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Autoantibodies to Zona Pellucida: A Possible Cause for Infertility in Women

Abstract. *Human and pig ovaries were tested by agar gel diffusion and found to contain several cross-reacting (common) antigens. At least one common antigen was located in the zona pellucida as determined by indirect immunofluorescence. Serum samples from 22 infertile women were tested on pig eggs by immunofluorescence, and six of these samples produced strong and nine produced moderate reactions with the zona pellucida. The autoantibodies may be responsible for infertility in these women.*

Mature mammalian eggs in the ovary or oviduct are enclosed by a noncellular, gelatinous-like layer known as the zona pellucida. Because of its position, anything passing into or out of the egg, including spermatozoa, must pass through this layer. Thus, a number of roles are ascribed to the zona, including sperm recognition, attachment, and penetration; block to polyspermy; protection of the egg and embryo; movement of the embryo in the reproductive tract; support of blastomeres; and osmotic regulation (*1*). The importance of these suggested roles make the zona pellucida an attractive target for manipulation in regulating fertility.

An immunological approach to the regulation of fertility has attracted much attention recently with the emphasis being on antibodies to hormones and spermatozoa (*2*). Antibodies produced against the zona pellucida alter the zona surface in such a way that receptor sites are no longer available to spermatozoa, so that attachment and penetration through the zona, and consequently fertilization, are inhibited (*3*). In addition to blocking fertilization, antibodies to the zona agglutinate zona-coated eggs; alter the light-scattering properties of the zona by forming a precipitate on the zona surface; block zona digestion by enzymes usually highly effective in dissolving the zona; and, finally, in the case of fertilized eggs prevent the embryos' escape from the zona (*4*), thereby inhibiting implantation (*5*). Since the zona is accessible to immunoglobulins in both the ovary and the reproductive tract (*6*), there are at least two focal points for attack in attempts to regulate fertility, the first being prior to sperm contact while the egg is in the ovary or oviduct, the second being after fertilization while the egg is in the oviduct or uterus but before it has undergone implantation.

The ovary appears to be a good source for large quantities of zona pellucida antigen or antigens, and a number of workers agree that the zona contains one of the strongest ovarian antigens (*3, 7*). It has been estimated that the human ovary may have as many as a million eggs at the time of birth, and a high proportion of these become zona-coated during maturation. Most eggs eventually become atretic and are absorbed while still in the ovary. Less than 1 percent of the eggs are ovulated during the reproductive life of the individual, and although most of these pass into the reproductive tract, they are probably absorbed in this location.

In view of the zona's strong antigenicity and the large amount of it that is synthesized and absorbed, autoimmune properties might be expected of the zona because it forms rather late in ontogenesis, during the diplotene stage of meiosis and after the time at which tolerance to the antigen might have developed. The breakdown and absorption of the zona in the ovary and reproductive tract could continually expose the zona antigen to the immune system and thus result in autosensitization. Autoantibodies are currently suspected as being responsible for a variety of immunologically related diseases. Indeed, auto- and isoantibodies to spermatozoa are believed to be responsible for some cases of infertility in both men and women (*8*). For these reasons we decided to examine serums for possible autoantibodies to zona pellucida in a group of women who were infertile for unknown reasons. The rationale was that autoantibodies to zona might react with the zona to produce infertility by preventing sperm-egg interaction at fertilization or by preventing the embryos' escape from the zona at implantation.

In order to test for autoantibodies to the zona pellucida in women, a large number of eggs were needed for exposure to the serums. The difficulty of obtaining enough human eggs to perform the tests led us to examine the zona pellucida of several mammals for antigens which might cross-react with the human zona. The pig turned out to be the animal of choice since large numbers of zona-coated eggs can easily be obtained by

Table 1. Antigens in pig and human ovaries and pig eggs. The results are expressed as the maximum numbers of precipitin bands formed in agar gel double diffusion tests. Whether the common antigens between pig and human ovaries are identical cannot be determined from the present experiments.

