GLUCOCORTICOID SUPPRESSION OF MACROPHAGE MIGRATION INHIBITORY FACTOR*

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The suppression of cell-mediated or delayed hypersensitivity reactions by glucocorticoids has led to their widespread use for modification of clinical and experimental immunological phenomena (1, 2). The precise mechanism(s) by which steroids suppress delayed hypersensitivity in vivo is not known. Moreover, there are significant species differences in lymphoid cell sensitivity to steroids, and extrapolation of data from studies in steroid-sensitive species (such as the mouse, rat, or rabbit) to predict responses in steroid-resistant species (such as the guinea pig and primates) is fraught with considerable uncertainty (3). Even in the steroid-sensitive species, it is unclear what relative role steroids play in vivo in alteration of the nonspecific inflammatory compared with the immunologically specific components of cell-mediated reactions.

The study of immunologic phenomena by use of in vitro correlates has permitted characterization of some of the complex cellular interactions necessary for the development and expression of delayed hypersensitivity (4–6). We have investigated the effects of steroids on cell-mediated immunity using an in vitro model, the assay of macrophage migration inhibitory factor (MIF)1 in the inbred guinea pig. Antigen-mediated generation of MIF in vitro by sensitized lymphocytes is an established and sensitive correlate of the presence of corresponding antigen-specific delayed hypersensitivity in vivo (7, 8). The use of the MIF assay in inbred guinea pigs provided a system for separation and recombination of the cellular components of this in vitro correlate and thus enabled us to determine the precise site of action of glucocorticoids. Our studies demonstrate that at concentrations equivalent to those obtained pharma-

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‡ Reprint requests should be sent to A.S.R., Section on Biologic Structure, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20004.
1 Abbreviations used in this paper: FCS, fetal calf serum; GPS, guinea pig serum; MIF, migration inhibitory factor; OHC, hydrocortisone; PEC, peritoneal exudate cells; PELS, peritoneal exudate lymphocytes; PPD, purified protein derivatives; S-MEM, Spinner modification of Eagle’s minimal essential medium.
cologically in vivo, glucocorticoids suppress antigen-induced MIF activity. These agents had no effect on antigen uptake by macrophages or on MIF synthesis by immune lymphocytes. Rather, glucocorticoids block MIF activity at the level of the interaction of MIF with macrophages.

Materials and Methods

Animals and Immunization.—Inbred strains 2 and 13 guinea pigs, weighing 300–500 g each, (Division of Research Services of the NIH) were immunized by footpad injection with a total of 0.4 ml of complete Freund’s adjuvant containing 2 mg/ml of killed Mycobacterium tuberculosis H37Ra (Difco Laboratories, Inc., Detroit, Mich.). Tuberculin purified protein derivative (PPD) skin tests with 20 μg/ml PPD were uniformly positive at 10–14 days.

Media.—Eagle’s minimal essential medium-Spinner modification (S-MEM) (Microbiological Associates, Inc., Bethesda, Md.) was used for preparation and culture of cells unless otherwise indicated. Each 500 ml of S-MEM was supplemented with 5 ml each of: nonessential amino acids (100 X; Grand Island Biological Co., Grand Island, N. Y.), 100 mM sodium pyruvate, 3% glutamine, 40% glucose, penicillin (10,000 U/ml), and streptomycin (10 mg/ml).

Peritoneal Exudate Cells (PEC).—Guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52; Humble Oil and Refining Co., Houston, Tex.) to induce exudates. 3 days later the animals were killed by a blow to the head, and their abdominal cavities were lavaged with 200 ml of cold Hanks’ balanced salt solution containing heparin (5 U/ml). After centrifugation, the cells were pooled and contaminating erythrocytes lysed by a modification of the method of Roos and Loos (9), and then washed three times with 10% fetal calf serum (FCS) in S-MEM.

Peritoneal Exudate Lymphocytes (PELS).—Lymphocytes were separated from the crude PEC by passage through glass bead columns (10). Briefly, PEC suspended in 100% FCS were incubated on the columns at 37°C for 45 min, after which the nonadherent cells were eluted with warm 10% FCS at a flow rate of 2 ml/min. The eluted population of cells contained approximately 90–95% small cells with characteristics of thymus-derived lymphocytes (10, 11).

