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Prospects and limitations of the rosette inhibition test to detect activity of early pregnancy factor in the pig

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Summary. After preincubation of lymphocytes in serum of non-pregnant pigs and using a standard anti-pig-lymphocyte serum the upper limit of the 99·9% confidence interval of rosette inhibition titres (RIT) for pig serum was calculated to be 11·4 and all titres >12 were defined as a proof of early pregnancy factor (EPF)-activity. The reproducibility of the RITs with serum samples of pregnant pigs was considered satisfactory (interassay coefficient of variation 23·8%), whereas their reproducibility was considered good with sera taken from non-pregnant animals (interassay coefficient of variation 6·5%).

Problems of the test were particularly evident due to the absence of increased RITs after previous incubation of lymphocytes in serum of pregnant pigs. Therefore, all EPF-negative samples were reanalysed up to two times; pregnancy was then correctly diagnosed for 88·7% of sows. There were 8·6% false positive results (N = 70) and 12·2% false negative results (N = 205).

During the first half of pregnancy RITs displayed periodic fluctuations which resembled the physiological cycle interval of the pig. Between Weeks 5 and 9 of pregnancy greater numbers of EPF-negative sows were detected. Subsequently, a continuous increase of mean RITs occurred, which then declined gradually from Day 80 of pregnancy. Similar changes were observed for progesterone values in the dialysed serum samples. However, highest progesterone concentrations always followed elevated RITs with a delay of 1·5–4 days. A subthreshold cyclicity of ovarian and luteal function probably persists despite pregnancy.

Introduction

In 1974, Morton, Hegh & Clunie reported that in mice the formation of spontaneous rosettes between lymphocytes and heterologous erythrocytes may be inhibited by lower concentrations of an antilymphocyte serum if cells of pregnant individuals are used instead of lymphocytes of non-pregnant animals. Since this phenomenon also occurred after preincubation of normal (non-pregnant and male) lymphocytes in serum of pregnant mice (Morton *et al.*, 1976), women (Morton, Rolfe, Clunie, Anderson & Morrison, 1977), sheep (Morton, Nancarrow, Scaramuzzi, Evison & Clunie, 1979) and pigs (Morton, Morton & Ellendorff, 1983), a pregnancy-associated serum constituent, termed early pregnancy factor (EPF) (Morton *et al.*, 1977), has been implicated.

Although several independent groups confirmed this observation for sheep (Nancarrow, Wallace & Grewal, 1981), cattle (Nancarrow *et al.*, 1981), pigs (Grewal, Wallace, Pan, Rigby & Nancarrow, 1981; Paisley, Davis, Anderson & Mickelsen, 1982) and humans (Smart, Roberts, Fraser, Cripps & Clancy, 1982), there was a consistent awareness of the inadequacies of the test system (Paisley *et al.*, 1982; Smart *et al.*, 1982; Koch, Morton & Ellendorff, 1983). As some authors

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were unable to detect any difference between the rosette inhibition titres of pregnant and non-pregnant women (Thomson, Milton, Campbell & Horne, 1980; Cooper & Aitken, 1981), and because a complete validation of the assay system is still required, the rosette inhibition test as well as EPF have remained controversial (Whyte & Heap, 1983).

Failures of the rosette inhibition test are usually attributed to aberrations from the original method (Morton, Tinneberg, Rolfe, Wolf & Mettler, 1982b; Smart *et al.*, 1982). By strictly following the instructions described to be successful for the detection of EPF (Morton *et al.*, 1982b, 1983), it was hoped that this investigation would show to what extent EPF activity may be found in the serum of pregnant pigs and would indicate the reproducibility of the titres, the reliability of pregnancy diagnosis and the practicability of the test system. Since major parts of the EPF complex may be produced in the ovary (Morton, Rolfe, McNeill, Clarke, Clarke & Clunie, 1980), pregnancy serum was also tested for progesterone, the main secretory product of the ovary during pregnancy in the pig.

