Immunity in barren and enriched housed pigs differing in baseline cortisol concentration

Johanna de Groot a,b,*, Ingrid C. de Jong b,c, Ina T. Prelle b, Jaap M. Koolhaas b

a Department of Immunology, Pathobiology and Epidemiology, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, Netherlands
b Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA, Haren, Netherlands
c Department of Behavior, Stress Physiology and Management, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, Netherlands

Received 22 February 2000; received in revised form 8 May 2000; accepted 25 May 2000

Abstract

It was shown in a recent study [7] that barren housed pigs (small pens, no substrate) have a blunted circadian rhythm of salivary cortisol as compared to enriched housed pigs (large pens with daily fresh bedding). In the light period, enriched housed pigs showed significantly higher concentrations of cortisol in saliva than barren housed pigs, whereas in the dark period, cortisol concentrations were low in both enriched and barren housed pigs. In the present study, the immunological consequences of the difference in baseline salivary cortisol concentration in the light period were evaluated. It appeared that leukocyte and lymphocyte distributions, and in vitro lymphocyte proliferation following ConcanavalinA (ConA) stimulation in the assay using purified lymphocytes were not affected. However, barren and enriched housed pigs did show a different proliferation response to ConA in the whole blood assay. At day 2 of culture, proliferation was higher in barren housed pigs than in enriched housed pigs, whereas at day 4 of culture, proliferation was higher in enriched housed pigs than in barren housed pigs. Lymphocyte proliferation at day 2 of culture in the whole blood assay correlated negatively with plasma cortisol levels, which might thus explain the higher proliferation in barren housed pigs at day 2 of culture. The in vivo humoral and cellular (delayed type hypersensitivity, DTH) immune response to KLH was not affected by housing conditions. We conclude that, although baseline salivary cortisol concentrations differ between enriched and barren housed pigs, immune function appears to be relatively unaffected.

1. Introduction

In intensive pig husbandry, pigs are often housed under barren conditions, in small pens without straw or other substrate. Many studies have been conducted to investigate the consequences of such barren housing conditions for welfare. It appeared that pigs reared under barren conditions perform more manipulative social behavior like biting, nosing and massaging of littermates, behave more aggressively and develop more abnormal agonistic behavior than pigs reared in an enriched environment [2,3,8]. Recently, it was shown that barren housed pigs have a blunted circadian rhythm of salivary cortisol [6,7], showing a decreased circadian peak as compared to enriched housed pigs. Blunted cortisol rhythms are also found in depressed patients (e.g. Refs. [10,24]), and in rodents (e.g. Ref. [17]) and pigs suffering from chronic stress (e.g. Ref. [13]), however in these situations there is an elevated circadian trough of cortisol.

Cortisol in saliva is essentially in the free biologically active form, and is a good indication of levels of cortisol in blood plasma [19]. Cortisol is known to affect several components of the immune system [5,12,23]. In pigs, an increase in cortisol caused a decrease in the number of lymphocytes and an increase in the number of neutrophilic granulocytes in the blood [18,21,27]. Lymphocyte proliferation to mitogens was suppressed under several conditions that increased plasma cortisol concentrations [4,14,27], and natural killer cell cytotoxicity and neutrophilic chemotaxis were suppressed after intracerebroventri-
tricular injection of corticotropin releasing hormone [21,22]. Thus, it is hypothesized here that enriched housed pigs (with their high daytime cortisol concentrations) might have a lower immune function than barren housed pigs (with low daytime cortisol concentrations). Leukocyte and lymphocyte subset distribution, lymphocyte proliferation to the mitogen ConcanavalineA (ConA) of purified lymphocytes and in a whole blood assay and the in vivo humoral and cellular immune response to KLH were compared between barren housed pigs and enriched housed pigs.

2. Materials and methods

The ID-Lelystad Animal Care and Use Committee (Lelystad, The Netherlands) approved all procedures in this study.

2.1. Animals and housing

Pigs (Great Yorkshire × (Great Yorkshire × Dutch Landrace)) used in this experiment were housed in either an enriched environment or in a barren environment from birth to slaughter. Three successive replicates of 24 pigs were used in the experiment. Within each replicate, three groups of four pigs (two barrows and two gilts) were assigned to the enriched (E) environment and three groups of four pigs (two barrows and two gilts) were assigned to the barren (B) environment. Salivary cortisol rhythms of the pigs from the first two replicates were previously published [7].

