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Application of a Radioimmunoassay (RIA) for Monitoring Immune Response to Porcine Zonae Pellucidae (41172)

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Abstract. Radioimmunoassay (RIA) methodology for purposes of monitoring immune response of female mice to injection with porcine zonae pellucidae was investigated. Titers of antibodies prepared against zonae pellucidae and, as measured by RIA, were compared to those obtained by the two frequently used zona antibody titration methods of (i) antibody produced precipitation layer formation on the zona surface (PPT titer), and (ii) indirect immunofluorescence (IF titer). RIA 30% binding and endpoint titers produced comparable immune profiles which indicated rapid rise in anti-zona activity occurring during the first 4 weeks following immunization, peak titer achieved between approximately 5 and 8 weeks followed by a very gradual decline in titer. Anti-zona titers remained at significantly high levels at 225 days following injection. RIA endpoint titers were approximately 200 times greater than the IF titers which, in turn, were 32–64 times greater than PPT titers. These data emphasize the greater sensitivity of the RIA over existing methodology for titrating zona antibodies and the feasibility for using RIA procedures for monitoring immune response following injection with zona material.

The zona pellucida contains one or more tissue-specific antigens capable of eliciting antibodies which can totally inhibit fertility and numerous studies demonstrating the contraceptive effectiveness of such zona antibodies have been reported (1). Consequently, considerable interest has been expressed in the utilization of the zona for immunocontraceptive purposes with particular emphasis being concentrated upon the porcine zona pellucida in view of the recent demonstration that pig and human zonae contain an immunologically similar or identical zona-specific antigen (2, 3).

The vast majority of immunocontraceptive studies involving zonae describe passive immunization procedures (1, 4–8) with investigations utilizing an active immunization approach being considerably fewer in number (9, 10). However, regardless of the immunization regime used, most studies concerning antibodies to zona have two common deficiencies: their lack of a sensitive assay system to adequately monitor immune response to zona antigens and their failure to provide adequate quantitative data describing the zona antibody titers.

Current methodology for assessing immune response to injection with zona material primarily involves using zona antibodies in assay systems such as (a) precipitation layer formation on the surface of the zona, (b) immunofluorescence, (c) inhibition of zona lysis by proteolytic enzymes, (d) inhibition of sperm attachment to zona, and (e) inhibition of *in vitro* fertilization (1, 11). While such assay systems are, and will remain essential for determining the contraceptive potential of zona antibodies, a considerably more sensitive, quantitative, and rapid assay procedure for the detection and titration of these antibodies is required. Obviously, a new and much more sensitive titration procedure would be tremendously useful both in immunocontraceptive investigations and in physiochemical and immunochemical studies designed to characterize individual zona antigens.

Recognizing the present need to develop new techniques to assess antibody activity against zona and the current lack of quantitative data regarding response to active immunization with zona material, the present report describes the modification of a recently published porcine radioimmunoassay (RIA) system (12) and its application for

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the detection and quantitation of immune response of female mice to injection with isolated porcine zonae pellucidae. Our data demonstrate that this RIA represents an extremely sensitive, reliable, and reproducible method for the quantitation of antibodies to zona.

Materials and Methods. *Immunogen preparation and immunization regime.* Intact, mechanically isolated porcine zonae pellucidae were used as the immunogen and were obtained following our previously described protocol (12). Briefly, the procedure involved mincing fresh pig ovaries in 0.1 M phosphate-buffered saline (PBS, 0.1 M PO_4; pH 7.2) and collecting the released follicular eggs from the buffer under a stereomicroscope. The pooled eggs were vigorously shaken for 30 sec in a culture tube (12 × 75 mm) containing 1 ml of PBS to remove cumulus cells. Isolated zonae pellucidae (IZP) were obtained by expelling eggs through a micropipet with an internal bore of a diameter slightly less than that of the egg plus its zona. Following isolation, the intact zonae were thoroughly washed in five changes of PBS and stored frozen until used.