Materials and Methods

Animals. Two groups of animals were investigated: (1) Group A consisted of 70 non-pregnant pigs and included 36 unmated, cyclic sows (25 animals in oestrus), 10 lactating sows, 10 boars and 7 female and 7 male gonadectomized animals. All pigs, except 7 ovariectomized miniature pigs, were of the German Landrace type, and were housed under different conditions at the Institute's farm. (2) Group B comprised 205 gilts and sows (German Landrace and crossbreds), which were kept on a commercial farm. All animals were tested daily for the onset of oestrus by a teaser boar and subsequently inseminated. The day of insemination was noted as Day 0 of pregnancy. All animals in Group B gave birth to live offspring between Days 113 and 119 after insemination. For a cross-sectional study a single blood sample was collected from these sows at different stages of pregnancy (Days 1–118). All samples were taken within 2 successive days.

Serum. Blood samples (about 3–8 ml) were collected from an ear vein (Hultsch & Ellendorff, 1979) or by puncture of the cranial vena cava. The blood was allowed to clot for about 1 h at room temperature, the serum was removed by centrifugation (20 min, 1000 ***g***) and frozen at −25°C. Before testing 5–10 serum samples were thawed at a time, inactivated for 30 min at 56°C and dialysed (Visking dialysing tube 8/32, Serva, Heidelberg, F.R.G.) against 1 l phosphate-buffered saline (PBS; 137 mM-NaCl, 8·1 mM-Na_2HPO_4, 2·7 mM-KCl and 1·5 mM-KH_2PO_4) overnight (about 16 h) at 4°C. Each sample was divided in three equal parts, stored at −25°C and thawed only once more before testing.

Lymphocyte suspension. Two or three castrated male German Landrace pigs (6–12 months of age), fitted with a chronic jugular vein catheter (Ellendorff, Parvizi, Elsaesser & Smidt, 1977), were permanently available for collection of lymphocytes. During the time course of the study lymphocytes were taken from a total of 25 donors. These animals were housed under standardized conditions (12 h light, temperature 22°C, relative humidity 65%) in the Institute's experimental animal house. A pelleted standard pig chow was provided twice daily, while water was freely available.

On each day of analysis 40 ml blood were collected from one of the lymphocyte donors and mixed with 400 i.u. heparin (Riker Pharma, Borken/Westf., F.R.G.). The lymphocytes were isolated by gradient centrifugation using a commercially available preparation (Lymphodex, Fresenius, Oberursel, F.R.G.). A centrifuge tube was filled with 8 ml lymphocyte separation medium and carefully overlayered with 12 ml blood. After centrifugation (20 min, 1000 ***g***) the lymphocyte-rich layer was removed using a Pasteur pipette, and the cells washed twice in calcium- and magnesium-free Hanks' balanced salt solution (HBSS; Serva, Heidelberg, F.R.G.). The solution was adjusted daily to pH 7·2 by the addition of sodium bicarbonate. If the lymphocyte

pellet was contaminated by red blood cells, the erythrocytes were lysed by the addition of 0·5 ml distilled H_2O for 5 sec. For use in the rosette inhibition test the lymphocyte suspension was finally made up to a concentration of 15 000 cells/μl HBSS. All work was carried out at room temperature.

Antilymphocyte serum. The preparation of rabbit anti-pig-lymphocyte sera has been published previously (Morton *et al.*, 1983). Of 6 originally prepared antisera one was used for all tests. The serum was stored at −25°C in 2·5 ml volumes. These samples were further divided into 20 μl aliquots as needed and again frozen (−25°C) until used.

Guinea-pig serum. Guinea-pig serum was used as a source of complement. Blood samples (about 10 ml) were collected by heart puncture from 6–12 adult male guinea-pigs at monthly intervals. Immediately after clotting the blood was kept on ice, the serum separated as soon as possible by centrifugation (20 min, 1000 ***g***, 4°C) and then absorbed for 2 h with equal parts of washed pig and sheep red blood cells at 4°C. After removal of the red blood cells (20 min, 1000 ***g***, 4°C), the serum was divided into 0·3 ml lots and stored at −25°C. The required quantity of serum was thawed only once immediately before use and diluted 1 in 5 with HBSS.

