REPEATED RESTRAINT AND ISOLATION STRESS IN LAMBS INCREASES PITUITARY-ADRENAL SECRETIONS AND REDUCES CELL-MEDIATED IMMUNITY

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ABSTRACT

Rambouillet crossbred ewe lambs were used to evaluate the effect of repeated restraint and isolation stress (RIS) on endocrinological and immunological functions. Lambs were blocked by weight and assigned to either RIS (n = 6) or to control (CON; n = 6) treatments. All lambs were tethered in environmentally controlled rooms at 22°C with constant light and, at this time (d 0), were given 1 mg of ovalbumin in adjuvant. On d 12, catheters were placed nonsurgically into the jugular vein of all lambs, and they were reimmunized with .5 mg of ovalbumin in incomplete adjuvant. Each lamb in the RIS treatment group was removed from its home stanchion, isolated from visual and tactile (but not auditory and olfactory) contact with other lambs, and restrained for 6 h on d 14, 15, and 16. Lambs in CON treatment remained undisturbed in their home stanchions. Lymphocyte blastogenic function and production of interleukin-2 (IL-2) were assessed in samples of blood collected before and at the conclusion (0 and 6 h) of each of the three stress bouts. In addition, ACTH and cortisol secretion in response to RIS was evaluated in samples of plasma and serum collected at .5-h intervals on the first and last days of stress (d 14 and 16). Finally, antibody production in response to immunization against ovalbumin was assessed in samples of serum collected 14 d after reimmunization on d 12 of the study. Polynomial curves fit to the ACTH and cortisol data differed (P < .005) on both d 14 and 16 between RIS and CON treatments. The area under the cortisol response curve in RIS lambs was reduced (P < .001) on d 16 compared with d 14, whereas the area under the ACTH response curve was not different between these 2 d. Lymphocyte blastogenic response to pokeweed mitogen was not affected by treatment. However, blastogenic response to phytohemagglutinin was lower (P < .005) in RIS lambs at the conclusion of each stress bout on d 14, 15, and 16 and even before the onset of stress on d 16 (P < .05). Blastogenic response also was reduced (P < .005) in response to concanavalin A at 6 h on d 15 and 16. No day x treatment interaction was noted for production of IL-2, but, overall, production of IL-2 by RIS lambs was reduced (P < .001). Antibody response to ovalbumin was not influenced by treatment. The data suggest that repeated application of a stressor repeatedly activates the pituitary-adrenal system and reduces some measures of cell-mediated immunological function.

Key Words: Lambs, Adrenal Glands, Corticotropin, Hydrocortisone, Immune Response


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Introduction

It is generally recognized that environmental or management-associated stressors activate the pituitary-adrenal axis and that such stressors may result in reduced immunological function in farm animals. Unmistakable reductions in immunological functions have been demonstrated in numerous studies in which either ACTH (Roth et al., 1982; Roth and Kaeberle, 1983; Roth, 1985; Blecha and Baker, 1986) or glucocorticoid (Collins and Suarez-Guemes, 1985; Roth, 1985) have been administered. However, there are only a few reports in which well-controlled application of a stressor has resulted in both activation of the pituitary-adrenal axis and reduced lymphocyte functions (Blecha et al., 1984; Westly and Kelley, 1984; Klemcke et al., 1990).

We have shown previously that both acute heat stress and a single 6-h episode of restraint and isolation stress (RIS) activated the pituitary-adrenal axis, but neither of these stressors resulted ultimately in changes in lymphocyte function (Minton and Blecha, 1990). These results led us to the conclusion that, although glucocorticoids are known immunosuppressive agents (Munck et al., 1984; Roth, 1985), not all stressors that result in greatly elevated cortisol result in reduced functioning of (at least some measures of) lymphoid cells. Therefore, the current study was designed to evaluate endocrinological and immunological functions in lambs presented with the same stressor on consecutive days. Restraint and isolation stress was chosen because it resulted in robust, immediate, and sustained activation of the pituitary-adrenal axis of lambs (Minton and Blecha, 1990).

