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10. A trial was begun by placing the animal in the start box. Five seconds later the guillotine door was raised, activating the discriminative cues (light and tone) for 5 seconds. At the termination of the cues, a 0.2-mA footshock was delivered through a scrambler into the grid floor for 10 seconds. Animals that escaped or avoided the shock successfully were permitted to remain for 20 seconds in the safe compartment (goal area).
11. G_{M1} ganglioside (purification 99+ percent, molecular weight 1546.9) was dissolved in Ringer solution at a concentration of 30 mg/ml. Animals received daily injections of 30 mg per kilogram of body weight starting on the day of surgery.
12. Behavioral results of the first testing period (the direction of difference is indicated for each comparison by the symbol <; $\alpha = 0.05$): (i) number of escape failures per reversal: C < L [$t(14) = 3.85$, $P < 0.01$], C < LG [$t(13) = 0.86$, NS (not significant)]; (ii) number of days to reach criterion after the first reversal: C < L [$t(14) = 6.15$, $P < 0.01$], C < LG [$t(13) = 1.47$, NS]; (iii) percentage of days criterion (9/10) was reached: C > L [$t(14) = 6.23$, $P < 0.01$], C > LG [$t(13) = 2.9$, $P < 0.01$].
13. A one-way analysis of variance for repeated measures was used for the statistical comparison of test-retest behavioral performance based on one-tailed probabilities. Behavioral improvement was apparent in every one of the following measures: (i) number of escape failures per reversal for group L [$F(1, 6) = 5.49$, $P < 0.05$], group LG [$F(1, 6) = 19.5$, $P < 0.01$], and group C [$F(1, 7) = 4.65$, $P < 0.05$]; (ii) number of days to reach criterion after the first reversal: group L [$F(1, 6) = 23.1$, $P < 0.01$], group LG [$F(1, 6) = 5.36$, $P < 0.05$], and group C [$F(1, 7) = 2.03$, NS]; (iii) percentage of days when criterion (9/10) was reached: group L [$F(1, 6) = 3.98$, $P < 0.05$], group LG [$F(1, 6) = 8.9$, $P < 0.01$], and group C [$F(1, 7) = 2.04$, NS].
14. To account for heterogeneous variances, Jonckeere-Terpstra's distribution-free test for ordered alternatives (one-tailed, $\alpha = 0.05$) [R. P. Runyon and H. Haber, *Fundamentals of Behavioral Statistics* (Addison-Wesley, Reading, Mass., 1971)] was used to analyze the number of escape failures per reversal: C < L ($U' = 43.5$, $P < 0.05$), C < LG ($U' = 35.5$, NS), L > LG ($U' = 36.0$, NS). In the analyses of the other measures, Dunnett's test was used ($\alpha = 0.05$). The results were as follows: number of days to criterion after the first reversal: C < L [$t(14) = 1.93$, $P < 0.05$], C < LG [$t(13) = 1.12$, NS], L > LG [$t(13) = 0.79$, NS); percentage of days on which criterion was reached: C > L [$t(14) = 2.63$, $P < 0.05$], C > LG [$t(13) = 0.75$, NS], L < LG [$t(13) = 1.81$, $P < 0.05$].
15. After the rats had been perfused transcardially with 0.9 percent saline followed by 10 percent Formalin in saline, the brains were cut coronally at 40 μm on a freezing microtome, and every sixth section was mounted on microscope slides and stained with cresyl-echt violet. To measure lesion size, the perimeter of the lesion extent was traced from successive, coronal sections with an overhead microprojector, and the lesion volume was determined by means of a Graphic tablet-menu on an Apple II plus computer.
16. Mean neuron-to-glia ratios and standard errors in caudate areas medial and adjacent to the lesion (L, 0.61 ± 0.18; LG, 0.64 ± 0.14; NS) and in substantia nigra pars compacta (L, 0.29 ± 0.04; LG, 0.23 ± 0.04; NS) were evaluated in both lesion groups ($n = 7$) [B. A. Sabel and D. G. Stein, *Exp. Neurol.* **73**, 507 (1981)].
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24. We thank E. Gants for her assistance in behavioral testing. Fidia Research Laboratories, Abano Terme, Italy, kindly provided us with the G_{M1} ganglioside. This work was supported by contract DAMD 17-82-C-2205 from the United States Army Medical Research and Development Command.

