Glucocorticoid-Induced Apoptosis in Early B Cells from Human Bone Marrow

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The sensitivity of normal human lymphoid precursor cells to glucocorticoid-induced apoptosis is a subject of controversy. The *in vitro* response of cells of the B lineage ($CD19^+$) from the marrow of 22 adult subjects to glucocorticoids was evaluated herein using both natural steroids and dexamethasone (Dex). When exposed to 1 μM Dex, 32% of the subjects exhibited high losses of $CD19^+$ B cells in the range of 45%. The remaining subjects exhibited more modest losses in $CD19^+$ cells of 26%–40%. Surprisingly, cortisol, a naturally produced glucocorticoid, produced B lineage losses nearly equivalent to Dex, which reached maximum by 12 hr. It was subsequently noted that the variances in losses of $CD19^+$ cells among the subjects correlated closely with the proportion of early $CD10^+$ $CD19^+$ B cells present in the initial population. The latter cells exhibited a high degree of sensitivity to glucocorticoids, with losses of 60%–80% noted. Mature B cells bearing IgD, on the other hand, were fairly resistant to glucocorticoids. Merocyanine 540, a membrane dye that fluoresces in the disordered membrane of apoptotic cells, confirmed that early or progenitor B cells in human bone marrow were indeed undergoing glucocorticoid-induced apoptosis, which could be blocked by the glucocorticoid antagonist RU38486. These data provide evidence that human marrow B cells, especially early B-cell progenitors, are quite sensitive to glucocorticoids and readily undergo apoptosis within a few hours of exposure to the steroids. Exp Biol Med 227:763–770, 2002

Key words: apoptosis; human bone marrow; glucocorticoids; merocyanine 540; progenitor B-cells.

The sensitivity of normal human bone marrow cells of the B lineage to glucocorticoids is of interest because of the increasing use of prednisone, dexamethasone (Dex), corticosterone, etc., in the treatment of allergies, rheumatoid arthritis, lupus, collagen-related diseases, leukemias, etc. (1–6). Moreover, endogenously produced glucocorticoids are chronically elevated by malnutrition, burns, trauma, etc., which appear to adversely effect lymphopoiesis (7, 8). Indeed, in mice, endogenously produced as well as synthetic steroids were able to induce substantial losses among precursor B cells in the marrow via apoptotic mechanisms both *in vivo* and *in vitro* (8–11). Thus, the lymphopenia associated with prolonged stress, malnutrition, or pharmacological regimens of glucocorticoids might be due to glucocorticoid-induced apoptosis in early developing lymphoid cells.

Conflicting reports were found in the literature regarding the sensitivity of human progenitor cells to glucocorticoids. Data from the 1970s and 80s have suggested that human leukocytes were not as sensitive to glucocorticoids as that of rodents and other species (12–15). However, many of these studies were performed on cell lines or peripheral blood lymphocytes, which contained mature lymphocytes as well as memory cells that would be more resistant to glucocorticoids (12–15). Subpopulations of early, precursor, and immature B cells from marrow were not examined. More recent reports indicate human cells such as thymocytes, activated T-cells, and normal tonsillar B cells are responsive to glucocorticoids (16–18). The purpose of this study was to examine the *in vitro* effects of natural and pharmacologically used glucocorticoids on normal human bone marrow cells of the B lineage.

In the past, difficulties have been encountered when trying to identify apoptotic cells in heterogenous human cell populations. Simple DNA dyes that worked well with murine cells give poor labeling of apoptotic human cells (19). End labeling of fragmented DNA via TUNEL precludes the phenotype labeling of subsets of cells, which is essential for studying a heterogenous tissue such as marrow. A recent series of studies in our lab indicated that merocyanine 540 (MC540), an inexpensive dye that binds to the outer leaflet of the cell membrane, was as effective and as reliable in identifying apoptosis as more standard methods using propidium iodide or annexin V (20). Moreover, MC540 was also very effective in identifying apoptotic human cells and could be used in multicolor protocols as shown herein (20).

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Therefore, MC-540 was used in conjunction with fluoresceinated antibodies to determine the effects of steroids on cells of the B lineage within the marrow. The results indicate that B cells from human marrow, especially early B cells ($CD10^+CD19^+$), are quite sensitive to glucocorticoids.

Materials and Methods

Materials. Dex, cortisol, RPMI-1640, and Histopaque were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated mouse-anti-human CD19, phycoerythrin (PE)-conjugated mouse anti-human CD19, FITC-conjugated mouse IgG_1, and PE-conjugated mouse IgG_1 antibodies (isotype-matched negative controls) were purchased from Becton-Dickinson (San Jose, CA). PE-conjugated mouse anti-human CD10, FITC-conjugated rabbit anti-human IgD, and FITC-conjugated $F(ab')_2$ fragment of rabbit immunoglobulin (negative control for anti-IgD) were purchased from DAKO (Carpinteria, CA). RU38486 was a gift from Roussel-UCLAF (Romainville, France). Merocyanine 540 was obtained from Molecular Probes, Inc. (Eugene, OR).