Sheep red blood cells. Two non-pregnant ewes served as erythrocyte donors. Blood (10 ml) was collected from both animals at 2-week intervals, mixed with 100 i.u. heparin and stored at 4°C. From this stock 3 or 4 drops were suspended daily in 10 ml HBSS, washed three times (3 min, 1000 ***g***) and finally made up to 10^5 red blood cells/μl HBSS.

Preincubation of lymphocytes in serum. To test for EPF activity in serum samples, 1·2 ml lymphocyte suspension (15 000 cells/μl) was centrifuged (5 min, 200 ***g***), mixed with 0·2 ml diluted test serum (1 in 2 with HBSS) and incubated for 30 min at 37°C. In two additional control tubes, the lymphocytes were incubated in 0·2 ml diluted serum (1 in 2 with HBSS) of a known EPF-positive pregnant and an EPF-negative (non-pregnant) pig. As there were no differences in the number of spontaneous rosettes and the RITs after preincubation of lymphocytes in sera of non-pregnant animals or HBSS alone (see 'Results'), the negative control sometimes consisted of lymphocytes preincubated in 0·2 ml HBSS. At the end of the incubation period the cells were washed twice in HBSS (5 min, 200 *g*) and resuspended in 1·2 ml HBSS.

Rosette inhibition test. For each test antilymphocyte serum was thawed and 10 μl were diluted in 10 ml HBSS. This basic dilution was followed by serial quadruple dilutions up to 1 in 256 × 10^6 to give 10 different dilutions of antiserum. For each serum to be tested 12 tubes were prepared: 10 tubes contained 250 μl diluted antiserum and 2 tubes contained 250 μl HBSS. To each of these tubes 50 μl diluted guinea-pig serum and 100 μl preincubated pig lymphocytes were added. These mixtures were incubated for 90 min at 37°C. After equilibration to room temperature for at least 10 min, 100 μl of a suspension of sheep RBCs was added to the 12 tubes of the first series. The tubes were centrifuged for 5 min at 200 ***g*** and resuspended on a wheel for 5 min (5 r.p.m.). A sample from each suspension was spread in both chambers of a haemocytometer (Neubauer improved, bright-line; Wertheim, F.R.G.) by use of a Pasteur pipette, the number of rosettes (lymphocytes with at least 3 attached red blood cells) within the ruled areas was counted (magnification × 100–150) and calculated for each antiserum dilution as a percentage of the number of rosettes in the two tubes containing only HBSS. After finishing the first series, investigation of the next test serum was started by the addition of sheep RBCs. On one day, 3–5 serum samples were counted by one person. The rosette inhibition titre (RIT) was defined as the highest dilution of antilymphocyte serum which caused, compared to the proportion of rosettes in the two tubes without antiserum, a reduction of the rosette number by at least 25%. This titre was recorded as the logarithm to base 2. If the basic dilution of the antiserum failed to induce a 25% rosette inhibition, a titre of 8 was chosen.

Progesterone estimation. Progesterone was measured in the dialysed and inactivated serum samples of pregnant pigs by radioimmunoassay (Elsaesser, 1980). The dialysis procedure decreased the progesterone values of control serum samples by about one third. Gestation time as well as the

original progesterone concentration had no significant effect on the dialysis rate. Therefore, the measured values do actually reflect, at a lower level, the time course of progesterone production during pregnancy (E. Koch, F. Elsaesser & F. Ellendorff, unpublished observation).

Statistical analysis. All results are presented as means ± s.e.m. To test for differences between means, data were tested for homogeneity of variance by the F-test followed by a Student's *t* test or a Behrens–Fisher-test. In cases of non-normal distribution the Whitney–Mann U-test was used. The *t* test was also used to test the statistical significance of correlation coefficients. Differences were considered to be significant at $P < 0.05$.

