The Effect of Epicutaneous Glucocorticosteroids on Human Monocyte and Neutrophil Migration in Vivo

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The effect of epicutaneous methyl prednisolone (MP) at $10^{-4}$, $10^{-5}$, and $10^{-6}$ molar concentration was studied in 54 normal, healthy volunteers using a new, in vivo microchemotaxis technique. Significant inhibition of monocyte chemotaxis occurred at all concentrations studied and persisted over a 24-hr period with $10^{-4}$ molar MP. Neutrophil chemotaxis was significantly inhibited only with $10^{-4}$ MP. The inhibitory effect of MP on neutrophil and monocyte chemotaxis occurred earlier and at lower concentrations if the skin sites were pretreated with steroid. Thus, when corticosteroids are applied on abraded skin in concentrations achievable with steroid, the skin sites were pretreated with steroid. Thus, when corticosteroids are applied on abraded skin in concentrations achievable in vivo, monocyte chemotaxis into tissue is inhibited for longer periods and at lower drug concentrations than is neutrophil chemotaxis. By avoiding the significant systemic effects of corticosteroids on circulating monocyte and neutrophil populations, these experiments establish that local inhibition of chemotaxis is an important anti-inflammatory effect of corticosteroids, with differential effect on monocytes and neutrophils.

During 20 years of clinical use, the efficacy of systemic and local glucocorticosteroids has been clearly demonstrated in the treatment of immunologic and inflammatory disease, but the major site(s) of action of steroids provide a complex combination of immunosuppressive and anti-inflammatory effects. Corticosteroids are effective inhibitors of specific T lymphocyte functions. In man, the ability of T lymphocytes to respond to antigenic and mitogenic stimuli with a blastogenic response and lymphokine release is diminished with long-term daily steroid treatment, [1-5] but these effects may be due largely to the profound redistribution of the recirculating small lymphocytes, [6-10] produced rapidly by even alternate-day steroids. B cell function as manifested by antibody production is inhibited in vitro and in vivo by steroids, [11,12] and the ability of specific antibody to induce tissue inflammation is also inhibited by steroids. [13]

In addition to these specific immunosuppressive effects of steroids, numerous significant anti-inflammatory effects of steroids alter the availability of nonspecific effector cells at tissue sites of inflammation and block the translation of specific immunologic signals into inflammatory responses. At clinically relevant doses, on alternate day regimens steroids produce a rapid but transient monocytopenia, lymphopenia, eosinopenia, and a neutrophilia. [8-14] This is associated with a decreased availability of monocytes at sites of tissue inflammation [15-18] and in some instances a paradoxical decrease in neutrophil availability in tissue secondary to a decrease in the marginal pool of neutrophils associated with diminished adherence of neutrophils to endothelial cells. [20-24] In contrast to the profound changes in cell distribution and availability induced by systemic steroids, direct inhibition of monocyte and neutrophil function is difficult to achieve at pharmacological concentrations of steroids. [24-27]

In evaluating the anti-inflammatory effect of steroids on tissue inflammation, it has been difficult to separate the systemic effects on cell populations from local effects on the migration of effector cells into tissue. Previous studies of tissue applications of steroids have been either semiquantitative, have failed to accurately differentiate neutrophil and monocyte migration, have used nonpharmacological levels of drug or have used assays with inappropriate nonphysiologic barriers to cell migration. [28-30] In the study presented here we have used a new in vivo microchemotaxis technique to quantitatively test the effect of local steroids in pharmacological concentrations on monocyte and neutrophil migration toward complement-derived chemotactants without the interfering influence of steroids on peripheral blood cellular populations. We have demonstrated that local inhibition of chemotaxis of monocytes and neutrophils in human tissue in vivo is a significant anti-inflammatory effect of steroids. Monocytes are clearly more susceptible to this effect than are neutrophils; the effect is dose and time dependent, and is achieved at steroid concentrations readily obtained in vivo.

METHODS

Subjects

Various aspects of the effects of epicutaneous corticosteroids were studied in 54 normal human volunteers. Subjects served as their own controls. Persons with recent illness, chronic skin disease, or who were taking medications were excluded.