Preparation of Marrow for Culture from Subjects. Bone marrow was removed by aspiration from the sternum of adults undergoing open-heart surgery by approval of the Michigan State University Committee on Research Involving Human Subjects. The 22 patients assessed ranged in age from 46 to 71 years, with the exception of one 75- and one 22-year-old patient. Subjects included members of both sexes (23% female, 77% male). Patients receiving glucocorticoids, catecholamine therapies, or immunosuppressive drugs were excluded from the study. Mononuclear cells were separated by density gradient centrifugation using Ficoll hypaque (1.077 g/cm^3). After washing, counting, and viability staining, the marrow cells were cultured at a concentration of 1×10^6 cells/ml in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 60 μmol/L 2-mercaptoethanol at 37°C in a humidified atmosphere of 5% carbon dioxide for 10–28 hr in the presence or absence of glucocorticoids. For the sake of consistency, the same lot of FBS was used throughout these studies. Solutions of the natural and synthetic glucocorticoids, cortisol, and Dex were added to cultures at final concentrations ranging from 10^{-8} to 10^{-4} *M*. RU38486, the glucocorticoid receptor antagonist, was added to cultures at a final concentration of 5 μ*M* just prior to addition of the glucocorticoids (21). At the end of the incubation period, cells treated with glucocorticoid exhibited an increase in apoptotic cell morphology via phase contrast microscopy, but cell viability remained high (>90%), as evidenced by trypan blue dye exclusion. Cells were harvested, phenotyped, fixed overnight, then analyzed by flow cytometry for the presence of cells bearing CD19 surface antigen, as well as CD10 and IgD. Murine thymocytes (A/J mice, 6–16 weeks old, Jackson Laboratory, Bar Harbor, ME) served as a control for Dex- and cortisol-induced apoptosis (19, 22).

Phenotypic Labeling of Bone Marrow B Lineage Cells. After incubation with glucocorticoid, cells were harvested, washed, and layered over a heat-inactivated FBS (HIFBS) gradient to remove cellular debris and to recover viable cells, which were incubated in 2% heat-inactivated mouse serum before phenotyping to reduce nonspecific antibody binding. The cells were then incubated with 0.50 μg of FITC-conjugated mouse anti-human CD19 (IgG_1 isotype) or 0.25 μg of PE-conjugated mouse anti-human CD19 (IgG_1 isotype) for 30 min, being maintained at 4°C for this and all subsequent steps. For dual-color phenotypic analysis, cells were subsequently labeled with 0.8 μg of PE-conjugated mouse anti-human CD10 (IgG_1 isotype) or with 2 μg of FITC-conjugated rabbit anti-human IgD [$F(ab')_2$ fragment]. In all instances cells were also incubated with isotype-matched nonspecific antibodies, which served as negative controls. All antibodies were diluted with modified Hanks' balanced salt solution containing 2% HIFBS and 0.15% NaN_3 to prevent capping. Labeled cells were washed and resuspended in 1:1 PBS: HIFBS and fixed in ice-cold ethanol to a final concentration of 52% and stored overnight. After removal of ethanol, cells were kept at 4°C until analyzed on the flow cytometer. Samples were analyzed in duplicate for 20 of 22 subjects. For the remaining two subjects, the small volume of marrow collected prevented duplicate determinations.

Detection of Apoptotic B-Lineage Cells from Bone Marrow. Cells were incubated in the presence or absence of glucocorticoid, harvested, washed, and layered over HIFBS to remove debris as described above. A stock solution of 1 mg/ml MC540 was prepared in 50% ethanol. To detect apoptosis, cells were phenotyped with FITC-CD19 without fixation and subsequently incubated with 30 μ*M* MC540 in Hanks balanced salt solution containing 4% HIFBS in the dark. The FBS was key to reducing low-affinity binding of MC540 to cells such that over a decade of fluorescence separated normal ($MC540^{dim}$) from apoptotic cells ($MC540^{bright}$) per previous methodology (20). The cells were stored on ice until fluorescence-activated cell sorter analysis, which was performed within 2 hr of staining. To differentiate viable cells from dead cells including necrotic cells and debris, samples were stained with 1 μg/ml PI as previously described (19, 20).

Flow Cytometric Analysis. Flow cytometry was performed using a Becton-Dickinson Vantage fluorescence-activated cell sorter equipped with an air-cooled argon laser for excitation at 488 nm with FITC and PE fluorochrome emission detected at 530 ± 15 nm and 575 ± 13 nm, respectively. MC540 was excited at 488 nm, and emission was detected at 575 ± 13 nm. Based on forward and side scatter characteristics, a gate was drawn to include the lymphoid population of the marrow where 15,000 events per sample were analyzed. FITC and/or PE positive cells were examined using a gating procedure, which eliminated debris,

dead and necrotic cells, cell doublets, and nonspecific antibody binding. The lymphoid population was analyzed for single antigen phenotyping (CD19, CD10, or IgD) by histogram analysis. Dual-labeled samples (CD19/CD10, CD19/IgD) were analyzed using a two-color cytogram. For detection of apoptosis, CD19-bearing cells that excluded PI were analyzed for binding of MC540 by histogram. Thus, dead cells, necrotic cells, and debris were excluded via use of PI and light scatter. All analyses were performed using Lysis II or PC Lysis software (Becton-Dickinson, San Jose, CA).

