as was the case with the transaminase value, the extent of liver damage was less in rats treated with immunopotentiators or vitamin E than in controls treated with saline. The liver tissue levels of TBA reacting substances were not elevated in rats treated with immunopotentiators or vitamin E even after injection of CCl_4, which fact suggested that lipid peroxidation in liver tissue by CCl_4 might be inhibited. The effect of immunopotentiators against CCl_4-induced lipid peroxidation was dose-dependent (table).

Discussion. It has been accepted that the liver injury caused by CCl_4 is due to lipid peroxidation in liver microsomes[1]. In the present study, experimental liver injury induced by CCl_4 could be inhibited by immunopotentiators such as BCG, levamisole, OK-432 and PS-K, and in spite of exposure to CCl_4 the liver tissue levels of TBA reacting substances were not increased in rats pretreated with such immunopotentiators. Levamisole has been shown to inhibit lipid peroxidation induced by X-irradiation[4]. This study suggests the possibility that such immunopotentiators might exhibit a protective action against lipid peroxidation damage induced by CCl_4. Lipid peroxidation mediated by free-radicals is believed to be one of the important causes of cell membrane destruction and cell damage, for the cell membrane contains much lipid, especially unsaturated fatty acids[7]. The possibility that immunopotentiators might inhibit the CCl_4-induced lipid peroxidation damage deserves special attention from the standpoint of protection against, and treatment of, various kinds of damage arising from lipid peroxidation.

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Immunogenic potency of the zona pellucida

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Summary. New Zealand white rabbits were immunized with low doses of pig zona pellucida material with the aim of reducing nonspecific antibodies in the antiserum. The antibody levels were assayed by the standard precipitation and immunofluorescence methods. The titers produced were comparable with those obtained using large amounts of zona material.

Injection of zona pellucida material into laboratory animals provokes antibody-formation against this membrane[1,2]. Treatment of the zona with antiserum containing antibody-activity against zona-antigens produces a precipitation layer on the surface of the zona pellucida (fig. 1). This precipitation line blocks zona digestion by trypsin[3]. The inhibition of fertilization by anti-zona serum has been reported in the mouse and hamster[4,5]. These anti-zona sera probably interfere with the so-called sperm-attachment sites, or sperm receptors, on the zona pellucida[6], and can be used as immunological vaccines for contraception[7]. Sacco[8] and Dietl et al.[9] demonstrated a crossreactivity between the porcine and the human zona pellucida. As pig zona material is easily obtainable it is therefore an ideal model for immunological studies of the human zona pellucida and can help us to analyse further the immunological properties of this extraordinary membrane.

The intention of the present study was to determine whether a low dose of 100–400 zonae pellucidae (equivalent to 330 ng to 1.32 µg protein) is enough to produce a detectable antibody-titer in a rabbit following immunization.

Fresh pig ovaries were obtained from a slaughterhouse and were kept frozen at −20 °C.

Follicular fluid was gained by aspiration with a fine glass pipette and the follicular eggs were collected from the pooled fluid using a stereomicroscope. Eggs were washed several times in PBS and then suspended in sodium citrate. In order to obtain isolated zonae the cumulus-free eggs were drawn into and expelled from a micropipette with an internal bore slightly less than the diameter of the oocyte plus its zona. The egg is destroyed during this procedure. Isolated zonae were collected and washed twice in PBS.

6 groups of New Zealand rabbits, each consisting of 3 females, 5–6 months old, were immunized as follows:

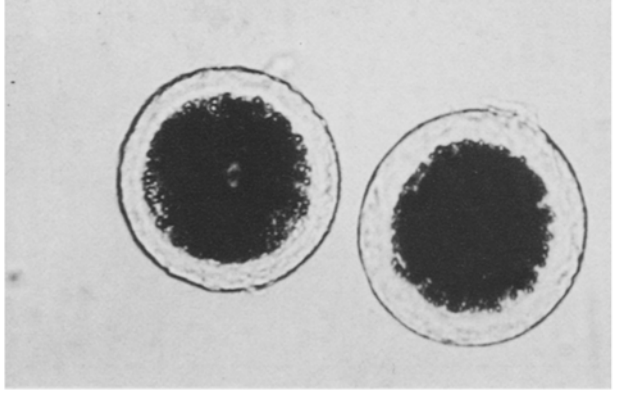

Figure 1. Precipitation layers on pig egg cells.

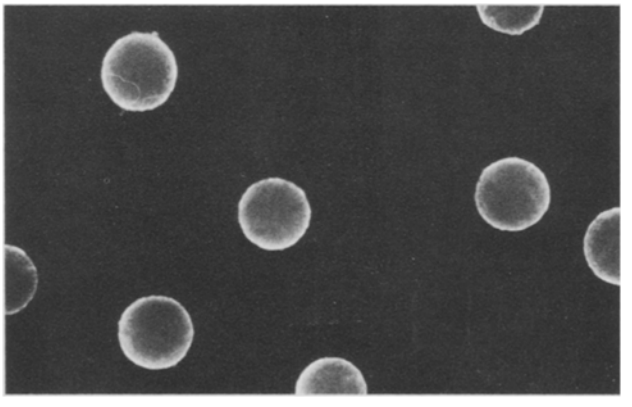

Figure 2. Positive immunofluorescence of pig zonae pellucidae.

