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Acrosomal Status Evaluation in Human Ejaculated Sperm with Monoclonal Antibodies

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ABSTRACT

An important question in mammalian gamete physiology concerns how capacitation and the occurrence of acrosome reactions in motile sperm relate to fertility. Evaluation of these relationships has been restricted by practical limitations because rapid, quantitative assays are unavailable. We have developed a rapid, reproducible assay for the evaluation of acrosomal status utilizing monoclonal antibodies specific to antigens localized in the acrosomal cap region of the sperm head. Mice were immunized with human ejaculated sperm preparations and the resultant hybridomas producing antisperm antibody were selected by solid-phase radioimmunoassay and indirect immunofluorescence (IIF). Two monoclonal antibodies (HS-19, HS-21) recognized target antigens restricted to the acrosomal cap by IIF, and 87 ± 8.5% of the sperm in fresh ejaculates from 10 different sperm donors showed positive cap fluorescence with these reagents. Loss of HS-21 binding as measured by IIF was correlated with disappearance of the acrosomal cap as observed directly by transmission electron microscopy. Acrosomal disappearance, artificially induced in vitro using the calcium ionophore A23187, also resulted in a loss of HS-21 binding. The induction of acrosomal loss by ionophore was dependent upon extracellular calcium. The data presented suggest that specific monoclonal antibodies can be used for the rapid evaluation of acrosomal status in mammalian sperm.

INTRODUCTION

Capacitation and the occurrence of an acrosome reaction in motile mammalian sperm are absolute prerequisites to fertilization since only capacitated sperm bind to the egg's zona pellucida and only motile acrosome-reacted cells penetrate this acellular encasement and undergo fusion with the egg (Yanagimachi, 1981). Capacitation per se is only poorly understood, but probably involves the removal and/or alteration of sperm membrane components in the anterior sperm head such that an acrosome reaction can occur, i.e., the loss of the outer acrosomal membrane and the overlying plasma membrane through membrane fusion, vesiculation, and sloughing. Since a precise definition of capacitation is unavailable, this process is usually defined in operational terms by in vitro bioassays that quantitate sperm-egg binding, zona penetration, and/or sperm-egg fusion. In humans, limitations associated with the use of mature human oocytes have resulted in assays measuring penetration of zonae of nonviable human ovarian oocytes (Overstreet and Hembree, 1976; Overstreet et al., 1980) or fusion with the zona-free hamster egg (Yanagimachi et al., 1976). These bioassays are cumbersome and suffer from the disadvantage that the entire sperm population is evaluated on the basis of the performance of only those few cells that actually undergo penetration or fusion.

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In capacitated sperm, loss of the acrosomal cap during the acrosome reaction is a morphologically distinct event that can be visualized readily by light microscopy in those mammalian species with large acrosomes, such as the guinea pig and the hamster (Yanagimachi, 1981). However, in many mammals, the human included, the acrosome is small and available staining techniques, including the use of labeled lectins (Koehler, 1978; Talbot and Chacon, 1980), have not met with widespread application. Monoclonal antibodies are recognized as highly specific reagents ideally suited to the detection of subcellular domains, and their application to the dissection of the individual events that comprise mammalian fertilization has been described (Feuchter et al., 1981; Myles et al., 1981; Bellvé and Moss, 1983; Moore and Hartman, 1984).

MATERIALS AND METHODS

Production of Monoclonal Antibodies

Monoclonal antibodies were produced by immunizing a young adult BALB/cByJ female mouse (Jackson Lab, Bar Harbor, ME) with human sperm obtained from proven fertile males: $1-3 \times 10^7$ washed sperm were injected subcutaneously on Days 0, 26, 42, and 114 [antigen in complete Freund's adjuvant, incomplete Freund's adjuvant, Dulbecco's phosphate-buffered saline (PBS; Dulbecco and Vogt, 1954), and PBS, respectively]. On Days 175, 176, and 177, 3×10^7 sperm, harvested from whole semen in a swim-up into an overlay of bovine serum albumin (BSA) and washed in PBS, were injected intravenously in PBS. Splenic lymphocytes were fused with the mouse myeloma cell line SP2/0 [which does not produce immunoglobulin (Ig) heavy or light chains (Shulman et al., 1978)] on Day 178 by the method of Kennett (1980). Antibody was produced in serum-free medium (Muraleami et al., 1982). HS-21 antibody was affinity purified on a protein A column according to Ey et al. (1978), with binding at pH 8.0 and elution at pH 5.5.