Results

Sensitivity of Human Bone Marrow CD19$^+$ B-Lineage Cells to Glucocorticoids. To determine the relative sensitivity of human B-lineage bone marrow cells to glucocorticoids, freshly isolated mononuclear cells from the bone marrow were incubated *in vitro* with 10^{-6} *M* Dex for 20 hr. Some variance in sensitivity of CD19$^+$ cells to Dex was noted among the 22 subjects who were analyzed (Fig. 1). Approximately 32% of all subjects tested were very sensitive to Dex (1 μ*M*), exhibiting losses of 40%–50% in CD19-bearing B cells after a 20-hr exposure period. A second group composed of about 45% of the subjects exhibited moderate losses of B-lineage cells of 25%–40%. Only 23% of subjects were resistant, exhibiting less than a 20% loss of B-lineage cells. Male and female subjects were distributed throughout the range of sensitivity, as were individuals of all ages. Previously, marrow from several subjects had been incubated with physiological (10^{-8} to 10^{-6} *M*), pharmacological (10^{-5} *M*), or suprapharmacological (10^{-4} *M*) concentrations of Dex for 20 hr and then labeled with CD19, the pan B-cell surface marker. Losses in cells of the B lineage plateaued at 10^{-7} through 10^{-5} *M* Dex. Only the suprapharmacological level of steroid caused further depletions of CD19$^+$ cells. Although the latter data is not shown, analogous results were obtained as will be seen in subsequent figures (see Figs. 2 and 3).

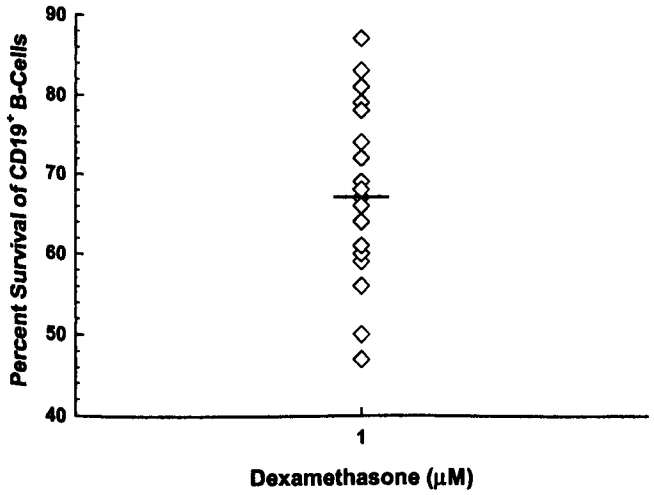

Figure 1. Effect of Dex on survival of CD19 bearing B cells from human marrow. Bone marrow mononuclear cells from 22 subjects were incubated in the presence or absence of 1 μ*M* Dex for approximately 20 hr using flow cytometry to measure changes in the percentage of CD19$^+$ B cells. Data are normalized to control cultures containing no steroid (100% for CD19$^+$). Data shown are the percentage of CD19$^+$ cells surviving Dex treatment. In the 1 μ*M* Dex-treated cultures, the mean value of CD19$^+$ B-cells for all subjects was 67% of control values. Symbols for four of the subjects overlap near the mean, and it is difficult to distinguish where the mean for duplicate samples are shown. The age range of subjects was 46 to 75 years, with one 22-year-old subject included in the study. Twenty-three percent of the subjects were women. Based upon forward and side scatter characteristics, a gate was drawn to include the lymphoid population of the marrow where 15,000 events per sample were analyzed. Cell viability was greater than 90% by trypan blue exclusion.

Comparison of Sensitivity of B-lineage Cells to Natural Versus Synthetic Glucocorticoids. Subsequently, Dex and cortisol, a natural or endogenously produced glucocorticoid, were evaluated simultaneously *in vitro* to compare their ability to induce losses among CD19$^+$ cells using three patients. Figure 2 indicates that cortisol was only moderately less potent than Dex. The effects of the two steroids were identical in patient 15, where substantial losses in CD19$^+$ cells were observed. In the remaining two patients, the potency of the steroids was similar, diverging only at 10^{-7} *M*. A concentration-dependent plateau for loss of CD19$^+$ cells was seen for both steroids in the range of 10^{-7} to 10^{-5} *M*, in agreement with earlier results. Thus, the data indicate that *in vitro*, the synthetic steroid, Dex, is only moderately more potent than cortisol in causing depletion of marrow of CD19$^+$ B lymphocytes.

Kinetic Response of CD19$^+$Cells to Dex. To determine the time frame in which glucocorticoids exert their effects upon bone marrow B-lymphocytes, the loss of CD19$^+$ B cells created by incubation with 1 μ*M* Dex was evaluated over a 20-hr period. A modest decrease in the percentage of CD19-bearing cells was observed at 8 hr (14% loss), followed by a significant decrease at 12 hr (36% loss). The losses then plateaued, remaining unchanged from 12 hr through 20 hr (data not shown). Thus, significant cell losses occurred within a few hours of exposure to glucocorticoids (12 hr). This same time course was noted for two different subjects (data not shown).