Chemotaxis Technique

Details of this quantitative in vivo neutrophil and monocyte chemotaxis technique have been described previously. [31] Betadine and alcohol-sterilized skin was tape abraded with Blenderm tape (3M Co., St Paul, MN) over 5-mm templates to produce 6 uniform glistening abrasions without macroscopic bleeding. Small plexiglass chambers of .2 ml volume containing 50% zymosan activated autologous serum (ZAS) [32] diluted in Hanks Balanced Salt Solution (HBSS) were recovered with a Nucleopore filter sandwich (Nucleopore, Pleasanton, CA) composed of a 3 μ and a 5 μ pore filter and inverted over the abrasion. In this way, cells migrating from the abrasion toward the attractant were required to pass first through the 5 μ pore filter and then through the 3 μ pore filter. Monocytes were relatively retarded within the filter sandwich, while neutrophils passed more freely through the 3 μ pore filter. Chambers were kept in place for various periods and removed for quantitation. To determine the total number of cells which migrated, the 2 filter sandwich was removed and treated as follows: the attractant side of the 3 μ filter was scraped until glistening to remove the mass of neutrophils accumulated there, and the number of cells in the scrapings were counted. The filter sandwich was separated and stained for monocyte esterase activity (alpha naphthyl acetate esterase) [31,32] and mounted for counting. This stain allows accurate differentiation of monocytes, neutrophils and lymphocytes. Differential counts of monocytes and neutrophils were performed on each filter, and the
total number of each type of cell in 10 random fields was multiplied by a conversion factor to give the total number of cells per 5-mm abrasion. The total numbers of monocytes and neutrophils in the resuspended scraping and on the filters were added to obtain total monocyte and neutrophil migration. The relative numbers of neutrophils in the scraping versus those within the filters were not altered by steroids, nor was the relative percentage of monocytes which crossed to the 5 μ and 3 μ filter. With the filter combination chosen, and at the time studied, greater than 90% of the cells which did not cross the 3 μ filter were monocytes, and fewer than 5% of the cells which did cross the filter were monocytes. Using this technique, lymphocytes were not shown to migrate in response to complement-derived chemotactants.

Experimental Design

In these experiments, subjects were used as their own controls by dividing 6 tape abrasions into 3 pairs. One member of each pair was covered with a chamber containing steroids or one containing HBSS diluent control. After the prescribed preincubation period, the chamber was removed and replaced with an attractant-containing chamber for the assay of subsequent monocyte and neutrophil migration. In this way the effect of varying steroid concentrations and periods of preincubation of steroids on abraded skin was studied over different intervals. Each data pair was analyzed independently and the significance of the effect of each variable was determined using a paired t-test. In this way, individual or anatomical variations in response were controlled.

The steroid used in these experiments was methyl prednisolone sodium succinate (SoluMedrol, Upjohn Co., Kalamazoo, MI), diluted in HBSS to concentrations that have been shown to be physiologic (10^-6M), pharmacological (10^-5M), and suprapharmacological (10^-4M). The suprapharmacological concentration was included because it has not been clearly shown whether a concentration of corticosteroids at 10^-4 Molar might be achieved locally in vivo with application of clinically available topical preparations (10^-3M) of corticosteroids.

Subjests were grouped and such variables as steroid concentration, duration of preincubation, and duration of inhibition were studied, using each subject as his own control. Data were compiled both as absolute cell migration with and without steroids and also as the inhibition of migration in thousands of cells, which represents the numerical difference between each experimental chamber and its paired diluent control.

RESULTS

The effect of preincubation of tape-abraded skin for 2 hr with steroids is shown on cell migration at 4, 8, and 12 hr in Fig 1-3 for groups of subjects in which methyl prednisolone (MP) at 10^-5, 10^-6, and 10^-7 Molar concentration was used. In Fig 1, significant inhibition by 10^-5 MP of both monocyte and neutrophil migration toward ZAS was seen at all times assayed. At 10^-5 MP, a concentration obtained with clinical systemic doses of steroids (Fig 2), both monocyte and neutrophil migration were still inhibited, but the duration and magnitude of inhibition were clearly greater for monocytes than for neutrophils. At 10^-6 MP (Fig 3), inhibition of chemotaxis was seen only with monocytes in experiments in which cells were collected for 12 hours.