Indirect Immunofluorescence

For all experiments in this study ejaculates were obtained from healthy adult donors by self-masturbation after 24–48 h of sexual abstinance, and allowed to liquify at 37°C for 0.5–1 h. All ejaculates displayed densities of 20 million/ml or greater with at least 60% normal morphology and at least 50% motile cells. Cells were washed 3 times by centrifugation-resuspension (700 × *g* for 7 min) in PBS, fixed for 15–30 min at 24°C in 1% paraformaldehyde in PBS (pH 7.4), and washed twice with 0.2 M glycine to block free aldehydes (Koehler et al., 1980). For indirect immunofluorescence sperm were air dried onto individual wells defined by Mylar tape on slides and washed 3–5 times with 0.01% NP-40/0.1% 2-mercaptoethanol/1% BSA. Aliquots (5 μl) of HS-21 antibody were added to each well, and the slides were incubated at ambient temperature for 1 h in a humidified chamber. Cells were washed as above, and 5 μl of second antibody were added (fluorescein isothiocyanate-goat antimouse IgG obtained as an affinity-purified product from Kirkegaard and Perry, Gaithersburg, MD). Cells were incubated for a further 1–2 h, washed as above, mounted in glycerol containing 0.2 M *n*-propylgallate (Giloh and Sedat, 1982), and examined under oil at 500× with a Leitz Dialux 20 microscope equipped with epifluorescence optics. Cells demonstrating uniform specific fluorescence restricted to the acrosomal cap region of the sperm head were considered positive (acrosome intact); cells with no detectable specific fluorescence over the acrosomal cap region were scored as negative (acrosome reacted).

Ionophore Induction of Acrosomal Loss

Ejaculated sperm were washed 3 times with BWW medium (Biggers et al., 1971) containing 0.3% human serum albumin (HSA). Cells were incubated for 3 h at 37°C under capacitating conditions (BWW with 3.5% HSA), washed, and then exposed to BWW containing 3.5% HSA/10 μM A23187 or BWW with 3.5% HSA and dimethylsulfoxide (carrier for A23187). To determine the extracellular calcium ion dependancy in ionophore-induced acrosomal loss, cells were washed 3 times with calcium-free BWW containing 0.3% HSA and then incubated for 3 h in: 1) calcium-free BWW containing 1.25 mM ethylene glycol-bis-(3-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; or 2) BWW containing 1.7 mM, 3.4 mM, or 5.1 mM calcium chloride. All incubation media contained 3.5% HSA. Following incubation, ionophore A23187 was added to a final concentration of 10 μM. Sperm were processed for indirect immunofluorescence evaluation of acrosomal status 3 h after ionophore addition.

Transmission Electron Microscopy

Three hours after ionophore addition, cells were washed 3 times with PBS, fixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (NaCac) at pH 7.4, washed at 4°C in 0.1 M NaCac, postfixed in 1% OsO_4 in 0.05 M NaCac, dehydrated in a graded series of alcohols and acetone, and embedded in Spurr's resin. Thin sections were stained with lead citrate and uranyl acetate, and examined with a JEOL 100C transmission electron microscope. Only sperm in which the entire head could be viewed in a sagittal or parasagittal plane were evaluated for acrosomal status.

RESULTS

In the present study, of the 10 stable hybridomas produced by immunization with ejaculated human sperm that showed antisperm antibody activity in a solid-phase radioimmunoassay using washed ejaculated sperm as the antigen source (Wolf et al., 1983a), two (HS-19 and HS-21) displayed target antigen localization, by indirect immunofluorescence (IIF) assay, to the anterior sperm head. The secreted antibody class for both of these antibodies, as determined by Ouchterlony

double diffusion using class-specific antiimmunoglobulins, was IgG_1 (Bionetics, Kensington, MD). Antibody HS-21 was employed in most of the experiments reported herein.

