THE EFFECT OF GLUCOCORTICOSTEROIDS ON THE PROLIFERATION AND KINETICS OF PROMONOCYTES AND MONOCYTES OF THE BONE MARROW*

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A previous paper described the effects of glucocorticosteroids on the kinetics of peripheral blood monocytes and peritoneal macrophages of mice during both normal steady-state conditions and acute inflammatory reaction in the abdominal cavity (1). The administration of glucocorticosteroids caused a rapid disappearance (within 6 h) of the monocytes from the circulation, most probably due to sequestration of these cells in a compartment of unknown localization. The duration of this effect was dependent on both the kind and the dose of the compound administered. After a single injection of the water-soluble dexamethasone sodium phosphate, the monocytes reappeared in the circulation within 12 h. Injection of the insoluble hydrocortisone acetate, which causes a sustained steroid release from a subcutaneous depot, resulted in prolonged monocytopenia, lasting at least 14 days after administration of a dose of 0.6 mg/g body weight.

The mechanism underlying this prolonged reduction of the monocyte population was not clear. Glucocorticosteroids are reported to inhibit cell division (2–7), the dividing cells being arrested in the G_1 phase (8–10), possibly as a result of interference with a chalone mechanism (11–15). The monocytopenia after hydrocortisone administration could therefore be brought about by diminished production of monocytes in the bone marrow, as a result of a cytostatic action on their direct precursor cells, the promonocytes, or on earlier precursors of this cell line. This would be reflected in alterations of the various parameters for the mitotic activity of these cells.

Alternatively, the monocytopenia could be due to inhibition of the release of monocytes from the bone marrow. If the monocytopoiesis persisted, these monocytes might accumulate in the bone marrow, since glucocorticosteroids have no lytic action on mononuclear phagocytes (1).

Recently, data on the proliferation and kinetics of promonocytes and monocytes in mouse bone marrow during normal steady-state conditions have been published (16). The present paper concerns effects of hydrocortisone administration on the production and kinetics of monocytes in the bone marrow and the release of these cells to the circulation.

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Materials and Methods

Animals.—This study was done in specific pathogen-free Swiss mice raised at the Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands. Only male mice with a body weight of between 25 and 30 g were used.

Bone Marrow Cultures.—Methods for culturing bone marrow have been described in detail elsewhere (17), and a brief description will suffice here. A suspension of the marrow from both femora was cultured on a flying cover slip in a Leighton tube for 6 or 48 h. Duplicate cultures were made for each animal. The culture medium consisted of medium 199 (Microbiological Associates, Inc., Bethesda, Md.), to which were added 20% newborn calf serum (Grand Island Biological Co., Grand Island, N. Y.), 2000 U sodium penicillin G (Mycofarm, Delft, The Netherlands) per ml, and 50 μg of streptomycin (Mycofarm) per ml. The cultures were gassed with 5% CO_2 in air. After 2 h of incubation, during which the mononuclear phagocytes were allowed to attach to the cover slip, the medium was removed, the cultures were thoroughly washed twice with medium 199, and fresh medium was added. The cultures were then incubated again, and after a total culture period of 6 h the cells on the cover slip were washed vigorously three times with medium 199, gently dried in an airstream, and fixed for 30 min in absolute methanol. When the culture was incubated for 48 h, the medium was renewed after 24 h. At the end of the incubation period these cultures were washed and fixed in the same way as the 6-h cultures.

Counting of Nucleated Bone Marrow Cells.—For determination of the total number of nucleated cells in the bone marrow, both femora were prepared and the adjacent soft tissues removed. After both ends of the bone had been cut off, the marrow was expressed from the shaft by flushing with 2 ml of medium 199, using a 2.5 ml disposable syringe and needle (25 gauge $\frac{5}{8}$ inch). The marrow of each femur was collected separately in a plastic tube and thoroughly suspended by repeated pipetting. Two samples of the cell suspension of each femur were taken in leukocyte pipettes, diluted 1:10 with Türk's solution, and counted in a Bürker hemacytometer. The four values obtained in this way for each mouse were used to calculate the total number of nucleated cells in the bone marrow (per mouse), the bone marrow in two femora accounting for 11.8% of the total bone marrow mass (18).

