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Abstract

Stress is a recognised problem in intensive pig husbandry, which might lead to changes in immune reactivity. To study the effect of stress on the development of an anti-viral immune response, we used a murine model in which mice were immunized with an attenuated strain of pseudorabies virus (PRV). The effect of two stress treatments, both relevant to intensive pig husbandry, on the development of the specific immune response against PRV was investigated. The stress treatments consisted of restraint, social isolation, and transport and they differed in predictability. The specific immune response against PRV, which developed in the draining lymph nodes, was measured by a lymphocyte proliferation assay and cytokine production assays. Our results showed that the unpredictable stress treatment had no effect on the development of the immune response against PRV in mice, whereas the predictable stress treatment actually hastened the immune response. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Stress; Pigs; Mouse; Anti-viral immunity; Pseudorabies virus; Herpes virus

1. Introduction

Stress is generally thought to suppress immunity in humans and animals. Many stressors have been shown to affect both humoral (Laudenslager et al., 1988) and cellular (Monjan and Collector, 1977) immune function. Pigs in intensive pig husbandry suffer...
from stress. Sows are continuously confined, finishing pigs are weaned at a very young age, and they are repeatedly mixed with strange pigs resulting in social stress. Pigs can also suffer from climatic stress and transport stress. Stress in pigs can lead to disturbed behavior, such as stereotypies, and to altered physiology and immunity (Morrow-Tesch et al., 1994; Scheepens et al., 1994).

Since viral infections are particularly problematic in intensive pig husbandry, causing major economic losses, the effect of stress on anti-viral immunity is of special interest. In studies using rodents it has been shown that stress can suppress anti-viral immunity, but these studies used very severe stress protocols, that is, 16 h of restraint stress per day for various numbers of days up to 14 days (Bonneau et al., 1991; Sheridan et al., 1991). Thus the results of these studies cannot be extrapolated to pigs in intensive pig husbandry, besides maybe to the situation of continuous confinement of sows.

Therefore, the purpose of our study was to investigate the effect of relevant stressors, that is, those that actually occur in intensive pig husbandry, on the immune response against a virus. To do this we used a model recently described by Bianchi et al. (1998), in which mice are immunized with an attenuated strain of pseudorabies virus. The mice then develop protective immunity against infection with the lethal wild-type virus. Two stress treatments were investigated, one consisted of confinement (restraint), social stress (individual housing) and transport stress and was administered unpredictably. The other consisted only of confinement and was administered predictably. All three stressors occur in intensive pig husbandry and have been shown to be stressful for pigs.

2. Materials and methods

2.1. Subjects

One-hundred and sixty specified pathogen-free male Balb/c mice (Charles Rivers, Germany) were obtained at 4 weeks of age, immediately marked (holes in ears) under Halothane/N₂O anaesthesia, and housed under conventionally clean conditions in groups of five with free access to sterilized food (Charles River) and water. The light–dark cycle was reversed, lights off: 5.00 a.m.–17.00 p.m. All manipulations with the animals were done at a fixed time of day during the dark phase under dim red light conditions. Animals from different groups (controls, predictable stress and unpredictable stress groups) were housed in separate animal rooms. The experiments were performed under supervision of the animal experimental committee in the institute.

2.2. Virus

Virus stocks of the virulent PRV Northern Ireland Aujeszky 3 (NIA₃) strain and the avirulent, thymidine kinase negative mutant of NIA₃ (NIA₃TK⁻) were prepared in monolayers of the porcine kidney cell line SK6, as has been described (Bianchi et al., 1998). Supernatant was collected and number of plaque-forming units (PFUs) determined by a titration on SK6 cells. Stocks were stored at −70°C until use.
2.3. Experimental design

