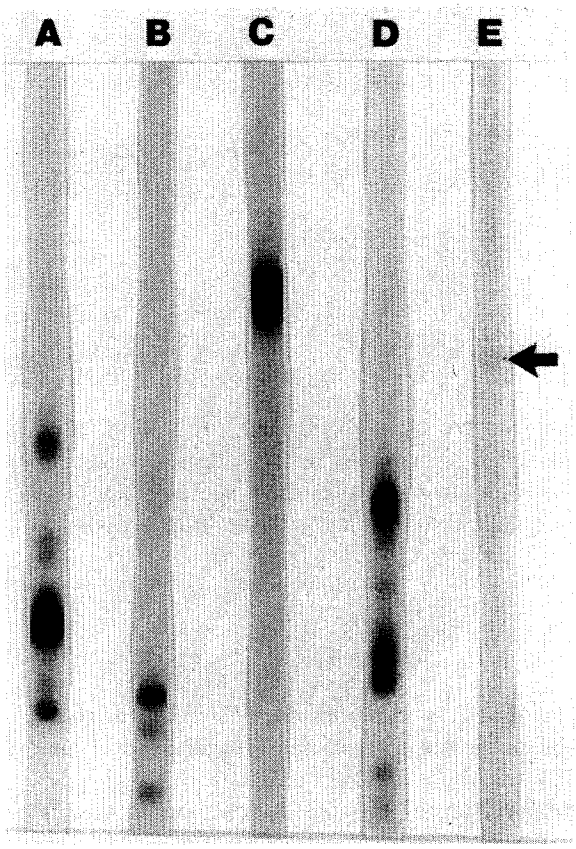

Fig. 2. Indirect immunofluorescence to reveal the localizations of binding of monoclonal antibodies on human spermatozoa upon incubations with the culture supernatants of the generated hybrid cell lines. (A) Staining on acrosomes (methanol-fixed sperm); (14D2). (B) Staining on equatorial segment (methanol-fixed sperm); (21D3). (C) Staining on neck, midpiece and tail (methanol-fixed sperm); (2A5). (D) Staining on whole sperm (viable sperm); (1A5). (E) Staining on swollen human sperm heads and tails (sperm pretreated with Triton X-100 and dithiothreoitol); (21A6).

reactions leaving a total of 60 supernatants that reacted with sperm only and not lymphocytes.

Similarly, by the same immunofluorescent assay the monoclonal antibodies were examined for binding to specific structures on spermatozoa from human, murine and rabbit (Table 3). The different regions of spermatozoa including the plasma membrane, head, acrosome, acrosomal cap, equatorial segment, post-acrosome area, neck, midpiece and tail were stained by the antibodies. Whereas, some antibodies reacted with one cytological domain of spermatozoa, the majority stained two or more sites in numerous combinations. None of the monoclonal antibodies was observed to stain the plasma membrane of viable sperm of the mouse or rabbit. A significant number of the antibodies, however, did cross-react with methanol-fixed sperm of these animals even though they did not always bind identical regions of the sperm cells among the three species (Table 4).

TABLE 3

Number of sperm-specific monoclonal antibodies that react with sperm cells by indirect immunofluorescent assays

Species of sperm	Methanol-fixed									Swollen sperm		Viable sperm	
	PM	HD	AC	AcCAP	ES	PAC	NK	MP	T	HD	MP/T	HD	MP/T
Human	18	4	8	4	7	6	20	6	24	15	9	8	11
Murine	6	0	1	1	0	0	0	9	2	NT		0	
Rabbit	0	0	1	3	1	0	0	6	2	NT		0	

Abbreviations used: PM = plasma membrane; HD = head; AC = acrosome; AcCAP = acrosomal cap; ES = equatorial segment; PAC = postacrosome; NK = neck; MP = midpiece; T = tail; NT = not tested.

Some of the monoclonal antibodies were against antigens of seminal plasma origin. After absorption (v/v basis for 1 h at 37°C) with seminal plasma from vasectomized patients, 7 out of 11 antibodies retained their sperm immobilizing activities but only one out of three still maintained its agglutinating properties to humam spermatozoa.

