BIOLOGY OF REPRODUCTION 56, 1142–1146 (1997)

Reversible Contraceptive Effect of PH-20 Immunization in Male Guinea Pigs[1]

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ABSTRACT

Sperm proteins are currently being studied as antigens on which to base a contraceptive vaccine. Sperm plasma membrane proteins offer the theoretical possibility of immunizing either males or females and achieving a contraceptive effect. In this study, we investigated the sperm plasma membrane protein PH-20 as an antigen for inducing infertility in males. We found that infertility can reproducibly be induced in male guinea pigs immunized with purified PH-20: 100% (29 of 29) of PH-20-immunized males became infertile, whereas all 22 controls were fertile. The males were extremely responsive to PH-20 immunization: infertility could be induced with a single injection of only 5 μg PH-20. Among males that received their initial injection when they were ~300 g (body weight), 14 of 15 had regained fertility at about 1 yr after initial injection. Surprisingly, in another group of males that received their first injection when they were ~650 g (body weight), only 1 of 5 had regained fertility about 1 yr after initial injection. Anti-PH-20 titers in antisera (2 mo after initial injection) were generally in the range 1.1–4.2 × 10^4 in twice-injected males and the range 1.8–9.4 × 10^3 in once-injected males. Over the next 6–11 mo, twice-injected males' titers decreased ≥ 4-fold, whereas once-injected males' titers decreased slightly (1.1- to 1.8-fold). After 6–11 mo, anti-PH-20 titers were in the range 1.0–4.8 × 10^3, and the precise residual titer did not correlate with fertility/infertility. The results show that immunization of males with PH-20, even at low doses, results in a reproducible, completely effective contraceptive action.

INTRODUCTION

As knowledge of the molecular basis of fertility grows, increasing numbers of potential targets for contraception become available. One approach to contraception that has received relatively scant attention is the approach of a contraceptive vaccine. Among the studies done on contraceptive vaccines, most have focused on achieving a contraceptive action in females, and only a few have investigated immunocontraception for males.

For females, possible target antigens for immunocontraception include reproductive hormones, zona pellucida proteins, and sperm surface proteins. The most studied candidate antigen for females is the hormone hCG, which has been demonstrated to have a contraceptive action in those women who have a strong immune response to it [1]. Immunization with zona pellucida glycoproteins results in high levels of infertility in test animals but leads in time to oophoritis and ovarian dysfunction [2, 3]. By immunizing female mice with a B-cell epitope from the zona protein ZP3, success has recently been achieved in inducing infertility without producing oophoritis [4]. A number of different sperm proteins have been tested as contraceptive immunogens in females, and immunization with the sperm surface protein PH-20 has been found to induce infertility in all tested female guinea pigs [5].

In theory, sperm surface proteins might also be useful as contraceptive immunogens in males. There is a substantial clinical literature on male infertility patients whose infertility apparently results because they produce antibodies to sperm surface proteins. These antibodies can be detected, by a variety of methods, bound to sperm in ejaculates from these men. The men are infertile but do not have other health problems and rarely (if ever) have orchitis [6]. Thus, an "Experiment of Nature" supports the idea that immunization with sperm surface proteins is a feasible male contraceptive approach.

The current study explores the possibility of male immunocontraception using the sperm surface protein PH-20 as a test antigen. PH-20 has been cloned and studied in various mammalian species including guinea pig [7], mouse [8], monkey, and human [9]. It has a glycosyl phosphatidylinositol membrane anchor and is present on the plasma membrane and inner acrosomal membrane [8, 10–12]. PH-20 has two required functions in fertilization. On the plasma membrane, it has a hyaluronidase activity that allows acrosome-intact sperm to penetrate the cumulus cell layer [8], and on the inner acrosomal membrane, it has a second, distinct activity required for acrosome-reacted sperm to bind to the zona pellucida [13, 14]. Considerable evidence indicates that PH-20 is tissue-specific, being expressed only in testis ([9, 15]; unpublished results). Thus, PH-20 has various properties desirable in an antigen for a contraceptive vaccine. In the present study and accompanying paper [16], we asked a variety of questions about the contraceptive effects of immunizing males with PH-20. These questions include the following: will immunization with PH-20 reproducibly yield infertility in males? What is the duration of the induced infertility? Does the infertile state correlate with a particular antibody titer? Does immunization result in orchitis? And what aspect of the immune response results in infertility?