Counting of Bone Marrow Monocytes.—A sample of bone marrow was taken from an obliquely cut femur and suspended between two injection needles in a drop of serum on a microscope slide. Smears made from this suspension were quickly air dried, fixed with absolute methanol for 15 min, and stained with Giemsa stain for 10 min. For each femur the percentage of monocytes was determined from counts of 500 nucleated cells. The total number of monocytes in the bone marrow was derived from the percentage of monocytes and the total number of monocytes in the bone marrow.

Counting of Bone Marrow Promonocytes.—Because promonocytes cannot be definitely recognized in bone marrow smears, these cells were indentified in the 6-h cultures. (19). The ratio between promonocytes and monocytes per mouse was determined in duplicate cultures from counts of a total of 400 cells. The total number of promoncytes was calculated on the basis of the promonocyte-to-monocyte ratio and the total number of monocytes in the bone marrow.

Labeling with [^{3}H]Thymidine.—The methods for cell labeling have also been described in detail elsewhere (16, 17). In vitro labeling of mononuclear phagocytes of the bone marrow was done by incubating the cells for 6 or 48 h in culture medium containing 0.1 μCi/ml [^{3}H]thymidine (specific activity 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.). In vivo labeling was done with one or repeated intravenous [^{3}H]thymidine injections in a dose of 1 μCi/g body weight.

The percentage of labeled cells was determined in autoradiographs. For the 6-h cultures the ratio between promonocytes and monocytes and the number of labeled cells of either category were determined in 400 cells in duplicate cultures, and these values were used to calculate the

labeling percentages of the promonocytes and monocytes. In the 48-h cultures the percentage of labeled mononuclear phagocytes was determined in 800 cells in duplicate cultures.

Autoradiography.—Autoradiography was performed with Ilford Nuclear Research Emulsion K 5 in gel form (Ilford Ltd., Ilford, Essex, England). The cover slips were dipped in the liquid emulsion, dried overnight, and stored in light-tight boxes. After 21 days of exposure, the autographs were developed with Kodak D19 for 2 min and fixed with Acidofix (Agfa/Gevaert, Germany).

Identification of Cells.—The bone marrow monocytes and promonocytes were identified according to the morphological criteria described previously (1, 19).

General Remarks.—Values given at each time point in figures and tables represent the mean values of at least four mice.

RESULTS

The Effect of Hydrocortisone on the Number of Monocytes in the Bone Marrow.—Since the injection of 15 mg of hydrocortisone acetate is followed by a rapid and prolonged decrease in the number of monocytes in the blood (1), it was relevant to determine whether the number of monocytes in the bone marrow was similarly affected. The total number of nucleated cells in the bone marrow showed an initial increase 6 h after the injection of hydrocortisone, followed by a gradual decrease to 75% of the initial value 96 h after the injection (Table I).

The percentage of monocytes remained almost unchanged (Table I). The total number of monocytes in the bone marrow, calculated from the total number of nucleated cells and the percentage of monocytes, rose initially after the administration of hydrocortisone, but thereafter gradually dropped to 75% of the initial value at 96 h (Table I). This decrease proved to be significant ($P < 0.001$).

The Effect of Hydrocortisone on the Number of Promonocytes in the Bone Marrow.— In 6-h cultures of the bone marrow of control mice the promonocyte-to-monocyte ratio was 15.8:84.2 (Table II). After the administration of hydrocortisone this ratio was altered. The percentage of promonocytes decreased, the lowest value being found at 6 h, and then gradually increased, but the normal ratio had not yet been reached at 96 h (Table II).

TABLE I

Effect of Hydrocortisone on Number of Nucleated Cells and Monocytes in Bone Marrow

Time after hydrocortisone*	Nucleated cells per femur‡		Monocytes	
	Total number	Percentage of controls	Percentage of nucleated cells	Total number in bone marrow‡
h	$\times 10^6$	%	%	$\times 10^6$
Controls	13.45		1.2	2.74
6	14.05	105	1.4	3.33
24	11.91	89	1.4	2.83
48	10.59	79	1.4	2.51
72	10.63	79	1.2	2.16
96	10.14	75	1.2	2.06

* 15 mg subcutaneously.