Outbred non-Swiss albino female mice (Sprague–Dawley; Sch:ARS $[CFL]_f$) were used for heteroantisera production. Isolated porcine zonae suspensions were emulsified with an equal volume of Freunds' complete adjuvant and mice were injected following the immunization method reported by Vaitukaitis *et al.* (13). Five groups of mice were injected, with each mouse receiving 0.2 ml of the emulsion containing isolated zonae material. Mice in Groups 1–4 (3 mice/group, total = 12 mice) each received, in a single injection, 100, 250, 500, and 1000 zonae, respectively. Mice in Groups 1–3 also received a booster immunization consisting of zonae quantities equal to their original immunization dosage and emulsified in Freunds' incomplete adjuvant at approximately 3.5 months following the initial immunization. Mice in Group 4 did not receive a booster immunization. Group 5 consisted of 10 mice with each mouse receiving a total of 1000 zonae distributed equally in four separate injections spaced 7–13 days apart. Group 5 mice received no booster immunization following this initial series of injections. Blood samples (~150 μl/bleeding) were collected via the orbital sinus commencing 2–3 weeks following the initial immunization and at weekly intervals thereafter for mice in Groups 1–4. Blood collection was initiated at 11 days following the initial immunization for Group 5 mice. Sera from the 3 mice in each of the first four groups were pooled whereas sera from each bleeding from the 10 mice in Group 5 were not pooled and were tested individually. A control group consisting of 10 mice was injected only with the adjuvants and their blood samples were collected as for Group 5. Immunization regimes for all groups are summarized in Table I.

Detection of anti-zona activity. Antibody activity directed against zona pellucida was titrated by three techniques: (a) formation of a precipitation layer on the zona surface following antiserum treatment (precipitation [PPT] titer), (b) the indirect fluorescent antibody technique (immunofluorescent

TABLE I. MOUSE IMMUNIZATION REGIMES

Group	No. of mice	Zona dosage	No. of injections	Booster dosage	Serum tested
1	3	100	1	100	Pooled
2	3	250	1	250	Pooled
3	3	500	1	500	Pooled
4	3	1000	1	0	Pooled
5	10	1000	4	0[a]	Individ.
Control	10	0	4	0[a]	Individ.

[a] For mice initially immunized with a series of injections, a booster is defined as an immunization injection given at some time beyond this primary series of injections.

[IF] titer), and (c) RIA. The first two procedures were performed as described elsewhere (14) with the highest serum dilution producing a precipitate and/or fluorescence on the zona surface, distinguishable from control serum-treated zona-coated eggs, considered as the final titer (i.e., endpoint titer). Zona-coated pig eggs for use in these tests were collected as described above; mouse eggs were obtained following established protocols for superovulation and cumulus cell removal.

Radioimmunoassay. Solubilized isolated pig zonae pellucidae (SIZP) were radioiodinated by the chloramine-T method as described previously (12). 400 freshly collected IZP were heat-solubilized in a volume of 50 μl Tris–EDTA buffer, pH 8.0, at 70° for 20 min. Iodination was carried out by the addition, in sequence, of the following: 30 μl of 0.5 M NaH_2PO_4, pH 7.5, 0.7 mCi (7 μl) of carrier-free sodium iodide ($Na^{125}I$, Amersham) and 10 μl freshly prepared chloramine-T solution (1 mg/ml 0.05 M NaH_2PO_4). The reaction was allowed to continue for 45 sec at which 500 μl of freshly prepared $Na_2S_2O_5$ (400 μg/ml 0.05 M NaH_2PO_4) were added to the reaction vial to terminate the reaction. The ^{125}I SIZP was separated from free $Na^{125}I$ by elution through a Biogel P-60 column equilibrated against 0.05 M NaH_2PO_4. For use in the RIA, the activity in the peak tube or the tube immediately following the peak was adjusted to 25,000 dpm/100 μl (equal to ~0.1 zona) by dilution with phosphate-buffered saline containing BSA (PBS-1; 0.01 M KH_2PO_4, 0.14 M NaCl, 1% BSA, 1:10,000 merthiolate, pH 7.0).

Sera for antibody evaluation were serially diluted in buffer containing mouse serum (PBS-2; 0.01 M KH_2PO_4, 0.14 M NaCl, 0.05 M Na_2EDTA, 1% normal mouse serum, pH 7.0). Aliquots from two pools of diluted antiserum were used in each assay to estimate the intra- and interassay variations. The precipitating antibody (2nd antibody) was goat anti-mouse γ-globulin (Cappel Laboratories) appropriately diluted in PBS-1 to that dilution which precipitated maximum radioactivity in test systems using fixed dilutions of labeled antigen and anti-IZP sera. Triplicate tubes devoid of anti-IZP serum were used in each assay to estimate nonspecifically bound (NSB) radioactivity.