Group 1: 100 pig zonae pellucidae in the initial injection, followed by 3 injections each of 50 zonae. Group 2: 200 pig zonae pellucidae in the initial injection, followed by 3 injections each of 100 zonae. Group 3: 400 pig zonae pellucidae in the initial injection, followed by 3 injections each of 200 zonae. Group 4: 400 heat-solubilized (75 °C/20 min) zonae in the initial injection, followed by 3 injections each of 200 solubilized zonae. Group 5: 0.5 ml volume of homogenized pig ovary in the initial injection, followed by 3 injections each of 0.25 ml volume. Group 6: control group - 4 injections containing only PBS and Freund's complete adjuvant.

Table 1. Average antibody titer in 2 groups of 3 rabbits each, following immunization with 100 and 200 isolated pig zonae pellucidae

Immunizations	Antibody titer determined by zona precipitate	indirect immunfluorescence
100 zonae		
1.	-	2^4
2.	2^3	2^6
3.	2^3	2^7
4.	2^3	2^6
200 zonae		
1.	2^2	2^4
2.	2^2	2^5
3.	2^3	2^6
4.	2^3	2^6

Table 2. Average antibody titer in 3 groups of 3 rabbits each, following immunization with 400 isolated pig zonae: 400 pig zonae heat solubilized; and homogenized pig ovary

Immunizations	Antibody titer determined by zona precipitate	indirect immunfluorescence
400 zonae		
1.	2^2	2^4
2.	2^3	2^6
3.	2^4	2^7
4.	2^5	2^8
400 zonae heat solubilized		
1.		2^3
2.	2^2	2^5
3.	2^3	2^7
4.	2^4	2^7
Ovary homogenized		
1.	-	2^3
2.	-	2^4
3.	2^2	2^5
4.	2^3	2^6

Table 3. Effect of absorption with tissue extracts on the titer of anti-pig zonae serum after 4 immunizations

Tissue	Antibody titer determined by indirect immunfluorescence			
	100 zonae	200 zonae	400 zonae	400 zonae heat solubilized
Unabsorbed	2^6	2^6	2^8	2^7
Liver Kidney Pig erythrocyte Follicular fluid	2^6	2^6	2^8	2^7
Zonae concentrate $\left[\frac{10000\,\text{zonae}}{1\,\text{ml}}\right]$	-	-	-	-

The immunization schedule used for each group was: the 2nd injection was given 9 days after the 1st injection, the 3rd, 15 days after the initial injection, and the 4th, 21 days after the initial injection.

Serum was obtained 1 day before each immunization, inactivated at 56 °C for 30 min and then stored at −20 °C.

All antisera were absorbed with pig liver and kidney cells, washed pig erythrocytes, follicular fluid and zona pellucida concentrate.

The titers of antisera were assayed by 2 methods: zona precipitation and indirect immunofluorescence. For the demonstration of zona precipitation a few denuded pig eggs were incubated in 20 µl PBS with 20 µl antiserum at room temperature for 30 min and examined under a phase-contrast microscope. The indirect immunofluorescence-test was carried out according to Gwatkin et al.[6].

The increase in titer of antisera according to time after immunization is shown in tables 1 and 2. There is no statistically significant difference between the immune responses obtained when 100 zonae and 400 zonae are used. The indirect immunofluorescence-test proved to be more sensitive for the detection of antibodies against isolated pig zonae pellucidae than the zona precipitation-reaction. It is not necessary to use large numbers of zonae pellucidae for immunization: 100–200 zonae are sufficient for the initial immunization with following booster injections of half the original dose. Low doses of the pure antigen from zona pellucida induce a distinct increase in the anti-zona titer.

The antibody titers after injection with 400 heat solubilized zonae compare with those after low dose immunization with 400 whole zonae, as determined by both assays. The injection with homogenized ovary caused a lower anti-zona titer in the serum. Of significance is that no precipitation layer was detected until after the 3rd immunization with pig ovary. Fluorescence and precipitation were not observed when the eggs were treated with anti-sera from the control animals (immunized with PBS and Freund's adjuvant).

The titers of the antisera did not change after absorption with pig liver or kidney cells, washed erythrocytes or follicular fluid, but disappeared after absorption with a concentrate of isolated pig zonae (table 3). This suggests that the anti-zona antibodies are very specific. Sacco and Palm[10] found, by immunodiffusion after absorption, that anti-pig zona serum produced 1 to 2 precipitin bands with all of these, except follicular fluid. Therefore the anti-zona pellucida sera may not have been monospecific for zonae; they may have been contaminated by other antibodies. Sacco and Palm used more than 2000 isolated pig zonae for each injection. In our opinion the possibility of contamination, in particular with follicular fluid, is increased with the number of aspirated follicular eggs. Therefore it is preferable to use as low a dose of isolated zonae as possible.

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