sulation of foreign substances or through coagulation processes. Although the exact role of the *H. polii* lyzosyme is still unknown, the observed level in coelomocytes, the cells involved in defensive processes[11], substantiates the hypothesis that the enzyme could participate in antibacterial defense mechanisms.

Lysozyme activity was also detected in *H. polii* cell-free coelomic fluid, though it was evident only when 50-fold concentrated coelomic fluid was used. This suggests that a low level of enzyme is naturally released by coelomocytes, probably to supply a basic defensive concentration of the antibacterial substance. As demonstrated by Cheng[8], lysozyme occurs in both serum and cells of *Mercenaria mercenaria*. The enzyme is normally released by the cells, but its hemolymph content is enhanced during phagocytosis[8,9]. As yet, we do not know whether any change in the lysozyme level of cell-free coelomic fluid occurs after injection of micro-organisms. It is possible that, as in other invertebrates[5–9], the holothuroid bacteriolytic enzyme could be enhanced by injections.

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Necessity of adjuvants for inducing effective antibody response to zona pellucida antigens

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Summary. Rhesus monkeys evoked a vigorous antibody response to a single heteroimmunization dose of zona pellucida antigens, when these were administered along with complete Freund's adjuvant. The antisera recognized all the three major porcine zona glycoprotein families and the animals were rendered amenorrheic after such immunization. Monkeys immunized with zona without adjuvant, however, failed to show any anti-zona antibody response and had normal menstrual cycles. Zona pellucida glycoproteins are thus not effective immunogens unless administered along with a powerful adjuvant.
Key words. Zona pellucida glycoproteins; adjuvant; immunoblotting; enzyme-linked immunosorbent assay; autoantibodies; infertility.

Zona pellucida (ZP), the extracellular glycoprotein coat enveloping the mammalian oocyte, has been the subject of extensive investigations as a candidate for immunological fertility control. A number of studies have emphasized the strong antigenicity and immunogenicity of this structure, and recently, studies on ZP have gained added importance since it has been established beyond doubt that active heteroimmunization with ZP glycoproteins results in the production of anti-zona antibodies which can inhibit fertility in many species[1–4]. The porcine ZP in particular has become the focus of such research owing to its cross-reactivity with human ZP and ready availability.

Autoantibodies to ZP have also been implicated in the development of infertility in women. Several studies employing immunofluorescence methods have demonstrated anti-ZP antibodies in the sera of infertile women[5,6]. Similar autoantibodies were also found in the sera of aging women as well as aging animals[6,7]. These studies led to the conclusion that such an autoimmune phenomenon might occur naturally in aging animals.

It has been postulated that breakdown products of ovum and zona may initiate an immune response due to their accessibility to the immune system for a long period[8]. Since zona is formed rather late in ontogeny, it may be recognized as foreign. Some reports dismissed this hy-

pothesis by demonstrating widespread anti-zona pellucida activity among sera from infertile and fertile human male and female subjects[9].

In the light of the above conflicting reports concerning autoantibody production in response to ZP antigens, the present study was designed with a view to determining whether antibodies to the highly immunogenic ZP antigens can be formed in the absence of an adjuvant in non-human primates, employing more specific techniques like enzyme-linked immunosorbent assay and radioautography.

Materials and methods

Porcine ovaries for ZP isolation were obtained from local slaughterhouses. Large-scale isolation of ZP was carried out as previously described by Wood et al.[4]. Heat solubilization was done according to the method of Dunbar et al.[10]. Acrylamide was obtained from Serva Chemicals, New York (USA) and wide-range ampholines (pH 3.5–10) from LKB, (Bromma, Sweden).