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs about 300 g (~4 wk old) or 650 g (~11 wk old) were obtained from Buckberg Laboratories. Female Hartley guinea pigs, 600–650 g, also from Buckberg Laboratories, were used for mating with immunized and control males.

Accepted December 6, 1996.
Received August 23, 1996.
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Purification of the PH-20 Protein

The PH-20 protein was purified from cauda epididymal sperm by affinity chromatography using an anti-PH-20 monoclonal antibody coupled to Sepharose (Sigma Chemical Co., St. Louis, MO) as described [5]. PH-20 was purified just before each injection, stored for one or a few days at 4°C, and then used for immunization.

Immunization of Male Guinea Pigs

Males were immunized as previously described for females [5]. Affinity-purified PH-20, in 0.375 ml PBS containing 3 mM octylglucoside (OG), was emulsified with complete Freund's adjuvant (CFA). Each animal received 0.5 ml of emulsion s.c. in the back and 0.25 ml i.m. in a rear leg. About 1 mo later, some animals (see *Results*) received a second injection, containing the same dose of PH-20 in PBS and 3 mM OG, emulsified with incomplete Freund's adjuvant (IFA). Control males received on the same schedule the same injections containing PBS and 3 mM OG and CFA or IFA, but lacking PH-20.

Mating Immunized and Control Males

At various times after the initial immunization (see *Results*), each immunized male or control was housed with two females for 3 wk. The females and males were then separated, and after an additional 5 wk the females were killed and the fetuses were counted.

Antiserum Titers

About 1 wk before matings, which occurred at 2, 6–7, or 11–13 mo after the first injection, immunized and control animals were bled. The antisera were titered in an RIA as previously described [5, 15]. Briefly, an OG extract of whole cauda epididymal sperm was used as an antigen on a microtiter plate. Serum dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were tested in duplicate. Binding of the serum dilutions to the sperm extract on the plate was measured using ^{125}I-labeled protein A as a secondary reagent. Binding of serum dilutions from a control male (measured on the same plate as serum dilutions from immunized males) was considered background binding. Specific binding was determined as counts per minute bound with immunized serum at a particular dilution minus counts per minute bound with control serum at the same dilution. Maximal specific binding was found at a dilution of 10^{-2} or 10^{-3}. Antiserum titer is defined as the dilution at which specific counts per minute bound is half maximal [15].

RESULTS

Induction of Infertility in Immunized Males

Male guinea pigs received injections of PH-20 affinity-purified using an anti-PH-20 monoclonal antibody [5]. PH-20, isolated by this protocol, is the only band seen when 5 μg of purified protein is examined by SDS-PAGE followed by silver staining [5], and PH-20 purity was confirmed by this test for each PH-20 preparation used in this study. We have also previously found that antisera from guinea pigs injected with purified PH-20 recognize only PH-20 in immunoblots of whole sperm cell extracts [15].

In earlier work we obtained the preliminary result that six male guinea pigs became infertile 2 mo after PH-20 immunization [5, 15]. Here we report on other consequences of immunization and long-term sequelae in these six males (group I) and on infertility induction and other short- and long-term effects of immunization in 23 additional males (group II and group III).

TABLE 1. Induction of infertility by PH-20 immunization in 300-g males.

