the 3D11 hybridoma or with the antiserum to *P. berghei* (tracks 7 and 8).

The Pb44 antigen was not detected on other developmental stages of *P. berghei*, by indirect immunofluorescence when monoclonal antibodies were used. Also, antibodies to sporozoites of two simian malaria parasites, *Plasmodium knowlesi* and *P. cynomolgi*, did not immunoprecipitate Pb44. This antigen is therefore probably stage- and species-specific.

Whether Pb44 constitutes the major, or even the single antigen on the outer membrane of the sporozoites, as is the case of the antigen from the surface coat of various African trypanosomes (*12–14*), is unknown. The availability of large amounts of this monospecific sporozoite antibody produced by hybrid cells will greatly facilitate its purification and subsequent characterization, and contribute to clarification of its role in protective immunity in vivo. Experiments show that mice bearing hybridoma solid tumors are resistant to sporozoite challenge and that their serum mediates passive protection (*8*).

Antibodies to sporozoites have been detected in humans (*2*) in spite of the small number of parasites inoculated by a mosquito bite and the brief passage of the parasites through the host's blood stream. This suggests that the sporozoites have a high degree of immunogenicity and could be used in the development of a malaria vaccine.

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15. We previously reported that antiserum to *P. berghei* immunoprecipitated a protein of molecular weight 41,000 from the same extracts (6). This probably represents a degradation product of Pb44, since in these initial experiments we did not add inhibitors of proteolysis after disruption of the parasites in the French pressure cell.
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## Sperm-Egg Interaction: Evidence for Boar Sperm Plasma Membrane Receptors for Porcine Zona Pellucida

Abstract. *Freshly ejaculated, noncapacitated boar sperm bind rapidly and in large numbers to pig egg zona pellucida in vitro. In the present study, the number of sperm bound decreased sharply when sperm motility was lowered by energy poisons or by reducing the temperature. Highly motile sperm from humans, guinea pigs, and rats, added at concentrations ten times higher than control sperm, did not bind to the porcine zona. At the same high concentration, a small number of hamster and bull sperm bound to the zona. Binding of boar sperm to the zona pellucida was blocked almost completely by diluted whole antiserum to sperm plasma membranes and by univalent (Fab) antibody to these membranes. When antibody to sperm plasma membrane was first absorbed with plasma membrane vesicles, sperm binding was not inhibited. These results provide direct evidence for the existence of sperm plasma membrane receptors for the zona pellucida of the pig.*

Gamete interactions have been studied in detail in some species of invertebrates, and a species-specific sperm protein, thought to be involved in the adhesion of sea urchin sperm to the egg surface, has been isolated (*1, 2*). In mammals, species specificity has been reported to reside in the ability of sperm to bind with the zona pellucida (*3*), an ability that is lost in the presence of antibody to eggs (*4–7*) or to whole sperm (*8*). An important inference that can be made from these studies is that receptors for the zona exist on the surface of the spermatozoon; the existence of such receptors, however, has not been directly demonstrated. In this report we present the results of experiments which characterize the binding of ejaculated boar sperm to the zona pellucida of porcine oocytes and show that this binding is inhibited by antiserum and univalent antibodies to boar sperm plasma membranes.

Ovaries obtained from sows and gilts after slaughter were minced with a sharp scalpel to free oocytes (*9*), which were collected in nylon screens (*10, 11*). Cumulus-free eggs in various stages of maturation (*11, 12*) were obtained by briefly rinsing the eggs with sodium citrate during isolation. Freshly ejaculated boar sperm were washed twice in a medium containing tris-HCl (20 m*M*), NaCl (120 m*M*), KCl (5 m*M*), $MgCl_2$ (1 m*M*), $CaCl_2$ (3 m*M*), glucose (5 m*M*), sodium pyruvate (3 m*M*), and bovine serum albumin (2 percent); the *p*H was 7.4 at 21°C.

In each experiment, about 50 eggs were added in a small volume (about 20 $\mu$l) to 0.2 ml of a sperm suspension ($0.2 \times 10^4$ to $0.6 \times 10^4$ cells) in buffer at room temperature and incubated in a petri dish for 60 minutes. Pasteur pipettes, drawn out to a narrow bore slightly larger in diameter than the eggs (about 150 $\mu$m), were used to transfer eggs to fresh, sperm-free medium. This was repeated three times to remove unbound or loosely bound sperm. [This method proved more reproducible and practical than a centrifugation method recently introduced to measure the extent of mouse sperm-egg binding (*13*).] Eggs were fixed in formalin, mounted on glass slides, and the number of sperm bound per egg counted by using phase-contrast microscopy. At least 20 eggs were counted on each slide.

Membrane vesicles from boar sperm were obtained by nitrogen cavitation and sucrose density centrifugation as described by Gillis *et al.* (*14*). Electron micrographs of these preparations and enzyme marker assays indicated that the plasma membranes were highly purified and free from other possible sperm membrane contaminants (*14*). Antiserums to these membranes were prepared by subcutaneous injection of 50 to 100 $\mu$g of


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membrane into rabbits every 3 to 4 weeks over an 8-month interval. Whole antiserum diluted in buffer was used in some experiments, and univalent antibody (Fab fragments) obtained by papain digestion of the immunoglobulin G (IgG) fraction of antiserum (*15*) was used in others.