The assay was carried out by incubating 200 μl of various dilutions of mouse anti-porcine IZP serum, 100 μl of ^{125}I-SIZP, and 500 μl of PBS-BSA at 37° for 4 hr followed by the addition of 200 μl of second antibody. The tubes were then incubated at 4° overnight, followed by the addition of 3 ml of cold PBS (0.01 M KH_2PO_4, 0.14 M NaCl, pH 7.5). The tubes were centrifuged (1100*g*) for 35 min, the supernatants discarded, and the precipitates drained for 15 min and then counted in a Searle Model 1185 gamma counter. The ^{125}I-SIZP precipitated in the nonspecific binding tubes and the anti-IZP sera containing tubes was expressed as the percentage of the total counts added to the tubes in the assay.

The antibody titers for the various bleedings were calculated by two methods: (a) 30% binding titer—defined as that dilution of antibody which precipitates 30% of ^{125}I-SIZP. This titer profile was obtained from the linear and thus most sensitive segment of the titration curve (Fig. 1). The more conventional 50% binding level could not be utilized to express titer for this study because the maximum ^{125}I-SIZP precipitated under our assay conditions was in the range of 55–60%; (b) endpoint titer—

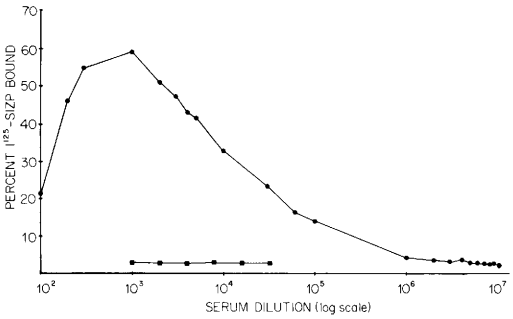

FIG. 1. Antiserum titration profile of a single bleeding from a mouse actively immunized with 1000 isolated porcine zonae pellucidae. Heat-solubilized isolated porcine zonae pellucidae (SIZP) were used as the labeled antigen. Final endpoint titer of this particular bleeding was established at 3.0×10^6. (●) Mouse anti-pig zonae serum; (■) control serum. Nonspecific binding level (NSB) for this particular assay was approximately 3%.

defined as the maximum dilution of antiserum which, when added in a 200-μl quantity and with a final incubation volume of 1.0 ml, precipitates significantly greater radioactivity than the NSB tubes. This method of titer determination was necessary to enable us to directly compare titers obtained by RIA methodology with final titers of antizona activity determined by the biologic assay methods (i.e., PPT and IF titrations) used in the present study and also, with those endpoint titer determinations reported in the literature (9, 10, 15–17).

Dilutions of sera ranging from 1:500 to 1:32,000 which were obtained from mice in the control group were also routinely assayed in the RIA for anti-zona activity.

Results. *Radioimmunoassay.* ^{125}I-SIZP consistently eluted off the Biogel P-60 column as a single sharp peak contained in 4 × 1-ml fractions. The calculated specific activity, expressed as the radioactivity (μCi) incorporated per 1000 zonae, was 164 ± 24 (mean ± SE) for 11 iodinations performed over an 11-month period. The NSB level for 19 assays over the same period was 4.49 ± 0.36% (mean ± SE) of the total ^{125}I-SIZP present in the assay. Intra- and interassay variations, expressed as the coefficient of variations, were 2.12 and 9.35%, respectively. A typical serial dilution titer profile of an individual bleeding from a mouse actively immunized with 1000 zonae is shown in Fig. 1. The diminished binding of antiserum to ^{125}I-SIZP at low serum dilutions (10^2-10^3) was determined to be due to deficiency of second antibody. Increasing the amount of second antibody concentration increased the amount of ^{125}I-SIZP precipitated. However, the maximum ^{125}I-SIZP precipitated in the antiserum dilution range of 10^2-10^3, by increasing either the amount of ^{125}I-SIZP, the second antibody (anti-mouse γ-globulin), or both did not exceed 50–55% of the total counts added to the assay. Furthermore, in the anti-IZP serum ranges at which 30% of ^{125}I-SIZP is precipitated (reflecting the linear portion of the titration curve) or at the highest anti-IZP serum dilutions capable of precipitating ^{125}I-SIZP significantly above the nonspecific binding level (endpoint dilutions), increasing the amount of ^{125}I-SIZP added, the second antibody, or both did not significantly alter the titer profile. Introduction into the assay of various dilutions of sera from control immunized mice or preimmune sera did not precipitate ^{125}I-SIZP above nonspecific binding levels (Fig. 1). Introduction into the assay of increasing concentrations of unlabeled SIZP produced an exponential decrease in percentage binding, similar to the dose–response curve previously published for this assay system (12).

