Adrenal Steroids and Neutrophil Mobilization

By GEORGE J. FRUHMAN

ALTHOUGH it is true that adrenal glucocorticoids have found wide clinical application as effective anti-inflammatory agents, they must be used with caution since they can also cause an increased susceptibility to infection. The present investigation was prompted by a desire to gain additional information regarding the role of adrenal steroids in relation to the extravascular migration of polymorphonuclear leukocytes. This is important because of mounting evidence that the ability of the organism to muster large numbers of functional neutrophils into an area of the body that has been invaded by bacteria may be crucial in determining subsequent events.1 Despite an ever-increasing emphasis upon the dynamic aspects of leukocytic functions, our knowledge of the extravascular movements of white blood cells remains fragmentary.

A number of reports have appeared stating that treatment with cortisone or similar steroids inhibits or prevents the appearance of neutrophils in the inflamed areas of the mouse,2,3 rat,4 guinea pig,5 rabbit,6,9 and man.10 On the other hand, other papers have indicated that adrenal steroids are ineffectual in modifying the tissue neutrophil response.11-13 The majority of observations have been of a qualitative or, at best, a semiquantitative nature. If, in addition, the problems inherent in equating and evaluating such variables as species differences, and differences in dosage and time of injection are considered, the extent of involvement of the adrenal steroids in neutrophil mobilization is difficult to evaluate. Classically, the peritoneal fluid of laboratory animals has been a favored site for the examination of white blood cells as evidenced by the publications of Durham (1897),14 Beattie (1903)15 and Metchnikoff (1905).16 However, these early workers and their followers described the cellular changes that occurred either qualitatively or semiquantitatively. Josey17 was aware that fluid shifts in the peritoneal cavity could change the cellular concentrations of various leukocytes without affecting their absolute numbers, but he was unable to devise a practical method of determining the total cell population. The necessity of more precise quantification was also noted by Webb18 who stated, “The significance of the quantitative changes per cubic millimeter of fluid must remain obscure until some method has been devised of measuring the entire amount of fluid within the body cavity.” One possible solution to this problem was by Seeley, Higgins and Mann19 who measured the absolute weight of the peritoneal fluid as well as the cellular concentrations and so were able to estimate the absolute numbers of cells present. More recently, the necessity for determining the absolute number of cells in the peritoneal fluid has been re-emphasized.20-23

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In the present experiments a standardized neutrophil mobilization test has been employed in which known trace amounts of certain bacterial extracts evoke a massive intraperitoneal neutrophilia following their injection into rats. A predetermined time is allowed to elapse after the intraperitoneal injection and the numbers and types of leukocytes that have appeared in the peritoneal fluid are then quantified. In addition to its basic simplicity, another advantage of the method is that even a slight influx of neutrophils into the peritoneal fluid can be detected readily since the peritoneal fluid of the unstimulated rat contains virtually no neutrophils.

METHODS

Male rats of the Sprague-Dawley strain (Charles River Laboratories) weighing 180–220 Gm. were pair-weighed for each experiment and were permitted to have food and water throughout. The methods used for challenging the rats, harvesting the leukocytes and determining their numbers and types have been described. In brief, a standard dose of 0.1 g. Piromen* (a Pseudomonas polysaccharide complex; Baxter Laboratories) dissolved in 10 ml. sterile, non-pyrogenic 0.9 per cent saline solution (Baxter Laboratories) was injected intraperitoneally into rats. After an appropriate time interval—usually 5 hours—the peritoneal cavity was washed out with isotonic saline and the total numbers and types of cells present were ascertained by conventional hemacytometric and staining technics. All doses of steroids employed have been expressed as milligrams per kilogram body weight. Cortisol (Hydrocortone acetate; Merck, Sharp and Dohme), in a commercial vehicle, was injected as a saline suspension. Control animals received the vehicle which was prepared in the same volume of pyrogen-free saline. In addition to the experiments with cortisol, a number of rats were given an injection of either prednisolone tertiary-butyl acetate (Hydeltra-T.B.A.; Merck, Sharp and Dohme), cortisone (Cortisone acetate; Merck, Sharp and Dohme), corticosterone as the free alcohol (Schering Corp.) or deoxycorticosterone acetate (Cortate, Schering Corp.).