All mice were immunized at 12 weeks of age, in both hind feet, subcutaneously at the dorsal side of the foot, with 25 μl 8.2 log PFU/ml NIA3TK-β. This area is drained by the popliteal lymph nodes, where the development of the PRV-specific immune response was studied. The immune response was studied in three different treatment groups (control, predictable and unpredictable stress) at four intervals (Days 2, 4, 6 and 9) after immunization. At each interval, 10 mice from each treatment were sacrificed, as well as five mice that had not been immunized to serve as negative controls for the immunological assays. The stress treatments lasted 3 weeks, and proceeded until 3 days after immunization. Both the predictable and the unpredictable stress treatment consisted of a total of 36 h of stress (Table 1). Restrained stress was applied by putting the mice into a plexiglass cylinder with ventilation holes, in which the mice could only move forward and backward, but they could not turn in the cylinder. Social isolation was done by taking the mice out of their home cage and putting them into a small and clean cage. They were physically separated, but could still hear and smell each other. Transport stress was performed using a big closed container on wheels (size 1 m × 0.75 m × 0.5 m). The cages of mice were put into the container, the door was closed, so that they remained in the dark (the stress was carried out in the dark phase) and the container was driven up and down the corridor for the amount of time as stated in Table 1. Control mice were not manipulated apart from the weighing procedure.

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Restraint</th>
<th>Transport</th>
<th>Isolation</th>
<th>Total duration</th>
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<tbody>
<tr>
<td>Mon</td>
<td>10.30–12.30</td>
<td>15.00–15.20</td>
<td>–</td>
<td>2 h 20 min</td>
</tr>
<tr>
<td>Tue</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thu</td>
<td>8.30–9.00</td>
<td>–</td>
<td>9.00–12.00</td>
<td>3 h 30 min</td>
</tr>
<tr>
<td>Fri</td>
<td>–</td>
<td>–</td>
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</table>

<table>
<thead>
<tr>
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<th>Transport</th>
<th>Isolation</th>
<th>Total duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mon</td>
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<td>–</td>
<td>17.00–8.30</td>
<td>16 h 30 min</td>
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<tr>
<td>Tue</td>
<td>–</td>
<td>8.30–9.00</td>
<td>–</td>
<td>30 min</td>
</tr>
<tr>
<td>Wed</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thu</td>
<td>–</td>
<td>16.00–16.10</td>
<td>–</td>
<td>10 min</td>
</tr>
<tr>
<td>Fri</td>
<td>14.00–14.15</td>
<td>–</td>
<td>–</td>
<td>15 min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>Transport</th>
<th>Isolation</th>
<th>Total duration</th>
</tr>
</thead>
<tbody>
<tr>
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<td>–</td>
<td>11.00–11.30</td>
<td>11.30–13.30</td>
<td>2 h 30 min</td>
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<tr>
<td>Tue</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wed</td>
<td>10.30–11.00</td>
<td>–</td>
<td>–</td>
<td>30 min</td>
</tr>
<tr>
<td>Thu</td>
<td>16.00–17.00</td>
<td>–</td>
<td>17.00–17.30</td>
<td>1 h 30 min</td>
</tr>
<tr>
<td>Fri</td>
<td>14.00–17.00</td>
<td>13.00–13.15</td>
<td>–</td>
<td>3 h 15 min</td>
</tr>
</tbody>
</table>

*Predictable stress consisted of 4 h restraint stress, three times a week. This was always applied at the same time of day (10:00–14:00 hour) by the same experimenter. The unpredictable stress schedule is outlined above. Both stress treatments consisted of a total of 36 h of stress.*
The animals were sacrificed by cervical dislocation under anaesthesia (Halothane/N₂O), their popliteal lymph nodes were removed aseptically, suspended in culture medium (DMEM-alpha, 10%FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin–sulphate, 6 mM L-glutamine, 25 U/ml Nystatin) and washed once with culture medium. After staining with Nigrosine, viable cells were counted using a microscope, cell yield was calculated and cell concentration was adjusted to $2.5 \times 10^6$ cells/ml. The cells were then ready for use in the immunological assays.

2.4. Lymphocyte proliferation assay

The proliferative capacity of the lymphocytes from the popliteal lymph nodes was determined immediately and after 2 and 4 days of culturing with and without antigenic restimulation (the NIA₃ strain was used as antigen (multiplicity of infection: 10)). The assay was performed in triplicate with 100 μl. ($2.5 \times 10^5$ cells) per well in a 96-well flatbottom microtiterplate as described by Bianchi et al. (1998).

2.5. IL-2 and IFN-gamma

Production of IL-2 and IFN-gamma was determined by putting the lymphocytes from the popliteal lymph nodes in culture as described for the proliferation assay. The supernatant was collected after 18 h of culturing in the presence or absence of antigen, and stored at $-70^\circ$C.