For comparison of EPF activities and progesterone values throughout pregnancy, the samples were divided into 24 groups, each group representing 5 consecutive days of pregnancy. The data of all groups were tested by analysis of variance. In addition, a Fourier regression was calculated for both observations to achieve a better perspective of the time course of EPF and progesterone production during pregnancy. The Fourier regression was selected since it allows the construction of a sine-like least-square fit between the RITs or the progesterone values and the gestation time. However, the memory of the computer system limited the number of harmonics to 7.

Results

Formation of spontaneous rosettes

The lymphocytes of all 25 donors formed rosettes with sheep red blood cells. After preincubation of lymphocytes in serum of pregnant pigs a proportion of 10·7 ± 1·13% (N = 18) rosette-forming cells was observed. The mean rosette count after incubation in non-pregnancy serum was 11·6 ± 0·64% (N = 20) compared with 11·4 ± 0·75% (N = 19) after incubation of lymphocytes in HBSS. The differences between these three means were not statistically significant. Using lymphocytes from the same donor on successive days, the number of rosettes ranged from 4·1 to 20·4% after incubation in different non-pregnancy sera. Because of this great variation the proportion of rosette-forming lymphocytes could not be used to discriminate between individual lymphocyte donors.

Determination of serum rosette inhibition titre

The lymphocytes of some donors never, and those of the remaining pigs only inconsistently, induced an enhanced rosette inhibition after preincubation in sera of pregnant sows. To avoid this failure of the assay system, known EPF-positive and EPF-negative controls were included in each test (see 'Materials and Methods'). Data were only utilized if these samples gave the expected results. After incubation of lymphocytes in serum of non-pregnant animals a mean titre of 10·7 ± 0·20 (N = 70) was determined. The upper limit of the 99·9% confidence interval was calculated to be 11·4. Therefore, all titres > 12 were defined as indicating the presence of EPF activity. However, EPF activity was also detected in 6 (8·6%) serum samples of non-pregnant pigs.

Compared with the preincubation of lymphocytes in serum of non-pregnant pigs, incubation in pregnancy serum caused a significant increase in the mean rosette inhibition titre, raising it to 14·5 ± 0·26 (N = 205). EPF-positive titres were found in the sera of 114 (55·6%) of these animals.

Reproducibility of determination of rosette inhibition titre

From 50 replicates an average inhibition titre of 10·4 ± 0·17 was found with lymphocytes from different donors after preincubation in HBSS (Table 1). This value was not significantly different from the mean titre after incubation of lymphocytes in sera from non-pregnant sows (10·7 ± 0·20, N = 70). With the exception of one test (2%; RIT 14) no EPF-positive titre was detected. Sera of two gonadectomized boars, each tested 10 times, gave mean inhibition titres of 9·8 ± 0·20 and 10·2 ± 0·20, respectively (Table 1). All titres were within the non-pregnancy range of 8 to 12.

Table 1. Synopsis of repeated determinations of rosette inhibition titre (RIT)

Preincubation of lymphocytes in:	No. samples	RIT 8	10	12	14	16	18	20	22	Mean ± s.e.m.	Coefficient of variation
HBSS	50	4/8·0	33/66·0	12/24·0	1/2·0	—	—	—	—	10·4 ± 0·17	11·6
Serum of gonadectomized boar A	10	1/10·0	9/90·0	—	—	—	—	—	—	9·8 ± 0·20	6·5
Serum of gonadectomized boar B	10	—	9/90·0	1/10·0	—	—	—	—	—	10·2 ± 0·20	6·2
Pregnancy serum (Day 4)	30	1/3·3	4/13·3	—	7/23·3	8/26·7	4/13·3	3/10·0	3/10·0	15·7 ± 0·68	23·8

Values are absolute no./relative no. (%).