RESULTS

Adrenalectomy and Neutrophil Mobilization

The ability of adrenalectomized rats to mobilize neutrophils was tested and compared with that of a group of sham-operated controls. Adrenalectomized animals were given 1 per cent NaCl as drinking water. Peritoneal fluid of both the adrenalectomized and sham-operated rats contained about two million neutrophils two days after surgery, but by the end of one week only insignificant numbers of neutrophils were present. Therefore, in the following experiments animals were not tested until two weeks after the operation in order to allow ample time for recovery and for re-equilibration of the peritoneal fluid cells. Results of the five-hour neutrophil mobilization test are summarized in table 1 and indicate that there was no significant impairment in the ability of adrenalectomized rats to develop a peritoneal fluid neutrophilia.

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* I am indebted to Baxter Laboratories for the Piromen used, and both to Merck, Sharp and Dohme Co. and the Schering Corp. for the adrenal steroids that they provided.

† The importance of using a standardized inflammatory response is emphasized, because it has been demonstrated that the view that almost anything injected into the peritoneal cavity will evoke a neutrophilia is no longer a valid concept.21,22

‡ Merck, Sharp and Dohme Research Laboratories. Sterile aqueous vehicle for steroid suspensions containing 0.9 per cent benzyl alcohol.
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Table 1.—Numbers of Peritoneal Fluid Neutrophils Five Hours Following Intraperitoneal Injection of 0.1 µg. Piromen

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>No. of Rats</th>
<th>Neutrophils millions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operation</td>
<td>10</td>
<td>54 ± 9*</td>
</tr>
<tr>
<td>Adrenalectomy</td>
<td>11</td>
<td>44 ± 12†</td>
</tr>
</tbody>
</table>

*Means ± standard errors.

Table 2.—Peritoneal Fluid Neutrophils: Effects of a Single Injection of Cortisol Followed Immediately by Intraperitoneal Injection of 0.1 µg. Piromen

<table>
<thead>
<tr>
<th>Cortisol mg./Kg.</th>
<th>No. of Rats</th>
<th>Neutrophils millions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcutaneous Administration</td>
<td></td>
</tr>
<tr>
<td>0 (vehicle)</td>
<td>6</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>.1</td>
<td>6</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>38 ± 5†</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>22 ± 6†</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal Administration</td>
<td></td>
</tr>
<tr>
<td>0 (vehicle)</td>
<td>9</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>.1</td>
<td>9</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>22 ± 4†</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>20 ± 3†</td>
</tr>
<tr>
<td>100</td>
<td>9</td>
<td>6 ± 1†</td>
</tr>
</tbody>
</table>

*Means ± standard errors.

Effects of Single Injections of Adrenal Corticoids upon Neutrophil Mobilization in Intact Rats

Graded amounts of cortisol were injected into rats as single doses and in all cases the animals were challenged immediately by the intraperitoneal injection of Piromen. Two types of experiments were performed. In the first series, animals were pretreated with subcutaneous injection of cortisol. In the second series, cortisol was administered intraperitoneally. Results of these experiments are listed in table 2 where it can be seen that single subcutaneous injections of cortisol had little apparent effect upon neutrophil mobilization in doses ranging from 0.1 to 20 mg. per Kg. body weight. However, with 50 mg. cortisol a significant depression was seen. Further depression was noted at the 100 mg. dose.

Table 2 also summarizes the striking response of intraperitoneal administration of cortisol. A single dose of only 1 mg. of cortisol was able to depress neutrophil numbers to about half the control values. Ten mg. was equally effective. Following 100 mg. of intraperitoneally administered cortisol, only six million neutrophils were mobilized. It is of interest that in neither set of experiments was it possible to block the neutrophil mobilization completely.

Several supplementary experiments were performed using other adrenal
Table 3.—Peritoneal Fluid Neutrophils: Effects of Subcutaneous Injection of Cortisol Administered at Different Times before Animals Were Challenged

<table>
<thead>
<tr>
<th>Cortisol mg./Kg.</th>
<th>No. of Rats</th>
<th>Time of Injection</th>
<th>Neutrophils millions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (vehicle)</td>
<td>6</td>
<td>immediately</td>
<td>48 ± 3*</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>immediately</td>
<td>18 ± 4†</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>18 hours before</td>
<td>24 ± 6†</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>48 hours before</td>
<td>39 ± 5</td>
</tr>
</tbody>
</table>

*Means ± standard errors.
†p < .01.