Materials and Methods

General. Fall-born, crossbred Rambouillet ewe lambs were blocked by weight and assigned within block to two treatments; stressed lambs (n = 6) were to receive three consecutive days of RIS and nonstressed control lambs (CON; n = 6) were to remain undisturbed. Lambs were housed in temperature-controlled rooms at 22°C in individual stanchions and were individually fed .5 kg of a 50% whole corn, 50% dehydrated alfalfa pellet diet twice daily. Lights remained on continuously throughout the study to minimize the potential for photic entrainment of the circadian rhythm of cortisol. The day on which lambs were housed was designated d 0, and the 3 d of application of stressor were d 14 to 16. The stress treatment consisted of removal of the lamb from its home stanchion, isolating it from visual and tactile contact with other lambs, and securing its legs with medical adhesive tape for restraint.

Endocrinological Endpoints. Catheters were placed into the jugular veins of all lambs on d 12 by nonsurgical, percutaneous venipuncture. On the morning of d 14, samples of plasma and serum were obtained before the onset of RIS and at .5-h intervals for 6 h. At the conclusion of the stressor treatment, RIS lambs were returned to their home stanchions. During the period of application of RIS, samples of blood were obtained from CON lambs; otherwise, these animals remained unhandled. The same protocol for blood sampling was repeated on d 16 (the final day of stress). Samples of plasma were assayed for ACTH and serum was analyzed for cortisol in an RIA previously validated in our laboratory (Minton and Blecha, 1990).

Immunological Endpoints. Lambs were immunized with 1 mg of ovalbumin in complete Freund's adjuvant on d 0 and were reimmunized with .5 mg (in incomplete Freund's adjuvant) on d 12 at catheterization. The treatments were applied on d 14 to 16, and antibody response to the reimmunization was evaluated in samples of serum collected 14 d after reimmunization. Sheep anti-ovalbumin antibody titers were determined by ELISA (Minton et al., 1991). The titer was defined as the greatest dilution of serum that gave a positive reaction (≥ twice the absorbance of control wells at 405 nm) in the ELISA. Lymphocyte blastogenic responses to mitogens (Blecha et al., 1984) and production of interleukin-2 (IL-2) (Blecha and Baker, 1986) were assessed in isolated peripheral blood mononuclear cells collected at 0 and 6 h of the stress treatments on d 14, 15, and 16. The assays were performed as published previously except that ovine lymphoblasts that had been stimulated for 3 d with concanavalin A (Con A) were used as effector cells in the IL-2 assay instead of an IL-2-dependent cell line.

Analyses of Data. The endocrine profiles in CON and RIS lambs were fit to polynomial equations and the time-trends, within day of the experiment, were tested for parallelism using a general method for testing models
The lymphocyte blastogenic response to mitogens and the production of IL-2 were analyzed using a similar model, except that time included data obtained on all 3 d of stress. Antibody responses to ovalbumin were transformed to log₂ and analyzed by one-way ANOVA.

Results

The pituitary-adrenal responses of lambs to treatment are illustrated in Figure 1. On both the initial (d 14) and the final day of stress (d 16), the fitted time-trends for both ACTH and cortisol differed (P < .005) for RIS and CON over the 6-h bleeding periods. The differences in the time-trends reflect the unmistakable and robust increases in ACTH and cortisol in RIS lambs. However, the area under the plotted cortisol responses was reduced (P < .001) on d 16 compared with d 14 in RIS lambs, even though the ACTH responses in RIS lambs on these days did not differ (Figure 2). The areas under both ACTH and cortisol profiles for CON lambs were similar on both days.

The lymphocyte blastogenic response (Table 1) to phytohemagglutinin (PHA) was reduced in RIS lambs at the conclusion of each stressor bout on d 14, 15, and 16 (P < .005) and even before the application of stress on d 16 (P < .05). In addition, proliferation of lymphocytes in response to Con A was reduced (P < .005) at the conclusion of the stressor bout on d 15 and 16. Lymphocyte blastogenic response to pokeweed mitogen (PWM) was not influenced by treatment.

The lack of a significant F-test for the treatment × time interaction for IL-2 precluded individual treatment comparisons before and at the conclusion of each stress bout on d 14 to 16. However, overall, RIS resulted in reduced (P < .001) production of IL-2 (Figure 3).

Antibody titers to ovalbumin were not detectable before immunization of lambs (data not shown). Titers of antibody to ovalbumin obtained 14 d after reimmunization on d 12 of the study were identical in CON (14.67 ± .92) and RIS (14.67 ± .92) treatments.