When examined by fluorescence microscopy after exposure to HS-21 and fluorescein isothiocyanate-conjugated anti-IgG (heavy and light chains), a high percentage (87 ± 8.5%; mean ± SD) of sperm cells recovered from fresh semen of 10 different donors displayed uniform and bright fluorescence over the acrosome (Fig. 1*A*). Those sperm that were negative were presumed to represent sperm with defective or absent acrosomes. This characteristic acroso-

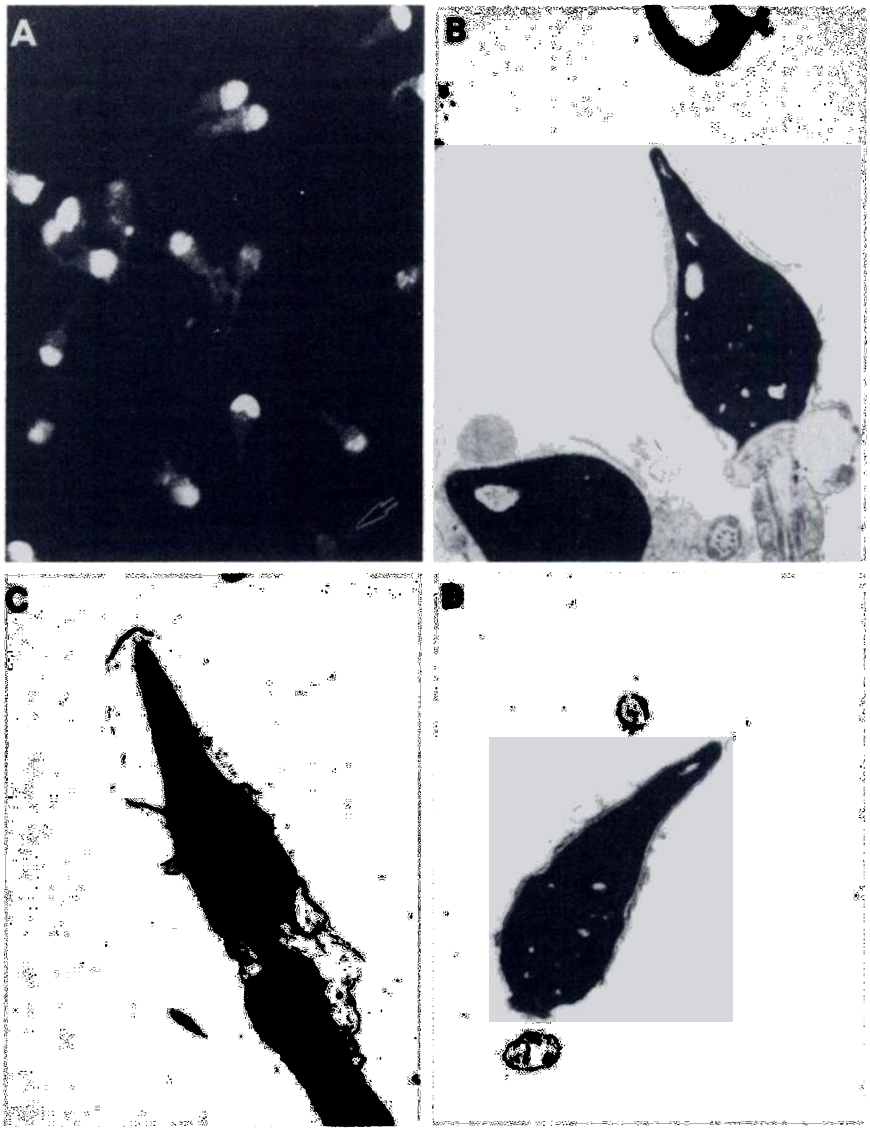

FIG. 1. *A*. Epifluorescence image of human ejaculated sperm. Freshly ejaculated sperm were washed, fixed for 15–30 min, and exposed to HS-21 monoclonal antibody. Following exposure to first antibody, cells were washed, exposed to fluorescein isothiocyanate–conjugated goat antimouse IgG, washed, and examined by epifluorescence microscopy. The *arrow* points to a sperm that is negative for acrosomal cap fluorescence. (×3200) *B–D*. Ultrastructure of human ejaculated sperm exposed to the calcium ionophore A23187. Cells were incubated and exposed to ionophore as indicated in *Materials and Methods*. Three types of cells were observed: *B*) acrosome intact–complete retention of acrosomal contents with intact plasma and outer acrosomal membranes; *C*) intermediate–vesiculated outer acrosomal membrane with retention of an intact plasma membrane; and *D*) acrosome reacted–absence of both plasma and outer acrosomal membranes. (×17,000–17,600)

mal cap staining pattern was completely eliminated when sperm were exposed to 0.04% Triton X-100 for as little as 5 min prior to immunofluorescence staining, a treatment known to result in quantitative acrosomal loss in human sperm (Langlais et al., 1981). No fluorescent staining was observed in the negative control, which was a supernatant from a hybridoma producing the same subclass of immunoglobulin but negative for antisperm antibody activity.