‡ Two femora = 11.8% of total bone marrow (18).

The total number of promonocytes in the bone marrow, calculated from the total number of monocytes and the promonocyte-to-monocyte ratio, also dropped after a hydrocortisone injection (Table II), the lowest value occurring at 6 h. This was followed by a slight increase, but over the entire period of the experiment the number of promonocytes remained almost constant at between 60 and 70% of the control value (Table II).

In Vitro Labeling of Promonocytes with [^{3}H]Thymidine during Treatment with Hydrocortisone.—The effect of hydrocortisone on the mitotic activity of the promonocytes was first assessed by determining its influence on the percentage of promonocytes synthesizing DNA in vitro. For this purpose, bone marrow was sampled at various intervals after hydrocortisone injection and cultured for 6 or 48 h in the presence of [^{3}H]thymidine. The 6-h cultures showed little influence of hydrocortisone on the in vitro labeling of the promonocytes, values at 48 and 72 h after injection being even higher than in the controls (Table III). In 48-h cultures, where all the cells have the appearance of macro-

TABLE II

Effect of Hydrocortisone on the Ratio of Promonocytes to Monocytes and Number of Promonocytes in Bone Marrow

Time after hydrocortisone*	Total number of monocytes in bone marrow	Ratio promonocytes/ monocytes	Promonocytes in bone marrow	
			Total number	Percentage of controls
h	$\times 10^6$		$\times 10^5$	%
Controls	2.74	15.8:84.2	5.14	
6	3.33	7.9:92.1	2.87	56
24	2.83	9.9:90.1	3.11	61
48	2.51	12.5:87.5	3.59	70
72	2.16	13.9:86.1	3.48	68
96	2.06	13.4:86.6	3.17	62

* 15 mg subcutaneously.

TABLE III

*Effect of Hydrocortisone on Labeling of Bone Marrow Promonocytes In Vitro**

Time after hydrocortisone‡	Promonocytes labeled 6-h culture	Total mononuclear phagocytes labeled	
		6-h culture§	48-h culture
h	%	%	%
Controls	56.8	9.5	22.4
24	54.8	5.3	12.6
48	60.8	8.3	13.5
72	63.3	9.1	19.5

* 0.1 μCi/ml [^{3}H]thymidine in medium.

‡ 15 mg subcutaneously.

§ Calculated from percentage of labeled promonocytes and percentage distribution of promonocytes and monocytes.

phages and promonocytes and monocytes can no longer be distinguished, a lower level of in vitro labeling was found after hydrocortisone treatment (Table III). For the 6-h cultures, however, when the labeling indices of all mononuclear phagocytes, i.e. promonocytes and monocytes, were calculated from the labeling indices of the promonocytes and the percentage distribution of the promonocytes and monocytes, lower values were also found after hydrocortisone administration (Table III). In these cultures only the promonocytes are dividing cells and can thus be labeled in vitro. Since the monocytes are nondividing cells, they are not labeled during the incubation with [^{3}H]thymidine (16). The fact that administration of hydrocortisone results in a lower percentage of promonocytes (Table II) would explain the lower in vitro labeling after hydrocortisone treatment. The labeling indices of 48-h cultures were about twice those of 6-h cultures (Table III), probably due to division of the promonocytes during the 48-h culture period (16).

Determination of DNA-Synthesis Time and Cell-Cycle Time during Treatment with Hydrocortisone.—Since the in vitro labeling gave no indication that hydrocortisone reduced the mitotic activity of the promonocytes, the effect of this compound on the proliferation of these cells was studied in more detail. The duration of the DNA-synthesis and cell-cycle times was determined 72 h after the injection of 15 mg of hydrocortisone acetate. This was done with serial labeling by two injections of [^{3}H]thymidine given 2 h apart. The prerequisites for the application of this method have been discussed elsewhere (16). The initial labeling index of the promonocytes (i) was determined on the basis of the mean of the labeling percentages of these cells determined 1 and 2 h after the first injection of [^{3}H]thymidine. The increment per hour of the promonocyte-labeling index (Δi) was calculated from the difference between the labeling index 1 h after the second [^{3}H]thymidine injection and the initial labeling index (i).