Figure 4 shows the monocyte and neutrophil migration after 24 hours in a group of subjects in which MP at 10^-5, 10^-6, and
10^{-4}M concentration or HBSS controls were applied to abrasions 2 hr before application of attractant. Although there is inhibition in numbers of monocytes accumulated over that period, with all MP concentrations the result is significant only with 10^{-6}M MP. With neutrophils, there was evident decrease in mean migration, but comparison to paired controls shows no significant inhibition. Close analysis of individual data pairs shows that with 10^{-5}MP and with concentrations of steroids at 24 hr, inhibition of neutrophil migration was highly variable from individual to individual. In other words, while neutrophil at 24 hr, inhibition of neutrophil migration was highly variable from individual to individual. In other words, while neutrophil migration is significantly diminished by adding steroid to attractant without pretreatment, pretreatment produced an earlier onset of effect. Inhibition of monocyte migration with 10^{-6}M MP is also delayed in onset and never reaches significant levels without pretreatment of the tissue before application of attractant. The absolute magnitude of inhibition of neutrophil chemotaxis is even more significantly diminished by adding steroid to attractant without pretreatment, and again the onset of inhibition is delayed. From this experiment, however, one cannot be sure whether the delay in onset of effect or the limitation in magnitude of steroid-induced inhibition of cell accumulation is due to direct interference of activated serum with steroid penetration or to an absolute requirement of pretreatment of migrating cells and skin with steroids for maximal effect.

**DISCUSSION**

The demonstration of a direct effect of epicutaneous corticosteroids on monocyte and neutrophil migration in vivo demonstrates an important effect of steroids not clearly defined by previous in vivo and in vitro experiments. The technique used here to identify the local effect of steroids on cellular migration provides several important advantages over the Rebuck Skin Window or other skin chamber techniques used previously to study cellular migration in vivo. This technique provides a physiological barrier to migration, a reservoir of a known chemotactant, and quantitative differential counts of monocytes and neutrophils studied at different times. The barrier to migration is anatomically intact: tape stripping removes stratum corneum and some cells of the stratum granulosum. [35] Cells migrating into the chemotaxis chamber must pass through the postcapillary venules and the papillary dermis, through the basement membrane of the dermato-epidermal junction, and through an intact epidermis before accumulating within the Nucleopore filter sandwich. The attractant is autologous activated serum complement, without which only a weak neutrophilic infiltrate develops. In the Rebuck technique, the abrasion of skin often removes most of the epidermis and itself induces specific. Inhibition of monocyte chemotaxis in vivo by local MP occurs at lower concentrations and persists longer at higher MP concentrations than does inhibition of neutrophil chemotaxis. Although the magnitude of inhibition of neutrophil migration in absolute numbers is far greater than the inhibition of monocyte migration, the relative percent inhibition is comparable between monocytes and neutrophils at concentrations where they are both susceptible.

The importance of pretreatment with MP is addressed in the Table. The inhibition in thousands of cells per chamber by MP at 10^{-4} and 10^{-5} concentration is expressed for chambers in which the MP was added with chemottractant or in which the abrasion site was treated with MP before application of chemottractant. Although significant inhibition of monocyte chemotaxis was achieved with 10^{-4}M MP with or without pretreatment, pretreatment produced an earlier onset of effect. Inhibition of monocyte migration with 10^{-6}M MP is also delayed in onset and never reaches significant levels without pretreatment of the tissue before application of attractant. The absolute magnitude of inhibition of neutrophil chemotaxis is even more significantly diminished by adding steroid to attractant without pretreatment, and again the onset of inhibition is delayed. From this experiment, however, one cannot be sure whether the delay in onset of effect or the limitation in magnitude of steroid-induced inhibition of cell accumulation is due to direct interference of activated serum with steroid penetration or to an absolute requirement of pretreatment of migrating cells and skin with steroids for maximal effect.

