Glucocorticoid-Induced Granulocytosis
Contribution of Marrow Release and Demargination of Intravascular Granulocytes

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Background—Glucocorticoid-induced granulocytosis has been attributed to enhanced release of polymorphonuclear leukocytes (PMNs) from bone marrow, delayed apoptosis, and reduced egress of PMNs into tissues. This study was designed to determine the relative contributions of PMNs released from the bone marrow and those entering the circulation from the marginated pool to the granulocytosis produced by a single dose of dexamethasone (2.0 mg/kg) in rabbits.

Methods and Results—PMN transit through the mitotic and postmitotic pools of the bone marrow and rate of release of PMNs into the circulation were measured by use of the thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) to pulse-label PMNs in the bone marrow. The shift of PMNs from the marginated to the circulating pool was measured with BrdU-labeled PMNs transferred from donor rabbits to recipients before dexamethasone was delivered. The data show that dexamethasone increased bone marrow release of PMNs and shortened their transit time through the postmitotic pool (P < 0.001) but not the mitotic pool of the bone marrow (P > 0.05). Dexamethasone slowed the clearance of BrdU-labeled PMNs from the circulation (P < 0.05) and lengthened their disappearance (half-life) from the circulation compared with control (half-life, 4.95 versus 9.45 hours). At 6 hours after dexamethasone, bone marrow release contributed ~10%, mobilization from the marginated pool ~61%, and a lengthened half-life in the circulation ~29% to the glucocorticoid-induced granulocytosis.

Conclusions—We conclude that a single dose of dexamethasone causes a granulocytosis primarily by a shift of PMNs from the marginated to the circulating pool, with a minor contribution from marrow release. (Circulation. 1998;98:2307-2313.)

Key Words: leukocytes ■ microcirculation ■ hormones ■ immunohistochemistry

Glucocorticoids are used to control the inflammatory response in many human diseases. Inhibition of phospholipases1 and inhibition of transcription of various cytokines2 have been reported to be major mechanisms for this anti-inflammatory action. Although glucocorticoids inhibit polymorphonuclear leukocyte (PMN) accumulation in inflamed tissue,3,4 they cause a marked increase in circulating PMNs available to participate in the inflammatory response.

Several possible mechanisms for the glucocorticoid-induced granulocytosis have been proposed, including enhanced release of PMNs from bone marrow,5 delayed apoptosis of PMNs in the circulation,6 and reduced egress of PMNs into inflamed tissues.5,7 The enhanced release of PMNs from the bone marrow after glucocorticoid administration has been demonstrated as an increased number of nonsegmented PMNs (band form) in the circulation,5,7 and by the dilution of infused radiolabeled PMNs5 after glucocorticoids. This is supported by patients with aplastic marrow, who have a poor response to glucocorticoids, suggesting that the enhanced release of PMNs from the bone marrow is an important mechanism of the glucocorticoid-induced granulocytosis.10

An alternative mechanism for the granulocytosis induced by glucocorticoids is an influx of PMNs from the intravascular marginated PMN pools (MPPs),11,12 similar to the effect of epinephrine and/or exercise.11 Although we have not found any reports that directly address this hypothesis, Bishop and colleagues5 suggested that PMNs in the MPP were mobilized into the circulation after glucocorticoids.

The purpose of this study was to determine the contribution of bone marrow release and demargination of PMNs to the glucocorticoid-induced granulocytosis. We used the thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) to pulse-label the myeloid precursors in the bone marrow and measured their transit time through the mitotic and postmitotic pools of the bone marrow by following the appearance and disappearance of the labeled cells in the circulation.13-15 We
have previously shown that this method can be used to measure the effect of an inflammatory stimulus such as pneumococcal pneumonia on the transit time of PMNs through the marrow pools. The chronic treatment of donor rabbits with BrdU also allowed BrdU-labeled PMNs (PMN-BrdU) to be transferred to recipients and the measurements of the effect of dexamethasone on the MPP and their half-life (t1/2) in the circulation. In this way, we were able to determine the relative importance of marrow release and demargination in the glucocorticoid-induced granulocytosis.