PPT and IF titer determinations. Figure 2 demonstrates the precipitation (PPT) and immunofluorescent (IF) titration levels obtained when the various pooled mouse anti-pig zona sera from Groups 1 to 4 were titered against both pig and mouse zonae. For all groups, the antibody titers as established by IF were consistently higher than the PPT titers when the sera were titrated against the same antigen (i.e., IF > PPT vs mouse zonae, and IF > PPT vs pig zonae; Fig. 2, Table II). Both the PPT and IF titers in respect to pig zonae (the homologous antigen) were consistently greater than corresponding PPT and IF titers against mouse zonae (the cross-reacting antigen; Fig. 2, Table II). For Groups 1–3, a slight increase in titer in response to booster injection was detected against pig zona antigen by both the IF and PPT procedures whereas only the IF procedure detected any increase in titer following booster immunization when mouse zonae were used as antigen (Fig. 2).

Peak titers of mice in Groups 2 and 3 were comparable with the exception that the Group 3 PPT titers against mouse zonae (Fig. 2C) were higher than those in Group 2 (Fig. 2B). Peak titers of Group 4 mice were comparable to Groups 2 and 3 except for the IF titer against mouse zonae (Fig. 2D) which was lower (Table II).

Bleedings from Groups 1 to 3 were not titrated prior to 61 days for Group 1 and not titrated prior to 54 days for Groups 2 and 3 due to insufficient volumes of sera to perform all tests. However, the PPT and IF assays indicated strong anti-zona activity against pig zona for all groups commencing with the first bleeding (23 days following immunization). Anti-zona activity, using

