Improved Effects of Novel Glucocorticosteroids on Immune-Induced Epithelial Pathophysiology

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ABSTRACT

Glucocorticosteroids are a mainstay therapy in inflammatory bowel disease and other chronic inflammatory conditions. However, severe systemic side effects are associated with their long-term use. The new generation of glucocorticosteroids have a high degree of topical activity with reduced systemic effects due to rapid metabolism. We previously described an in vitro model of inflammation in which monolayers of the human T84 colonic epithelial cell line displayed altered ion secretion and increased permeability after coculture with endotoxin-activated monocytes/macrophages (MΦ). Here, we tested the effects of budesonide and two novel analogs, D5519 and S1316, on MΦ-induced epithelial changes. Filter-grown T84 monolayers were cocultured with activated MΦ and single daily doses of drug were added to the luminal (physiological) side of the monolayer. Basal and stimulated epithelial ion transport [baseline short-circuit current (Isc) and ΔIsc to forskolin, respectively] and barrier (transepithelial resistance) parameters were measured 48 h later in Ussing chambers. D5519, S1316, and budesonide (10⁻⁷ to 10⁻⁹ M) dose dependently inhibited the MΦ-induced epithelial abnormalities, restoring normal resistance, decreasing the elevated baseline Isc, and improving the reduced Isc response to forskolin. Of the drugs tested, D5519 was consistently the most potent and effective in inhibiting the MΦ-induced epithelial irregularities. Coupled with a further improvement in their rate of hepatic inactivation, our findings indicate that the novel steroids, particularly D5519, will be a valuable addition to current treatment strategies for inflammatory bowel disease and other chronic inflammatory conditions.

Glucocorticosteroids (GC) have been a major anti-inflammatory therapy for over four decades. However, despite their impressive immunosuppressive properties, the therapeutic value of GCs is counterbalanced by a number of deleterious side effects including osteoporosis, adrenal insufficiency, hypertension, and growth retardation in children. Attempts to overcome the systemic side effects of steroid therapy, while maintaining therapeutic benefits, have led to the development of novel GCs for topical treatment of inflammatory diseases. These new drugs are characterized by a high affinity for the GC receptor and an enhanced hepatic first-pass metabolism, resulting in products with an improved ratio between desirable high topical efficacy at the target and unwanted systemic steroid activity (Brattsand, 1990). The properties of these new GC make them particularly suitable for treating inflammation locally at mucosal surfaces, such as in the gastrointestinal tract and airways. In inflamed airways/lungs, the high affinity of these new GCs for the GC receptor compensates for the great dilution of the drug over a large surface area, whereas in the inflamed intestine, the enhanced hepatic first-pass inactivation is extremely important for reducing GC systemic side effects after leaving the target organ. The prototype of these new GCs, budesonide, has proven to be beneficial in treating airway inflammation in patients with asthma and rhinitis (Broden et al., 1992; Pederson and O’Byrne, 1997). Budesonide has also been found to be effective in the short-term induction of remission during active Crohn’s disease (Lofberg et al., 1993; Greenberg et al., 1994; Rutgeerts et al., 1994; Campieri et al., 1997). In an experimental study, we showed that budesonide could broadly inhibit T cell-mediated epithelial pathophysiology (McKay et al., 1996a).

Despite its reported >90% first-pass metabolism, side effects such as adrenal insufficiency have been found to be associated with budesonide administration (Cui et al., 1994). Recently, structural modifications to budesonide have led to newer GCs that combine an even higher receptor affinity with a nearly complete first-pass hepatic inactivation rate. The synthesis and basic pharmacological properties (affinity for the cytosolic GC receptor and inactivation rate) of one of

ABBREVIATIONS: MΦ, monocytes/macrophages; GC, glucocorticosteroids; IBD, inflammatory bowel disease; Isc, short-circuit current.
these newer GCs has been described in a recent paper by Thalén et al. (1998). This analog, D5519, together with another derivative, S1316, were shown to have twice the GC receptor affinity while being biotransformed 10 times more rapidly in human liver than budesonide, emphasizing a much lower systemic bioavailability after oral administration. However, although D5519 and S1316 display the desired physicochemical characteristics, their usefulness in ameliorating immune-mediated disease symptoms (i.e., intestinal epithelial dysfunction) has not been examined.