**Effect of 2 hr preincubation of methyl prednisolone on chemotaxis: ΔM in thousands (mean ± SEM)**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>MP Concentration</th>
<th>4 hr</th>
<th>8 hr</th>
<th>12 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>10^{-4}M (n=9)</td>
<td>6.0 ± 2.1</td>
<td>14.0 ± 8.0</td>
<td>18.4 ± 5.0</td>
</tr>
<tr>
<td>0 hr</td>
<td>10^{-4}M (n=10)</td>
<td>1.7 ± 4.3</td>
<td>20.7 ± 7.6</td>
<td>15.8 ± 6.1</td>
</tr>
<tr>
<td>2 hr</td>
<td>10^{-5}M (n=6)</td>
<td>4.9 ± 2.9</td>
<td>12 ± 1.6</td>
<td>25.8 ± 6.7</td>
</tr>
<tr>
<td>0 hr</td>
<td>10^{-5}M (n=8)</td>
<td>1.4 ± 1.0</td>
<td>10.9 ± 6</td>
<td>3.9 ± 4.6</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>10^{-4}M (n=9)</td>
<td>80.3 ± 19</td>
<td>107 ± 26</td>
<td>168 ± 34</td>
</tr>
<tr>
<td>0 hr</td>
<td>10^{-4}M (n=8)</td>
<td>6.4 ± 7.1</td>
<td>27 ± 10</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>2 hr</td>
<td>10^{-5}M (n=6)</td>
<td>31 ± 15.4</td>
<td>32 ± 29</td>
<td>20 ± 69.9</td>
</tr>
<tr>
<td>0 hr</td>
<td>10^{-5}M (n=8)</td>
<td>6.2 ± 9</td>
<td>44 ± 23</td>
<td>69 ± 45</td>
</tr>
</tbody>
</table>

* Compared with paired medium control.
the migration of inflammatory cells even without the addition of exogenous attractants. Quantitation in the Reubuck Window technique requires migrant cells to attach to a glass coverslip applied to sites of abrasion. Since steroids affect cell adhesion, the decrease in numbers of cells counted with the Reubuck Window may merely represent a decrease in the ability of cells to attach to glass surfaces, rather than diminished migration. Conversely, in most skin chamber techniques, the cells are counted in the chamber fluid after detaching from the abraded surface through which they migrate. Steroid-induced detachment from the abraded surface might cause the inhibitory effects of steroids on cell migration to be underestimated. In our microchemotaxis chamber technique, cells are counted as they migrate through a filter trap placed between attractant and abrasion—thus decreasing the effects on quantitation of steroid-induced reduction in cell adhesiveness. The use of uniform reservoirs for application of known steroid concentration in our technique allows the induction of significant local effect without the necessity of systemic administration of steroids.

Such deficiencies in technique are reflected in previous reports of the effects of steroids on cellular migration in vivo. Reubuck and Mellinger [29] reported significant inhibition of cellular infiltrates by local steroids, but the concentrations used were apparently suprapharmacological, the barrier to migration was limited, and quantitation was semi-quantitative. Peters et al [30] reported that the absolute numbers of leukocytes accumulating in skin chambers in response to clotted serum actually decreased with dexamethasone and decreased somewhat with prednisone; however, leukocyte clearance (migrated cells/peripheral blood WBC) was diminished in both, reflecting the profound neutrophilia produced by both steroid preparations. In that study, differential migration of monocytes and neutrophils was not measured, the effect of steroids on the systemic availability of cells was misleading, and the measured end-point—cells in suspension in the skin chamber after detachment from the abrasion—may have been differentially affected by such steroids with known differences in glucocorticoid potency.

Several in vivo techniques have shown that systemic steroids have a profound effect on monocyte availability at sites of tissue inflammation [k5,18] but again, it is not clear whether the effect on cellular availability was systemic or local. The most successful attempt to address the effect of steroids on monocyte and neutrophil chemotaxis in vivo was the investigation by Dale, Fauci, and Wolff of the Reubuck Skin Window response of 20 patients with systemic inflammatory disease treated with daily and alternate day steroid therapy. [19] They found profound monocytopenia and decreased monocyte chemotaxis in patients on daily steroids on the "Day On" alternate day steroids. Neutrophilia and an elevated circulating neutrophil half-life was produced by daily steroids on the "Day On" alternate day steroids, but neutrophil chemotaxis in vivo was only depressed on daily steroids. Monocyte and neutrophil numbers and functions were normal on the "Day Off" alternate day steroids. They established both a differential effect of steroids on monocyte and neutrophil availability in tissue inflammation, and the apparent safety of alternate day steroid regimens in allowing effector cells to be available at sites of inflammation. However, the obligatory use of patients with significant immunologic disease as subjects likely selected a population with altered reactivity, as evidenced by the rather large differences in monocyte chemotaxis in controls and patients on the "Day Off" steroids. Also, although the marginal pool of neutrophils was apparently decreased on the "Day On" alternate day steroids, neutrophil chemotaxis was normal. While monocyte accumulation as judged by a weighted response over 24 hr was normal on the "Day Off" steroids, migration between cells 12 and 24 hr appeared to decrease for unexplained reasons. In the final analysis this classic study did not clearly define the differences between local and systemic effects of steroids on monocyte and neutrophil migration in vivo, and encompassed the shortcomings of the Reubuck Window technique: non physiological barriers, poor attractant specificity, inadequate differentiation of monocytes and neutrophils, and dependence on cell adherence for counting and a semiquantitative endpoint. In contrast, the experiment which we present uses normal subjects who serve as their own controls, measures local and not systemic effects of corticosteroids, more clearly defines tissue dose response, provides quantitative specific differentiation of monocytes and neutrophils, and provides a timed picture of both induction and duration of inhibition.