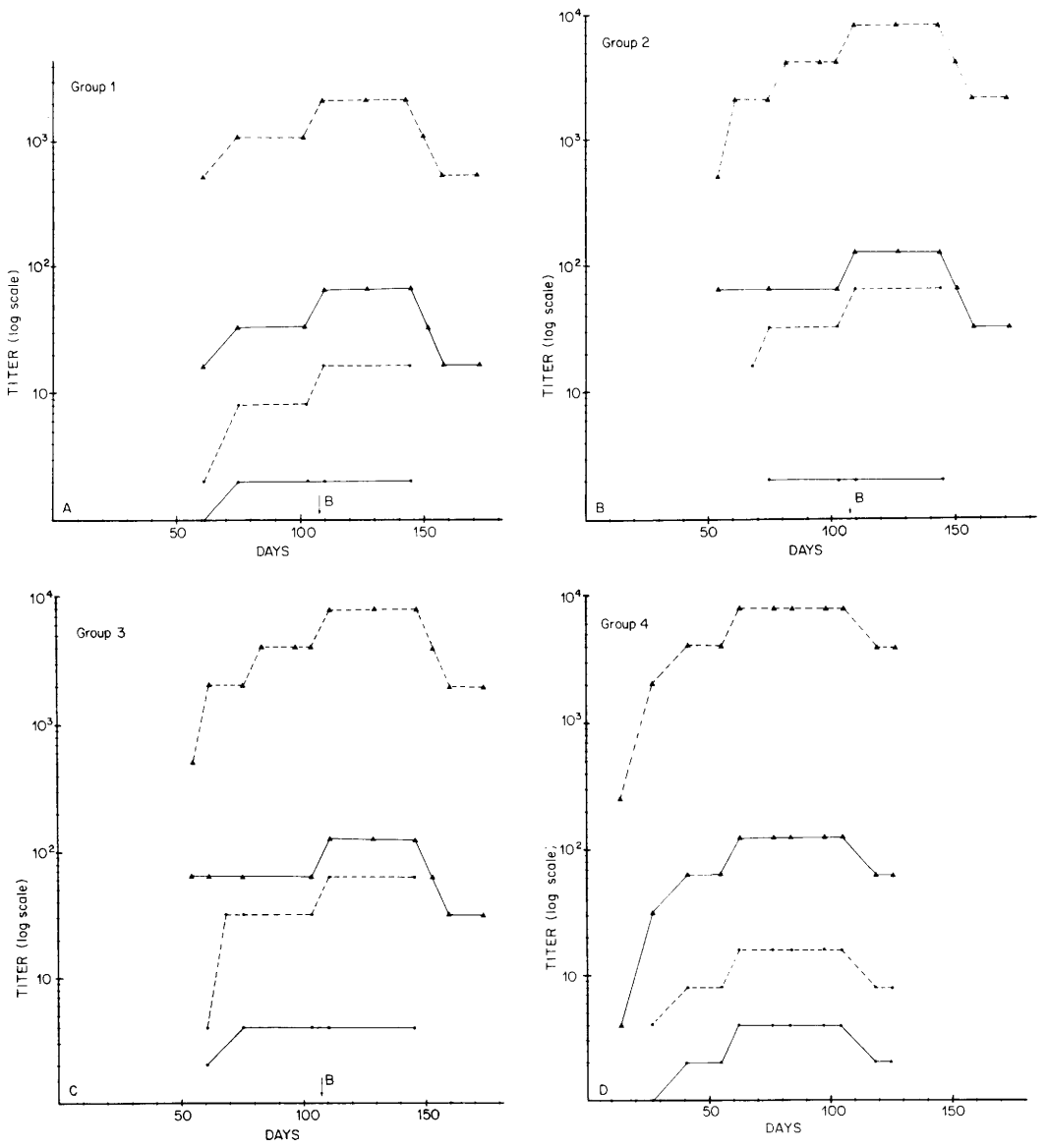

FIG. 2. Precipitation (PPT) and immunofluorescent (IF) titration profiles of mouse anti-porcine zonae pellucidae sera versus porcine zonae and mouse zonae. (●——●) PPT profile versus mouse zonae; (●– – –●), IF profile versus mouse zonae; (▲——▲) PPT profile versus pig zonae; (▲– – –▲) IF profile versus pig zonae. (A) Group 1 mice immunized with 100 zonae; (B) Group 2 mice immunized with 250 zonae; (C) Group 3 mice immunized with 500 zonae; (D) Group 4 mice immunized with 1000 zonae. Each point represents pooled sera from three mice. ↓B indicates time when booster immunization was administered.

mouse zonae as target antigen, was detectable by IF at 30 days (second bleeding) and by the PPT assay at 54 days (fifth bleeding) for all groups. Complete PPT and IF titer profiles using both pig and mouse zona target antigens and inclusive of the first bleeding, were obtained from sera of Group 4 mice (Fig. 2D).

RIA titer determinations. RIA endpoint and 30% binding titer profiles of pooled

TABLE II. COMPARISON OF PEAK ENDPOINT TITERS OF MOUSE ANTI-PORCINE ZONAE PELLUCIDAE SERA VERSUS PORCINE AND MOUSE ZONA PELLUDICA AS DETERMINED BY VARIOUS TITRATION METHODOLOGIES

	Titer[a]		
Group	PPT	IF	RIA[b]
1	64 (2)	2048 (16)	1.0
2	128 (2)	8192 (64)	2.2
3	128 (4)	8192 (64)	1.8
4	128 (4)	8192 (16)	1.6
5	ND	ND	2.95[c]

Note. Abbreviations: IF, immunofluorescent titer; ND, not determined; PPT, precipitation titer; RIA, radioimmunoassay titer.

[a] Reciprocal of highest serum dilution giving a positive reaction. Numbers in parentheses represent peak titers against mouse zonae. Control and preimmune sera did not indicate anti-zona activity by any methodology.

[b] Titers expressed as $\times 10^6$.

[c] Mean titer of 10 animals.

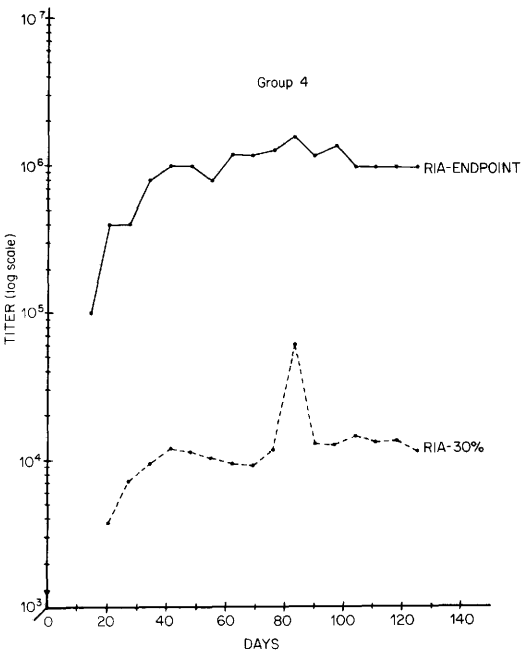

FIG. 3. RIA titration profiles of mouse anti-porcine zonae pellucidae serum obtained from Group 4 mice. Each point represents pooled sera from 3 mice. (●——●) endpoint titer profile; (●– – –●) 30% binding titer profile; (▼) time of immunization. A 30% binding level was not achieved for the first bleeding.