Immunized male #	μg of PH-20 injected (# of times injected)	Fertility status 2–3 mo postimmunization
Group I		
1	50 (2×)	Infertile
2	30 (1×)	Infertile
3	20 (2×)	Infertile
4	10 (2×)	Infertile
5	5 (2×)	Infertile
6	2.5 (2×)	Infertile
(7 controls, #1c–7c)	0 (2×)	All fertile
Group II		
1	30 (1×)	Infertile
2	30 (1×)	Infertile
3	20 (2×)	Infertile
4	20 (2×)	Infertile
5	20 (2×)	Infertile
6	20 (1×)	Infertile
7	20 (1×)	Infertile
8	10 (2×)	Infertile
9	10 (2×)	Infertile
10	10 (2×)	Infertile
11	10 (1×)	Infertile
12	10 (1×)	Infertile
13	5 (2×)	Infertile
14	5 (2×)	Infertile
15	5 (2×)	Infertile
16	5 (1×)	Infertile
17	5 (1×)	Infertile
10 controls (#1c–10c)	0 (2×)	All fertile

At the time of their initial injection, PH-20-immunized and control males in groups I and II (Table 1) were about 300 g in weight (~4 wk old) and thus were about the age at which they could first be fertile [17]. Two months after the initial injection, each PH-20-immunized male or control was a mature adult and was tested for fertility by mating with two females for 21 days. The number of fetuses found in each female was counted to score fertility. A male was considered infertile if neither female had fetuses and fertile if one or both females had fetuses. No fetuses were found in the 46 females mated with the 23 immunized males of groups I and II. Fetuses were found in at least one and in most cases (14 of 17) both of the females mated with the 17 controls of groups I and II. Thus, all the males (23 of 23) immunized with PH-20 were infertile, whereas all the controls (17 of 17) were fertile (Table 1).

To test larger, older males, ~650 g (~11 wk) at the time of initial injection, and to determine the time required to induce infertility, we immunized another 6 males (group III, Table 2). These 6 males received a first injection of PH-20 at 0 mo, and 3 of them received a second injection at 1 mo. Testing for fertility by mating with two females (over a 3-wk period) beginning at 0 mo showed that 5 of the 6 were fertile; a second mating beginning at 1 mo (over the subsequent 3 wk) showed that 6 of the 6 immunized males were fertile (Table 2). (It is not known why immunized male no. 4, group III, appeared infertile in the first mating (0 mo) and fertile in the second mating (1 mo). By 2 mo after the initial injection, all 6 PH-20-immunized, 650-g males had become infertile (Table 2). In contrast, the 650-g controls were fertile at all times tested (Table 2). Therefore, grouping together the results on 300- and 650-g males, we

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TABLE 2. Fertility of 650-g males at different times relative to PH-20 immunization.

Immunized male #	μg of PH-20 injected (# of times injected)	Fertility status 0 months	1 mo postimmunization	2 mo postimmunization
Group III				
1	100 (2×)	Fertile	Fertile	Infertile
2	100 (1×)	Fertile	Fertile	Infertile
3	50 (2×)	Fertile	Fertile	Infertile
4	50 (1×)	Infertile	Fertile	Infertile
5	20 (2×)	Fertile	Fertile	Infertile
6	20 (1×)	Fertile	Fertile	Infertile
5 controls (#1c–5c)	0 (1×: #3c, #4c #5c) (2×: #1c, #2c)	All fertile	All fertile	Fertile (3/3 tested)

found that 0 of 29 immunized males were fertile whereas 22 of 22 controls were fertile (Tables 1 and 2).

The male guinea pigs were extremely sensitive to induction of infertility: a single injection of as little as 5 μg PH-20 (males no. 16 and no. 17, group II) was sufficient to produce infertility (Table 1). Therefore, we tested even lower doses of PH-20, in the range 0.25–2 μg, to see if very low-dose immunization would yield infertility. Among 9 males immunized in the very low-dose range, 3 were infertile (Table 3).

Duration of Infertility in Immunized Males

In considering immunization with sperm proteins as a practical contraceptive method, it is important to determine the duration of the induced infertility. In group I, (~300 g at time of first injection) we found that 4 of 6 males had regained fertility 6–7 mo after immunization (Table 4). Three of these four (group I, males nos. 1, 4, and 5) were tested again at 11–13 mo after fertilization, and their return to fertility was confirmed in this additional mating. Also at 11–13 mo after immunization, the other group I males (nos. 3 and 6) regained fertility, so 6 of 6 group I males returned to fertility. In group II at 6–7 mo after immunization, 6 of 10 immunized males had returned to fertility while 4 of 10 remained infertile. When 3 of these 4 infertile males (group II, nos. 4, 6, and 16) were retested at 11–13 mo after immunization, 2 of 3 had regained fertility (Table 4). Thus, 8 of 9 PH-20-immunized males that were tested in group II regained fertility by about 1 yr (Table 4).