Six sows per replicate bred the piglets used in this experiment. One week before the expected date of farrowing, the sows were housed in the farrowing pen. E piglets were born in farrowing pens (7.2 m²) with a concrete lying area covered with straw (1.75 × 2.4 m) and a concrete slatted area (1.25 × 2.4 m). B piglets were born in standard farrowing pens where the sows were crated (3.1 m², half concrete area, half metal slats). Castration of male piglets, teeth clipping, ear tattooing, and tail docking were carried out at 3 days of age, following standard animal husbandry procedures at the experimental farm. Piglets were weaned at 28 days of age and six piglets per sow (three barrows, three gilts) were randomly selected within a litter for use in this experiment. Piglets stayed in the same pen at weaning, and the sow and non-selected piglets were removed. At 10 weeks of age, a final random selection within a litter of four experimental pigs per sow (two barrows, two gilts) was done. E pigs were relocated to enriched fattening pens (4.64 m²) with half concrete area covered with straw and half concrete slats. B pigs were relocated to barren fattening pens (3.36 m²) with half concrete lying area and half concrete slatted floor. E and B fattening pens were in the same room. All pens were cleaned daily and fresh straw was provided in the E pens at 0830 h. Throughout the experiment, water and food were available ad lib. Environmental temperature was kept between 19°C and 21°C in each room. Artificial lights were on from 0600 to 1800 h., with no daylight visible in the rooms.

Individual pigs could be recognized by a plastic ear tag and a number painted on their back. All pigs were accustomed to the experimenter by weekly handling from 5 weeks of age to avoid unwanted stress reactions to saliva sampling.

2.2. Blood sampling

Pigs were restrained to obtain blood by venapuncture between 0900 and 1000 h. when they were 22 weeks of age (replicate 1 and 2) or 20 weeks of age (replicate 3). All pigs from one pen were captured in succession, and blood was collected within 2 min after capture in 10-ml heparinized tubes and stored on ice until processing.

2.3. Saliva sampling and cortisol measurement

Saliva was collected during a 24-h period by allowing pigs to chew on two large cotton buds until they were thoroughly moistened (about 30–60 s per sample). Samples were collected every hour in replicates 1 and 2 [7], and every 2 h in replicate 3. Therefore, results for all three replicates are shown only for samples that were taken every 2 h. Saliva samples were stored at −20°C until analysis. Cortisol concentration in saliva was determined by radioimmunoassay as described [7]. Cortisol in plasma was measured using a time-resolved fluorometric assay (TR-FIA). The assay was performed as described for ovine plasma [9]. Cortisol binding plasma proteins were inactivated, thus total cortisol concentrations were measured.

2.4. Immunological measurements

2.4.1. Leukocyte differentials

The total number of white blood cells was counted using an automatic cell counter (Sysmex, type F-800, TOA, Japan). Blood smears were stained with modified Giemsa (Hema-tek stain pack, Ames) and the percentages of lymphocytes, neutrophilic, eosinophilic and basophilic granulocytes were determined by counting 100 cells in total.

Fig. 1. Baseline salivary cortisol concentrations (mean ± SEM) at 2 hour intervals during a 24 hour period in enriched and barren housed pigs at 22 weeks of age (replicates 1 and 2) or 20 weeks of age (replicate 3). Black bars indicate the dark period. *p < 0.10; **p < 0.05 for comparison of enriched and barren housed pigs.

Graphs showing changes over time: replicate 1 enriched and replicate 1 barren, replicate 2 enriched and replicate 2 barren, replicate 3 enriched and replicate 3 barren.
2.4.2. Purification of lymphocytes

For use in flow cytometry and lymphocyte proliferation, peripheral blood mononuclear cells (PBMCs) were enriched for lymphocytes by centrifugation on a Ficoll-paque discontinuous one-step density gradient (Pharmacia Biotech). For use in the lymphocyte proliferation assay, cells were resuspended to a concentration of 5 x 10^6 cells/ml in culture medium (DMEM-alpha + 10% pig serum (obtained from SPF-pigs from the herd of the institute and filtrated through a 0.45-µm filter) + 5% antibiotic mix (containing penicillin, streptomycin, amphoterrin, polymycin and kanamycin) + 2 mM glutamin + 5 x 10^-5 M β-mercaptoethanol). For flow cytometric analysis, cells were resuspended to a concentration of 5 x 10^5 cells/ml in phosphate buffered saline + 0.1% NaN3 + 1% bovine serum albumin.