To establish these monoclonal antibodies as reagents suitable for use in the quantitation of acrosomal status in human ejaculated sperm, transmission electron microscopy (TEM) was utilized for independent corroboration of IIF observations. In some cases the calcium ionophore A23187, which is known to induce acrosome reactions in human sperm (Russell et al., 1979; Jamil and White, 1981), was employed to increase the population of acrosome-reacted cells. Following incubation under capacitating conditions, sperm were exposed to 10 μM ionophore in BWW medium (Biggers et al., 1971) containing 3.5% HSA. The motility of sperm exposed to ionophore is dependent upon time and the concentration of ionophore and HSA (Byrd and Wolf, 1984a). In the present instance, the percentage of motile cells dropped to approximately 5.0% by 60 min of treatment. All sperm displaying an entire head in either a sagittal or parasagittal plane were evaluated for acrosomal status by TEM.

Three categories of sperm were distinguished by TEM following exposure to ionophore: 1) acrosome intact (Fig. 1*B*), 2) acrosome reacted (Fig. 1*D*), and 3) an intermediate type in which the outer acrosomal membrane vesiculated with apparent retention of an intact plasma membrane (Fig. 1*C*). This latter response, which constituted a variable but small percentage of the cells examined, was scored as an unreacted cell because the plasma membrane remained intact. For the comparative experiments, summarized in Fig. 2, fresh and in vitro capacitated sperm from three different donors were exposed to ionophore, to media containing only the carrier (dimethylsulfoxide), or to media alone. The cells were then fixed and processed independently for acrosomal status evaluation by TEM or by IIF assay with antibody HS-21. An excellent correlation (r=0.96) was observed between the two approaches, and it is concluded, therefore, that IIF with HS-21 allows the accurate quantitation of acrosomal status.

In this experimental series, a small population of cells was scored as reacted by IIF but not by TEM. This discrepancy between IIF and TEM scoring of acrosomal status was probably due to the sensitivity of the HS-21-recognized antigen to paraformaldehyde. In subsequent experiments, it was shown that, in contrast to methanol fixation, paraformaldehyde exposure exceeding 15 min resulted in a limited loss of antigenicity and HS-21 binding that became severe (50%) upon prolonged exposure to the fixative (24 h). In these studies, all sperm were fixed prior to immunochemical staining in the presence of 0.01% NP-40. This nonionic detergent was included initially to lower nonspecific antibody binding; however, its ability to render cells permeable is recognized. Although it is not our intent to define the subcellular domain(s) of the target antigen here, it is important to note that at least some of the antigen is present on the cell surface, as evidenced by the successful immunochemical staining of living, motile sperm.

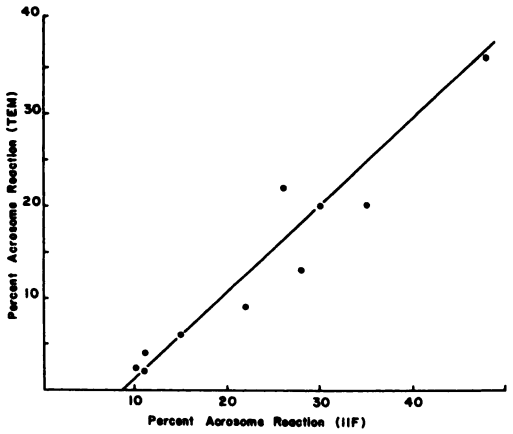

FIG. 2. Evaluation of acrosomal status in human ejaculated sperm by two independent methods. Sperm (20–27 × 10^6/ml) were washed and incubated under capacitating conditions as described in *Materials and Methods*. Cells were then incubated in BWW with 3.5% HSA, BWW with 3.5% HSA and 10 μM A23187, or BWW with 3.5% HSA and dimethylsulfoxide (carrier for A23187). After 3 h, sperm were independently processed for either indirect immunofluorescence or transmission electron microscopy, for which a minimum of 400 or 100 sperm were scored for each point, respectively.