A different result was obtained in the males that received their initial immunization when they were older (group III, ~650 g [~11 wk old] at first injection). Only 1 of 5 of these immunized males had regained fertility by 11–13 mo after immunization (Table 4). Each of these 5 immunized males had been proven fertile before immunization (Table 2), and 3 of 4 control-injected group III males were fertile at 11–13 months postinjection (Table 4). These data indicate that, in a high percentage of 650-g males, PH-20 immunization results in either long-term (> 1 yr) or possibly irreversible infertility.

TABLE 3. Induction of infertility by low-dose PH-20 immunization in 300-g males.

Immunized male #	μg of PH-20 injected (# of times injected)	Fertility status 2–3 mo postimmunization
Group IV		
1	2 (2×)	Infertile
2	2 (1×)	Fertile
3	1 (2×)	Fertile
4	1 (2×)	Fertile
5	1 (1×)	Fertile
6	0.5 (2×)	Fertile
7	0.5 (1×)	Fertile
8	0.25 (2×)	Infertile
9	0.25 (2×)	Infertile

Antiserum Titers in PH-20-Immunized Males

The antiserum titers in PH-20-immunized males were measured at various times (Table 5). All males were bled

TABLE 4. Return to fertility of PH-20 immunized males.

Immunized male #	μg PH-20 injected (# of times injected)	Number of fetuses in each mated female: 6–7 mo post-immunization	11–13 mo post-immunization
Group I[a]			
1	50 (2×)	4,2	3,0
2	30 (1×)	3,4	n.t.
3	20 (2×)	0,0	2,3
4	10 (2×)	3,6	4,3
5	5 (2×)	2,3	5,4
6	2.5 (2×)	0,0	0,1
Group II[a]			
2	30 (1×)	0,0	n.t.
3	20 (2×)	4,3	n.t.
4	20 (2×)	0,0	0,0
6	20 (1×)	0,0	3,4
9	10 (2×)	4,2	n.t.
10	10 (2×)	0,1	n.t.
11	10 (1×)	3,1	n.t.
14	5 (2×)	2,2	n.t.
15	5 (2×)	0,5	n.t.
16	5 (1×)	0,0	2,3
Group III[a]			
1	100 (2×)	n.t.	0,0
2	100 (1×)	n.t.	4,0
3	50 (2×)	n.t.	0,0
4	50 (1×)	n.t.	0,0
5	20 (2×)	n.t.	0,0
6	20 (1×)	n.t.	n.t.
Group III (control males)			
1c	0 (2×)	n.t.	5,4
2c	0 (2×)	n.t.	3,4
3c	0 (1×)	n.t.	3,4
4c	0 (1×)	n.t.	n.t.
5c	0 (1×)	n.t.	0,0

[a] Summary: total Group I return to fertility: 6/6 tested by 11–13 months postimmunization; total Group II return to fertility: 8/9 tested by 11–13 months postimmunization; total Group III return to fertility: 1/5 tested by 11–13 months postimmunization; n.t., not tested.

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TABLE 5. Antiserum titers in PH-20 immunized males.