Table 4.—Effects of Daily Subcutaneous Injections of Cortisol upon Ability of Rats to Mobilize Neutrophils into Peritoneal Fluid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Neutrophils millions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-injected controls</td>
<td>6</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>Cortisol, 10 mg./Kg. daily</td>
<td>6</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>for 6 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle-injected controls</td>
<td>6</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>Cortisol, 25 mg./Kg. daily</td>
<td>6</td>
<td>19 ± 1*</td>
</tr>
<tr>
<td>for 8 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < .01.

steroids. Single doses of 10, 25 and 50 mg. prednisolone, injected intraperitoneally, caused a marked inhibition of neutrophil influx into the peritoneal fluid. Both cortisone and corticosterone injected subcutaneously in single doses of either 25 mg. or 50 mg. also caused a significant depression of neutrophil mobilization. On the other hand, single subcutaneous injections of deoxycorticosterone of 10, 25 or 50 mg. were ineffectual in altering the numbers of neutrophils that appeared in the peritoneal fluid of challenged rats.

Demonstration that a massive injection of cortisol administered subcutaneously immediately prior to challenging the animal would arrest the influx of neutrophils into the peritoneal cavity led to an experiment to determine the duration of the effect of a single dose. Groups of single-injected animals were challenged immediately, after 18 hours, and after 48 hours. The results (table 3) indicate that the rats challenged immediately or after 18 hours were both under the influence of the cortisol injection. After 48 hours the numbers of neutrophils mobilized by cortisol-treated rats in response to Piromen injection were not significantly different from those of control animals.

Effects of Multiple Injections of Cortisol upon Neutrophil Mobilization in Intact Rats

Two experiments were performed assessing the effectiveness of multiple doses of cortisol upon impeding the extravascular migration of neutrophils. These data appear in table 4. Rats received daily subcutaneous injections of 10 mg. cortisol and were challenged on the sixth day, immediately following the last injection. The numbers of neutrophils that were harvested from such rats approached normal values despite the fact that these animals exhibited
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typical effects of the steroid treatment, including impairment of weight gain, lymphopenia, eosinopenia and thymic and splenic atrophy. The inability to modify the neutrophil mobilization pattern with this dose of cortisol led to a second experiment in which patently pharmacologic doses of steroids were employed. These animals received, subcutaneously, 25 mg. cortisol daily for eight days and were challenged by the intraperitoneal injection of Piromen immediately following the last steroid injection. Under this severe regimen, the steroid-injected rats failed to muster normal numbers of neutrophils into the peritoneal fluid.

Additional Control Experiments

A number of animals were examined in order to establish supplementary controls for some of the foregoing experiments. For example, it was necessary to determine whether large subcutaneous injections of cortisol would cause an influx of neutrophils into the peritoneal fluid of rats otherwise untreated. Single doses of cortisol as high as 100 mg. were unable to cause a peritoneal fluid neutrophilia after 5, 24, 48 or 72 hours. Moreover, daily injection of up to 25 mg. cortisol for eight days was, in itself, unable to bring about the appearance of significant numbers of peritoneal fluid neutrophils. Finally, neither the saline solutions injected nor the abdominal massage that followed resulted in the appearance of significant numbers of peritoneal fluid neutrophils.

DISCUSSION

The ability of salt-maintained adrenalectomized rats to mobilize large numbers of neutrophils into the peritoneal fluid in response to the local injection of bacterial polysaccharide supports the concept that the adrenal glands do not play a key role in the phenomenon of neutrophil mobilization. It is pertinent that Delaunay, Pages and Martinet24 reported that adrenalectomized rats were capable of evoking a tissue neutrophilia following toxic doses of typhoid vaccine and, indeed, were able to do so somewhat better than intact rats. Moreover, Speirs, Wenck and Dreisbach reported that 4 and 12 hours after the intraperitoneal injection of either albumin or pollen, the concentration of neutrophils was found to be significantly greater in adrenalectomized mice than in intact mice.25

It is also plain from the present experiments that although small and moderate doses of subcutaneously injected cortisol are ineffectual, pharmacologic doses of cortisol and related steroids can impede the diapedesis of neutrophils into the peritoneal fluid. A survey of the literature reveals that, in general, those reports asserting a striking arrest of tissue neutrophilia following adrenal steroid treatment have dealt with animals receiving very large doses of adrenal steroids. In one such experiment, workers injected as much as 1,000 mg. cortisol per Kg. body weight into mice for five days. The possibility of obtaining meaningful data using such massive doses cannot be dismissed, but it is felt that such effects cannot be equated with the better known effects of adrenal corticoids that require much smaller doses. Certain effects of these steroids are separable. Edema and diapedesis of leukocytes are separate phenomena and it has been noted27 that a given dose of cortisone may have
significant effects upon suppressing edema and other aspects of inflammation but at the same time may have slight or no effects upon the diapedesis of leukocytes.