anti-zona sera obtained from Group 4 mice are presented in Fig. 3. Both titration profiles exhibited a comparable trend with a rapid rise during the first 40 days following immunization, reaching the peak at 83 days, followed by a decline, and then leveling off (100–125 days).

Figure 4 presents RIA endpoint and 30% binding profiles for Group 5 mice with each point representing the mean titer of 10 mice. Once again, both titration profiles were similar with a rapid increase in titer during the first 31 days, peak titer achieved between 31 to 56 days, followed by a slight decrease and leveling of titer with an indication of a very gradual decrease up through 225 days when titrations were terminated. However, at 225 days following the initial immunization, anti-zona activity still remained at significantly high levels.

Peak RIA endpoint titers achieved for Groups 4 and 5 were 1.6 and 2.95 million, respectively (Figs. 3 and 4; Table II). Peak individual response for Group 5 mice was 5.5 million. A limited number of IF titrations performed on individual bleedings from mice injected with 1000 zonae indicated peak IF titers of 25,000 and 75 versus pig and mouse zonae, respectively (data not shown).

Discussion. The tremendous interest recently shown in use of the porcine zona pellucida as a potential target antigen for immunocontraceptive purposes has necessitated the requirement for new assay techniques for the measurement of both zona antigens and the immune response following injection with zona material. The recently published porcine zona pellucida RIA (12), a competition-type assay, has satisfied the former need. However, a considerably more rapid and sensitive procedure for quantitating zona antibody activity was required, even though biologic assay systems currently in use will always continue to remain essential for demonstration of the biologic efficacy and contraceptive potential of such antibodies.

The modified RIA described in the present paper represents a rapid, quantitative, extremely sensitive, and reproducible technique for measuring antibody response to zona pellucida antigens and should be eas-

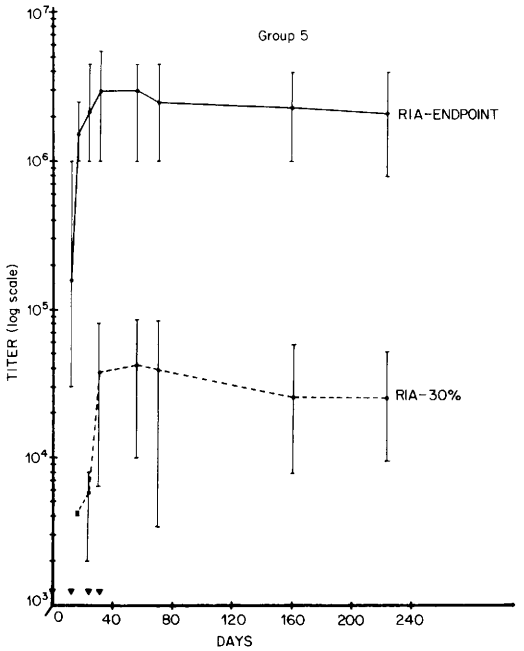

FIG. 4. RIA titration profiles of mouse anti-porcine zonae pellucidae sera obtained from Group 5 mice. All mice were bled on the same day and each point represents the mean titer of 10 animals. Brackets for each point indicate the range of titers obtained for the 10 mice for that particular bleeding. A 30% binding level was not achieved for any mouse for the first bleeding. (●——●) endpoint titer profile; (●- - -●) 30% binding titer profile; (▼) time of immunization.

ily adapted to assay systems utilizing individual purified zona antigens when such immunogens become available. A previous report from our laboratory (12) has demonstrated the specificity of the porcine RIA zona–anti-zona reaction, and other studies (10, 18) have indicated that use of heat-solubilized isolated zona material as immunogen results in the production of antibodies specific to only zona. Therefore, binding of the antisera raised against IZP in the present study to heat-solubilized, radiolabeled zona (^{125}I-SIZP) and the resultant precipitate likely represents a specific zona–anti-zona immunoreaction. Failure to detect any anti-zona activity in either control or preimmunization serum (Fig. 1) provides additional support for this view.