Effect of Maturation State on Sensitivity of Marrow B Cells to Glucocorticoids. It was suspected that variations in the proportion of pro-B, pre-B, immature-B, mature, and memory B cells found in the marrow of these adults might account for their differences in sensitivity to glucocorticoid induced losses in CD19$^+$ cells as it did in the mouse (9, 23, 24). It was known that murine pre-B cells and pre-B leukemic equivalents in acute lymphoblastic leukemia patients are very responsive to glucocorticoids (8–11, 16, 25), suggesting that differentiation state affects glucocorticoid sensitivity. To test this hypothesis, bone marrow mononuclear cells were cultured in the presence or absence of Dex, then labeled with the antibodies to the following markers: CD19 only (all B cells), CD19$^+$ and CD10$^+$ (pro-, pre-B), or CD19$^+$ and IgD$^+$ (mature-B; Ref. 26). The early B-lymphocytes, which express CD10, were very responsive

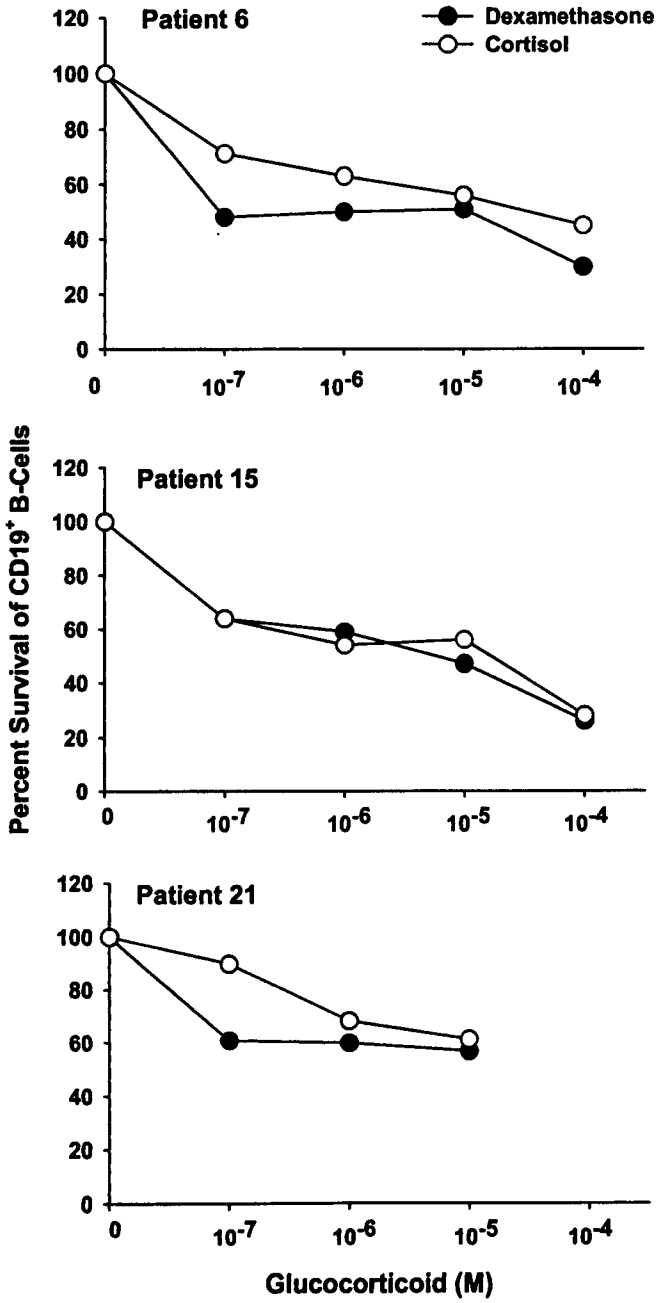

Figure 2. Comparison of sensitivity of $CD19^+$ B cells from human bone marrow to cortisol versus Dex. Exposure to various concentrations of each steroid was monitored for 20 hr, with the proportion of surviving $CD19^+$ cells determined by flow cytometry. Data are normalized to values obtained from cultures containing no steroid and represent mean values of duplicate samples with comparisons made for three subjects where 15,000 cells per sample were evaluated. Based upon forward and side scatter characteristics, a gate was drawn to include the lymphoid population of the marrow. Cell Viability was greater than 90% by trypan blue exclusion. Overlapping data points appear as a single symbol for patient 15.

to the glucocorticoids (Figs. 3 and 4). Loss of 60%–80% of all $CD19^+$ cells was observed in all four subjects at a low concentration of Dex (10^{-7} *M*). As would be expected from previous data, losses in this population plateaued at 10^{-7} *M* to 10^{-5} *M* Dex (Fig. 3). At suprapharmacological doses (10^{-4} *M*), 90% losses were observed in the $CD19^+CD10^+$ subset of the three patients in which sufficient cells were available to test the full range of concentrations. Mature cells expressing the $CD19^+IgD^+$ phenotype were more resistant. Patients 13 and 15 exhibited only 16%–25% losses of IgD-bearing cells at 10^{-7} *M* Dex, whereas patients 20 and 22 exhibited losses of 25%–35%.