Data are presented in this report indicating that the levels of cortisol required to inhibit neutrophilic migration are much larger than those reported to be necessary to reduce a generalized inflammation. Ten mg. cortisol per Kg. body weight daily did not affect neutrophil mobilization. The same dosage employed by Jasmin, Bois and Mongeau caused a dramatic decrease in fluid exudate formation in the standardized granuloma pouch technic. In addition to the amount of steroid injected, both the site of injection and the time interval between the injection of the hormone and the challenging injection of the bacterial polysaccharide are of importance. The finding that a relatively small dose of cortisol (in terms of body weight) inhibited neutrophils locally supports the results of Rebuck and Mellinger, who used the "skin window" technic and reported that a single topical application of cortisone caused a diminished neutrophilic leukocyte response and affected other types of leukocytes as well. The great differences in dose requirements between a topical injection and an indirect one suggest that adrenal steroids may be exerting a direct, local action upon neutrophil migration into the tissues. Moreover, a single dose of cortisol has a relatively short-lived effect and this finding is in general agreement with similar observations of Meier and Ecklin. The mechanisms by which cortisol and similar hormones are able to depress extravascular accumulations of neutrophils are still largely a matter of speculation. Rapid onset of this effect makes it unlikely that these steroids are acting by suppressing either formation of neutrophils or their release from the bone marrow. Furthermore, the well-known blood neutrophilia that is seen following cortisol treatment indicates that the circulating supply of these cells is adequate. A unique but unsubstantiated explanation of steroid-induced neutrophil inhibition in tissues is that of Menkin who proposed that repeated injections of cortisol into an inflamed area induce an "inactive leukotaxine" and a reduction in the potency of a "leukocytosis promoting factor." However, most of the evidence available at present concerns the action of corticosteroids upon small blood vessels. Accordingly, it has been stated that cortisone lessens the degree of "endothelial sticking" of leukocytes normally seen following thermal injury, bacterial infection, croton oil inflammation, and local trauma. In addition, the following related actions also have been ascribed to the adrenal corticoids: suppression of vasodilation, better maintenance of vascular tone, and vasoconstriction. If the primary action of cortisol in inhibiting neutrophil exit from the vascular bed is related directly to vascular integrity and permeability, it might prove profitable to examine the morphologic aspects of this problem with the electron microscope. Along these lines, recent studies have shown that, in certain types of inflammation, plasma escapes primarily through endothelial gaps on the venous side of the circulation, and that the basement membrane appears to be capable of a filtering action. It would be of great interest to see how these vascular components would behave under the dual impact of cortisol and bacterial polysaccharide.
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Summary

Rats were challenged to mobilize neutrophils by intraperitoneal injection of a bacterial extract. Adrenalectomized rats were able to accomplish this as well as their sham-operated controls. Furthermore, pretreatment of intact animals with moderate doses of cortisol injected subcutaneously did not produce a detectable effect upon neutrophil mobilization. On the other hand, when massive, pharmacologic doses were injected subcutaneously, significantly fewer neutrophils appeared in the peritoneal fluid. Inhibition of neutrophil accumulation was noted when the steroid was injected locally; this was accomplished with relatively small doses when calculated in terms of body weight. The relative ineffectiveness of subcutaneous injection compared with topical administration suggests a direct effect of the hormone, probably upon small blood vessels.

Summario in Interlingua

Rattos esseva provocate al mobilisation de neutrophilos per le injection intraperitoneal de un extracto bacterial. Rattos adrenalectomisate poteva compler isto tanto ben como le rattos de control subjicite a operationes ficticie. In plus, le pretractamento de animales intacte per injectione subcutanee de moderate doses de cortisol produceva nulle detegibile effecto super le mobilisation de neutrophilos. Del altere latere, quando massive doses pharmacologic esseva injicite subcutaneemente, significativemente reducite numeros de neutrophilos appareva in le fluido peritoneal. Inhibition del accumulation de neutrophilos esseva notate quando le steroide esseva injicite localmente; isto esseva complite per doses que esseva comparativemente micre in relation al peso corporee. Le relative inefficacia de injectiones subcutanee in comparation con administrationes topic suggere un effecto directe del hormon, probabilmente super le micre vasos sanguinee.

References

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