Immunized male	μg of PH-20 injected (# of times injected)	Antiserum titers: 2 mo post-immunization	6 mo post-immunization (fold decrease)	11 mo post-immunization (fold decrease)[a]
Group I				
1	50 (2×)	3.2×10^4	2.3×10^3 (13.9)	
2	30 (1×)	3.7×10^3	3.7×10^3 (1.1)	
3	20 (2×)	1.6×10^4	3.2×10^3 (5.0)	
4	10 (2×)	3.5×10^4	4.8×10^3 (7.3)	
5	5 (2×)	1.4×10^4	2.9×10^3 (4.8)	
6	2.5 (2×)	1.3×10^4	3.5×10^3 (3.7)	
Group II				
1	30 (1×)	1.6×10^3		n.t.
2	30 (1×)	4.1×10^3		3.1×10^3 (1.3)
3	20 (2×)	1.3×10^4		2.2×10^3 (5.9)
4	20 (2×)	1.6×10^4		3.3×10^3 (4.8)
5	20 (2×)	4.0×10^4		n.t.
6	20 (1×)	5.3×10^3		1.7×10^3 (3.1)
7	20 (1×)	9.4×10^3		n.t.
8	10 (2×)	1.6×10^4		n.t.
9	10 (2×)	5.9×10^3		1.3×10^3 (4.5)
10	10 (2×)	3.4×10^4		4.5×10^3 (7.6)
11	10 (1×)	1.8×10^3		1.0×10^3 (1.8)
12	10 (1×)	6.3×10^3		n.t.
13	5 (2×)	8.9×10^3		n.t.
14	5 (2×)	1.1×10^4		2.5×10^3 (4.4)
15	5 (2×)	1.1×10^4		4.5×10^3 (2.4)
16	5 (1×)	3.1×10^3		2.3×10^3 (1.3)
17	5 (1×)	1.8×10^3		n.t.
Group III				
1	100 (2×)	4.2×10^4		
2	100 (1×)	1.8×10^4		
3	80 (2×)	2.3×10^4		
4	80 (1×)	7.3×10^3		
5	20 (2×)	2.6×10^4		
6	20 (1×)	4.0×10^3		

[a] n.t., Not tested.

before their first mating at 2 mo after immunization. Serum titers were determined in an RIA that we have previously described [5, 15]. The sera from immunized males showed saturating or high binding in the assay at dilutions of 10^{-2}–10^{-4} and had measurable binding above background even at dilutions of 10^{-6} or 10^{-7}. Titer is defined as the serum dilution that gives half-maximal binding.

At 2 mo after immunization, among males injected twice with PH-20, 2 (of 17) males had relatively low titers, $< 10^4$ (group II, nos. 9 and 13, titers = 5.9×10^3 and 8.9×10^3, Table 5). All other groups I, II, and III males (15 of 17) that received two injections of PH-20 at any dose from 2.5–100 μg had titers $> 1.0 \times 10^4$. The range of titers 2 mo after immunization in these 15 twice-injected males was 1.1×10^4–4.2×10^4 (Table 5). In contrast, 25 female guinea pigs (300 g at time of first injection) that received two injections of PH-20 at doses 5–50 μg all had titers 2 mo after immunization in the range 3.1×10^4–1.5×10^5 [5, 15]. Thus, females immunized with PH-20 appear to be capable of responding with an antiserum titer about three times higher than the males.

Twelve groups I, II, and III males that received a single injection of PH-20 all had titers at 2 mo after immunization $< 1.0 \times 10^4$ (range 1.8×10^3–9.4×10^3) with one exception: the male that received the highest dose, 100 μg in one injection, had a titer of 1.8×10^4. The full range of titers (after one or two injections) at 2 mo after immunization in the 29 immunized males was 1.8×10^3–4.2×10^4 (Table 5). All these males were infertile.

To determine how much titer decreased over time and whether the decrease correlated with return to fertility, group I males' titers were determined again at 6 mo after immunization, and group II males' titers were determined again at 11 mo after immunization (Table 5). In general, tested males that had received two injections and had comparatively high titers (usually $> 1.0 \times 10^4$) at 2 mo experienced relatively large declines in titer at 6 or 11 mo after immunization (usually ≥ 4-fold reduction). In contrast, tested males that had received a single injection and at 2 mo had titers in an initially lower range (3.1–5.3×10^3) did not experience as much of a decrease in titer (usually 1.1- to 1.8-fold). These trends can be readily seen in the example of the group I males' titers at 6 mo after immunization. Group I male no. 2, which received a single injection, had essentially no decrease in titer between 2 and 6 mo, whereas the other 5 group I males, twice injected, showed a substantial decrease in titer (Table 5).