Thus, the variance in the sensitivity to steroid noted among subjects could be attributed, in part, to differences in the proportion of $CD10^+$ cells in the initial population. For example, 50% of the $CD19^+$ cells were $CD10^+$ for patient 15. Thus, in this subject a greater overall decrease in the $CD19^+$ population was observed after steroid treatment. In the case of patient 13, only 15% of $CD19^+$ cells were $CD10^+$, which appears to explain at least in part, the greater resistance of the $CD19^+$ B-cell compartment of this subject to Dex. Therefore, the response to glucocorticoids is dependent upon maturation stage in which $CD19^+CD10^+$ cells are particularly sensitive to steroid-induced apoptosis.

Potential Role of Apoptosis in Loss of Bone Marrow Cells of the B Lineage. It was suspected that losses among cells of the B lineage could be attributed to apoptosis. Previous studies in our lab of pro- and pre-B cells from murine marrow indicated they readily underwent glucocorticoid-mediated apoptosis both *in vitro* and *in vivo* (9–11). Apoptotic human cells can readily be quantified with merocyanine 540, a dye that binds to the outer membrane of cells (19, 20). Because apoptotic cells exhibit markedly disordered membranes, they are more intensely fluorescent ($MC540^{bright}$) than normal cells ($MC540^{dim}$) when labeled with MC540 (20, 27, 28). In these experiments, cells were cultured with or without Dex and $CD19^+$ lymphocytes were identified and were evaluated for membrane changes associated with apoptosis by measuring the percentage of $CD19^+$ cells that appeared in the $MC540^{dim}$ (normal) and $MC540^{bright}$ (apoptotic) histogram regions. Thus, the focus here was on all cells of the B lineage because subsets such as $CD10^+$ cells represented a small fraction of the total population. As shown in Figure 5, the percentage of cells falling into the $MC540^{bright}$ region nearly triples in patients 17, 18, and 19 and almost quadruples in patient 16 at 1 μM Dex. This is indicative of an accelerated apoptotic process. A suprapharmacological level of Dex increased the percentage of apoptotic cells in the $MC540^{bright}$ region by 11-fold in patient 19 (Fig. 5 insert). Thus, much of the observed losses in the B-cell compartment of the marrow can probably be attributed to glucocorticoid-induced apoptosis.

To further verify the presence of glucocorticoid-induced apoptosis, cells were also incubated with and without RU38486, the glucocorticoid antagonist and well-known inhibitor of glucocorticoid-induced apoptosis in murine thymocytes (21, 22). RU38486 prevented the glucocorticoid-induced losses of $CD19^+$ cells, which was also indicative of an apoptotic mechanism for cell death (Fig. 6; Refs. 21, 22). As per past observation RU 38486,

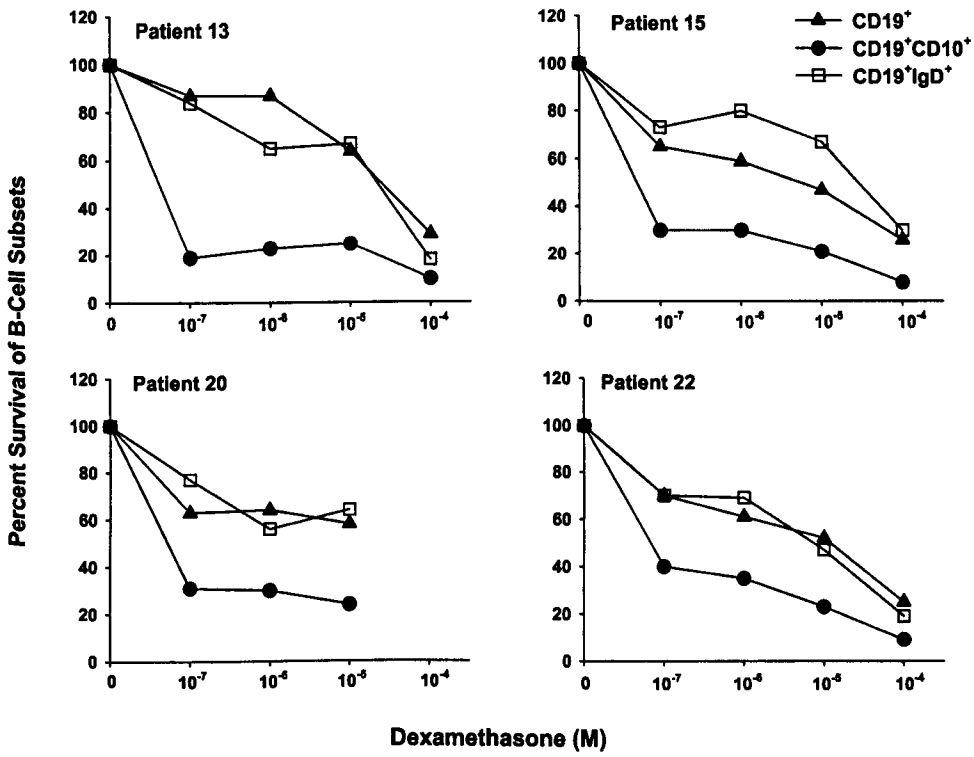

Figure 3. Effect of state of maturity on ability of bone marrow B cells to survive exposure to glucocorticoids. Bone marrow from four subjects were incubated with varying concentrations of Dex, and the effect of maturity of cells of the B lineage to glucocorticoid-induced cell loss was examined after a 20-hr incubation period. Cells were phenotyped and labeled with CD19 only (all B-lineage cells), $CD19^+CD10^+$ (pro- and pre-B cells), and $CD19^+$ IgD^+ (mature B-cells) and analyzed by flow cytometry. Limited sample volume prevented analysis of patient 20 for effects of 10^{-4} *M* Dex (supra-pharmacological). All data were normalized to values obtained from control cultures containing no steroid. Data are the mean of duplicate samples. Based upon forward and side scatter characteristics, a gate was drawn to include the lymphoid population of the marrow where 15,000 events per sample were analyzed. Cell viability was greater than 90% by trypan blue exclusion.