Antigen Stimulation.—Tuberculin PPD from M. tuberculosis (Connaught Medical Research Labs, Toronto, Canada) was used for lymphocyte activation in concentrations indicated in each experiment. An antigen pulse exposure technique was used for lymphocyte activation (12, 13). PELs were incubated for 30 min at 37°C with 100 μl/ml PPD in 10% FCS in S-MEM. These cells were then washed four times to remove nonbound PPD (< 10⁻³ μg/10⁶ cells remained). These antigen-pulse lymphocytes were then used for MIF assay by mixing with PEC from nonimmunized guinea pigs (transfer MIF assay), or alternatively cultured for 18 h in S-MEM with 15% heat-inactivated guinea pig serum (GPS) at 5 × 10⁶ cells/ml at 37°C in an atmosphere of 5% CO₂–95% air to produce MIF-rich supernatants that were subsequently assayed on PEC from nonimmunized guinea pigs (indirect MIF assay). The antigen pulse system in conjunction with the transfer and/or indirect MIF assays eliminated the inhibition of macrophage migration by antigen-antibody complexes that can simulate cellular immunity when testing antigen sensitivity in the direct MIF assay (14).

Dialysis.—In experiments requiring dialysis of cell supernatants, 5% GPS in S-MEM was used for lymphocyte cultures. The culture supernatants were pooled, centrifuged to remove cells, and dialyzed in cellophane tubing (Union Carbide Corp., Chicago, Ill.) against serum-free S-MEM at 4°C for 24 h. After this, the supernatants were sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.), and 10% fresh GPS was added before testing by the indirect MIF assay.

In Vitro MIF Assays.—60 × 10⁴ PEC/ml were suspended in S-MEM, drawn into 50-μl capillary pipettes (Scientific Products Division, McGaw Park, Ill.), one end of which was sealed by flaming, and then centrifuged into a pellet at 130 g for 5 min. The capillaries were
cut at the cell-fluid interface and duplicates fixed to the bottom of Sykes-Moore chambers (Bellco Glass, Inc., Vineland, N. J.) with sterile stopcock grease (Dow Corning Corp., Midland, Mich.). Corticosteroid and/or additional antigen were added to the media or supernatant used for filling the chambers as described in each experiment. After incubation at 37°C for 18 h, the areas of macrophage migration were projected, 20 times enlarged, on a Nikon profile projector (Nippon Kogaku K.K., Japan), traced, and measured by a planimeter. The areas of migration of four capillaries (duplicate chambers) were averaged, and the results expressed as percent migration inhibition, where:

\[
\text{Migration inhibition (\%)} = \left( 1 - \frac{\text{mean area}_{\text{test}}}{\text{mean area}_{\text{control}}} \right) \times 100.
\]

**Steroids.**—The following steroid compounds were tested in the concentrations indicated in each experiment: (a) hydrocortisone sodium succinate (Solu-Cortef), The Upjohn Co., Kalamazoo, Mich.; (b) testosterone, Eli Lilly and Co., Indianapolis, Ind.; (c) dexamethasone sodium phosphate (Decadron), Merck Sharpe and Dohme, West Point, Pa.; (d) progesterone, The Upjohn Co.; (e) conjugated estrogens (Premarin), Ayerst Laboratories, New York; and (f) desoxycorticosterone pivalate (Percorten), Ciba Pharmaceutical Co., Summit, N. J.

**RESULTS**

**Effect of Various Classes of Steroids on Antigen-Mediated Inhibition of Macrophage Migration.**—Initial experiments surveyed a variety of steroid compounds for their ability to alter macrophage migration in the presence or absence of antigen. PELS from immune animals were pulsed with antigen, washed, and \(3 \times 10^6\) mixed with \(60 \times 10^6\) PEC/ml from nonimmunized guinea pigs for the transfer MIF assay. Macrophage migration was inhibited 74% of control value in the absence of any steroid (Table I). Various steroid preparations were then added in equivalent concentrations (10 \(\mu\)g/ml) to assess their effects on antigen-induced inhibition of macrophage migration. Only the glucocorticoids, hydrocortisone (OHC) and dexamethasone, were found to significantly reduce the degree of migration inhibition. Moreover, no steroid at the concentrations employed altered macrophage migration in the absence of antigen stimulation.