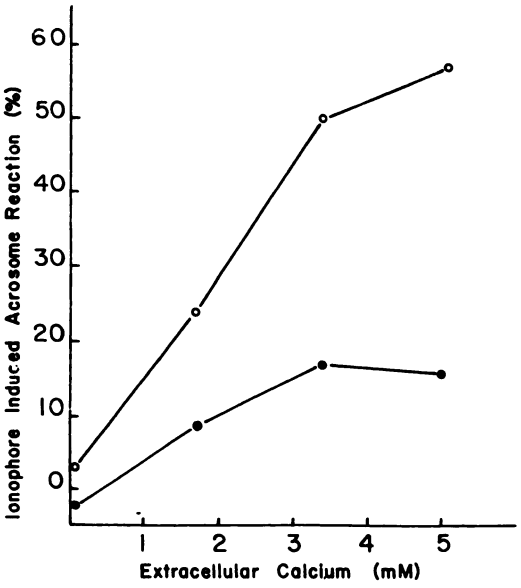

FIG. 3. Extracellular calcium ion dependency in calcium ionophore induction of the acrosome reaction. Cells were washed and incubated in calcium-free medium or in calcium-substituted medium before exposure to 10 μM ionophore. All media contained 3.5% HSA. Sperm were processed for indirect immunofluorescence evaluation of acrosomal status 3 h after ionophore addition. Ionophore-induced reactions have been corrected in all cases by subtracting the percentage of spontaneously reacted cells. The symbols represent two different donors.

The acrosome reaction in all mammalian sperm studied displays an absolute extracellular calcium ion dependency (Yanagimachi, 1981). Consistent with this tenet, chemical induction of the reaction in human sperm did not occur in calcium-free media but was maximal at 3–5 mM extracellular calcium (Fig. 3). A correlate of the results presented in Fig. 3 is that the sperm response to ionophore is donor dependent. For comparative purposes, most culture media for mammalian in vitro fertilization including BWW contain 1–2 mM total calcium. A description of the parameters influencing chemical induction of acrosomal loss in human sperm will be presented elsewhere (Byrd and Wolf 1984b, manuscript in preparation).

DISCUSSION

The results presented demonstrate that indirect immunofluorescence with specific monoclonal antibodies provides an accurate method for assessing acrosomal status in human sperm. Loss of specific antibody binding as measured by IIF was correlated with complete acrosomal loss as quantitated by TEM. It is recognized, of course, that target antigen loss may actually precede complete acrosomal loss depending upon the antigen's subcellular localization. Additionally, although considered unlikely, the possibility exists that acrosomal status evaluation by these techniques may suffer from fixation artifacts. To distinguish between acrosomal loss in dead or dying sperm and physiologic acrosome reactions using this approach, living motile sperm must be scored directly or acrosome status evaluation on fixed cells must be done in conjunction with viability and objective motility determinations. The former of these approaches can be accomplished readily by direct immunofluorescence microscopy with fluorescein-conjugated monoclonal antibody given a sufficient population of exposed cell surface antigen. Preliminary biochemical characterization of the HS-21 recognized antigen supports the existence of a minor pool of surface antigen amenable to detection in the living cell (this study; Wolf and Ochs, 1984). Recently, Kallajoki and Suominen (1984) described an intracellular acrosomal antigen of human sperm that may also be useful as an acrosomal marker.

The relationship between acrosomal status and fertility in man remains an open question. Apparently spontaneous, physiologic reactions do not occur with high frequency in capacitated cells (Byrd and Wolf, 1984b) and attempts to correlate acrosomal status in free-swimming sperm with fertility status of husbands participating in human in vitro fertilization programs have been unsuccessful (Plachot et al., 1984). However, it is anticipated that the ability to quantitate acrosomal status on large numbers of cells or individually on living motile sperm will find application in a clinical as well as a research setting, because it is likely that a subset of infertile human males can attribute their reproductive deficit to inadequate or subnormal acrosomal response. At present, we are contrasting the ability of noncapacitated and capacitated sperm to respond to ionophore (A23187) induction of acrosomal loss (Byrd and Wolf, 1984b) with the intent of establishing an assay for human sperm capacitation.

REFERENCES

Bellvé, A. R. and Moss, S. R. (1983). Monoclonal antibodies as probes of reproductive mechanisms.

Biol. Reprod. 28:1–26.

Biggers, J. D., Whitten, W. K. and Whittingham, D. G. (1971). The culture of mouse embryos *in vitro*. In: Methods in Mammalian Embryology (J. C. Daniel, ed.). Freeman, San Francisco, pp. 86–116.

Byrd, W. and Wolf, D. P. (1984a). Evaluation of capacitation in human sperm using monoclonal antibodies against acrosomal cap antigens. Biol. Reprod. 30:90.

Byrd, W. and Wolf, D. P. (1984b). Chemical induction of the acrosome reaction in human ejaculated sperm. J. Cell Biol. 99:260a.