Preparation and characterization of antisera. a) *Immunizations*: Antisera were prepared by immunizing two groups of healthy cycling female rhesus monkeys (*Macaca mulatta*) with 250 µg of heat-solubilized porcine zona (HSPZ) glycoproteins. The first group (Group I) received the antigen emulsified with 0.5 ml of complete Freund's adjuvant at multiple sites intradermally, and the second (Group II) received antigen without any adjuvant. Animals were bled sequentially at 4-week intervals and sera were tested for the presence of anti-HSPZ antibodies using counter-immunoelectrophoresis and enzyme-linked immunoassay[11]. Preimmune bleedings served as negative controls.

b) *Enzyme-linked immunosorbent assay*: A modification of the technique described by Voller et al.[12] was used in the present study. A total of 400 ng of HSPZ protein per well was coated on the microELISA plate (Dynatech Labs, Virginia, USA). Peroxidase-conjugated goat anti-human IgG (Dakopatts, Denmark) was used as the second antibody. Plates were read on a Titertek Multiscan ELISA reader (Flow Labs, Finland) 30 min after adding orthophenylinediamine dihydrochloride as the substrate.

c) *High resolution two-dimensional PAGE*: Two-dimensional electrophoresis was carried out as outlined by Dunbar et al.[13] and Dunbar[14]. Isoelectric focusing (first-dimensional electrophoresis) was carried out at 25 °C for 18 h at 650 V for a total of about 12,000 V hours, after solubilization with IEF-solubilization buffer containing 2% SDS, 2% β-mercaptoethanol and 1% cyclohexylaminoethanesulfonic acid (CHES), pH 9.5 (95 °C, 10 min). Second dimension slab gels of 10 to 20% polyacrylamide (including bis crosslinker) linear gradient were prepared using the Pace linear gradient maker (Electronucleonics, Inc., Tennessee, USA). Electrophoresis was carried out using the Anderson DALT electrophoresis tank at 550 V for 5 h. The color-based silver stain[15,16] was used to detect the ZP proteins and peptides as described by Dunbar[14].

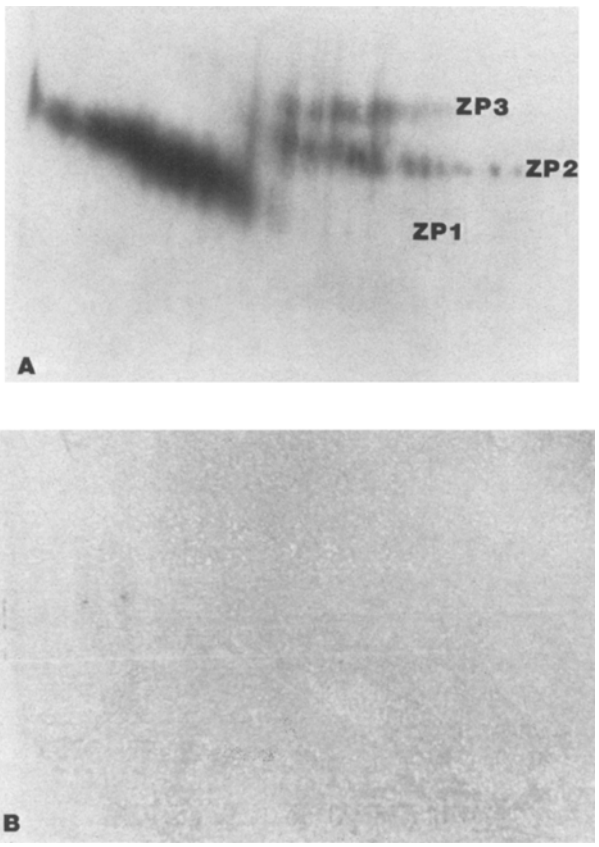
d) *Immunoblotting*: Immunoblotting was performed according to a modification of the method of Timmons et al.[17] to identify specific glycoprotein families. Unstained and unfixed 2D-PAGE gels were placed on the cathode side of nitrocellulose paper (BioRad, California, USA) and transferred for 2.5 h at 1.2 A using E-C Electroblot (Florida, USA) transfer unit. The nitrocellulose paper was then blocked in 10 mM tris-saline, pH 7.2, containing 3% (w/v) BSA, overnight, following washings with two changes of tris-saline (-BSA).