After incubation of lymphocytes in serum of a pregnant sow (Day 4 of pregnancy) EPF activity was detected in 25 (83·3%) of 30 repeated assays. The mean titre was 15·7 ± 0·68 and the interassay coefficient of variation was calculated to be 23·8%. When only the EPF-positive titres were considered, the coefficient of variation was 16·0% (Table 1).

Modification of the determination of rosette inhibition titre

As shown by the repeated assays, problems of the rosette-inhibition test were particularly evident due to the absence of increased titre values after incubation of lymphocytes in pregnancy serum. Therefore, all EPF-negative samples were, independently of their origin from pregnant or non-pregnant pigs and provided no EPF activity was found in a second test, reanalysed up to a total of three times. Since the analysis of a pregnancy serum gave EPF-positive results in only about four-fifths of 30 repeated assays (see above), it should be possible by this modification to detect EPF-activity in a particular serum with a very high probability. The highest titre observed during the repeated analyses was always selected for evaluation.

Table 2. Synopsis of determination of rosette inhibition titre (RIT) after preincubation of lymphocytes in serum of pigs in different reproductive condition

Reproductive status	No. of animals	RIT 10	12	14	16	18	20	22	Mean ± s.e.m.
Cyclic sows (oestrus)	25	17/68·0	6/24·0	2/8·0	—	—	—	—	10·8 ± 0·26
Cyclic sows (dioestrus)	11	8/72·7	2/18·2	1/9·1	—	—	—	—	10·7 ± 0·41
Lactating sows	10	8/80·0	1/10·0	1/10·0	—	—	—	—	10·6 ± 0·43
Boars	10	6/60·0	4/40·0	—	—	—	—	—	10·8 ± 0·33
Castrated boars	7	3/42·8	3/42·8	—	1/14·3	—	—	—	11·7 ± 0·81
Ovariectomized sows	7	4/57·1	2/27·6	—	—	1/14·3	—	—	11·7 ± 1·11
Pregnant sows	205	13/6·3	12/5·9	44/21·5	51/24·9	31/15·1	38/18·5	16/7·8	16·5 ± 0·22

Values are absolute no./relative no. (%).

The mean titre for sera from non-pregnant sows (N = 70) was only slightly influenced by repeated investigation (11·0 ± 0·19). This increment was exclusively caused by 7 sera, which yielded an increased titre within the non-pregnancy range (RIT 8 to 12). Since the upper limit of the 99·9% confidence interval was only elevated to 11·6, the definition of EPF activity did not need to be changed. The results of the analysis are summarized in Table 2. No differences existed between the individual groups of non-pregnant pigs for the mean rosette inhibition titre and the frequency of EPF-positive results.

The first test of 205 pregnancy sera resulted in only 114 cases (55·6%) with an EPF-positive titre. This percentage increased to 77·1% (N = 158) during a second analysis of the remaining EPF-negative samples and reached 87·8% (N = 180) following a third determination. The mean titre was raised after the repeated tests to 16·5 ± 0·22. However, despite repeated evaluations, EPF activity was undetectable in sera of 25 (12·2%) pregnant pigs. The results are given in detail in Table 2.

No significant difference could be found between the EPF-positive (N = 180) and the EPF-negative (N = 25) pregnant pigs for the total number of piglets born (9·4 ± 0·24 *vs* 9·6 ± 0·70) or those born alive (9·1 ± 0·23 *vs* 9·3 ± 0·68). These two values also showed no significant correlation with the rosette inhibition titre (total piglets born, $r = -0{\cdot}04$; piglets born alive, $r = -0{\cdot}05$).