We recently described an in vitro model of inflammation in which coculture of confluent monolayers of human T84 intestinal epithelial cells with endotoxin [lipopolysaccharide (LPS)]-activated monocytes (MΦ) resulted in significant abnormalities in epithelial ion transport and barrier functions (Zareie et al., 1998). These changes were largely abrogated by neutralization of tumor necrosis factor-α (TNFα). Using this in vitro model, the present study was designed to compare the effects of D5519, S1316, and budesonide on immune-mediated changes in epithelial function. Our findings demonstrate beneficial properties of all three corticosteroids in this model system. However, D5519 was the most effective in reducing both TNFα production and immune-mediated epithelial pathophysiology.

**Materials and Methods**

**Cell Culture**

**Epithelial Cells.** Human colonic epithelial cells (T84, passage 45–65) were seeded onto tissue culture-treated semipermeable filter supports (0.4 μm pore size, 1.0 cm² surface area; Costar Corporation, Cambridge, MA) at 10⁶ cells/filter and grown in culture media [equal volumes of Dulbecco’s modified eagle medium and F12 medium, supplemented with 1.5% (v/v) HEPES, 2% (v/v) penicillin-streptomycin, and 10% newborn calf serum; all purchased from Gibco Laboratories, Grand Island, NY]. The culture media was changed daily. After culture for 7 days, confluent T84 monolayers consistently displayed transepithelial electrical resistances of greater than 1,000 Ω/cm² as measured by a voltmeter (Millicel-ERS; Millipore Corpora-

**Immune Cells.** Human peripheral blood mononuclear cells from healthy volunteers (male and female, ages 23–45 years) were isolated by one-step density centrifugation of whole blood over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and resuspended in fresh media at 10⁶ cells/ml. The MΦ population was obtained by plastic plating of peripheral blood mononuclear cells (4 h at 37°C) and subsequent removal of the nonadherent T and B cells. Fresh media was added to the adherent cells, which were then incubated for 18 h at 37°C before use in coculture studies. We have previously shown that >95% of the adherent immune cell population express CD14 (the LPS receptor) and have the appropriate size and granularity characteristics of MΦ (Zareie et al., 1998). Trypan blue exclusion revealed that >90% of MΦ were viable following the coculture period.

**Immune Cell Activation**

MΦ were activated by *Salmonella minnesota* LPS (10 ng/ml; Sigma Chemical Co., St. Louis, MO) added to the culture media at the time of coculture. Activation was assessed by the production of TNFα measured in culture media by enzyme-linked immunoassay (ELISA DuoSet, Genzyme Diagnostics, Cambridge, MA). Determinations were performed in duplicate using serial dilutions; the assay had a detection limit of 4 pg/ml.

**Coculture Studies**

Confluent T84 monolayers were cocultured for 48 h with MΦ (~200,000 cells/well) placed in the basal compartment of the culture wells, as previously described (Zareie et al., 1998). Control groups included 1) T84 monolayers, 2) T84 monolayers treated with GC, and 3) T84 monolayers cocultured with nonactivated MΦ.

**Preparation of Corticosteroid Solutions**

Budesonide, D5519 and S1316 were dissolved in absolute ethanol at a concentration of 10⁻² m and stored as stock solutions at −20°C. Each drug was diluted to its required concentration (10⁻³ to 10⁻⁹ m) in fresh media on the day of use. Drugs were added to the apical compartment of the culture well in single daily doses during the 2-day coculture period.