The DNA-synthesis time (t_S) was calculated by dividing the initial labeling index by the increment of the labeling index per hour; the cell-cycle time (t_C) was computed by dividing the DNA-synthesis time by the initial labeling index. The results showed that the duration of both t_S and t_C was shorter during hydrocorticose treatment than in normal mice (Table IV), but the difference was not significant ($P > 0.70$).

The Effect of Hydrocortisone on the Kinetics of Promonocytes and Bone Marrow Monocytes after a Single Injection of [^{3}H]Thymidine.—To gain insight into the effect of glucocorticosteroids on the release of newly formed monocytes from the bone marrow, animals were labeled with [^{3}H]thymidine and hydrocortisone was injected 1, 24, or 48 h later. The normal mice were injected with [^{3}H]thymidine. In these experiments a possible interference of the glucocorticosteroids with the release of monocytes could be expected to be reflected in an altered trend of the labeling curve of these cells.

In the normal mice, 69% of the promonocytes were labeled 6 h after [^{3}H]thy-

TABLE IV

*Effect of Hydrocortisone on DNA-Synthesis Time (t_S) and Cell-Cycle Time (t_C) of Promonocytes in Bone Marrow**

	Normal‡	Hydrocortisone§
i	69.8%	62.5%
Δi	5.2%	6.1%
t_S	13.6 h	10.3 h
t_C	19.5 h	16.4 h

* Mice labeled with 2 × 25 μCi [^{3}H]thymidine i.v. at 2-h interval: i = initial labeling index after first [^{3}H]thymidine injection; Δi = hourly increment of promonocyte labeling after second injection; $t_S = i/\Delta i$; $t_C = t_S/\Delta i$.

‡ Data from van Furth and Diesselhoff-Den Dulk (1970).

§ 72 h after injection of 15 mg of hydrocortisone acetate subcutaneously.

midine injection; the labeling index gradually dropped to 29% at 96 h (Figs. 1–3) due to mitosis of labeled promonocytes, these cells being replaced by unlabeled cells deriving from dividing precursors.

Division of labeled promonocytes gives rise to labeled monocytes. Consequently, the percentage of labeled monocytes in the bone marrow increases, reaching a maximum of 32.3% at 24 h after labeling. Thereafter this percentage decreases, because the labeled monocytes leave the bone marrow and are replaced by newly formed, unlabeled monocytes (16) (Figs. 1–3).

In hydrocortisone-treated mice the percentage of labeled promonocytes, although differing at individual points, runs a course that is generally similar to that found in mormal mice (Figs. 1–3). However, the labeling indices of the monocytes were altered by hydrocortisone. When [^{3}H]thymidine and hydrocortisone were injected 1 h apart, the labeling percentage of the monocytes increased to 39.2% at 24 h, indicating that new monocytes had been formed. Thereafter, in contrast to the decrease of the labeling percentage observed in the normal mice, the percentage of labeled monocytes in the hydrocortisone-treated mice remained almost constant during the next 48 h, the difference at time point 72 h being significant ($P < 0.001$). From 72 to 96 h the labeling percentage dropped to 19 (Fig. 1).

In the experiment with an interval of 24 h between the [^{3}H]thymidine and hydrocortisone injections, the percentages of labeled monocytes again remained almost constant after hydrocortisone administration until the labeling index decreased after 72 h (Fig. 2).

When hydrocortisone was injected 48 h after [^{3}H]thymidine, the percentage of labeled monocytes was higher than in the normal animals at 60 and 96 h; the value at 72 h did not differ from that in animals not treated with hydrocortisone (Fig. 3).