Methods

Animals
Female New Zealand White rabbits (n=27; weight, 2.3 to 3.2 kg) were used in this study. Rabbits were anesthetized by a subcutaneous injection of fentanyl (0.02 mg/kg) and droperidol (1.0 mg/kg) at each time point of blood collection. All these studies were approved by the Animal Experimentation Committee of the University of British Columbia.

Study Design

Effect of Dexamethasone on the Release of PMNs From the Bone Marrow
BrdU (100 mg/kg IV; Sigma Chemical Co) dissolved with saline was given to 13 rabbits. Twenty-four hours later, 2.0 mg/kg of dexamethasone (Sabex, QC) in 3 mL of saline solution was given intravenously to 7 rabbits and 3 mL of saline solution was given intravenously to 6 additional rabbits that served as controls. Blood samples were obtained from the central ear artery just before the BrdU and dexamethasone or saline injection and at 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours after dexamethasone or saline injection. Blood (1 mL) was collected in tubes containing EDTA for leukocyte counts, and an additional 1 mL was collected in tubes containing acid-citrate-dextrose (ACD) for the detection of PMN-BrdU.

Effect of Dexamethasone on the Release of PMNs From the MPP
The PMNs of donor rabbits (n=5) were labeled with BrdU (25 mg kg−1 · d−1 for 7 days), and the BrdU-labeled leukocytes were transferred to recipient rabbits as 15 mL/kg of whole blood by a modified alkaline phosphatase/anti–alkaline phosphatase technique previously described in detail. Briefly, the slides were digested at 37°C for 15 minutes in a 0.04% pepsin solution acidified to pH 2.5. DNA in the samples was denatured by incubating slides in 2N HCl, which was neutralized by 0.1 mol/L borate buffer, pH 8.5. Mouse monoclonal antibody against BrdU (2 μg/mL; DAKO Laboratories) was applied for 60 minutes. Nonimmune mouse IgG (5 μg/mL) and omission of the primary antibody were used as negative controls. A 1:20 dilution of rabbit anti-mouse IgG (DAKO) was applied for 30 minutes, followed by the anti-mouse IgG alkaline phosphatase–conjugated complex (DAKO) in a 1:50 dilution for 30 minutes. The alkaline phosphatase was developed for 20 minutes, and endogenous alkaline phosphatase was blocked by addition of levamisole (Sigma) to the color reaction. The preparations were counterstained with Mayer’s hematoxylin.

Evaluation of PMN-BrdU Released From Bone Marrow
PMN-BrdU was evaluated as previously described. Briefly, PMN-BrdU were divided into 3 groups according to the intensity of nuclear staining: weakly positive (staining of <5% of the nucleus, G1), moderately positive (staining of 5% to 80% of the nucleus, G2), and highly positive (staining of >80% of the nucleus, G3). This grading system was designed to evaluate the transit time of the myeloid cells that were in their last division in the mitotic pool when exposed to BrdU (G3), those that were in middle (G2), and those that were in their first division (G1). Fields were selected in a systematically randomized fashion, and 100 cells were evaluated per specimen. Intraobserver and interobserver reliabilities of this grading system were estimated by 2 different observers’ grading of PMN-BrdU on 10 randomly selected slides and 1 observer’s grading of the same 10 slides 3 weeks apart without knowledge of the identity of slides.

Transit Time of PMN-BrdU Through Bone Marrow
Transit time of PMN-BrdU through the bone marrow was calculated as previously described. Briefly, the number of PMN-BrdU was corrected for the disappearance (t0) of cells in the circulation. In previous studies, we have reported that the t0/2 of PMN-BrdU in rabbits was 4.5 hours. We applied this rate of exponential loss of PMN-BrdU from the circulation to calculate the number of PMN-BrdU released from the bone marrow and the transit time through the different pools in the bone marrow in the following manner:

\[
\Delta N(t) = N_0 - N(t)e^{-k(t-t_0)}
\]

where N is the number of labeled cells released from the bone marrow in the interval t; t0 and t are the initial and successive times, and k=ln2/t1/2.