**TABLE I**

| Steroid* | Percent of migration inhibition† |
|----------|----------------------------------|
| None     | 74 ± 3.5                         |
| Hydrocortisone | 22 ± 4.5                     |
| Dexamethasone    | 27 ± 9.5                        |
| Desoxycorticosterone | 76 ± 1.5                    |
| Estrogens   | 70 ± 5.5                         |
| Testosterone | 72 ± 3.5                        |
| Progesterone  | 76 ± 7.0                         |

* All steroids were tested at a concentration of 10 \(\mu\)g/ml in the transfer MIF assay.
† Mean ± 1 SEM.
The Effect of Hydrocortisone and Antigen Concentrations on the Detection of Steroid Suppression of MIF Activity.—The effects of OHC concentration (0–100 μg/ml) on suppression of MIF activity were further investigated using the transfer MIF assay. Progressive reduction of the percent inhibition of macrophage migration from 61% without OHC, to 22% at 10 μg/ml OHC was observed (Fig. 1 a). There was no further reduction of MIF activity at 100 μg/ml OHC. While these data demonstrated that OHC reduced MIF activity, in a dose-related fashion, this effect could have been due to either inhibition of MIF synthesis or direct antagonism of MIF itself. Therefore, the capacity of OHC to reduce MIF activity was tested in the absence of MIF-generating lymphocytes by using the indirect MIF assay system. Increasing concentrations of OHC were added to MIF-rich supernatants derived from PPD pulse-activated PELS cultures. Supernatants alone from antigen-pulsed PELS produced 63% inhibition of macrophage migration (Fig. 1 b). In the presence of increasing levels of OHC, a concentration dependence similar to that of the transfer system was observed; near maximal reversal of macrophage migration inhibition occurred with as little as 1 μg/ml OHC.

Since a previous study (15) failed to demonstrate glucocorticoid suppression of MIF activity produced by continuous exposure to a single concentration of PPD (10 μg/ml), the relationship between antigen concentration and the capacity of OHC to suppress MIF activity was assessed in both the transfer and indirect MIF systems. As shown in Fig. 2 a, increasing concentrations of PPD

![Graphs showing the effect of hydrocortisone on MIF activity](image)

**Fig. 1.** Effect of hydrocortisone on MIF activity. Changes in percent migration inhibition as a function of increasing concentrations (micrograms per milliliter) of hydrocortisone were assessed on the transfer MIF assay where PPD-pulsed PELS were mixed with PEC from nonimmune animals (Fig. 1 a), and on the indirect MIF assay, where MIF-rich supernatants were tested on PEC from nonimmune animals (Fig. 1 b).
Fig. 2. Effect of additional antigen on the capacity of hydrocortisone to suppress MIF activity. At the level of maximum hydrocortisone effect (10 µg/ml), increasing concentrations (micrograms per milliliter) of PPD reversed the steroid-induced reduction of macrophage migration inhibition in the presence of immune lymphocytes (transfer MIF assay), Fig. 2 a; however, no change in steroid reduction of the activity of MIF-rich supernatants (indirect MIF assay) was observed with additional PPD, Fig. 2 b.

significantly reduced the effectiveness of the OHC blockade in the transfer MIF system (MIF-generating lymphocytes present). However, identical concentrations of added PPD had no effect on OHC blockade of MIF activity in the indirect MIF assay (lymphocyte-free MIF-rich supernatants), Fig. 2 b. These data demonstrated a competitive and reversible relationship between glucocorticoid and antigen concentrations in the presence of MIF-generating lymphocytes. Since additional antigen had no effect on level of MIF activity in the indirect assay, antigen neither potentiated MIF nor bound and inactivated OHC. Finally, while the data in this section demonstrated that OHC blocked the effect of MIF itself on macrophage migration, it does not exclude a concomitant effect of steroids on MIF production, at the level(s) of either antigen recognition or lymphokine synthesis.

The Effect of OHC on Antigen “Processing” by Macrophages.—Previous studies from this and other laboratories indicate an obligatory role for macrophages in the in vitro activation of immune lymphocytes (16–18, 40). These studies suggest that the thymus-derived lymphocyte may be unable to recognize soluble protein antigens that are not bound or “processed” by macrophages. Briefly, it has been shown that PEC, a population of cells rich in macrophages, can be pulsed with antigen and mixed with antigen-sensitive lymphocytes to stimulate blast transformation and secretion of lymphokines. To test
the effect of glucocorticoids on this macrophage activity, PEC from nonimmune guinea pigs were preincubated with 0–1,000 µg/ml OHC at 37°C for either 1 or 24 h, after which they were pulsed with PPD (100 µg/ml) for 30 min at 37°C. Subsequently, 6 × 10⁶ PEC were mixed with 60 × 10⁶ PEC/ml from PPD-sensitive animals for MIF assay. Treatment of PEC with 100 or 1,000 µg/ml OHC for 1 h and 10 or 100 µg/ml OHC for 24 h before antigen pulse did not diminish the resultant MIF activity, compared with that of PEC pulsed with antigen alone (Table II). Thus, the glucocorticoid suppression of MIF activity does not appear to result from a block in antigen uptake or processing by macrophages.