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Monoclonal Antibody to a Human Germ Cell Membrane Glycoprotein That Inhibits Fertilization

Abstract. *A monoclonal antibody to an antigen in the human germ cell membrane did not agglutinate or immobilize sperm but inhibited binding and penetration of zona-free hamster ova by human sperm and blocked murine fertilization in vitro. The antibody, of the 2a subclass of immunoglobulin G, was germ cell–specific but not species-specific. It recognized a single antigen of 23 kilodaltons that has been isolated from human germ cells. This fertilization antigen, located on the postacrosome, midpiece, and tail of human sperm, is a glycoprotein of testicular origin associated with some types of human involuntary immunoinfertility.*

Immunization of male or female animals of different species with homogenates of mature sperm, testis, or their extracts results in infertility (*1*). Antigenicity of sperm has also been implicated in involuntary infertility in human beings (*2*) and is the cause of continued infertility after vasovasostomy (*3*). However, use of whole sperm and testis homogenates is not appropriate for immunization since (i) many antigens of the germ cells are likely to be shared with other somatic tissues (*4, 5*) and (ii) immunization could result in undesirable immune complexes. Other germ cell–specific antigens do cause a reduction, although not a complete block, of fertility. Lactate dehydrogenase C_4 reduced fertility in mice, rabbits, and baboons (*6*), and antiserum to a rabbit sperm 13-kilodalton (kD) autoantigen inhibited fertility in female rabbits (*7*).The advent of hybridoma technology has made it possible to identify and purify sperm-specific antigens (*8, 9*) that are involved in fertility and infertility.

In our study, BALB/c mice were immunized intraperitoneally with 10^6 sperm (washed ejaculated or epididymal human sperm) in Freund's complete adjuvant. Hybridomas were prepared by fusion of the mouse myeloma line P3-NS1/1-Ag4-1 (*10*) with spleen cells from mice with high antibody titers to sperm. The hybridomas secreting these antibodies were selected and cloned. The preliminary screening was performed by an indirect enzyme-linked immunosorbent assay (ELISA) with whole washed human sperm or a membrane preparation solubilized with LIS (0.3*M* lithium diiodosalicylate) (*11*). Positive clones (reading higher than 2 standard deviations above the mean for controls) were selected and recloned, and 10^7 hybrid cells were injected into BALB/c mice previously treated with pristane to generate ascites fluid.

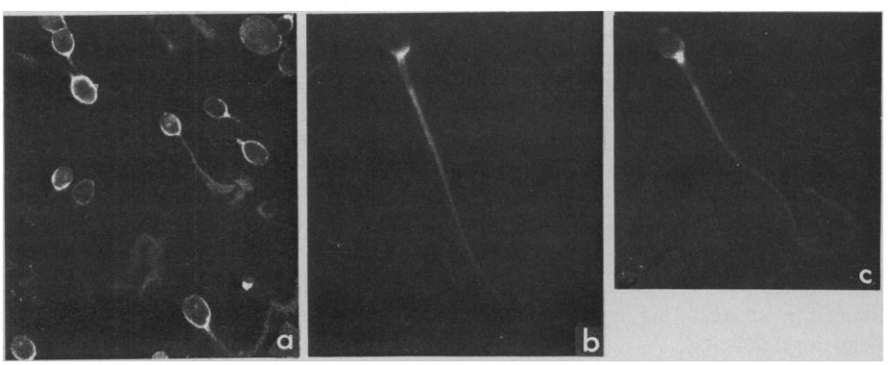


Fig. 1. Location of the FA-1 antigen by indirect immunofluorescence on (a) human sperm that have been reduced (swollen), (b) a methanol-fixed spermatozoon, and (c) a capacitated spermatozoon. The MA-24 clone reacted with the postacrosomal, tail, and (to a limited extent) midpiece regions of the sperm (×1100).