Antiserums	Antigens		
	Pig ovary	Human ovary	Pig eggs*
Antiserum to pig ovary			
Unabsorbed	8 to 12	4 to 6	2
Absorbed with pig kidney and spleen	4	2	2
Antiserum to human ovary			
Unabsorbed	2 to 4	9 to 12	2
Absorbed with pig kidney and spleen	2	3	2

*Saline homogenates of zona-coated eggs were tested against antiserums. Approximately 200 eggs were used per well in diffusion tests.

rupturing follicles of ovaries from freshly slaughtered pigs. We have obtained as many as 100 zona-coated eggs from a single ovary by this method. In addition, heteroantibodies produced in rabbits against human ovary (RAHO) strongly cross-reacted in agar gel diffusion tests with saline extracts of pig ovary, and rabbit antibodies to pig ovary (RAPO) likewise cross-reacted with human ovary (see Table 1). These results show that the pig and human ovaries have several serologically related (common) antigenic components. At least two of these common antigens are associated with the egg since two precipitin bands were produced in gel diffusion tests when either RAHO or RAPO was reacted with freeze-thawed, sonicated, saline extracts of isolated zona-coated pig eggs.

To isolate zona-coated eggs, we removed cumulus and follicular cells from ovarian eggs by shaking the eggs vigorously for 1 minute in 0.1 percent sodium citrate in 0.05*M* phosphate-buffered saline (PBS) at *p*H 7.0. Removal of these cells by citrate was superior to the usual method of using hyaluronidase because all of the cells, even in immature eggs, were removed by the citrate. The denuded eggs were washed in several changes of PBS by transfer with a micropipette, and were then used either in gel diffusion experiments or in indirect fluorescent antibody tests to localize the antigens (*7*).

For localization of the antigens, the eggs were incubated in a 1:1 mixture of serum (rabbit control, RAHO, or RAPO) and PBS for 30 minutes (25°C) and finally washed with several changes of PBS to remove excess serum and unreacted antibodies. The immunoglobulin fraction of serum was used in some tests and gave identical results to whole serum. After exposure to serums, the eggs were incubated for 10 minutes in fluorescein-conjugated sheep serum (control) or sheep antiserum to rabbit IgG (Nutritional Biochemicals). The eggs were washed several more times by transfer into buffer and were allowed to remain in the buffer for 1 hour with occasional stirring to remove any unreacted antibody. The washed eggs were transferred to microscope slides and were observed with Zeiss fluorescence optics (barrier filters - 65/27 and excitation filter UG5); permanent records were made with Kodak High Speed Ektachrome film.

Table 2. Autoantibodies in the zona pellucida in serums from 22 infertile women (*11*). These serums were tested on pig eggs by the immunofluorescent antibody technique.

Strength of reaction*	Number	Individual samples†
++++	7	1,3,4,20,35,36,42
+++	9	9,12,13,14,22,24,27,33,43
++ to 0	6	6,7,8,10,23,24

*Symbols: ++++, strong reaction; +++, moderate to weak reaction; ++ to 0, no reaction. †Patient numbers assigned by the World Health Organization Serum Bank.

Only weak or in most cases no fluorescence was seen in the zona pellucida after treatment of the eggs with the control serum. In contrast, strong fluorescence was observed on the pig zona (see Fig. 1a) after exposure to antiserum to pig or human ovary, indicating that at least one common antigenic component between pig and human ovary is in the zona. It has been shown that RAPO produces strong fluorescence on the human zona (*9*). The antigens reacting with these antibodies are restricted to or at least have their highest concentration on the zona surface, since fluorescence was strongest in this area. This correlated with the location of specific zona antigens reported for the mouse, hamster, and rabbit (*3, 7, 10*). Little or no fluorescence due to antibody treatment was seen within the egg. A few tests made on pig eggs flushed from oviducts produced results identical to ovarian eggs.