Time course of EPF activity during pregnancy

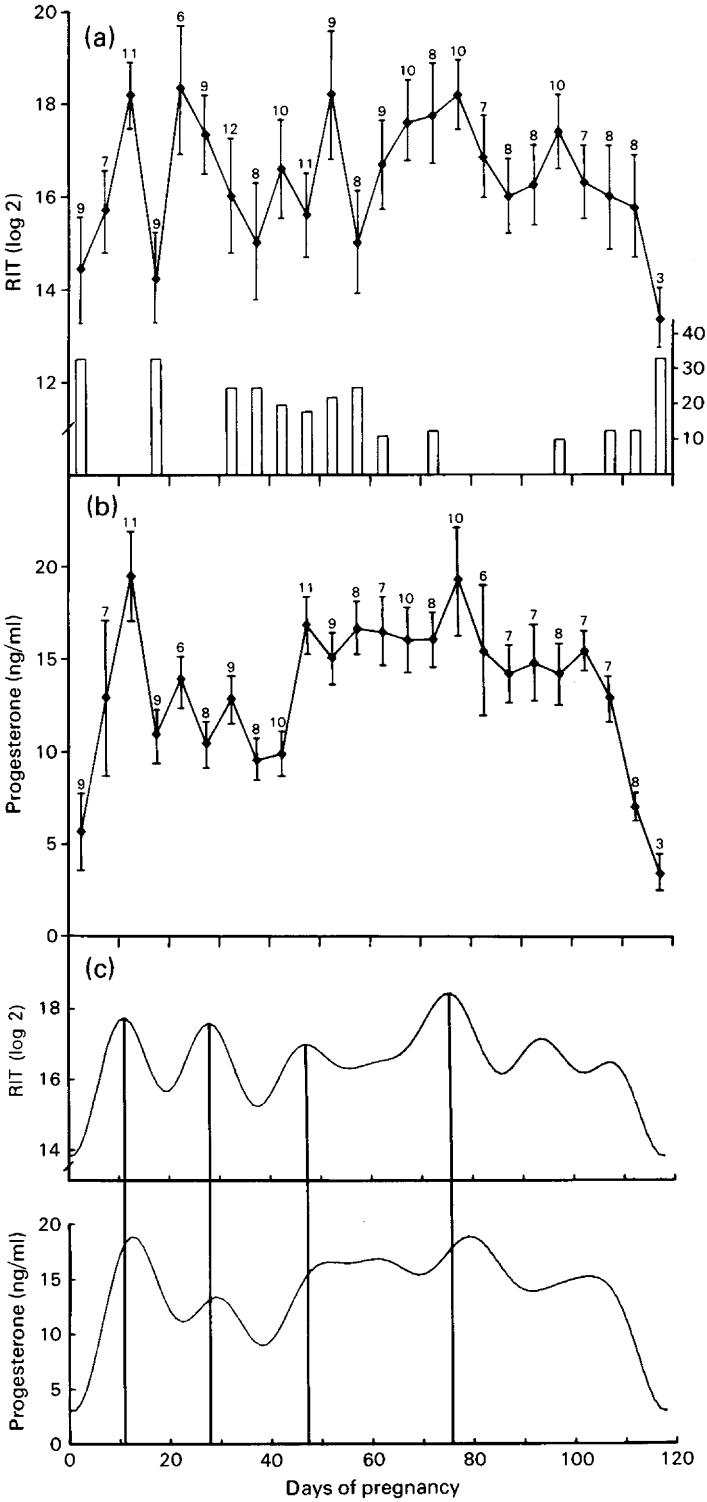
After grouping serum samples of pregnant sows according to gestation time and calculation of the group means, the time course of EPF activity was as shown in Text-fig. 1(a). During the first half of pregnancy the inhibition titre displayed periodic fluctuations which resembled those of the oestrous cycle of the pig: peaks were always followed by a marked decline of EPF activity around Days 20, 40 and 60 of gestation. Especially at these pregnancy stages, but generally between Days 35 and 65, an increased number of EPF-negative animals was observed. In the second half of pregnancy the proportion of EPF-negative animals as well as the fluctuation of the inhibition titres was diminished. A continuous rise of mean titre up to Day 80 of gestation changed to a largely uniform decrease towards the time of delivery. The differences between the titres of all groups did not prove to be significant (F = 1·42, $P = 0{\cdot}1$). However, after direct comparisons statistically significant differences were detected.