A unique feature in the description of this RIA procedure was the use of endpoint titers, necessitated for comparative purposes since all previously published titrations of antizona sera involving biologic-type assay systems used this method of titer determination (1, 15–17). The similarities in RIA titration profiles obtained using either the endpoint or 30% binding methods (Figs. 3 and 4) indicate that in this particular application, use of RIA endpoint determinations does present a valid profile of the immune response and provides accurate and useful information for purposes of comparing the various endpoint titers obtained by different methodologies. However, since the titers obtained using the 30% binding method reflect values derived from the more linear portion of the titration curve (Fig. 1) and, therefore, represent a more precise measurement of the binding dynamics of this system, for titer determinations other than those used for comparative purposes (e.g., to only obtained immune profiles), the 30% binding procedure should be the method of choice.

Of the three titration methodologies used, the RIA was obviously the most sensitive, detecting antizona activity at serum dilutions approximately 200 times greater than those of the IF procedure (Table II). Zona precipitation represented the least sensitive of the three techniques tested (Figs. 2D, 3; Table II), being 32–64 times less sensitive than IF. This low sensitivity level of the zona precipitation method when used for purposes of titering zona antibodies has been observed by others (15–17) in studies involving comparisons of endpoint titration values obtained using various biologic type assay systems.

The discrepancy in final peak RIA endpoint titers between Groups 4 and 5 mice is likely a reflection that Group 4 data represent values of pooled sera from only three mice and that one or possibly more of these mice represented a poor responder. The large degree of biologic variation observed between different mice in this study, and which is not unusual when comparing individual immune responses, is indicated by the broad titration ranges obtained for Group 5 mice (Fig. 4). However, use of different immunization regimes between

the two groups may also have had an effect on final endpoint titer values. These RIA data also suggest that within the immunogen dosage range studied (100–1000) increasing zonae dosage results in a dose-dependent increase in titer with the 250 and 500 zonae dosage levels producing comparable results (Table II, Groups 1–3, 5). However, additional studies, currently in progress, involving more animals, larger immunogen dosages, and different immunization regimes should provide a more detailed and precise indication of the immune response to this contraceptive target antigen. Nonetheless, the cumulative immune profile as indicated for Group 5 mice (Fig. 4) readily demonstrates the application and feasibility of utilizing the described RIA for assessing immune response to injection with zona material. In addition, several encouraging aspects for the continued consideration of the porcine zona pellucida as an immunocontraceptive target antigen are emphasized in this study. These include (a) the potent immunogenicity of the porcine zona, with a dosage as little as 100 zonae (which represents approximately 5 μg total protein (19)) producing a significant immune response, and (b) the high level of titers reached and the maintenance of these high titers for an extended period of time.

PPT and IF titration studies (Fig. 2; Table II) quantitatively indicate the response of mouse anti-pig zona sera was significantly greater against the homologous antigen (pig zonae) than the heterologous or cross-reacting antigen (mouse zonae). These findings are similar to those of Gwatkin and Williams (9) who performed limited numbers of IF titration studies on mouse anti-hamster zona sera versus hamster and mouse zonae and reported considerably higher titration values obtained against hamster zonae. While inhibition of fertility has been previously reported in heterologous anti-zona systems (9, 10), these findings emphasize that in situations involving heteroimmunization with zona material for immunocontraceptive purposes, the degree of similarity or dissimilarity between zona antigens of the two species involved is likely of paramount importance in determining the contraceptive efficacy of the zona antibodies resulting from that particular system. Although not applicable to the pig–mouse combination chosen for this investigation, in systems involving species possessing more closely immunologically similar or identical zona material, the titration of the resulting zona antibodies by RIA against both homologous and heterologous zona material may be feasible.

In summary, development of this sensitive porcine zona RIA for the detection and titration of antibodies to zona pellucida provides a useful new tool for evaluating the immune response to zonae pellucidae antigens. Its use, in conjunction with the existing porcine zona RIA for detection of zona antigens (12) and the biologic assay techniques for assessing the contraceptive efficacy of zona antibodies (1, 11, 15–17) should provide a comprehensive technique for evaluating this very promising immunologic approach to fertility control.

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