when incubated with control cells, did not initiate any apoptosis (data not shown; Ref. 9).

Discussion

These studies indicate that human bone marrow cells of the B lineage exhibited substantial sensitivity *in vitro* to both physiological and pharmacological concentrations of glucocorticoids. The extent of the response to the steroid was affected by differentiation state with $CD19^+CD10^+$ cells, exhibiting greater sensitivity than mature B cells. Because experiments were performed on bone marrow obtained from hospitalized patients undergoing elective open-heart surgery and taking various types of cardiac and non-cardiac medications, there was some concern that these factors could have contributed to some of the experimental variance. Hence, a large number of subjects (22) were evaluated. It became evident that those patients with a higher proportion of $CD19^+CD10^+$ early B cells exhibited the greater losses upon exposure to glucocorticoids. The concentration of Dex needed to induce a response in human marrow B cells as well as the time frame in which cell loss occurred were surprisingly similar to results for murine marrow (9–11). Evidence from our lab indicates that marrow from mice exposed to glucocorticoids for 12–16 hr resulted in 60%–80% apoptosis among early B cells (9). The studies herein also show extensive sensitivity of early stage human B cells because 60%–80% losses were observed for $CD19^+CD10^+$ cells in a similar time frame. Moreover, the $CD19^+CD10^+$ phenotype, which includes pro- and pre-B cells, were more susceptible to the effects of glucocorticoid than the $CD19^+IgD^+$mature B cells, also mimicking observations made in the mouse (9–11). It should be acknowledged that these IgD^+ cells may be surviving within the marrow or they may be circulating into the marrow from the periphery (29). The loss of human B cells also could be attributed, at least in part, to an apoptotic death mechanism, as evidenced by the ability of RU38486 to block glucocorticoid-induced $CD19^+$ cell loss and the appearance of higher proportions of $MC540^{bright}$ apoptotic cells in populations exposed to glucocorticoids. Both the natural and synthetic glucocorticoids were surprisingly similar in potency.

As discussed earlier, studies of splenocytes, lymph node lymphocytes, and peripheral blood lymphocytes have suggested to some that the human lymphocytes might be resistant to glucocorticoids (12–14). This was undoubtedly because these populations contain mature B cells as well as memory cells now known to be resistant to glucocorticoids (9–11, 24). Furthermore, in these earlier studies, cells that had been incubated with glucocorticoids were often examined for viability by trypan blue dye exclusion. When the cells did not lyse nor turn blue, they were considered resistant to glucocorticoids. However, it is now known that as a cell becomes apoptotic, it will continue to exclude trypan blue for extended periods of time (22). Clearly, the developing B cells of the marrow constitute a very different group than mature B cells in terms of their apoptotic response. These early B cells appear to be highly programmed to die as a result of perhaps to the high proportion that make nonsense rearrangements of the immunoglobulin genes and those with anti-self configuration that must be eliminated apoptotically (23). In the case of murine marrow B cells,