These calculations were made for each 6-hour interval, and a histogram was drawn showing the distribution of the PMN-BrdU released from the bone marrow during each 6-hour interval. The mean transit times for all the PMN-BrdU and the different populations of PMN-BrdU (G1, G2, and G3 cells) were calculated individually in each rabbit.

Number of PMN-BrdU in Circulation of Recipients
The number of PMN-BrdU in the circulation of each recipient was expressed as a fraction of the total number of labeled PMNs originally infused and corrected for the calculated blood volume of the recipient in the following manner:

\[
\%PMN-BrdU_{circ} = \frac{(PMN_{circ} \times BV \times fractionPMN-BrdU_{recipient})}{PMN-BrdU_{infused} \times 100}
\]

where %PMN-BrdU_{circ} represents the number of PMN-BrdU in the circulation as a fraction of the total number of PMN-BrdU infused; PMN_{circ}, the calculated number of PMNs (×107/mL) in the circulation (WBC count×fraction of leukocytes that are PMNs); BV, calculated blood volume (mL); fractionPMN-BrdU_{recipient}, the fraction of PMN-BrdU in a cytospin of peripheral blood in the recipient; and PMN-BrdU_{infused}, the number (×107/mL) of PMN-BrdU infused (PMN count/mL×mL of fluid infused×fractionPMN-BrdU).
Calculation of $t_{1/2}$ of PMN$^{BrdU}$ in Circulation of Recipients

The number of PMN$^{BrdU}$ in the circulating blood of recipients reached a steady state 1 hour after transfer of labeled cells followed by a decay. Dexamethasone was given at this time point, which was designated as 0 hours, from which the rate of decay of PMNs in the circulation was calculated ($t_{1/2}$) by the following equation:

$$N_t = N_0 e^{-kt}$$

where $k$ is the rate of loss of PMN$^{BrdU}$ from the circulation, $t$ is the time after 0 hours, $e$ is 2.71828, $N_t$ is number of PMN$^{BrdU}$ in the circulation at time $t$, and $N_0$ is the number of PMN$^{BrdU}$ in the circulation at time 0 hours.

Because the $t_{1/2}$ can be estimated as the time at which $N_t$ is $N_0/2$, the rate-decay equation for $t_{1/2}$ becomes $t_{1/2} = \ln(2)/k$. The constant $k$ was calculated with the random-effects regression method. In dexamethasone-treated rabbits, time 12 hours was used as $t$.

Statistical Analysis

All values are expressed as mean±SEM except as otherwise mentioned. Temporal changes in circulating PMN counts, percentage of band cells, PMN$^{BrdU}$ counts, and %PMN$^{BrdU}$ were evaluated by ANOVA for repeated measurements. Transit times of PMN$^{BrdU}$ were compared by 2-sample $t$ test, and Bonferroni corrections were made for multiple comparisons. Statistical significance was defined as a value of $P<0.05$. One-way random-effects ANOVA was used for estimating the intraclass correlation coefficient of reliability, $R$, within 1 observer’s evaluations as well as between those of 2 different observers.

Results

Effects of Dexamethasone on the Release of PMNs From the Bone Marrow

WBCs, PMNs, and Band Cells in the Circulation

Figures 1 and 2 show the effects of dexamethasone on circulating WBCs, PMNs, and band cells. Circulating WBC and PMN counts at baseline (0 hours) were similar in the dexamethasone-treated rabbits and control rabbits. Dexamethasone increased the WBC counts from 8.9±2.0×10^9/L to 13.4±1.1×10^9/L at 6 hours ($P=0.028$ at 4 to 6 hours), and counts returned to the baseline value by 48 hours (Figure 1A). Dexamethasone increased the PMN counts from 4.6±0.8×10^9/L to 11.6±1.1×10^9/L at 6 hours ($P=0.004$ at 4 to 24 hours), and counts returned to the baseline value by 48 hours (Figure 1B). Dexamethasone increased the band cell counts from 0.28±0.05×10^9/L to 1.28±0.25×10^9/L at 6 hours ($P=0.016$ at 4 to 12 hours), and counts returned to the baseline value by 48 hours (Figure 2A). The percentage of band cell counts increased from 6.1±1.0% to 11.0±1.6% at 6 hours and 11.0±1.4% at 12 hours ($P=0.044$ at 2 to 12 hours), and values returned to the baseline value by 48 hours (Figure 2B). Leukocyte counts in the control rabbits did not change over the study period.