Dulbecco, R. and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167.

Ey, P. L., Prowse, S. J. and Jenkin, C. R. (1978). Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 15:429–436.

Feuchter, F. A., Vernon, R. B. and Eddy, E. M. (1981). Analysis of the sperm surface with monoclonal antibodies: Topographically restricted antigens appearing in the epididymis. Biol. Reprod. 24:1099–1110.

Giloh, H. and Sedat, J. W. (1982). Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. Science 217:1252–1255.

Jamil, K. and White, I. G. (1981). Induction of acrosomal reaction in sperm with ionophore A23187 and calcium. Arch. Androl. 7:283–292.

Kallajoki and Suominen (1984). An acrosomal antigen of human spermatozoa and spermatogenic cells characterized with a monoclonal antibody. Int. J. Androl. 7:283–296.

Kennett, R. H. (1980). Fusion by centrifugation of cells suspended in polyethylene glycol. In: Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analysis (R. H. Kennett, T. J. McKearn and K. B. Bechtol, eds.). Plenum, New York, pp. 365–367.

Koehler, J. D. (1978). The mammalian sperm surface: studies with specific labeling techniques. Int. Rev. Cytol. 54:73–107.

Koehler, J. K., Nudelman, E. D. and Hakomori, S. (1980). A collagen-binding protein on the surface of ejaculated rabbit spermatozoa. J. Cell Biol. 86:529–536.

Langlais, J., Zollinger, M., Plante, L., Chapdelaine, A., Bleau, G. and Roberts, K. D. (1981). Localization of cholesteryl sulfate in human spermatozoa in support of a hypothesis for the mechanism of capacitation. Proc. Natl. Acad. Sci. (USA) 78:7266–7270.

Moore, H.D.M. and Hartman. T. D. (1984). Localization by monoclonal antibodies of various surface antigens of hamster spermatozoa and the effect of antibody on fertilization in vitro. J. Reprod. Fertil. 70:175–183.

Muraleami, H., Masui, H., Sato, G. H., Sueoka, N., Chow, T. P. and Kano-Sueoka, T. (1982). Growth of hybridoma cells in serum-free medium: ethanolamine is an essential component. Proc. Natl. Acad. Sci. (USA) 79:1158–1162.

Myles, D. G., Primakoff, P. and Bellvé, A. R. (1981). Surface domains of the guinea pig sperm defined with monoclonal antibodies. Cell 23:433–439.

Overstreet, J. W. and Hembree, W. C. (1976). Penetration of the zona pellucida of nonliving human oocytes by human spermatozoa in vitro. Fertil. Steril. 27:815–831.

Overstreet, J. W., Yanagimachi, R., Katz, D. F., Hayashi, R. and Hanson, F. W. (1980). Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: a study of fertile donors and infertile patients. Fertil. Steril. 33:534–542.

Plachot, M., Mandelbaum, J. and Junca, A. (1984). Acrosome reaction of human sperm used for in vitro fertilization. Fertil. Steril. 42:418.

Russell, L., Peterson, R. N. and Freund, M. (1979). Morphologic characteristics of the chemically induced acrosome reaction in human spermatozoa. Fertil. Steril. 32:87–92.

Shulman, M., Wilde, C. D. and Kohler, G. (1978). A better cell line for making hybridomas secreting specific antibodies. Nature 276:269–270.

Talbot, P. and Chacon, R. S. (1980). A new procedure for rapidly scoring acrosome reactions of human sperm. Gamete Res. 3:211.

Wolf, D. P. and Ochs, D. (1984). Biochemical characterization of a specific human sperm head antigen. Biol. Reprod. 30:76.

Wolf, D. P., Sokoloski, J. E., Dandekar, P. and Bechtol, K. B. (1983a). Characterization of human sperm surface antigens with monoclonal antibodies. Biol. Reprod. 29:713–723.

Wolf, D. P., Sokoloski, J. E. and Quigley, M. M. (1983b). Correlation of human in vitro fertilization with the hamster egg bioassay. Fertil. Steril. 40:53–59.

Yanagimachi, R. (1981). Mechanisms of fertilization in mammals. In: Fertilization and Embryonic Development *In Vitro* (L. Mastroianni and J. D. Biggers, eds.). Plenum Press, New York, pp. 81–182.

Yanagimachi, R., Yanagimachi, H. and Rogers, B. J. (1976). The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biol. Reprod. 15:471–476.