Discussion

We have previously observed robust increases in both ACTH and cortisol in lambs in response to a single RIS bout (Minton and Blecha, 1990); however, we had not evaluated the response of lambs to repeated RIS treatment. This report demonstrates clearly that the pituitary-adrenal axis of lambs continues to be activated with three consecutive 6-h RIS bouts. However, the secretion of cortisol, but not of ACTH, diminished from the first to the third exposure. This observation does not suggest that lambs “habituate” to the stressor (because the secretion of ACTH remained unchanged from the 1st to the 3rd d); rather, the ability of the adrenal to secrete cortisol seems to be compromised upon subsequent exposure to stress. In contrast to our observation is a report suggesting that ewes given three bouts of 5 h of isolation from the flock spaced at 3-d intervals actually had increased cortisol secretion with each successive isolation (Niezgoda et al., 1987). The different findings of these two studies on the adrenal may reflect the obvious difference in timing between the stressor bouts or (unknown) differences in the effects of restraint and isolation together vs isolation alone. In agreement with our results are studies in rats in which corticosterone responses to restraint (De Souza and Van Loon, 1982; Pitman et al., 1988) and noise (De Boer et al., 1988) stressors decreased in response to repeated exposure. In addition, repeated application of restraint stress did not affect glucocorticoid catabolism or clearance (De Souza and Van Loon, 1982).

Neither 72 h of exposure to heat stress nor a single, 6-h bout of RIS affected lymphocyte blastogenic function in sheep, even though cortisol was unmistakably increased by both stressors (Minton and Blecha, 1990). Results of this study suggest that the duration of stressor may affect subsequent measures of immune function. The ability of elevated cortisol to suppress lymphocyte blastogenic
responses to mitogens has been demonstrated in sheep (Collins and Suarez-Guemes, 1985) and cattle (Roth et al., 1982); however, in both studies, elevated cortisol was maintained by administration of cortisol (four times daily for 12 d) (Collins and Suarez-Guemes, 1985) or ACTH (at 12-h intervals for 3 d) (Roth et al., 1982) rather than by stressors. However, our results are also consistent with stress-induced physiological elevations in cortisol and subsequent reduction in blastogenic responses of lymphocytes in transportation-stressed calves (Blecha et al., 1984) and in restraint-stressed pigs (Westly and Kelley, 1984).

Of interest in the current study is the observation that (with only one exception) reduced lymphocyte proliferative responses to PHA occurred at the conclusion of the RIS on d 14, 15, and 16 and that reduced blastogenic response to Con A did not occur until the conclusion of the RIS bouts on d 15 and 16. Furthermore, proliferative responses to PWM (generally considered to be a mitogen predominantly for B-lymphocytes) were not affected by treatment, consistent with results for ACTH-injected cattle (Roth et al., 1982). Taken together, these findings suggest that T-lymphocytes may be more susceptible to cortisol-induced suppression than B-lymphocytes; this supposition would generally support our observation that antibody production in response to ovalbumin was not affected by RIS.

Figure 1. Adrenocorticotrophic hormone (ACTH) and cortisol responses to restraint and isolation stress. (A) Hormonal responses to the first stress bout (d 14 of the experiment). (B) Hormonal responses to the third stress bout (d 16 of the experiment). Both ACTH and cortisol responses differ ($P < .005$) between lambs given restraint and isolation stress (broken line with open circles) and control lambs (solid line with solid circles). Each stress bout lasted for 6 h. In panel A, the pooled SE for the time trends describing ACTH in stressed and control lambs is 22.54 pg/ml and that for cortisol is 3.62 ng/ml. In panel B, the pooled SE for the time trends describing ACTH in stressed and control lambs is 21.68 pg/ml and that for cortisol is 5.14 ng/ml.
treatment (although the effect of RIS may have been different had adjuvant not been used to stimulate the immune response). Finally, the mechanism underlying differential suppression by cortisol of lymphocyte proliferation in response to selected mitogens is unknown but may reflect changes in the proportions of subpopulations of lymphocytes, which have been described recently in sheep infected with Bluetongue virus (Ellis et al., 1990). The effect

![Area Under ACTH Response](image1)

![Area Under Cortisol Response](image2)