Of 40 putative clones secreting antibodies to sperm, one (MA-24) was selected for detailed analysis. The MA-24 clone was determined to be of the 2a subclass of immunoglobulin G (IgG_{2a}) by the agar-gel double diffusion technique (*12*). This clone was negative in the agglutination (*13*) and immobilization assays (*14*), two methods that we used to test for antibodies to sperm in serum of humans with involuntary infertility. MA-24 was germ cell–specific and did not bind to lymphocytes, erythrocytes, fibroblasts, lymphoblastoid cells, K562 leukemia cells, or other somatic cells by indirect immunofluorescence (IIF) (*8*). The results of IIF were further checked in the absorption assay (*8*) and in a test of residual activity in the ELISA after the antibodies had been incubated with various somatic tissues (*15*). In all of these and subsequent studies, both the supernatant from myeloma cell cultures and ascites fluids obtained from rats injected with other hybridoma clones were used as controls.

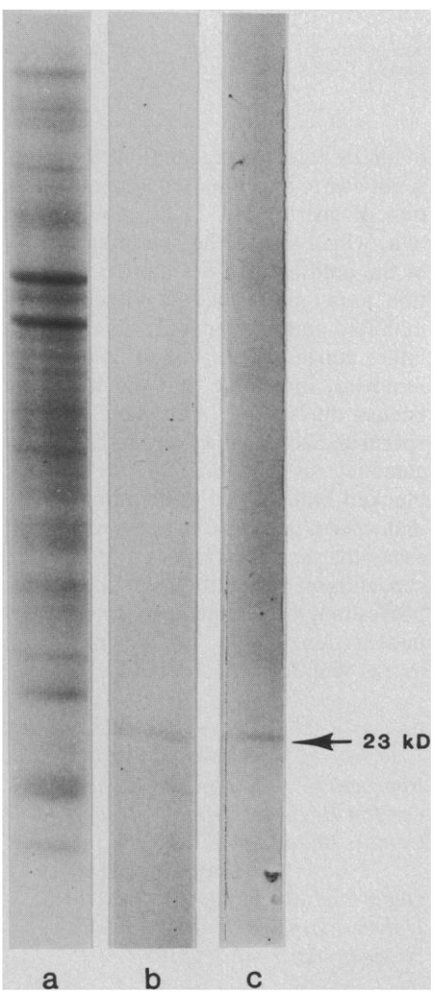
The MA-24 clone showed binding to the postacrosome, midpiece, and tail of viable and methanol-fixed human spermatozoa (Fig. 1) by IIF. It showed the same binding pattern with capacitated, noncapacitated, and reduced (swollen-head) sperm treated with dithiothreitol and Triton X-100 (*16*). It also reacted with murine epididymal sperm and ejaculated rhesus monkey sperm. It did not react with seminal plasma from vasectomized humans. In the Western blot enzyme immunobinding assay (*17*), the MA-24 clone reacted with a single 23-kD protein band when a detergent-solubilized membrane preparation of human testis was used (Fig. 2, lane c). Thus, MA-24 was specifically directed to an intrinsic membrane protein of sperm and not to one adsorbed onto the sperm surface from accessory gland fluids.

The tissue-specific but not species-specific MA-24 antibody to the 23-kD protein completely blocked binding and penetration of zona-free hamster ova by human sperm (Table 1). MA-24 also significantly inhibited fertilization in vitro of mouse eggs by murine sperm (Table 2).