To look for autoantibodies to zona, we used 22 serum samples (*11*) from infertile women (SIW). Pig eggs were treated with these serums according to the indirect immunofluorescent antibody technique as described above. The eggs were exposed to SIW or to appropriate control serums and then exposed to fluorescein-conjugated sheep antiserum to human IgG. Controls included serums from women of known fertility, antigen-absorbed serums, and labeled serums alone. None of the controls produced fluorescence on the pig zona. Seven samples of SIW produced strong fluorescence in the zona, nine produced moderate to weak, and six no fluorescence (see Table 2 and Fig. 1b). The fluorescence was due to an antigen-antibody reaction because the immunoglobulin fraction of serum gave a positive test and only the antigen (zona or ovary) was capable of neutralizing the antibody. We interpret the binding of antibody to zona in these experiments as indicating the presence of autoantibodies to zona in the serum of these infertile women.

Whether the autoantibodies are the main factors responsible for infertility in these women is not known. However, it is tempting to speculate that autoantibodies could react with zona to prevent sperm attachment and penetration, or that the antibody could block implantation by preventing the embryos' escape from the zona, as has been shown to occur in other animal species following exposure of eggs to heteroantibodies (*3, 5, 10, 12*). It would be of interest to test eggs from these women to determine whether antibodies are present on the zona and whether these antibodies block sperm to zona attachment, in vitro.

We were somewhat surprised at the high percentage (7 of 22, or 32 percent) of serum samples which showed high titers of antibody to the zona; however, this was a selected group of women who may be prone to autosensitization. These SIW were kept in the World Health Organization Serum Bank because the infertility of the women from whom the serums were collected is believed to be due to immunological factors, primarily antibodies to spermatozoa (*8*). This leads us

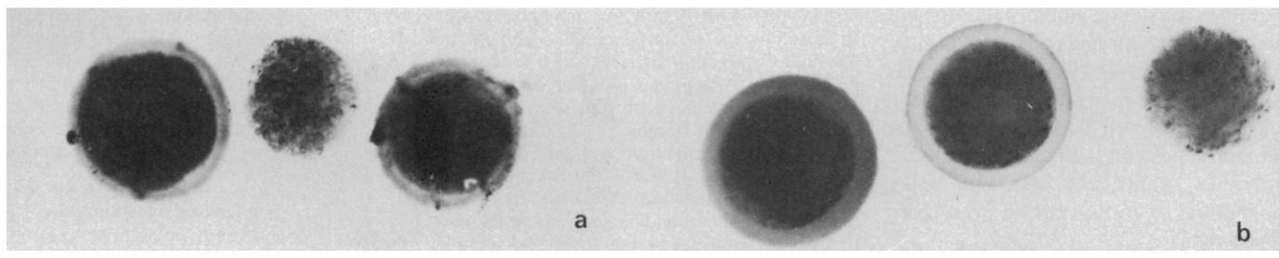

Fig. 1. (a) Zona-coated pig eggs were exposed to rabbit control serum (center), or antiserum to pig (left) or to human (right) ovary, and were then exposed to fluorescein-conjugated sheep antiserum to rabbit immunoglobulin. Note the strong fluorescence in the zona of both eggs treated with ovary antiserum. The egg cytoplasm shows a blue autofluorescence in control or antibody-treated eggs and can easily be distinguished from the apple-green fluorescence caused by the antibody (× 640). (b) Zona-coated pig eggs were exposed to serum from infertile women and then to fluorescein-conjugated sheep antiserum to human IgG. Examples of strong (left), moderate to weak (middle), and no reactions (right) are shown (× 640).

to ask why these women have antibodies to their own zona. One explanation is that repeated exposure of the immune system to zona for several years through egg atresia in the ovary and absorption of ovulated eggs in the reproductive tract may account for the production of antibodies to the zona. If this is true, then a higher frequency and titer of antibody might be expected in older (menopausal) women compared to younger ones, since the former would have had a longer exposure to the zona antigen. We are now testing serums from menopausal women for possible antibodies to the zona pellucida. Another possible explanation is that these women might have been sensitized to an antigen (from an unknown source) which cross-reacts with the zona; however, no such antigen has been identified. In fact, all studies to date point to the conclusion that the zona antigen is tissue-specific.