The nitrocellulose transfer was then incubated with ^{125}I-protein A. A total of 1.1×10^6 cpm in 50 ml of tris-saline (containing BSA and sodium azide) was added to each transfer and incubated overnight. Extensive washing in tris-saline (-BSA) was carried out before air drying and exposure to Kodak XAR-5 X-ray film for autoradiography using intensifying screens, for 72 h at − 70 °C.

Results and discussion

The porcine zona pellucida, when analyzed by 2D-PAGE, resolved into three major glycoprotein families as reported earlier[13,18,19]. Rhesus monkeys evoked a vigorous antibody response to immunization with pig zona antigens, when these were administered with CFA (Group I). The antibody titers were very high, as determined by ELISA, despite the fact that no booster immunizations were given. This response is similar to that observed in rabbits[11,20]. The sera recognized all the three major glycoprotein families (fig., A) as described earlier using coomassie blue staining or iodination procedures for protein identification[13,15,21]. The group of monkeys immunized with zona glycoproteins without any adjuvant (Group II), however, failed to elicit any detectable antibody response that could be measured by ELISA or immunoblot techniques (fig., B). The mean peak OD values of the immune sera, evaluated by ELISA at 1:500 dilution, were 1.172 ± 0.03 and 0.105 ± 0.02 for group I and group II, respectively. The differences were highly significant statistically (table). The mean number of menstrual cycles in group I and group II were 2 ± 0.82 and 19.25 ± 5.44, respectively.

The anti-zona pellucida activity detected in the sera of some infertile women has been proposed to be due to an autoimmune phenomenon against a human zona-specific antigen, which cross-reacts with porcine zona due to the well-documented antigenic sharing between the two[22]. During the last decade, many elaborate studies have been published, both in favor of and against this hypothesis. Studies supporting the concept of autoimmunity to ZP have shown the presence of anti-zona activity in infertile sera even after absorption with pig and human erythrocytes. A recent report[8] demonstrated the presence of anti-zona activity in 53.3% of unabsorbed sera and 26.7% of absorbed sera from tubectomized women. The



Autoradiographs showing the three major glycoprotein families of porcine zona pellucida labelled I, II, and III (having relative molecular weights ranging from 45 K to 120 K; 70 K to 101 K; and 95 K to 118 K, respectively). Intact pig zonae were separated by 2D-PAGE and after transfer to nitrocellulose paper, were probed with anti-HSPZ antiserum from monkeys immunized with (*A*) HSPZ + CFA, (*B*) HSPZ – CFA, using ^{125}I-Protein A as the second antibody, followed by exposure to Kodak XAR-5 film for 72 h at – 70 °C.

authors failed to detect any fluorescence in positive sera when it was further absorbed with zona-coated eggs, thus indicating a zona-specific antibody response.

The mere presence of anti-ZP activity in female sera, however, does not imply that it is due to an autoimmune response to her zonae. This view has been supported by the findings of Sacco and Moghissi[9], who reported a high incidence of anti-zona antibodies in human sera from male and female fertile and infertile subjects. The authors were convinced that such antibodies could initially be produced against a presently unidentified antigen(s) which shares antigenic determinants with the zona or a contaminant (follicular fluid or egg components) absorbed to the zona[23]. Moreover, the presence of anti-zona antibodies does not necessarily correlate with infertility, as is clear from the experiments of Sacco[24] and Shivers and Dunbar[5], which showed that antibody-bound zona-coated eggs were still fertilizable.

The results of the present study also confirm the findings of Sacco and Moghissi[9], and cast suspicion on the validity of the postulate that 'autoantibodies' to ZP are formed in aging or infertile women because they are exposed to the zonae of the numerous ovulated ova. If a high concentration of foreign ZP protein without adjuvant does not elicit an immune response, even though it contains 'foreign' as well as 'cross-reactive' determinants, it is highly unlikely that ZP from a few ovulated ova would induce such a response in a normal situation. In fact, high titer alloantibodies are not generated even if rabbits are immunized with rabbit ZP in the presence of CFA, and such antibodies do not block fertility[4].