The antigen (FA-1) to which the MA-24 antibody is directed has been isolated from both human ejaculated sperm and testis by immunoaffinity chromatography (*9*). Freshly isolated FA-1 (before freezing) binds to lectin concanavalin A and shows a single band of 23 kD during polyacrylamide slab gel electrophoresis (*18*) when stained with Coomassie blue as well as with an ultrasensitive silver

Table 1. Effects of monoclonal antibodies on the interactions between human sperm and zona-free hamster ova. Superovulation was induced in female hamsters (*21*), and mature unfertilized ova were collected and separated from the surrounding cumulus cells by incubation in 0.1 percent hyaluronidase and from the zona pellucida by treatment with 0.1 percent trypsin. Semen collected from a healthy human male was incubated for 30 minutes at 37°C, washed three times, adjusted in BWW medium (*22*) to 10^7 sperm per milliliter, and incubated overnight at 37°C. Ascites fluid from different mice containing monoclonal antibody or control ascites fluid was added to sperm for 15 minutes. Subsequently, 30 ova were added to 100 μl of the sperm preparation and incubated under mineral oil at 37°C in a CO_2 incubator. Ovum penetration was assessed at 3 hours by phase microscopy. The end point was defined as swollen sperm head with a tail discernible within the cytoplasm of the ovum.

Hybridoma	Assays (No.)	Ova examined (No.)	Spermatozoa bound per ova (No.)	Postincubation sperm motility (%)	Ova penetration (%)
Control ascites	2	34	20	55	88 (30 of 34)
MA-24	2	33	0	56	0 (0 of 33)
Myeloma ascites	5	60	15	52	53 (32 of 60)



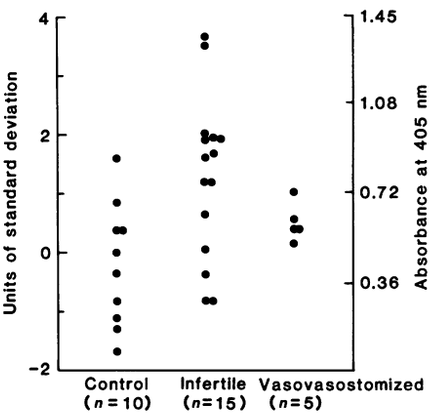


Fig. 2 (left). Sodium dodecyl sulfate–polyacrylamide slab gel (10 percent) electrophoresis of FA-1 isolated with the use of an immunoglobulin G–Sepharose 4B immunoaffinity column. The preparation of human testis was solubilized with detergent [15 m*M* sodium deoxycholate in 0.05*M* tris-HCl buffer (*p*H 8.0) containing 1 m*M* phenylmethylsulfonyl fluoride and 5 m*M* soybean trypsin inhibitor]. The solution was then passed through a lectin concanavalin A–Sepharose 4B column (Sigma). The fraction enriched in glycoprotein (lane a) was incubated with the immunosorbent affinity column prepared by coupling IgG [isolated from the MA-24 supernatant or ascites fluid by stepwise sodium sulfate precipitation (*25*)] to Sepharose-4B activated by CNBr. After washing the column with buffer (*p*H 8), the bound proteins were subsequently eluted at *p*H 10 and 11.4 (*9*). The freshly isolated (not frozen) fraction (lane b) eluted at *p*H 11.4 shows a single band of 23 kD on staining with silver stain (*26*). The molecular identity of the antigen was confirmed by Western blot enzyme immunobinding procedure (*9*, *17*) with the detergent-solubilized human testis preparation. MA-24 reacted with a single protein band of the same molecular identity (lane c). Fig. 3 (right). Reaction of FA-1 with sera from infertile and vasovasostomized patients having sperm antibodies by LIS-ELISA. Approximately 5 μg of FA-1 were diluted in carbonate buffer (0.1*M*, *p*H 9.6) and left overnight. The antigen-coated trays were incubated for 2 hours at 37°C with sera from normal, infertile, and vasovasostomized patients (diluted 1:20 in 4 percent polyethylene glycol 6000 in Tween buffer), washed, and incubated for 1 hour at room temperature with β-D-galactosidase protein A conjugate (1:500 in Tween buffer). The trays were washed again, the wells were incubated with a solution of ρ-nitrophenyl-β-D-galactopyranoside in buffer (1 mg/ml in 10 m*M* tris, 1 m*M* $MgCl_2$, and 10 m*M* NaCl) at 37°C for 30 minutes, and the absorbance was determined at 405 nm. To compare the results, we changed optical readings to units of standard deviation on the basis of the absorbance in controls. The serum of some infertile patients showed a strong reaction with FA-1.