Sperm bound rapidly and in large numbers (an average of 50 sperm per egg; range, 30 to 80) in ten different experiments carried out on different days. Motility and rate of forward progression strongly influenced binding, since sperm rendered poorly motile by the uncoupling agent carbonyl cyanide, *p*-trifluoromethylphenylhydrazone, by lowering the temperature of the medium, or by aging for several hours at room temperature failed to bind eggs in significant numbers (20 percent or less than controls). Sperm whose acrosomes had been removed by addition of calcium and the divalent cation ionophore A23187 (*14, 16*) were also rendered immotile and failed to bind in significant numbers to eggs. The data relevant to decreased motility suggest that contact of sperm with the zona was not forceful enough for a sperm-egg complex to form or that fewer contacts of sperm and egg occurred and resulted in the decreased numbers of bound sperm. Another observation was that sperm binding showed limited species specificity (Table 1). When human ejaculated sperm, or rat or guinea pig cauda epididymal sperm, were added at high concentration (ten times higher than the boar sperm controls) and stimulated to high motility by caffeine, they failed to bind to porcine oocytes. Golden hamster epididymal sperm and ejaculated bull sperm did bind to the porcine zona pellucida but in smaller numbers than the boar sperm.

Antiserum (diluted 1:1000) almost completely blocked the binding of boar sperm to the zona after incubation periods from 10 minutes to 1 hour. Agglutination of most sperm occurred after prolonged incubation (1 hour), but binding was still blocked in the presence of caffeine, which stimulated motility and considerably reduced the rate of agglutination. However, in order to remove any ambiguity about the effect of agglutination on motility, univalent antibody was prepared by papain digestion of the IgG fraction of antiserum and tested for its effects on the formation of sperm-zona complexes (Table 2). Agglutination of sperm in the presence of this type of antibody was negligible and was not a factor in inhibiting sperm motility (and thus sperm-egg binding). Binding of highly motile sperm to eggs was blocked at antibody concentrations as low as 5 μg/ml. However, when membrane vesicles were used to absorb the antibody preparation, the absorbed preparations were incapable of blocking sperm penetration and binding to the zona pellucida (Table 2).

These experiments provide what may be the first direct evidence that specific antigenic receptors on the surface of the mammalian spermatozoon are responsible for sperm binding to the zona pellucida. A recent study by Gwatkin and Williams (*17*), based on the effects of the absorption of heat-solubilized zona to hamster sperm on the formation of sperm-egg complexes, suggested that different sites may be involved in the binding of noncapacitated and capacitated sperm to the zona of that species. Porcine oocytes appear to contain a limited number of proteins in complex association with carbohydrates (*12, 18*), and several distinct antigenic sites appear to be associated with this arrangement (*10*). Since successful binding of sperm and egg is the first step in the fertilization process, the isolation of the surface antigens of sperm involved in binding to the egg investments will increase our understanding of this key reaction in fertilization. Recently developed techniques for isolating large amounts of porcine zona (*10*) and boar sperm plasma membranes (*14*) should facilitate the isolation and identification of these antigens.

Table 1. Species specificity of sperm binding to porcine eggs.

| Species* | No. of bound sperm per egg† | Motile sperm‡ (%) |
|---|---|---|
| *Experiment 1* | | |
| Boar | 37.4 ± 7.4 | 40(3) |
| Human | 0.1 ± 0.0 | 40(3) |
| Human (+ 8 m*M* caffeine) | 0.5 ± 0.1 | 50(5) |
| *Experiment 2* | | |
| Boar | 50.2 ± 10.7 | 90(5) |
| Guinea pig (+ 8 m*M* caffeine) | 0.2 ± 0.1 | 80(5) |
| Rat (+ 8 m*M* caffeine) | 0.0 ± 0.0 | 60(2) |
| Hamster (+ 8 m*M* caffeine) | 11.9 ± 2.0 | 80(6) |
| *Experiment 3* | | |
| Boar | 79.2 ± 8.5 | 70(3) |
| Bull | 7.1 ± 1.2 | 70(3) |

*Concentration of boar sperm, $3 \times 10^5$ per milliliter; all other sperm, $3 \times 10^6$ per milliliter. †Each datum is the average of three assays ± standard error. ‡Numbers in parentheses are estimates of rates of forward movement of sperm, subjectively evaluated on a scale of 0 to 10.

Table 2. Effect of univalent antibody (Fab) to boar plasma membranes on sperm-egg binding.

| Addition* | Motile sperm (%)† | No. of bound sperm per egg‡ |
|---|---|---|
| *Experiment 1* | | |
| None (control) | 70(3) | 63.1 ± 6.7 |
| Fab (50 μg/ml) | 70(2) | 0.8 ± 0.2 |
| Absorbed Fab§ (50 μg/ml) | 70(3) | 47.3 ± 1.5 |
| *Experiment 2* | | |
| None (control) | 70(5) | 79.2 ± 8.5 |
| Fab (10 μg/ml) | 70(5) | 5.4 ± 1.9 |
| Fab (5 μg/ml) | 70(5) | 2.6 ± 1.0 |
| Absorbed Fab (10 μg/ml) | 70(5) | 39.7 ± 10.7 |

*Caffeine (8 m*M*) was added to the buffer in all treatments. †Numbers in parentheses are estimates of rates of forward movement of sperm, subjectively evaluated on a scale of 0 to 10. ‡Each datum is the average of three assays ± standard error. §Absorption of antibody was carried out by mixing 25 μg of Fab with 100 μg of membrane vesicles in 50 μl of buffer for 60 minutes. Vesicles were removed by pelleting at 100,000*g* for 30 minutes; the supernatant was incubated with sperm for 1 hour in 0.2 ml of buffer before adding eggs.

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