**Ussing Chamber Experiments**

**Epithelial Ion Transport.** Following coculture, T84 monolayers were mounted in Ussing chambers as previously described (McKay et al., 1996b). Epithelial monolayers were bathed in oxygenated Krebs buffer (37°C) containing 10 mM glucose as an energy source in the serosal buffer, which was osmotically balanced by 10 mM mannitol in the mucosal buffer. The epithelial spontaneous potential difference was maintained at zero volts by the continuous injection of an external current by an automated voltage clamp (World Precision Instruments Inc., Sarasota, FL). This short-circuit current (Isc, in μA/cm²) reflects net active ion transport across the preparation. Baseline Isc was recorded after a 15-min equilibration period. STimulated ion secretion was measured by adding the adenylyl cyclase-activating agent, forskolin (10⁻⁵ m), or the cholinergic agonist, carbachol (10⁻⁴ m) (both from Sigma Chemical Co.), to the serosal side of the T84 monolayers and recording the maximum increase in Isc.

**Epithelial Permeability.** Electrical resistance is a measure of the barrier property of the epithelium to passive ion movement. Decreased resistance indicates an increase in permeability. At intervals during each experiment, potential difference across the monolayer was clamped at 1.0 mV (differential pulse method, 1 pulse/30 s), and the resulting change in current was used to calculate the transepithelial ion resistance (R, in Ω/cm²) according to Ohm’s law (Powell, 1981).

**Cell Viability**

T84 monolayer viability was assessed by measuring the release of lactate dehydrogenase (LDH) as described by Madara and Stafford (1989). After coculture, T84 monolayers were removed and rinsed three times in fresh PBS. Epithelial monolayers were lysed by immersing each filter in 0.1% (v/v) Triton-X 100 (Sigma Chemical Co.)/PBS for 30 min at room temperature followed by vigorous manual pipetting. The lysate was centrifuged at 500 rpm for 5 min and the supernatant was analyzed for LDH activity using an automated multiple point rate test (Kodak, Rochester, NY).

**Statistical Analysis**

Results are presented as mean ± S.E.M. Due to variability in absolute values between different batches of T84 cells, data were normalized to control values in each experiment (expressed as percentage of control). N values represent the number of experiments (different blood donors) in which two to four monolayers were examined for each condition. Data were analyzed using one-way ANOVA followed by Newman-Keuls comparison. Student’s t test was used for individual comparisons. Statistically significant differences were accepted at p < 0.05.

**Results**

**TNFα Production by MΦ**

Stimulation of MΦ with 10 ng/ml LPS for 48 h induced a significant increase in TNFα production (533 ± 68 pg/ml; n =
8) compared with donor-matched nonactivated MΦ (4–68 pg/ml). Activated MΦ cultured in the presence of T84 cells receiving daily applications of D5519, S1316, and budesonide (10⁻⁷ M) displayed a significant reduction in TNFα production compared with activated MΦ with no GC added (Fig. 1). At one log lower concentration (10⁻⁸ M), however, D5519 proved to be the most effective in reducing the TNFα production by 86 ± 2%, whereas budesonide was the least effective, reducing TNFα by 28 ± 5% (Fig. 1). At the lowest concentration (10⁻⁹ M), D5519 was the only GC to significantly inhibit the TNFα production.

**MΦ-Induced Epithelial Pathophysiology**

**Ion Transport Abnormalities.** T84 monolayers grown in the presence of nonactivated MΦ or GC alone displayed a baseline Isc of 0.8 ± 0.3 μA/cm²; n = 12 (range from 0.6–1.2 μA/cm²), a value not significantly different from that of naïve monolayers. Therefore, naïve T84 monolayers were used as controls in further experiments. Coculture with activated MΦ for 48 h evoked a significant increase in baseline Isc to 284 ± 315% of control values, indicating stimulated Cl⁻ secretion (Fig. 2), as in our previous findings (Zareie et al., 1998). Treatment of the monolayers with D5519, S1316, or budesonide dose dependently inhibited this immune-mediated epithelial abnormality. Again, D5519 was the most potent GC compared with S1316 and budesonide under these conditions. T84 monolayers cocultured with activated MΦ plus D5519 at 10⁻⁸ M displayed completely normal baseline Isc values (Fig. 2A). At the same concentration, S1316 partially corrected the elevated baseline Isc (Fig. 2B), while budesonide showed partial diminution of the elevated baseline only at 10⁻⁷ M (Fig. 2C).