2.4.3. Lymphocyte proliferation

Lymphocytes from the ficoll purification were cultured in triplicate in 96-well culture plates with or without the T-cell mitogen ConA (5 µg/ml, Sigma; final cell concentration 2.5 x 10^6 cells/ml) for 2 and 4 days at 37°C, 5% CO2 and 95% humidity. During the last 4 h of culture 3H-thymidine (Amersham, The Netherlands) was added (0.4 µCi per well). For determination of lymphocyte proliferation to ConA in whole-blood cultures, heparinized blood was diluted 1:15 with complete culture medium and cultured for 2 or 4 days with or without ConA (5, 10 and 20 µg/ml).

2.4.4. Flow cytometric analysis

Lymphocyte subpopulations were determined by flow cytometric analysis: 5 x 10^5 lymphocytes (from the ficoll-purification) were stained with mouse-anti-pig IgM (stains B-cells; ID-Lelystad, mab 28.4.1, [26]) and mouse-anti-pig CD6 (stains T-cells; MCA 1221 (MIL8) Serotec, Oxford, UK), or mouse anti-pig CD4 (stains T helper cells; ID-Lelystad, mab 74.12.4, [20]) and mouse-anti-pig CD8 (stains cytotoxic T-cells (CTLs) and NK-cells; ID-Lelystad, mab SL2 (295/33), [15]). Anti-IgM and -CD6 were combined in a double staining procedure, and developed with conjugates goat-anti mouse IgG1-Fitc (for anti-IgM; Southern Biotechnology Associates (SBA), Birmingham, AL, USA) and goat-anti-mouse IgG2a-PE (for anti-CD6; SBA). Anti-CD4 and-CD8 were also combined in a double staining procedure, and developed with conjugates goat-anti mouse IgG1-Fitc (for anti-CD4; SBA) and goat-anti-mouse IgG2a-PE (for anti-CD8; SBA). Since in pigs peripheral blood lymphocytes cannot be gated purely based on their forward–sideward scatter profile, positive staining was expressed as a proportion of the total amount of stained cells. In the IgM/CD6 analysis all lymphocytes thus indicate proportions of the T-lymphocyte+ NK-cell population [1].

2.4.5. Specific in vivo immune response to KLH

Pigs were immunized at 22 weeks of age with 50 µg keyhole limpet hemocyanin (KLH, Calbiochem, mixed 1:1 with Al(OH)3; 1 day before use). Three weeks later, the delayed type hypersensitivity (DTH) response was determined by injecting 10 µg KLH in 100 µl phosphate buffered saline intradermally in the outer skin of the ear. The thickness of the injection spot was measured in duplicate with a technical slide-rule, especially adapted for this purpose, before and 24 h after local injection of KLH. The DTH was expressed as millimeters increase in ear thickness. For determination of antibody titers, blood was collected in non-heparinized tubes at slaughter (25 weeks of age). After clotting and subsequent centrifugation, serum was collected and stored at −20°C. The specific IgG-antibody titer against KLH was measured by ELISA. Briefly, 5 µg/ml KLH in PBS was coated, serum samples were incubated and developed with peroxidase conjugated mouse-anti-swine IgG (ID-Lelystad, 23.3.1.aPO [26]). In each plate a positive and negative control serum were included, and the titer of serum samples was calculated as the dilution having an optical density (OD) closest to 50% of ODmax of the positive sample.

2.5. Statistical analysis

Differences in immunological parameters, salivary cortisol and plasma cortisol concentration between enriched and barren housed pigs were tested with a two-sided Student’s t-test. Salivary cortisol concentrations were also expressed as integrated mean of the light period (mean of samples of 8, 10, 12, 14, 16 and 18 h) and the dark period (mean of samples of 20, 22, 24, 2, 4 and 6 h), and the effect of housing was tested with a two-sided Student’s t-test. Pearson’s correlation coefficient between plasma cortisol levels and proliferation was calculated. The level of significance was set at 5%.