Figure 2. Area under plotted adrenocorticotrophic hormone and cortisol responses to restraint and isolation stress. Values represent means ± SEM. Solid bars represent hormonal responses to the first stress bout (d 14 of the experiment) and shaded bars represent hormonal responses to the third stress bout (d 16 of the experiment). Cortisol response in stressed lambs was reduced (*P < .001) on d 16 compared with d 14.
TABLE 1. LYMPHOCYTE BLASTOGENIC RESPONSE TO MITOGENS IN LAMBS ON CONSECUTIVE DAYS OF STRESS

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Days of treatment</th>
<th>0 h</th>
<th>6 h</th>
<th>0 h</th>
<th>6 h</th>
<th>0 h</th>
<th>6 h</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWM&lt;sup&gt;a&lt;/sup&gt;, cpm × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>14</td>
<td>9.9</td>
<td>13.1</td>
<td>7.8</td>
<td>19.5</td>
<td>12.0</td>
<td>15.4</td>
<td>2.3</td>
</tr>
<tr>
<td>CON&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
<td>5.5</td>
<td>8.0</td>
<td>6.2</td>
<td>7.6</td>
<td>11.8</td>
<td>9.7</td>
<td>2.3</td>
</tr>
<tr>
<td>RIS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16</td>
<td>6.6</td>
<td>9.4</td>
<td>7.2</td>
<td>11.3</td>
<td>10.2</td>
<td>11.0</td>
<td>2.3</td>
</tr>
<tr>
<td>PHA&lt;sup&gt;a&lt;/sup&gt;, cpm × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0 h</td>
<td>185</td>
<td>193</td>
<td>144</td>
<td>187</td>
<td>209</td>
<td>212</td>
<td>11</td>
</tr>
<tr>
<td>CON&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15</td>
<td>194</td>
<td>145&lt;sup&gt;d&lt;/sup&gt;</td>
<td>171</td>
<td>126&lt;sup&gt;e&lt;/sup&gt;</td>
<td>178&lt;sup&gt;d&lt;/sup&gt;</td>
<td>106&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11</td>
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<td>16</td>
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<td>169</td>
<td>130</td>
<td>163</td>
<td>213</td>
<td>213</td>
<td>12</td>
</tr>
<tr>
<td>Con A&lt;sup&gt;a&lt;/sup&gt;, cpm × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0 h</td>
<td>156</td>
<td>138</td>
<td>157</td>
<td>110&lt;sup&gt;e&lt;/sup&gt;</td>
<td>190</td>
<td>131&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12</td>
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<tr>
<td>CON&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>190</td>
<td>131&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup>PWM = pokeweed mitogen; PHA = phytohemagglutinin; Con A = concanavalin A. Units are net counts per minute × 10<sup>3</sup> (mitogen-stimulated counts per minute minus background counts per minute).

<sup>b</sup>CON = control lamb; RIS = lambs treated with restraint and isolation stress.

<sup>c</sup>Within day of treatment and hour, reduced compared with CON lambs (P < .005).

<sup>d</sup>Within day of treatment and hour, reduced compared with CON lambs (P < .05).

of stressors, such as RIS, on the subpopulations of lymphocytes is unknown but is under investigation currently in our laboratories.

The production of IL-2 was measured in this study as another index of T-lymphocyte function. Administration of ACTH to calves at 12-h intervals for 2 d reduced the production of IL-2 in both the 1st and 2nd d of treatment (Blecha and Baker, 1986). Furthermore, acute restraint stress reduced production of IL-2 in barrows (Klemcke et al., 1990). Our results support and extend those observations and

![Bar graph showing production of interleukin-2 (IL-2) by lymphocytes of control (solid bars) and stressed (shaded bars) lambs. Values represent means ± SEM. No treatment x time interaction was detected (P > .10); therefore, no comparisons between treatments at individual sampling times were performed. Over all days of treatment, production of IL-2 was reduced (P < .001) in lambs subjected to restraint and isolation stress.](image)
suggest that stressor-induced pituitary-adrenal activation also results in suppression of IL-2 synthesis in sheep.

Implications

The results of this study suggest that repeated application of a stressor repeatedly activates the pituitary-adrenal axis, although the ability of the adrenal to secrete cortisol may be reduced upon subsequent application of the stressor. In addition, repeated application of RIS reduced some measures of T-lymphocyte function, whereas only a single application of stressor previously failed to alter similar measures of immune function. Thus, all short-term management or environmental stressors to which farm animals are often exposed may not alter immunological function.

Literature Cited