PMN$^{BrdU}$ in the Circulation

The first PMN$^{BrdU}$ appeared in the circulation 24 hours after labeling of the bone marrow, rapidly rose, and peaked at 48 (dexamethasone-treated rabbits) and 72 (control rabbits) hours (Figure 3A), and then disappeared over the next 4 to 5 days. Figure 3B shows the transit of highly stained PMNs (G3 cells), and Figure 3C shows weakly stained PMNs (G1 cells) through the circulation. G3 cells appeared in the circulation first and G1 cells appeared last in each group. We assume that the G3 cells represent cells labeled during their last myeloblast division and that the G1 cells represent cells labeled in their first myeloblast or promyelocyte division. The earlier peak time of PMN$^{BrdU}$ in dexamethasone-treated rabbits was mostly a result of the earlier release of G3 cells (Figure 3B). The estimated intraclass correlation coefficients for the evaluation of PMN$^{BrdU}$ indicated very high reliability within 1 observer ($R=0.95$) and between 2 observers ($R=0.94$).

Transit Time of PMN$^{BrdU}$ Through the Bone Marrow

The Table shows the calculated transit time of all the PMN$^{BrdU}$ and the different subpopulations of PMN$^{BrdU}$ (G3, G2, and G1 cells). The transit time of all the PMN$^{BrdU}$ through the bone marrow was shortened by dexamethasone ($P<0.01$). This shortened transit time was due to a shorter transit time through the postmitotic pool (G3 cells, $P<0.001$). The transit time through the mitotic pool (G1 to G3) was not changed by dexamethasone.

Effects of Dexamethasone on the Mobilization of PMN$^{BrdU}$ From the MPP

Circulating PMN Counts

PMN counts at baseline were similar in both dexamethasone-treated rabbits and control rabbits (Figure 4A). Dexamethasone caused a rapid increase in circulating PMN counts.
within 2 hours. Counts remained high up to 6 hours \( (P = 0.027 \text{ at } 4 \text{ to } 6 \text{ hours}) \) and then decreased to control values by 12 hours.

**Clearance of PMN\textsuperscript{BrdU} From the Circulation**

After transfusion of the PMN\textsuperscript{BrdU}, 1 hour was allowed to achieve a steady state.\textsuperscript{14} The fractions of the transfused PMN\textsuperscript{BrdU} in the circulating PMN pool (CPP) of recipients at 1 hour were similar in both dexamethasone-treated rabbits (40\( \pm \)2.8\%) and control rabbits (38\( \pm \)9.1\%, Figure 4B). In control rabbits, the fraction of PMN\textsuperscript{BrdU} decreased throughout the 24-hour study period, and the calculated \( t_{1/2} \) of PMN\textsuperscript{BrdU} from the circulation was 4.75 hours. Dexamethasone treatment increased the fraction of PMN\textsuperscript{BrdU} present in peripheral blood \( (P = 0.016 \text{ at } 2 \text{ to } 6 \text{ hours}) \), which increased the \( t_{1/2} \) of PMN\textsuperscript{BrdU} to 9.45 hours. Because the circulating PMN counts in the dexamethasone-treated rabbits returned to control values within 12 hours, the \( t_{1/2} \) in both groups were calculated between steady state (1 hour after transfusion) and 12 hours.