3. Results

3.1. Effect of housing conditions on salivary cortisol concentrations

The circadian rhythm of salivary cortisol concentrations in pigs from all three replicates is shown in Fig. 1. The integrated mean in the light period was compared between enriched housed pigs and barren housed pigs, and this revealed a significantly higher mean salivary cortisol concentration in E pigs than in B pigs during the light period: replicate 1: E, 3.90 ng/ml (SEM: 0.65); B, 2.13 (0.21); p = 0.026, replicate 2: E, 3.38 (0.51); B, 1.58 (0.12); p = 0.002; replicate 3: E, 3.79 (0.61); B, 2.34 (0.24);
p = 0.046. In replicates 1 and 3, E pigs showed the same concentration of salivary cortisol as B pigs in the dark period, but in replicate 2, E pigs still had a significantly higher cortisol concentration than B pigs in the dark period: replicate 1: E, 1.73 ng/ml (SEM: 0.23); B, 1.61 (0.29); p = 0.74; replicate 2: E, 1.80 (0.24); B, 1.15 (0.09); p = 0.019; replicate 3: E, 1.31 (0.18); B, 1.46 (0.15); p = 0.51.

### 3.2. Replicate 1: Leukocyte and lymphocyte distribution

Housing conditions had no significant effect on leukocyte distribution: there was a tendency for a higher number of leukocytes and a lower percentage of eosinophils in B pigs, but no effect on the percentages of lymphocytes, and neutrophilic and basophilic granulocytes (Table 1). The same was true for the lymphocyte subset distributions, which showed a tendency for a lower relative frequency of B-cells and a higher relative frequency of T-cells in B pigs, but no difference in the CD4/CD8 distribution of T-cells (Table 1).

#### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Enriched</th>
<th>Barren</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td># Leukocytes*</td>
<td>17.4</td>
<td>0.77</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>68.8</td>
<td>3.44</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>28.0</td>
<td>3.22</td>
</tr>
<tr>
<td>% Basophils</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>2.3</td>
<td>0.64</td>
</tr>
<tr>
<td>% IgM⁺ (B-cells)</td>
<td>35.7</td>
<td>2.69</td>
</tr>
<tr>
<td>% CD6⁺ (T-cells)</td>
<td>64.3</td>
<td>2.69</td>
</tr>
<tr>
<td>% CD8⁺ (cytotoxic T-cells and NK-cells)</td>
<td>58.2</td>
<td>1.80</td>
</tr>
<tr>
<td>% CD4⁺ (T helper cells)</td>
<td>19.3</td>
<td>0.90</td>
</tr>
<tr>
<td>% CD4⁺ CD8⁻ (memory T-cells)</td>
<td>22.5</td>
<td>1.53</td>
</tr>
</tbody>
</table>

* Number of leukocytes × 10⁶/ml blood.

b Tendency for a difference between enriched and barren housed pigs.

#### 3.3. Replicate 2: Immune function

No effect of housing conditions was found in the ConA-induced lymphocyte proliferation assay using purified lymphocytes (day 2 of culture: E, 119,146 corrected counts per minute (cpm) (SEM: 9.109); B, 121,500 (9.371); p = 0.859; day 4 of culture: E, 32,473 (6.753); B, 21,797 (7.116); p = 0.282). Pigs were immunized with KLH to investigate the in vivo specific immune response. At 3 weeks after immunization, there were no differences between E and B pigs in anti-KLH IgG titers (E: 1953 (SEM: 265.1), B: 1595 (271.6); p = 0.351), nor in DTH responses to KLH (E: 0.66 mm (0.14); B: 0.68 (0.14); p = 0.905).

#### 3.4. Replicate 3: Whole blood lymphocyte proliferation

After 2 days of culture in the whole blood lymphocyte proliferation assay, lymphocytes from B pigs showed a higher proliferation response to ConA than lymphocytes from E pigs, whereas after 4 days of culture, proliferation was higher for lymphocytes from E pigs than for lymphocytes from B pigs (Fig. 2). In plasma from the same blood samples that were used for the whole blood proliferation assay, cortisol concentrations were measured, and although there was no significant difference between E and B pigs in

![Fig. 2. Whole blood lymphocyte proliferation (mean ± SEM) in pigs from replicate 3. Filled bars: barren housed pigs; hatched bars: enriched housed pigs. *p < 0.05; #p < 0.10 (enriched vs. barren housed pigs).](image-url)
plasma cortisol concentration (E: 17.34 ng/ml (SEM: 3.84); B: 12.66 (2.35); \( p = 0.32 \)), the plasma cortisol concentration was negatively correlated with whole blood proliferation at day 2 of culture: 5 \( \mu \)g/ml ConA: \( r = -0.31, p = 0.16 \); 10 \( \mu \)g/ml ConA: \( r = -0.35, p = 0.10 \); 20 \( \mu \)g/ml ConA: \( r = -0.42, p = 0.05 \), indicating that the higher proliferation in B pigs at day 2 of culture might at least partially be caused by the lower cortisol levels in these animals.