The amount of IL-2 and IFN-gamma in the supernatant was measured using ELISA. Briefly, high-binding microtiter plates (Greiner) were coated with rat–anti-mouse IL-2: 2 μg/ml (Southern Biotechnology Associates (SBA), Birmingham, USA) or rat–anti-mouse IFN-gamma: 10 μg/ml (R46A2, supplied by Dr. H. Savelkoul, Rotterdam). The plates were then incubated for 2 h with serial dilutions of supernatants or standard solutions containing IL-2 (0.04–5 ng/ml; SBA) or IFN-gamma (0.4–50 ng/ml; SBA). Biotine conjugates (rat–anti-mouse IL-2-bio: 1/1000 (SBA) or rat–anti-mouse IFN-gamma-bio: 3 μg/ml (XMG1.2, Coffman, DNAX, USA)) were used for detection.

2.6. Physiological parameters

To measure the degree of stress caused by the two stress treatments, body weight and thymus weight were determined. Body weight of all animals was determined throughout the experiment, from 4 until 9 weeks of age once a week, and from then until sacrifice three times a week. Thymus weight was determined for the animals sacrificed at Day 6 after immunization, which was 3 days after the last stress session.

2.7. Statistics

To test the effects of treatment on the development of the immune response (lymphocyte proliferation response, cell yield from popliteal lymph nodes), an ANOVA with main effects and interactions for factors time (days after immunization) and treatment (predictable stress, unpredictable stress or control) was used. Other differences
between treatments (lymphocyte proliferation at one time point, cytokine production, thymus weight, body weight) were tested with a two-tailed Student’s \( t \)-test. Significance was set at 5% for both the ANOVA and \( t \)-test.

3. Results

3.1. Effects of stress on the development of the immune response

After immunization with PRV, the cell yield from the popliteal lymph nodes increased 10 times between Days 0 and 9. The cell yield was not affected by either stress treatment at any of the intervals after immunization (data not shown). The lymphocytes from the popliteal lymph nodes were tested functionally in a lymphocyte proliferation assay. When restimulated in vitro with antigen, the lymphocytes from the popliteal lymph nodes started to proliferate specifically to PRV antigen between Days 2 and 4 after immunization (Fig. 1). ANOVA revealed that this response developed significantly faster in the predictable stress group than in the unpredictable stress group and the control group, as shown by a significant interaction effect between factors treatment and time.

![Lymphocyte proliferation graph](image)

Fig. 1. Lymphocyte proliferation after being cultured 4 days in the presence of antigen, at various days after immunization in controls (crosses), predictably (filled triangles) and unpredictably (open triangles) stressed mice. Each point represents the mean of 10 mice \( \pm \) SEM. * \( p < 0.05 \) compared to unpredictable stress on the same day (\( t \)-test). ANOVA revealed a significant effect for time (\( F(3) = 95.87 \) \( p < 0.001 \)) and a significant interaction between treatment and time (\( F(2,3) = 2.71 \) \( p < 0.05 \)).
When lymphocytes were not restimulated with antigen in vitro, their proliferation was not affected by the stress treatments (data not shown). At Day 4 after immunization, lymphocytes from the popliteal lymph nodes produced IL-2 and IFN-gamma specifically in response to PRV antigen. The mice from the predictable stress group produced higher amounts of IL-2 than the controls or unpredictably stressed mice, but this difference was not significant. (Table 2).

### 3.2. Physiological effects of the stress treatments

Predictable stress decreased thymus weight at 3 days after the last stress session (26.8 mg (SEM 1.54)) compared to controls (34.5 mg (SEM 1.35)) \((p < 0.01)\), whereas unpredictable stress had no effect on thymus weight (33.3 mg (SEM 2.73)). Both stress treatments affected body weight. At 4 days after the start of the stress treatments the predictably stressed mice had a significantly lower body weight than control mice, whereas the unpredictably stressed mice showed a trend to a lower body weight than controls. From Day 4 until 14 days after the start of the stress treatments the difference between stressed and control groups declined (data not shown).