Time course of progesterone values during pregnancy

Progesterone values were determined in the serum of 194 out of the 205 pregnant animals (Text-fig. 1b). Like the rosette inhibition titres the progesterone concentrations peaked around Day 15, whereas lower values were measured between about Days 20 to 45 of pregnancy. Subsequently, a continuous increase in the mean levels occurred, which then declined gradually from Day 80 of pregnancy. The analysis of data revealed significant differences between groups (F = 3·77, $P < 0{\cdot}001$).

Relation between inhibition titres and progesterone values

No significant correlation could be found between the progesterone concentrations and the inhibition titres of individual pregnant pigs ($r = 0{\cdot}13$). Even though EPF-negative animals showed a lower mean progesterone value than did EPF-positive sows (12·0 ± 1·64 ng/ml, N = 22 *vs* 14·0 ± 0·50 ng/ml, N = 172), this difference was not significant ($P = 0{\cdot}17$). However, when the mean titres and progesterone concentrations of the 24 pregnancy groups were compared, a correlation coefficient of 0·66 was obtained ($P < 0{\cdot}001$). Although similarities between the time course of the inhibition titres and progesterone values were obvious after the distribution of samples into groups, they were particularly evident from direct comparison of the corresponding Fourier regression curves (Text-fig. 1c). However, the highest progesterone concentrations always followed elevated rosette inhibition titres with a delay of about 1·5–4 days.



Text-fig. 1. Serum rosette inhibition titres (RIT) and progesterone concentrations throughout pregnancy in the pig. (a) Mean RIT after incubation of lymphocytes in serum samples of sows at different stages of pregnancy. The percentage of animals without detectable EPF activity (RIT < 14) is shown in the bars. (b) Mean progesterone concentrations in dialysed serum samples of sows at different stages of pregnancy. (c) Comparison of RITs and progesterone values throughout pregnancy after transformation of data by Fourier regression. All points in (a) and (b) are expressed as mean ± s.e.m. for the no. of animals indicated. Each point summarizes 5 consecutive days of pregnancy.

Discussion

In contrast to the formation of spontaneous rosettes, a property shown by lymphocytes of all the donors tested, the lymphocytes of some pigs never and those of the remaining animals only inconsistently induced an increased rosette inhibition titre after preincubation in pregnancy serum. In addition, many pregnancy sera caused EPF-positive as well as EPF-negative titres, although both controls included in these tests did not reveal any fluctuation in the assay system. Therefore, failures of the rosette inhibition test are not always recognizable by the inclusion of control samples as stated by Morton *et al.* (1982b). Therefore, to improve the reliability of the test system a modification of the assay with up to 3 analyses of all EPF-negative samples was chosen.

The factors responsible for the inconsistency of the rosettes inhibition test are not known, but similar difficulties have been reported by other groups (Paisley *et al.*, 1982; Smart *et al.*, 1982). It is our experience that besides the technical details recommended previously (Morton *et al.*, 1982b; Smart *et al.*, 1982), even a slight disturbance of the health state of the lymphocyte donors as well as the storage time of the red blood cells might be responsible for some variation. While these factors perhaps can explain a total breakdown of the assay system, they do not really offer an explanation for the failure of only a few samples within an otherwise valid test. Morton *et al.* (1982b) observed variable numbers of rosettes after incubating the same lymphocyte suspension in sera or media of different protein concentration, although this variation was not significant in the rosette inhibition test. Because in our hands the fluctuation of rosette numbers after incubation in different sera was rather high, we cannot deny a negative effect on the assay system.