Should these autoantibodies prove to be responsible for infertility, the same system might be exploited in the future for contraceptive purposes. Utilization of zona antibodies as a means of contraception would appear to have several attractive features. Because only a single cell in the oviduct, or at most a few in the ovary, would be involved and because of the long time that the eggs would be available for exposure to the antibody in the ovary or oviduct, a low titer of antibody would, presumably, be required. Research on the mouse and hamster (*10*) has shown that a single injection of serum containing antibodies to the zona results in the antibody binding to the surface of ovarian zona-coated eggs but not to the immature, nonzona-coated ones. This binding to the zona prevented sperm-egg interaction. Infertility in these antibody-injected animals lasted for four estrous cycles (*12*), and thereafter fertility returned to normal levels, suggesting that the contraceptive effects of the antibody do not result in permanent sterility.

Much more work is required before immunocontraception by antibodies to the zona can be envisioned for humans, but all studies to date on animal species have been most encouraging. The identification in women of naturally occurring autoantibodies to the zona pellucida, the lack of any known side effects of these autoantibodies, and the ability of these antibodies to produce infertility make this method of contraception even more promising.

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Purine Nucleoside Phosphorylase Deficiency: Altered Kinetic Properties of a Mutant Enzyme

Abstract. *Erythrocyte purine nucleoside phosphorylase from two brothers had 0.5 percent of normal activity. It differed from the normal enzyme by a tenfold increase in the Michaelis constant for inosine, an inability of inosine to protect against thermal lability, and a more positive net charge. The altered kinetic properties may account for the milder disease in the patients compared to the previously described cases. The data provide evidence for a structural gene mutation and genetic heterogeneity in the new disease of purine nucleoside phosphorylase deficiency and T cell dysfunction.*

Purine nucleoside phosphorylase (E.C. 2.4.2.1) catalyzes the phosphorolysis of purine nucleosides to the corresponding purine base by the following reactions:

Inosine + $P_i \rightleftharpoons$
hypoxanthine + ribose-1-phosphate

Guanosine + $P_i \rightleftharpoons$
guanine + ribose-1-phosphate

This enzyme reaction is an essential component of the pathway by which purine nucleotides are degraded to uric acid in man. A genetic deficiency of this autosomally inherited enzyme is associated with a disorder of cellular immunity and a block of purine catabolism, characterized by hypouricemia, hypouricosuria, elevated plasma inosine, and increased urinary excretion of inosine, deoxyinosine, guanosine, and deoxyguanosine (*1*).

A milder form of T cell dysfunction with an almost normal serum and urinary uric acid and only a partial block of purine nucleoside degradation occurred in two brothers, ages 8 and 10, with purine nucleoside phorphorylase deficiency (*2*).

Table 1. Comparison of purine nucleoside phosphorylase in normal and enzyme-deficient hemolyzate. Diluted hemolyzate (50 μl) was incubated at 37°C with 0.2 m*M* [8-^{14}C]inosine (5 μc/μ*M*) and 50 m*M* sodium phosphate buffer, *p*H 7.4. Normal hemolyzate was diluted (1 to 1500) and was incubated for 10 minutes, and hemolyzate containing the deficient enzyme was diluted by 1 to 20 and incubated for 60 minutes. The reaction was stopped by heating to 85°C for 2 minutes. The tubes were centrifuged at 1500*g* for 5 minutes. A portion (20 μl) of supernatant was spotted on sheets of Whatman 3 MM chromatography paper, already spotted with nonisotopic hypoxanthine and inosine, each 1 mg/ml. The sheets were subjected to electrophoresis for 30 minutes in 50 m*M* sodium borate, *p*H 8.9, at 4000 volts and 250 ma. The hypoxanthine spots were located by ultraviolet light and cut out; radioactivity was counted in a Packard liquid scintillation spectrometer system. The reaction was linear with protein and with time up to 60 minutes. The mean enzyme activity in 65 patients was 2368 ± 614 nmole/mg per hour. With 2.0 m*M* inosine the same procedure was followed. The activity of the control enzyme increased by a factor of 3.4 upon increasing the inosine concentration tenfold. The mutant enzyme activity increased by factors of 4.1 and 3.8.

Inosine (m*M*)	Nucleoside phosphorylase as hypoxanthine (nmole/mg per hour)		
	Control	Patient 1	Patient 2
0.2	2955	9.9	9.9
2.0	9929	40.2	37.3