### 4. Discussion

After immunization in the hind feet, the specific immune response against PRV developed in the popliteal lymph nodes. The cell yield from the popliteal lymph nodes increased after immunization, and the lymphocytes located there responded to PRV antigen with proliferation and production of IL-2 and IFN-gamma. Neither of the two stress treatments that were investigated, inhibited the development of the immune response against PRV. In fact, the predictable stress treatment even hastened the PRV-specific lymphocyte proliferation response. This finding suggests that the formation of immunological memory developed faster in mice after predictable stress.

The finding that predictable stress did not suppress the immune response indicates that the mice were able to habituate to the stress treatment. However, habituation was not found consistently in the two physiological parameters measured. Body weight was only

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**Table 2**

Effect of predictable and unpredictable stress treatments on IL-2 and IFN-gamma production

<table>
<thead>
<tr>
<th>Type of stress</th>
<th>IL-2 (ng/ml)</th>
<th>IFN-gamma (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Predictable</td>
<td>3.2</td>
<td>0.50</td>
</tr>
<tr>
<td>Unpredictable</td>
<td>2.5</td>
<td>0.55</td>
</tr>
<tr>
<td>Control</td>
<td>2.3</td>
<td>0.35</td>
</tr>
</tbody>
</table>

\(^a\)As determined 4 days after immunization, in supernatant of lymphocytes from popliteal lymph nodes cultured overnight with viral antigen. In supernatant of lymphocytes from non-vaccinated mice or cultures without viral antigen, IL-2 and IFN-gamma were below detection limit (IL-2: 0.31 ng/ml; IFN-gamma: 0.78 ng/ml). No significant differences between the two treatments were found \((t\text{-test})\).
slightly affected during the first week after the start of the treatment and not thereafter, showing habituation. Thymus weight, however, was found to be reduced 3 days after the end of the stress, which argues against habituation. That predictable stress can actually enhance immune function confirms reports by others, who showed that predictable or repeated stress enhances aspecific immune function, such as the activity of NK cells (Irwin and Livnat, 1987), or has no effect on immune function, such as the proliferation in response to ConA (Lysle et al., 1987; Mormede et al., 1988). In our results there appears to be a discrepancy between the effects on thymus weight and body weight on the one hand and lymphocyte proliferation on the other hand. How can it be that the same stress treatment suppresses thymus weight and body weight, whereas it enhances lymphocyte proliferation? This might be explained by assuming that a habituation on the level of the immune system does not necessarily mean that there is also habituation on a physiological level. Mormede et al. (1988), for example, found that unpredictable and predictable stress both led to a rise of corticosterone levels, but only the unpredictable stress treatment reduced lymphocyte proliferation.

Although unpredictable stress is believed to lead to sensitisation (Mormede et al., 1988), in which the stress response increases with ongoing stress treatment, we found that the effect of unpredictable stress on body weight was slight and brief, and it had no effect on thymus weight. Surprisingly no sensitisation occurred, despite the unpredictable nature of the stress treatment. An important factor in this may be that the mice were housed in groups. It has been shown in rats that animals housed in groups are better able to cope with stress than individually housed animals, which might be due to social support (Ruis et al., in press). Since the physiological parameters revealed that the unpredictable stress treatment caused only a low (or no) degree of stress, it is not surprising that the unpredictable stress treatment was unable to alter the immune response in any way, negative or positive.

5. Conclusion

We conclude that stress, applied in types and intensities commonly found in intensive pig husbandry, does not have a negative effect on the immune response against PRV in mice, and may even improve the immune response. Extrapolating these results to pigs, suggests that the everyday stress experienced by pigs in intensive pig husbandry may not inhibit anti-viral immunity.

It should be noted, however, that although we tried to design stress treatments that were more relevant for pig husbandry than the treatments currently used in rodent studies, the effect of the applied stressors may differ between mice and pigs. As far as we know, no studies have been performed to compare stress-responses in mice and pigs, but it is known that confinement is stressful in pigs leading to stereotypical behaviour and both social isolation and transport cause a stress response in pigs (pers. commun., I.C. de Jong, ID-DLO). Maybe even more important than the relevancy of the type of stressor may be the severity and frequency of the stressor. With regard to this aspect of the stress treatments, our stress treatments had a low frequency and severity, and are thus relevant for the situation in intensive pig husbandry. However, specific studies in pigs are needed.
to investigate which situations are regarded as stressful and how these situations affect immune function.

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