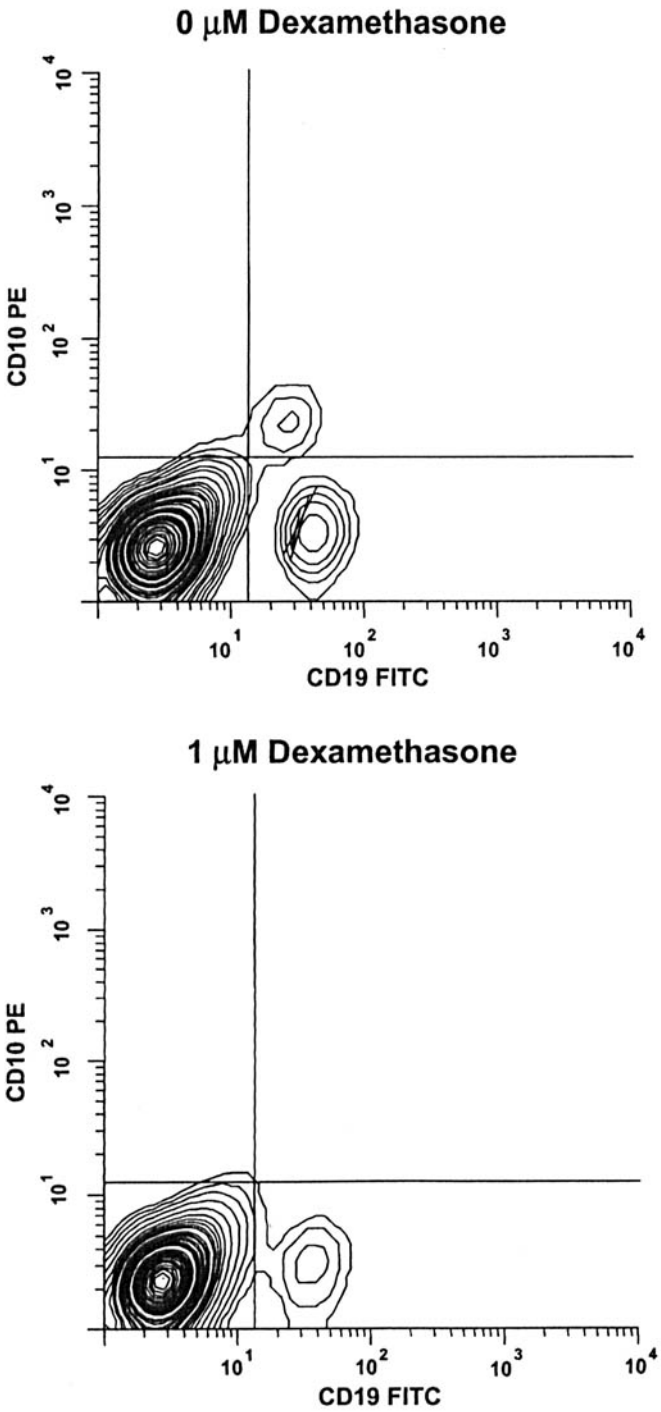

Figure 4. Flow cytometric visualization of the sensitivity of early B-cells ($CD19^+CD10^+$) to Dex treatment. A typical profile is presented where 29% of the $CD19^+CD10^+$ survived 20 hr of incubation with 1 μM Dex. Bone marrow was cultured for 20 hr in the presence or absence of Dex, layered over a heat-inactivated FBS gradient to recover viable cells, phenotyped with FTIC-CD19 and PE-CD10, and fixed as described in the Methods section. A light-scatter gate was used to restrict analysis to lymphocytes, including all $CD19^+$ cells with exclusion of debris and doublets as described in methods. A contour for surviving $CD19^+CD10^+$ cells was not drawn because this data was below the 3% probability level required to plot a contour in this data set.

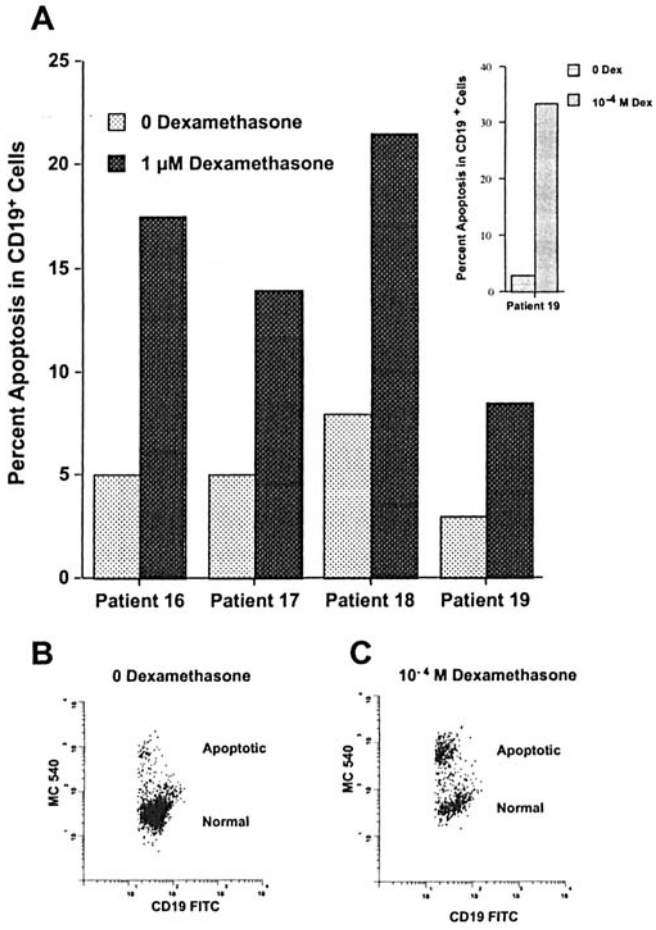

Figure 5. Occurrence of apoptosis among $CD19^+$ marrow B cells exposed to glucocorticoids as determined by flow cytometry for four patients (A). Bone marrow cells were stained with merocyanine 540 (MC540) to determine whether $CD19^+$ B-cells from human marrow were subject to glucocorticoid-induced apoptosis. Cells were cultured with or without 1 μM Dex for 16–18 hr, then subsequently labeled with CD19 and stained with MC540 to measure the occurrence of apoptosis where normal cells are $MC540^{dim}$ and apoptotic cells are $MC540^{bright}$ being well separated by over a log of fluorescence. Representative dual-color flow cytometric cytograms are shown in (B) and (C). Propidium iodide was used to assess viability. Thus, light scatter and propidium iodide were used to exclude dead or necrotic cells and debris. The insert shows degree of apoptosis among cells cultured with or without suprapharmacological concentrations of Dex (10^{-4} M).

resistance to apoptosis correlates with the expression of Bcl-2, a proto-oncogene that enhances cell survival (24). Bcl-2 was highly expressed in mature B-cells, but nearly undetectable in murine pre-B cells with low levels in early pro-B cells (24). DNA fragmentation (200 bp) in leukemic cells treated with Dex and methylprednisolone suggests that induction of apoptosis may account, at least in part, for their effectiveness as chemotherapeutic agents (15, 16, 25). Some leukemic equivalents of normal B cell precursors are very susceptible to glucocorticoid-induced apoptosis, which may parallel the sensitivity of normal human B cell precursors (15, 16, 25).