The rosette inhibition test proved to be more reliable for the analysis of non-pregnancy sera when compared to pregnancy serum samples. In contrast to Morton *et al.* (1982b), who never obtained known false-positive results, we observed EPF-positive titres with 8·6% of non-pregnant sera. This rate of false-positive results was contrasted with a proportion of 12·2% false-negative titres during the whole gestation time, but because false-negative titres were especially observed at certain stages of pregnancy, it is likely that failures did not exclusively result from insufficiencies of the test system.

Because the titre value for EPF activity was arbitrarily established at a 25% rosette inhibition, it is possible that only a slight crossing of this limit in either direction may cause false results. For example, inhibition titres of 14, found with sera of 4 non-pregnant pigs, could be attributable to this particular situation. Titres of 16 and 18, detected in 2 gonadectomized animals, are more difficult to explain. However, one of these animals had received repeated injections of rolitetracyclin (Reverin: Hoechst, Frankfurt, F.R.G.) up to the 2nd day before blood collection. Tests to reveal a possible relation between such treatment and the increased serum titre showed that the addition of 0·05 mg rolitetracyclin/ml to a non-pregnancy serum (RIT 12) caused a titre of 18. As with pregnancy serum, but in contrast to other immunosuppressive substances which have inherent rosette-inhibiting activities (Bach, Dardenne & Fournier, 1969), tetracyclin decreased the rosette number only in the presence of antilymphocyte serum. Since we did not follow this aspect in detail, further experiments are required to clarify possible interference. Up to now no other substance has been described which acts solely in co-operation with antilymphocyte serum in producing an elevated rosette inhibition titre. Therefore, the test system must still be regarded as suitable for the detection of EPF activity.

In accordance with previous longitudinal investigations (Morton *et al.*, 1983), the present cross-sectional study shows that EPF activity in the pig is detectable throughout the whole of gestation. Nevertheless, the previously supposed biphasic EPF production now appears to be a polyphasic phenomenon. During the first half of pregnancy the inhibition titres displayed periodic fluctuations reminiscent of the physiological cycle interval of the pig. Similar changes were observed for progesterone concentrations in the dialysed serum samples. However, the highest progesterone values always followed elevated RITs with a delay of 1·5–4 days. Obviously a subthreshold cyclicity of ovarian and luteal function persists despite gestation during at least the

first half of pregnancy in the pig (MacDonald, 1979). Since there is some evidence that the conceptus itself or its surrounding membranes can produce EPF during advanced stages of gestation (Morton, Rolfe & Cavanagh, 1982a), these sources of EPF could perhaps be responsible for the absence of RIT fluctuations during the second half of pregnancy.

Immunosuppressive effects of progesterone have been described for a number of in-vitro situations (see Siiteri & Stites, 1982). As there was no correlation between the rosette inhibition titre and the progesterone value in individual animals, the hypothesis that progesterone might also influence the rosetting capacity of lymphocytes and thereby cause different titre values can be rejected. Experiments with ovariectomized sheep (Nancarrow *et al.*, 1981) and mice (Morton *et al.*, 1982a) have shown that, if this operation is performed in early stages of pregnancy, the serum inhibition titres decrease to the non-pregnancy range within a short time after surgery, while pregnancy can be maintained by progesterone replacement therapy. Provided EPF possibly bound to lymphocytes does not possess a prolonged action *in vivo*, then the essential presence of EPF activity in the serum during pregnancy is only explicable if it has a luteotrophic function (Nancarrow *et al.*, 1981). The slightly different time courses of rosette inhibition and progesterone concentrations perhaps point to such a physiological role of EPF.

In summary, the rosette inhibition test proved cumbersome, time-consuming and was easy to disturb. Of particular disadvantage is the indirect character of the test and its possible interference by other substances. However, the rosette inhibition test uses normal cells and sera which makes it particularly sensitive to natural fluctuations. The detection of EPF activity by use of the rosette inhibition test may therefore be suitable for limited investigations under well-defined experimental conditions. Practical application, such as for routine pregnancy diagnosis, is not possible at present. Highest priority should be placed on the isolation and biochemical characterization of EPF to allow development of a specific, reliable and rapid test system.

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