The ability of the epithelium to respond to forskolin was significantly reduced to ~70% of the control value: 63 ± 6 μA/cm²; n = 12 (range from 56–72 μA/cm²) by coculture of the monolayers with activated MΦ. D5519 and S1316 (10⁻⁸ M) were equally effective in almost completely normalizing the reduced secretory response of the epithelium to forskolin to control values (Fig. 3, A and B). In contrast, a beneficial effect of budesonide was observed only with a concentration of 10⁻⁷ M (Fig. 3C). Carbachol-induced ΔIsc was unaffected by coculture of the monolayers with activated MΦ (113 ± 16 μA/cm² versus 97 ± 18 μA/cm² for controls).

**Barrier Abnormalities.** Control T84 monolayers displayed a transepithelial electrical resistance of 1557 ± 226 Ω/cm²; n = 12 (range from 1106–1784 Ω/cm²). After 48 h of coculture with activated MΦ, the barrier function of T84 monolayers was significantly altered, as indicated by an ~40% reduction in transepithelial resistance of the monolayers compared with control monolayers. D5519 was the most effective GC tested, significantly inhibiting the activated MΦ-induced reduction in epithelial resistance at concentrations ≥10⁻⁸ M (Fig. 4A). In contrast, S1316 was effective only at 10⁻⁸ M (Fig. 4B). A 10-fold higher concentration of budesonide (10⁻⁷ M) was required to improve T84 monolayer resistance (Fig. 4C).

**Epithelial Viability**

After 48 h, there was no significant difference in LDH released from T84 epithelial cells cultured in media alone or cocultured with activated MΦ (1497 ± 71 versus 1560 ± 88 U/liter). Therefore, the effect of the GC was not investigated.
Discussion

Inflammatory bowel disease (IBD) is characterized by altered epithelial physiology, typically increased permeability and diarrhea that may be due, at least in part, to altered regulation of ion secretion. Evidence from a number of studies has established that epithelial pathophysiology can be caused by activated immune cells (Madara et al., 1993; Perdue and McKay, 1994; McKay et al., 1996b). Among the immune cells, MΦ play a central role in immune and inflammatory events in the intestinal mucosa. Recent studies have demonstrated that unlike in the intestine of normal individuals, resident macrophages in the intestinal lamina propria of patients with IBD express unusually high levels of CD14 (LPS receptor) on their cell surface, presumably due to rapid recruitment of monocytes from the circulation to the gut (Baldassano et al., 1993; Rugtveit et al., 1994; Grimm et al., 1995). The newly recruited cells are more easily activated resulting in the production of excessive amounts of potent inflammatory mediators (Baldassano et al., 1993; Rugtveit et al., 1994). In accordance with these observations, we recently described an in vitro model of inflammation in which coculture of T84 human intestinal epithelial monolayers with activated MΦ for 48 h resulted in stimulated Cl–secretion and impaired epithelial barrier function. In this model, MΦ-derived TNFα was identified as a key factor in mediating these abnormalities (Zareie et al., 1998). Here, we tested the effects of budesonide and two novel analogs, D5519 and S1316, on MΦ-induced epithelial changes. Our data clearly demonstrate that D5519 was the most effective GC in normalizing the MΦ-induced epithelial ion transport and permeability irregularities, and this result is in accordance with its higher receptor affinity (Thalén et al., 1998).

Budesonide has been used successfully in the treatment of asthma and allergic rhinitis (Pederson and O’Byrne, 1997). It has also been found to be effective in oral or rectal treatment of patients with IBD, most commonly Crohn’s disease and ulcerative colitis, respectively (Campieri et al., 1997; Greenberg et al., 1994; Hanauer et al., 1998). However, budesonide generally has similar efficacy to conventional steroids with high systemic availability (Greenberg et al., 1994; Rutgeerts et al., 1994; Lofberg et al., 1994; Campieri et al., 1997; Hanauer et al., 1998) and it does not appear to markedly reduce the number of patients experiencing relapse after 1 year of treatment (Greenberg et al., 1996; Gross et al., 1998). Compared with other steroid regiments, fewer adverse ef-

Fig. 3. Percentage (%) of change from control values (T84 cells alone) of epithelial secretory responses (ΔIsc) to forskolin (10−5 M) after 48 h of coculture with LPS-activated monocytes ± GC (10−7 to 10−9 M) added to the apical compartment of the culture well in single daily doses (n = 5–6 experiments with 2–4 monolayers per experiment). Values represent mean ± S.E.M.; #p < .05 compared with no GC addition (0), *p < .05 compared with control (100% = 63 ± 6 μA/cm²).