Discussion

Producing monoclonal antibodies against a complex antigen mixture, such as sperm cells, the problem arises of obtaining a sufficient number of different hybridomas secreting antibodies toward those cellular antigens that may be of interest. If antibodies against a wide spectrum of antigens are obtained they can be

TABLE 4

Comparison of the immunofluorescent staining of sperm structures of human, murine, and rabbit by monoclonal antibodies against human sperm

Clone	Species of sperm		
	Human	Murine	Rabbit
2A5	NK, MP, T	HD, MP	Neg
3C1	MP, T	HD, MP	MP
4D1	MP, T	MP	Neg
7A3	NK, MP	MP	Neg
14D4	MP, T	HD, MP	T
17B5	PM, NK, MP, T	HD, MP, T	MP, T
17B6	AC, ES, MP, T	HD, MP, T	Neg
19D5	PM, NK, MP	HD, MP	MP
20C4	PM, AC, MP	Neg	AcCAP, MP
20D3	AC, ES	AC	AC
21A3	NK, MP, T	Neg	MP
21D2	PM, AC, NK, MP, T	Neg	AcCAP
21D3	ES	AcCAP	AcCAP
22A3	NK, MP	MP	MP

Abbreviations from Table 3.

screened by relatively easy and quick assays, i.e. in our studies by immunofluorescence, Western-blot, sperm agglutination and sperm immobilization, to select promising clones for further investigation utilizing biological or functional assays and finally immunoaffinity techniques to isolate specific antigens for characterization.

We have employed a modified procedure (Davis et al., 1982) to generate more than one-hundred hybrid cell lines that secrete monoclonal antisperm antibodies. After cell fusion, hybrid cells were cloned in a semi-solid selection medium using methylcellulose as the support instead of the conventionally used liquid medium (Kohler, 1978). Since after fusion, each hybrid cell grows independently as a single colony in a semi-solid phase, no mutual interference among colonies occurs in contrast with liquid media in which the slow-growing clones are often taken over by the fast-growing ones. Furthermore, this modified procedure minimizes the laborious work of limiting dilution for subclonings (Kohler, 1978; Davis et al., 1982). Colonies removed for subculture are clearly distinguishable under the dissecting microscope, if they are at least 0.75 mm apart. Under these conditions, the probability that a picked clone is not monoclonal was estimated to be less than 4% (Davis et al., 1982).

By the SDS gel/protein blot radioimmunobinding method, molecular size of sperm antigens that react specifically with monoclonal antibodies can be determined (Lee et al., 1982b). Similar to the previous observations (Lee et al., 1982a), the majority of the generated antibodies in supernatants of the hybrid cell lines revealed no apparent binding to sperm antigens on nitrocellulose strips, even though they showed significant binding to sperm antigens by the microplate enzyme-linked immunoassay and indirect immunofluorescence study. Several possibilities were proposed previously (Lee et al., 1982a) to explain this phenomenon.

On the other hand, some monoclonal antibodies exhibited binding to more than one sperm protein band. This observation clearly indicated that the same antigenic determinant(s) is frequently shared by more than one sperm protein. After extensive subclonings by limiting dilution, the derived antibodies had identical immunoblot binding patterns to those of the pre-subcloned cell lines substantiating the monoclonal nature of the antibodies generated by the present procedure.

The indirect immunofluorescent procedure demonstrated that at least one-half of the generated monoclonal antibodies reacted with different surface regions of human spermatozoa. Interestingly, while none appeared to react with surface antigens from rabbit or mouse spermatozoa some showed cross-reactivity with subsurface antigens suggesting that a high degree of homology of sperm antigens or antigenic determinants exists among different mammalian species. Since these antibodies were of mouse origin it is apparent that some of the human sperm antigens (or their respective antigenic determinants) are autoimmunogenic in mice. The results of this study raise further questions regarding the conservative nature of sperm antigenic determinants among different mammalian species.

The cytotoxic and indirect immunofluorescent assays revealed that the majority of these monoclonal antibodies were sperm-specific and few showed cross-reactivity with human lymphocytes. Further assessment of the sperm-specificity of these antibodies needs to be determined using other tissues before final studies are undertaken.

Some monoclonal antibodies were shown to cause agglutination and/or comple-

ment-dependent immobilization of live human spermatozoa in vitro. The results suggest that monoclonal antibodies can exhibit similar actions and specificities to those of the naturally occurring antisperm antibodies in human beings; even though they only react with one single determinant of sperm surface antigens.

In summary, a collection of monoclonal antisperm antibodies was generated by this facile procedure from which a selected group will be further evaluated for effects on sperm function utilizing assays with cervical mucus and zona-free animal ova. Those antibodies demonstrating significant effects will serve as tools for isolation of sperm antigens for future investigations on immunologic infertility and fertility control.

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