It is important to note that almost all the reports claiming the occurrence of anti-zona autoantibodies have employed immunofluorescence techniques for antibody detection. Moreover, ZP is a complex extracellular matrix, and may easily trap immunoglobulin molecules (i.e. immune complexes formed from Fc aggregation). Since it is difficult to wash the ZP free of these antibodies, non-specific fluorescence of non-immune sera frequently results, if experimental conditions are not stringent. Such assays should therefore be interpreted carefully and must be supported by alternative methods of antibody detection. Dunbar[1] used radioimmunoassay to screen anti-ZP antibodies in sera of infertile women, but failed to detect significant quantities of antibodies which recognize pig zonae.

In summary, the results of the present study demonstrate that the constituent molecules of zona pellucida are not

Effect of immunization with or without adjuvant on antibody production and ovarian cyclicity.

	Peak ELISA value (at 450 nm)	Cycles post immunization	Observation period (years)
Group 1: Immunization with HSPZ + complete Freund's adjuvant			
Animal 1	1.156	2	2
Animal 2	1.202	2	2
Animal 3	1.194	1	2
Animal 4	1.134	3	2
Mean*	1.172 ± 0.03	2 ± 0.82	
Group 2: Immunization with HSPZ – complete Freund's adjuvant			
Animal 1	0.110	27	2
Animal 2	0.085	15	1.8
Animal 3	0.127	16	2
Animal 4	0.098	19	2
Mean*	0.105 ± 0.02	19.25 ± 5.44	

*$p < 0.001$ (paired 't' test).

effective immunogens unless administered along with a powerful adjuvant. Based on these findings, it is very unlikely that the anti-ZP antibodies found in the serum of the human female are a normal physiological consequence of isoimmunization to her own ovulated zona components.

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Suppression of phytohemagglutinin induced splenocyte proliferation during concurrent infection with *Eimeria nieschulzi* and *Nippostrongylus brasiliensis*[1]

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Summary. Results suggest that infection with *Eimeria nieschulzi* (Protozoa) interferes with splenocyte proliferation induced by infection with *Nippostrongylus brasiliensis* (Nematoda).
Key words. Eimeria nieschulzi; Nippostrongylus brasiliensis; splenocyte; blastogenesis; PHA.

Numerous studies have demonstrated that some parasites can alter immune function in infected hosts[3]. However, with the exception of *Toxoplasma gondii*[4] few studies have been published demonstrating depression of mitogen-induced lymphocyte blastogenesis by coccidia. Recently, however, Rose and Hesketh[5] showed that serum from *Eimeria tenella* infected chickens is capable of depressing mitogen induced T- and B-lymphocyte blastogenesis in vitro. Since previous research has demonstrated delayed expulsion of helminths during concurrent infections with *Eimeria nieschulzi* and *Nippostrongylus brasiliensis*[6,7] as well as decreased numbers of intestinal polymorphonuclear leukocytes, suppressed systemic granuloma formation, and a suppression of eosinophil release from the bone marrow during *E. nieschulzi* infections[7,8] it was of interest to us to explore whether serum from rats infected with *E. nieschulzi* is capable of altering splenocyte blastogenesis since these cells are involved in immune regulation.

Materials and methods

Forty specific-pathogen-free male TEX: (SD) AM Sprague-Dawley outbred rats (Harlan Breeding Laboratories, Houston, TX), weighing 200–250 g each, were housed in autoclaved cages with sterile wood shavings for bedding. Rats were given commercial rodent chow and water ad libitum, and kept on a 12-h light/dark cycle. After allowing the animals to acclimate to the cages for 2 weeks, feces were examined for extraneous protozoa and helminth ova by flotation[6]. Rats were then divided on day 0 into 4 groups of 10 rats each. Group 1 served as uninfected controls. Group 2 rats were each inoculated per os with 2.5×10^5 sporulated oocysts of *E. nieschulzi* and sacrificed on day 8 postinoculation (PI). Rats in group 3 were each administered subcutaneously 2000 L_3 larvae of *N. brasiliensis* and sacrificed on day 16 PI. Group 4 animals were inoculated with *N. brasiliensis* larvae and on day 8 PI, *E. nieschulzi* was administered; rats were sacrificed on day 16 PI of the helminth infec-