Although in the experiment in which [^{3}H]thymidine and hydrocortisone were

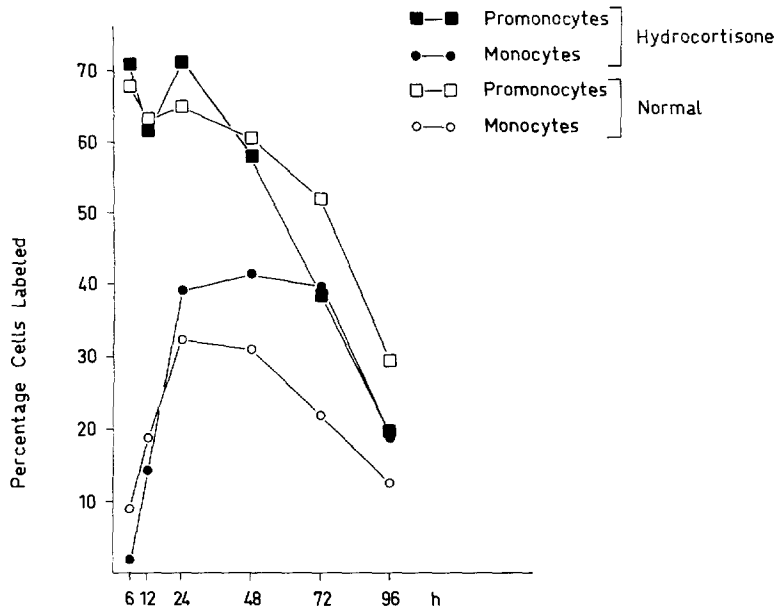

FIG. 1. The effect of a subcutaneous injection of 15 mg of hydrocortisone acetate administered 1 h after 25 μCi of [^{3}H]thymidine intravenously on the percentage of labeled promonocytes and monocytes in the bone marrow.

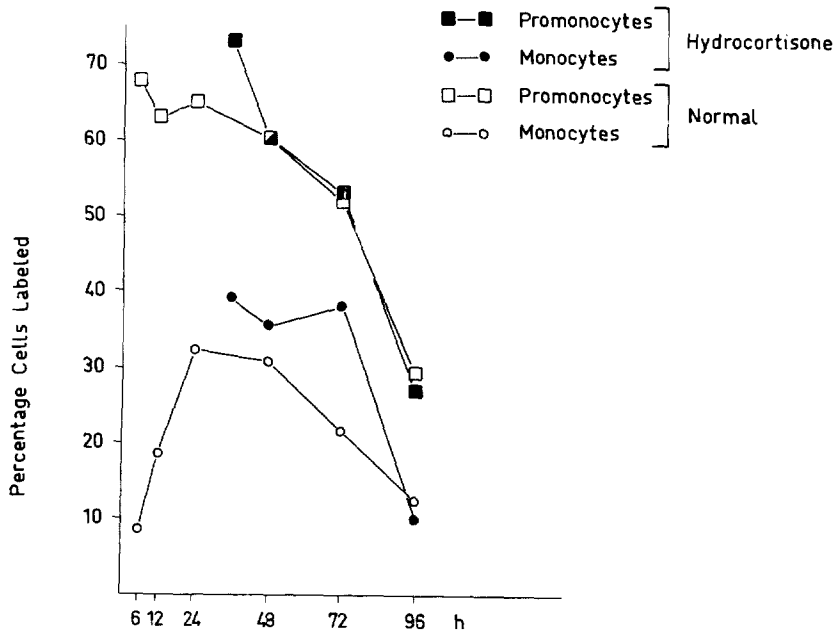

FIG. 2. The effect of a subcutaneous injection of 15 mg of hydrocortisone acetate administered 24 h after 25 μCi of [^{3}H]thymidine intravenously on the percentage of labeled promonocytes and monocytes in the bone marrow.