Fig. 4. Percentage (%) of change from control values (T84 cells alone) of transepithelial resistance after 48 h of coculture with LPS-activated monocytes ± GC (10−7 to 10−9 M) added to the apical compartment of the culture well in single daily doses (n = 5–6 experiments with 2–4 monolayers per experiment). Values represent mean ± S.E.M.; #p < .05 compared with no GC addition (0), *p < .05 compared with control (100% = 1557 ± 226 Ω/cm²).
fects, particularly less impact on the hypothalamic-pituitary-adrenal axis, have been reported in association with budesonide administration (Campieri et al., 1997; Hanauer et al., 1998). However, despite its high (>90%) first-pass metabolism in healthy people, as much as a 40% depression of plasma cortisol levels has been observed in Crohn’s disease patients following administration of 9 mg/day of budesonide. This suggests that the extent of first-pass metabolism is insufficient, especially in patients with active Crohn’s disease who may have an impaired metabolism due to cytokine spillover from the intestine (Cui et al., 1994; Greenberg et al., 1994). The need to produce potent GCs that exhibit both greater receptor affinity (resulting in higher topical anti-inflammatory activity) and an enhanced hepatic inactivation rate (resulting in less systemic side effects) has led to the development of two new analogs of budesonide, D5519 and S1316. Using our simplified model of intestinal inflammation, we have shown for the first time in functional terms, that the new analogs of budesonide, particularly D5519, are more effective than budesonide itself in inhibiting the M<sup>F</sup>-mediated epithelial abnormalities that are characteristic of intestinal inflammation. The steroid treatment protocol used in our coculture system is compatible with that used in clinical settings, because the steroids were added to the physiological (luminal) side of the epithelial monolayers.

Treatment of the T84 monolayers in the absence of M<sup>F</sup> with budesonide, D5519, or S1316 did not alter epithelial ion secretion and did not affect the transepithelial resistance. This suggests that in our study, the inhibition of the immune-mediated epithelial pathophysiology was due to the effect of the drugs on M<sup>F</sup> and not on the epithelial cells directly. In support of these observations, it has been reported that M<sup>F</sup> activity, as measured by cytokine production, is steroid sensitive (Waage and Bakke, 1988; Linden and Brattsand, 1994; Oddera et al., 1995).

We have recently shown that M<sup>F</sup>-derived TNFα is a critical mediator of epithelial pathophysiology in this in vitro model of intestinal inflammation (Zareie et al., 1998). In the present study, the production of TNFα by M<sup>F</sup> was significantly inhibited by steroid treatment, with D5519 being the most potent drug tested. The potency of the three GCs tested to inhibit TNFα production by M<sup>F</sup> correlated with their ability to prevent the epithelial dysfunction.

In summary, we have shown that M<sup>F</sup>-induced epithelial abnormalities were inhibited in a dose-dependent manner by the addition into the coculture of D5519, S1316, or budesonide. Our data also suggest that prevention of M<sup>F</sup>-mediated epithelial pathophysiology was due to GC inhibition of M<sup>F</sup> activation as shown by suppression of TNFα production. Finally, the novel analogs of budesonide, D5519 in particular, exhibited greater potency and efficacy in preventing epithelial dysfunction, when compared with budesonide. In conjunction with its higher GC receptor affinity and enhanced hepatic inactivation rate, our results indicate a better therapeutic ratio, especially for D5519, which should be of great advantage in topical therapy of inflammatory conditions of the intestine.

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References


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