Glucocorticoids are known to bind readily to the plasma proteins, transçortin and albumin. It is thought that only the unbound steroid is active (30). Synthetic glucocor-

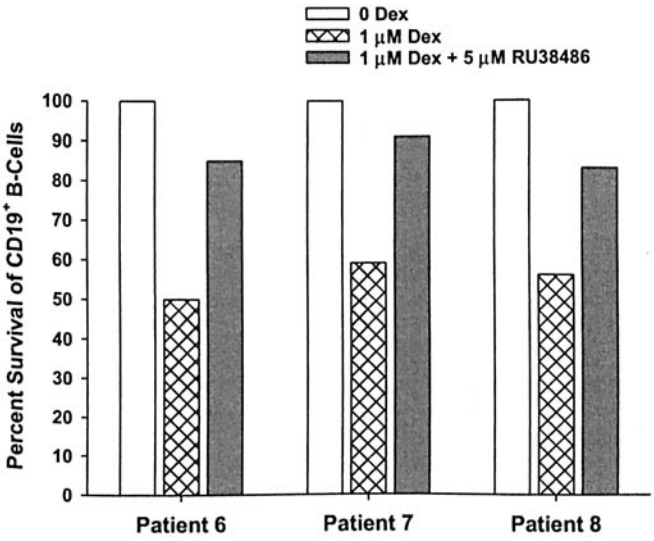

Figure 6. Verification of ability of Dex to induce apoptosis in $CD19^+$ human B cells from the marrow. To further demonstrate that human bone marrow B cells were undergoing glucocorticoid-mediated apoptosis, cells were incubated with either 5 μ*M* RU38486 (a glucocorticoid-receptor antagonist) and 1 μ*M* Dex, 1 μ*M* Dex alone, 5 μ*M* RU38486 alone (not shown), or were untreated. Cells were phenotypically labeled with anti-CD19 antibody and assayed by flow cytometry. Data was normalized to $CD19^+$ values obtained from control cultures containing no steroid (100%) and reported as the mean of duplicate samples for each subject tested. Based upon forward and side scatter characteristics, a gate was drawn to include the lymphoid population of the marrow where 15,000 events per sample were analyzed. Cell viability was greater than 90% by trypan blue exclusion. No induction of apoptosis was noted in control cultures treated with RU38486.

ticoids are often more potent *in vivo* because they bind to these proteins less readily and can diffuse into tissues. All of our experiments were performed *in vitro*; however, the same lot number of FBS was used to prevent variations in the binding of glucocorticoids to plasma proteins. There may be other factors contributing to glucocorticoid metabolism and clearance occurring *in vivo* that are not manifest in these *in vitro* studies. Nevertheless, for the *in vitro* conditions used herein, cortisol and Dex were surprisingly similar in potency with regard to effects on $CD19^+$ cells. This was also the case in the murine marrow studies and suggests that endogenously produced and synthetic glucocorticoids could cause significant disruption of B-cell lymphopoiesis in human as was the case for the rodent (8, 10).

The study of glucocorticoid sensitivity of a heterogeneous tissue, such as bone marrow, is facilitated by the use of multicolor flow cytometry. Use of one- and two-color phenotypic analysis made it possible to examine subsets of B cells in the marrow for their response to the glucocorticoids. Our lab and others have experienced difficulty in measuring apoptosis among human cells using standard DNA binding dyes (unpublished results). Using the lipophilic membrane dye, MC540, in conjunction with phenotypic labeling for the CD19 surface marker, we were able to provide evidence that at least part of the losses in these cells were due to an apoptotic mechanism. Apoptotic cell membranes undergo dynamic changes, becoming more disordered (20, 27, 28) with increasing MC540 fluorescence ($MC540^{bright}$) when compared to that of normal cells ($MC540^{dim}$). By measuring the percentage of $CD19^+$ cells that were $MC540^{bright}$ after exposure to steroids, it was possible to ascertain the degree of apoptosis among these cells. It should be noted that MC540 gave the same quantitative results over time and dose for Dex-treated thymocytes as annexin-V, PI (used as a DNA cell cycle stain) and light scatter (20). Concomitant incubation of human bone marrow lymphocytes with Dex and RU38486 prevented development of the $MC540^{bright}$ population, also indicative of apoptosis (21, 22). Thus, MC540, in conjunction with phenotypic markers, may make possible the study apoptosis in a wide range of human cell types for diseases such as cancers, AIDS, autoimmune diseases, toxicology, etc.

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