At 6 mo after immunization, group I males (nos. 1, 2, 4, and 5, titers 2.3–4.8×10^3) regained fertility whereas group I males (nos. 3 and 6, titers 3.2–3.5×10^3) remained infertile (Tables 4 and 5). Thus, in group I at 6 mo, fertility could not be correlated with titer by comparing the 4 fertile males to the 2 infertile males. Also, the 4 fertile males had a range of titers that was the same range as in some males that had been infertile when mated at 2 mo. Similarly, by the 11–13 mo mating, 8 of 9 group II males (titers 1.0×10^3 to 4.5×10^3) had regained fertility while the 9th tested male (no. 4, titer 3.3×10^3) had not. Again the infertile male had a titer in the range of titers in fertile ones at 11 mo. Furthermore, these fertile males at 11 mo still maintained titers as high as those in some infertile males at 2 mo (Tables 1, 4, and 5). Thus, in no case did fertility correlate with precise titer in the titer range 1.0 to 4.8×10^3 exhibited by males 6 or 11 mo after immunization. This appears to be a titer range compatible with either fertility or infertility, and whether the male remains infertile or not may be determined by additional factors.

PH-20 Immunization-Induced Changes in the Reproductive Tract

Many of the PH-20-immunized, infertile males were examined for histological changes in the reproductive tract. The results of these studies are presented in the accompanying paper [16].

DISCUSSION

Our results in this study show that PH-20 is consistently highly immunogenic in male guinea pigs and, given at a total dose of ≥ 5 μg, elicited infertility in all animals tested (29 of 29). Males receiving a single injection of ≥ 5 μg PH-20 generally had titers in the range 1.8–9.4×10^3 while those receiving two injections (≥ 2.5 μg each) generally had even higher titers in the range 1.1–4.2×10^4. Male fertility is very sensitive to immunization with PH-20; one injection of 5 μg PH-20 resulted in infertility.

The average serum anti-PH-20 titer in male guinea pigs given two injections of ≥ 5 μg PH-20 was about 3-fold lower than the anti-PH-20 titer in female guinea pigs given the same doses [5, 15]. The male and female guinea pigs tested were the same strain (Hartley) and the same age (~4 wk old, at first injection), and titers were determined in the same assay, so the difference appears to depend on gender.

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It is unknown whether the same 3-fold difference would be observed in antibody responses to various other antigens or is related to PH-20's being a sperm-specific antigen. If the latter case is correct, it is possible that some regulatory feature of the male immune system dampens the response to sperm proteins even when they are administered by systemic injection.

The duration of the induced infertility seems to depend upon the body weight (age) of the male at the time of initial injection. Male guinea pigs receiving their initial injection when they weighed ~300 g or ~650 g all became infertile. Unexpectedly, the males that were smaller (~300 g) at the time of their first injection showed a shorter period of infertility. Among these ~300-g males, 10 of 16 tested had regained fertility 6–7 mo after the initial injection, and 14 of 15 tested had regained fertility after ~1 yr. In contrast, among the ~650-g males, only 1 of 5 had regained fertility after ~1 yr (3 of 4 of the ~650-g controls were fertile at this time). This apparent difference in duration of infertility is difficult to explain with our current level of knowledge. Possibly the immune responses that result in infertility are longer-lasting in the ~650-g males, or processes that protect epididymal sperm [16] come into play sooner in the ~300-g males. Further work will be necessary to test for any such differences and to determine when (or if) the ~650-g males regain fertility at times later than 1 yr after immunization.

Serum titers were determined at the times of the initial and subsequent matings. At ~6 mo or ~1 yr after initial injection, titers for all the males were in the range 1.0–4.8 $\times 10^3$. Both the "regained fertility" and the "still infertile" animals were spread throughout this range. Thus we found no correlation of infertility with a precise serum titer threshold, suggesting that additional factors are operative in determining fertility status.

The finding that immunization of male guinea pigs with PH-20 reproducibly leads to infertility suggests that further investigation of this approach is warranted. Tests of other sperm surface proteins, other species, and other adjuvants are potentially of interest to determine whether immunocontraception in the male will be feasible.

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