Examination of the effects of steroids on monocyte function in vitro has produced a confusing mosaic of positive and negative effects. Rhinehart et al. have shown that suprapharmacological concentrations of corticosteroids in vitro inhibit monocyte chemotaxis, random migration, and staph bactericidal activity but do not affect neutrophil chemotaxis or monocyte cryptococcal phagocytosis, IgG receptor function or glass adherence. [36] On the other hand, when normal healthy subjects were fed significant clinical doses of steroids (prednisone 50 mg po q12h x 6), chemotaxis, cryptococcal phagocytosis, hexose monophosphate shunt activity, and ultrastructural features of harvested peripheral blood monocytes were not altered [37], only Staph Aureus and Candida tropicalis killing were depressed. It has also been shown [14] in patients receiving clinically relevant doses of alternate day steroids that chemotaxis, phagocytosis, and bacterial killing by peripheral blood monocytes were normal although profound monocytopenia occurred even at low doses of steroids. Other investigators have also shown variable effects on phagocytosis and killing of different organisms [38] and on monocyte maturation into macrophages [33] by steroids. Thus while it is easy to decrease the peripheral availability of monocytes with pharmacological doses of steroids, overt inhibition of isolated in vitro monocyte functions is difficult. A crucial problem in many experiments studying the effects of steroids on monocyte function in vitro is how to present the drug with appropriate carrier to potentiate cell absorption; [12] if this problem is not overcome, the levels of drug required to achieve effect in vitro are often much greater than those which in vivo will produce the same degree of effect [40]. Comparison of the difficulties in inhibitory monocyte chemotaxis in vitro with the same results presented here underscore this problem.

We have demonstrated that steroids presented locally in vivo in the milieu of serum and extracellular proteins clearly inhibit monocyte migration at pharmacological levels without any effect on the total monocyte pool available to tissue. In addition, this chemotaxis technique—which measures the ability of cells to cross vascular endothelium, the dermo-epidermal junction, and the epidermis—is a more critical and sensitive means of judging the total chemotactic response of monocytes than is measuring the cells' ability to cross or enter a thin filter in response to attractant. However, the effects attributed to steroids by these experiments may not be due to alteration of the migratory machinery of inflammatory cells but may instead affect local margination or the cells' ability to cross endothelium, dermo-epidermal junction or epidermis. The local effect of steroids on neutrophil availability in tissue is clearly less profound than the effect on monocytes. In vitro inhibition of neutrophil chemotaxis is achieved only at suprapharmacological levels [41,43]. At pharmacological levels, neutrophil adherence [24] but not chemotaxis [26,27] or phagocytosis [43] are affected.

The standard technique for evaluation of the potency of topically applied steroids is the vasoconstrictor assay of McKenzie and Stoughton [44] which utilizes graded degrees of vasoconstriction to evaluate corticosteroid potency. The demonstration of differential effects of steroids on monocyte and neutrophil accumulation in microchemotaxis chambers strongly suggests that vasoconstriction alone is not necessarily a representative measure of the anti-inflammatory effects of steroids and further techniques for evaluating steroid potencies in vivo must consider the important biological differences in monocyte and neutrophil susceptibility documented here.
REFERENCES

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