Table 2. Effects of MA-24 monoclonal antibody on fertilization in vitro in mice 6 to 8 weeks of age (*23*). Ovulation was induced with 5 U of pregnant mare serum administered interperitoneally. After 48 hours, 5 U of human chorionic gonadotropin was administered peritoneally. One cauda epididymis was removed from a male mouse, snipped, and placed in 0.5 ml of Toyoda's medium under oil (*24*). After 10 to 15 minutes sperm were collected, and a portion (4×10^3 sperm) was added to a fertilization dish containing, under oil, 180 μl of Toyoda's medium and 20 μl of ascites fluid with monoclonal antibody or control ascites from different mice. Eggs in cumulus mass were collected from the oviduct and added to the fertilization dish. After 6 to 8 hours, the cumulus cells were removed with hyaluronidase; the eggs were then fixed in 1 percent paraformaldehyde at 4°C, stained with 0.25 percent lacmoid in 45 percent acetic acid, destained with 20 percent acetic acid plus 20 percent glycerol, and mounted. Eggs were considered fertilized if two of the following criteria were met: (i) two pronuclei were present, (ii) sperm tail was present in the cytoplasm, or (iii) the second polar body with condensed chromatin was present. The cumulus mass was removed with hyaluronidase (300 U/ml) before fertilization to determine whether the site of inhibition was at the interaction of sperm and cumulus. The zona pellucida was removed with acidified Tyrode's (*24*) before fertilization to determine whether the site of inhibition was at the interaction of sperm and zona pellucida.

Hybridoma	Assays (No.)	Ova examined (No.)	Ova fertilized (No.)	Fertilization rate (%)*
Control ascites	4	113	69	61†
MA-24	10	415	61	15‡
Medium control	9	320	223	70§

*Significance tested by chi-squared analysis. †Not significantly different. ‡$P < 0.001$; purified IgG (~15 μg) showed a complete block of fertilization (0 percent). §$P < 0.001$.

stain (Fig. 2, lane b). This glycoprotein was stained by periodic acid–Schiff reagent and was stable at room temperature (~22°C) for at least 4 days. The FA-1 isolated from human sperm is identical to that isolated from testis, although the concentration of antigen was greater in the testis where it constituted approximately 5 percent of the membrane protein solubilized with sodium deoxycholate.

Preliminary data implicated FA-1 in human involuntary immunoinfertility. Sera from infertile patients with immunologic involvement showed a strong binding to the purified antigen in the ELISA (Fig. 3). The binding pattern was comparable to that seen with the LIS extract of human sperm (*11*), a soluble preparation reported to contain surface antigens acting in fertilization (*19*). This finding also indicates indirectly that the FA-1 antigen is immunogenic in humans.

The mechanism or mechanisms by which MA-24 inhibits fertilization are not clear. Since both binding and penetration are inhibited in fertilization systems in vitro, these antibodies may hinder interactions between sperm and ovum or receptor recognition; the block is not due to agglutination or immobilization of sperm. MA-24 did not bind to ova, which shows the sperm specificity of the antibody. In the murine fertilization assay performed in vitro, MA-24 inhibited fertilization only in ova with intact zonae and not when zonae were removed, indicating that the inhibition occurs during the interaction between sperm and zona. However, in the heterogametic assay, MA-24 completely blocked binding and penetration of human sperm in zona-free hamster ova, an assay that has been reported to measure capacitation indirectly (*20*). These antibodies may be directed to a sperm component (enzymatic or nonenzymatic) that is vital for capacitation.

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26. The antigen has a tendency to autopolymerize and form a dimer of 46 kD in detergent solutions and when it is frozen. The same dimer is recognized by MA-24 in Western blots (*17*) of frozen testis preparations solubilized with deoxycholate. The molecular weight mentioned here is for freshly isolated FA-1 that has not been frozen (R. K. Naz and N. J. Alexander, unpublished data).
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