4. Discussion

As was shown previously [7], E pigs display a distinct circadian rhythm in salivary cortisol, whereas this rhythm is blunted in B pigs. During the light period, E pigs had a higher mean salivary cortisol concentration than B pigs. Since salivary cortisol is in the free biologically active form [19], it might be expected that E pigs have suppressed immune function when compared to B pigs.

There were however only slight differences in the investigated parameters of immunity between E and B pigs. Proliferation to ConA in whole blood cultures was higher in B pigs than in E pigs after 2 days of culture, but the opposite was seen after 4 days of culture. Interestingly, there was no difference between B and E pigs in proliferation of purified lymphocytes to ConA. This might be explained by the fact that no plasma cortisol was present in purified lymphocyte cultures due to extensive washing of the cells, whereas in whole blood cultures plasma cortisol was still present. Indeed, there is a negative correlation between plasma cortisol concentration and proliferation to ConA at day 2 of culture in whole blood cultures. Another explanation might be that the number of leukocytes in the blood tended to be higher in B pigs than in E pigs, which might contribute to a higher proliferation in whole blood cultures. In the cultures of purified lymphocytes, cell suspensions were corrected for the absolute number of lymphocytes present such that each cell suspension had the same lymphocyte concentration. It has been observed previously that lymphocyte proliferation to ConA and PHA in a whole blood assay was suppressed after ACTH treatment of pigs, probably due to high levels of plasma cortisol [27]. As in our study, no effect of ACTH treatment on proliferation of purified leukocytes to ConA and PHA was seen.

An acute increase in cortisol shifts the lymphocyte/neutrophil ratio towards a higher percentage of neutrophils and a lower percentage of lymphocytes. This was demonstrated following cortisol injection, cortisol induction by stress or by injection of ACTH or CRF [18,22,27]. In our experiment, the distribution of leukocytes was not significantly affected by housing conditions, despite the difference in salivary cortisol concentrations between E and B pigs. It might be that the magnitude of the cortisol difference between E and B pigs at baseline is insufficient to induce such a shift in lymphocyte/neutrophil ratio, but since in the above-mentioned studies cortisol concentrations were measured in plasma and not in saliva, the increase in cortisol concentrations induced by acute stress or by CRF or ACTH injection cannot be compared to the differences we found in salivary cortisol concentrations between E and B pigs. However, we observed that shipping of pigs in combination with pre-slaughter treatment (waiting in lairage) increased the salivary cortisol concentration to ±9 ng/ml, which had major effects on the leukocyte distribution (unpublished results). Thus, this might imply that the salivary cortisol concentrations seen in enriched housed pigs during the light period (mean of light period 3–4 ng/ml) are indeed insufficient to induce an effect on leukocyte distribution. Alternatively, cortisol may only exert effects on leukocyte distribution when it is acutely increased, whereas continuously high baseline levels of cortisol, as were found in enriched housed pigs (in the light period), represent a level of homeostasis to which the animal is adapted by changes at other levels in the regulatory system. The latter is similar to the situation in depressed patients, which show hypercortisolemia. No differences between depressed patients and healthy controls (in the majority of the studies) in the number of white blood cells and the lymphocyte/neutrophil ratio were found [25]. Indeed, it has been shown that the number of glucocorticoid receptors on lymphocytes was decreased in depressed patients showing hypercortisolemia as compared to healthy controls [11,28], which indicates that adaptation to the higher baseline levels of cortisol takes place.

5. Conclusions

Since most immune parameters and the in vivo immune response to KLH are not affected by housing conditions, we conclude that the difference in baseline salivary cortisol concentration between E and B pigs does not induce major immunological differences. Apparently prolonged increase of cortisol/corticosterone concentrations does not necessarily impair immune function, as has been shown in rats with a chronic social stress model [16]. Although studies with pathogens are needed for a conclusion about effects of housing conditions on disease resistance, the present study implicates that the difference between barren and enriched housed pigs in baseline salivary cortisol concentration has no important consequences for disease resistance.

Acknowledgments

This work was funded by the EU (AIR3-CT94-2304) and the Dutch Ministry of Agriculture and Fisheries. We thank Arie Hoogendoorn for performing the white blood cell counts and leukocyte differentials, Bernie Moonen-Leusen for assistance with the lymphocyte proliferation assays, Marjan Benning, Joop te Brake, Dinand Ekkel, Nicole
References