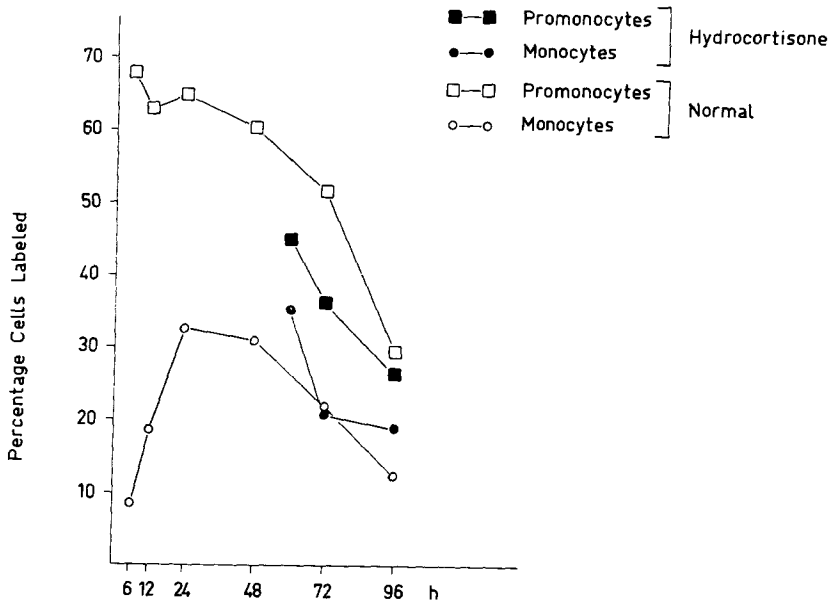

FIG. 3. The effect of a subcutaneous injection of 15 mg of hydrocortisone acetate administered 48 h after 25 μCi of [^{3}H]thymidine intravenously on the percentage of labeled promonocytes and monocytes in the bone marrow.

injected 1 h apart the percentage of labeled monocytes remained almost constant from 24 to 72 h after labeling (Fig. 1), it was possible that monocytes had left the bone marrow during this period and were replaced by newly formed cells. To check this possibility the grain counts were determined over 200 monocytes. At time points 24 and 72 h the mean grain count was 10.1 and 7.9, respectively, indicating that during that period monocytes were replaced by new cells with a lower [^{3}H]thymidine content.

DISCUSSION

There are two main conclusions to be drawn from this study. The first concerns the effect of hydrocortisone on the production of monocytes in the bone marrow, the second the release of newly formed monocytes from the bone marrow.

A quantitative evaluation of the effect of hydrocortisone on the cells in the bone marrow showed that administration of this compound resulted in a moderate decrease in the total number of nucleated cells as well as of promonocytes and monocytes.

The total number of nucleated cells found in normal mice and the gradual decrease to 75% of the initial value over a period of 96 h after hydrocortisone injection are in good agreement with the results of other studies in which comparable doses of glucocorticosteroids were used (20–22).

The decrease of the promonocyte number was found to be maximal shortly after hydrocortisone injection. Thereafter, this number remained almost constant, and about 65% of these cells were still present in the bone marrow.

The scanty data in the literature concerning the effect of glucocorticosteroids on the number of monocytes in the bone marrow give no indication of major changes (23–25). In this study, a gradual decrease of the monocytes in the bone marrow to 75% of the initial value was found over a period of 96 h after hydrocortisone injection. This is in contrast with the rapidly induced monocytopenia in the peripheral blood, where only about 10% of the monocytes are left 6 h after glucocorticosteroid injection (1).

Previous experiments led to the conclusion that a lytic action of glucocorticosteroids on monocytes is unlikely (1). The persistence of the monocytes in the bone marrow after hydrocortisone injection seems consistent with this conclusion.

Do glucocorticosteroids influence the production of mononuclear phagocytes in the bone marrow? In various systems glucocorticosteroids have been claimed to influence both the number and proliferation of hemopoietic cells (26–30). On this basis, the reduced number of promonocytes after a hydrocortisone injection could be due to diminished production of these cells, as a result of either a smaller number of precursor cells or a decreased mitotic activity of these cells. However, since the direct precursor cell of the promonocytes has not yet been definitely identified, these possibilities could not be checked.

A decreased production of monocytes could be caused by a reduced mitotic activity of their immediate ancestors, the promonocytes. However, in vitro labeling with [^{3}H]thymidine resulted in normal or even slightly increased labeling indices of the promonocytes after hydrocortisone injection. Furthermore, the DNA-synthesis and cell-cycle times determined 72 h after hydrocortisone administration were found to be shorter than in normal mice. If glucocorticosteroids diminished promonocyte proliferation, a longer cell-cycle time would be expected. It may therefore be concluded that glucocorticosteroids do not cause decreased mitotic activity of the promonocytes.