**Distribution of CPP and MPP**

In the control rabbits, the calculated percentage of PMN\textsuperscript{BrdU} remaining in the circulation immediately after the injection of PMN\textsuperscript{BrdU} was 44\% by use of a \( t_{1/2} \) of 4.75 hours. This means that the total blood PMN pool (TBPP) consists of 44\% CPP and 56\% MPP. We assume an equilibration between the CPP and MPP similar to that in control rabbits in the dexamethasone-treated rabbits at 12 hours, because the circulating PMN counts in dexamethasone-treated rabbits returned to control values within 12 hours (Figure 4A). On the basis of these assumptions, the calculated percentage of PMN\textsuperscript{BrdU} in the TBPP of dexamethasone-treated rabbits was 91\% at 0 hours and 38\% at 12 hours (Figure 5). With these data, the calculated \( t_{1/2} \) of PMN\textsuperscript{BrdU} in the TBPP of dexamethasone-treated rabbits was 9.45 hours. It follows that the calculated percentage of PMN\textsuperscript{BrdU} in the TBPP of dexamethasone-treated rabbits at 6 hours after treatment should be 59\%. The directly measured percentage of PMN\textsuperscript{BrdU} in the CPP of dexamethasone-treated rabbits at 6 hours after treatment was 46\%. This means that the TBPP in dexamethasone-treated rabbits at 6 hours after treatment consists of 78\% CPP and 22\% MPP. The calculated distribution of CPP and
MPP in the same way at 4 hours after treatment was 70% and 30%, respectively. The effect of dexamethasone on demargination was maximal at 6 hours after treatment. From these results, the calculated contribution of bone marrow release to the dexamethasone-induced granulocytosis at 6 hours after treatment was 10%, demargination was 61%, and other mechanisms that prolonged the t\(_{1/2}\) of PMNs (delayed apoptosis, reduced egress from blood) were responsible for the remaining 29% (Figure 6).

Discussion

This study shows that glucocorticoid-induced granulocytosis results from multiple mechanisms; it enhances the mobilization of PMNs from the bone marrow, it shifts PMNs into the CPP, and it causes prolongation of their intravascular half-life. Demargination of PMNs accounts for two thirds of the granulocytosis, with a minor contribution from bone marrow release of new PMNs (10%). This observation contrasts with previous reports in which the granulocytosis induced by dexamethasone is attributed primarily to the release of PMNs from the bone marrow.

Several studies showed that glucocorticoids cause a granulocytosis by enhancing the release of PMNs from the bone marrow. Our data support this concept and extend it by showing that the effect of dexamethasone is to shorten the transit time of PMNs through the postmitotic pool of the bone marrow. However, this shortening of the transit time of PMNs is smaller (83% of control values) than the effect of an inflammatory stimulus such as pneumococcal pneumonia (56% of control values). Furthermore, there were no differences in the release of PMNs from bone marrow into the circulation between the dexamethasone-treated and the control rabbits at the peak of the dexamethasone-induced granulocytosis (6 hours, P=0.15). This suggests that dexamethasone provides a weak stimulation for the bone marrow to release new PMNs into the circulation. Only 10% of the total number of PMNs added to the intravascular pool can be accounted for by bone marrow release. This contrasts sharply with the release from the marrow induced by an inflammatory stimulus such as pneumococcal pneumonia, in which transit times through both the mitotic and postmitotic pools are shortened and there is a much greater marrow release.

Mishler and Emerson suggested that a shift of PMNs from the MPP into the CPP could contribute to the granulocytosis induced by dexamethasone. Although PMNs from the bone marrow, it shifts PMNs from the MPP into the CPP, and it causes prolongation of their intravascular half-life. Demargination of PMNs accounts for two thirds of the granulocytosis, with a minor contribution from bone marrow release of new PMNs (10%). This observation contrasts with previous reports in which the granulocytosis induced by dexamethasone is attributed primarily to the release of PMNs from the bone marrow.

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Mechanism of Glucocorticoid-Induced Granulocytosis

Figure 6. Contribution of bone marrow release, demargination, and disappearance of PMNs in glucocorticoid-induced granulocytosis at 6 hours after treatment of rabbits with 2.0 mg/kg of dexamethasone (Dex). Numbers show PMN counts (×10⁹/L). See Results for assumptions made to calculate these fractions. C indicates CPP; M, MPP.

In summary, the data reported here show that dexamethasone causes PMNs to enter the circulation from both the bone marrow and the MPP. However, the effect of dexamethasone on the bone marrow was minimal and confined to an effect on the transit time through the postmitotic pool. Dexamethasone also prolonged the survival of PMNs in the circulation, but its major effect was on the demargination of PMNs, which accounted for the bulk of the PMNs that entered the circulation after treatment.

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References


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