The effect of hydrocortisone on the production of monocytes in the bone marrow can be assessed quantitatively in two ways. In normal mice during the first 24 h after a single injection of [^{3}H]thymidine, 1.45×10^6 labeled monocytes are formed (16). The number of monocytes in the bone marrow 24 h after the injection of hydrocortisone is 2.83×10^6, and almost no monocytes are present in the circulation (1). Since 39.2% of the monocytes are labeled at this time point when [^{3}H]thymidine is injected 1 h before hydrocortisone, at least 1.11×10^6 labeled monocytes must be formed during the first 24 h after hydrocortisone administration. This is 77% of the production in normal mice.

The production of monocytes can also be calculated from the total number of promonocytes in the bone marrow and the cell-cycle time of these cells. In normal mice $(5.14 \times 10^5 \times 2)/19.5 = 5.3 \times 10^4$ monocytes/h are formed. 72 h after the injection of hydrocortisone, when monocytes are virtually absent in

the circulation, the production amounts to $(3.59 \times 10^5 \times 2)/16.4 = 4.2 \times 10^4$ monocytes/h. This is 80% of the production in normal mice. From these calculations it may be concluded that hydrocortisone causes only a moderate reduction of monocyte production. It is therefore unlikely that the prolonged monocytopenia in the peripheral blood induced by hydrocortisone is brought about by a diminished production of monocytes in the bone marrow.

Although the production of monocytes continues after hydrocortisone administration, no accumulation of these cells is found in the bone marrow, which shows that these newly formed cells disappear from this compartment. This was confirmed by the in vivo labeling experiments, in which both monocyte-labeling curves and grain count determinations demonstrated that labeled cells disappear from the bone marrow. Nevertheless, hardly any labeled monocyte can be found in the peripheral blood of hydrocortisone-treated animals (1). Whether these newly formed cells are destroyed within the bone marrow or continue to enter the circulation and are then immediately removed into a sequestration compartment is not yet known, however.

Comparison of the labeling curves of the bone marrow monocytes shows that after hydrocortisone injection the labeling indices of these cells were initially significantly higher than those found at the same time point in the normal animals. This could be due to either an increased production or a slower release of these cells. Since, however, a somewhat diminished monocyte production was found after hydrocortisone injection, it may be concluded that hydrocortisone interferes with the release of monocytes in a way resulting in a prolonged sojourn of these cells in the bone marrow. How this is brought about remains to be determined. It is conceivable that hydrocortisone affects the production or the action of factors normally regulating the mobilization of the monocytes from the bone marrow, but since the mechanisms responsible for the regulation of the kinetics of the mononuclear phagocytes are still unknown, the mode of action of glucocorticosteroids remains speculative.

SUMMARY

To elucidate mechanisms underlying the prolonged monocytopenia induced in the peripheral blood of mice by injection of a subcutaneous depot of hydrocortisone acetate, the effect of this compound on the production of monocytes and their release from the bone marrow was studied.

Hydrocortisone was found to cause a rapid reduction of the bone marrow promonocytes to about 65% of their initial number. The number of monocytes in the bone marrow decreased gradually, over a period of 96 h, to 75% of the initial value.

The mitotic activity of the promonocytes was not diminished, as judged from the labeling in vitro with [^{3}H]thymidine and the DNA-synthesis and cell-cycle times of these cells. The production of monocytes was only moderately diminished, i.e., to about 80% of the normal amount.

The release of monocytes from the bone marrow was found to be influenced

by hydrocortisone. After in vivo labeling with [^{3}H]thymidine the monocyte-labeling indices were initially significantly higher in hydrocortisone-treated than in normal mice.

It is concluded that a decreased production of monocytes in the bone marrow cannot account for the prolonged monocytopenia in the peripheral blood after hydrocortisone administration. However, hydrocortisone interferes with the release of newly formed monocytes from the bone marrow, resulting in a prolonged sojourn of these cells in this compartment.

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