**The Effect of Glucocorticoids on the In Vitro Production of MIF by Immune Lymphocytes.**—The ability of OHC to inhibit MIF production by antigen-

| Conc. (µg/ml) | Time (h) | Percent of migration inhibition |
|--------------|----------|-------------------------------|
| 0            | 1        | 71 ± 1.7                      |
| 100          | 1        | 73 ± 1.0                      |
| 1,000        | 1        | 79 ± 0.5                      |
| 0            | 24       | 50 ± 2.0                      |
| 10           | 24       | 61 ± 4.3                      |
| 100          | 24       | 48 ± 2.4                      |

*PEC from nonimmunized guinea pigs were preincubated with OHC for the times and concentrations indicated, pulsed with 100 µg/ml PPD for 30 min at 37°C, and washed before their addition to PEC from PPD-sensitive animals for MIF assay.
†Mean ± 1 SEM.
TABLE III
Effect of Hydrocortisone on MIF Production by Immune Lymphocytes

| Cell culture* | Supernatant dialysis | Additional OHC† | Percent of migration inhibition§ |
|---------------|----------------------|-----------------|-------------------------------|
| No OHC        | 0                    | 0               | 65 ± 2.6                      |
| No OHC +      | 0                    | 0               | 71 ± 1.0                      |
| OHC           | 0                    | 0               | 6 ± 3.5                       |
| OHC +         | 0                    | 0               | 56 ± 5.8                      |
| OHC + +       | 17                   | 0               | 17 ± 8.4                      |

* Indicates presence or absence of OHC (10 μg/ml) in antigen pulse-activated lymphocyte cultures.
† Readdition of OHC (10 μg/ml) after supernatant dialysis.
§ Mean ± 1 SEM.

parallel culture without OHC). Dialysis of the supernatants cultured with OHC revealed the presence of significant MIF activity (56%); dialysis of the supernatant cultured without OHC caused no reduction of MIF activity (71%). Readdition of OHC to its original concentration (10 μg/ml) again reduced the percent inhibition to insignificant levels (17%). Thus, we have demonstrated that pharmacologic concentrations of OHC block MIF activity but had no effect on the synthesis of MIF by immune lymphocytes.

DISCUSSION

The mechanism by which glucocorticoids suppress immunological functions is unknown despite two decades of widespread empirical use of these therapeutic agents. Claman has recently reviewed the effects of these agents on immunological phenomena and indicated many of the critical obstacles that exist in identifying their specific mechanism(s) of action (3). First, significant species differences exist in vivo and in vitro with the mouse, rat, and rabbit exhibiting a high degree of corticosteroid sensitivity, while the guinea pig and primates are highly steroid resistant. Second, functionally distinct subsets of lymphocytes within the lymphoid cell populations of a given species exhibit varying degrees of steroid sensitivity. This differential sensitivity has been extensively studied in the mouse where steroids induce rapid loss of cortical thymocytes (21), sequestration of mature thymus-derived lymphocytes into bone marrow (22), and lysis of thymocytes in vitro (23). Third, corticosteroids have been shown to alter nonspecific inflammatory responses in vivo (24) and to suppress complement-mediated neutrophil chemotaxis in vitro (25). We have noted that OHC treatment of guinea pigs reduced the total number of oil-induced PEC to 25% of nontreated controls (unpublished observations). These observations indicate that steroids may compromise the ability of the host to mobilize leukocytes into inflammatory sites, and thus indirectly modify certain early components of the delayed hypersensitivity reaction. Finally, macrophages are necessary for the development and expression of cellular immunity (26) and
steroids are already known to affect several physiologic activities of these cells (27, 28). A specific effect of glucocorticoids on macrophages relevant to their critical role in delayed hypersensitivity reactions has not been excluded.

North has investigated the effects of OHC on nonspecific bacterial resistance in the mouse (29). He concludes that corticosteroids increase susceptibility to infection by suppression of the proliferation of those splenic lymphocytes necessary to activate macrophages for enhanced bactericidal capacity. Whether this apparent reduction of splenic lymphocytes by OHC was a result of local inhibition of cell division or the removal of a subset of lymphocytes from splenic foci, i.e., distant sequestration, was not determined.

Casey and McCall observed an in vivo effect of steroids on the development of delayed hypersensitivity in rabbits if methylprednisolone was given before or immediately after immunization with bacille Calmette-Guérin (BCG) but observed no effects of this drug on established BCG immunity (15). These authors did not detect an in vitro effect of methylprednisolone on the MIF activity produced by alveolar cells from rabbits sensitive to PPD, and concluded an exclusive effect of corticosteroids on the induction of cellular immunity. The data in the present study have shown that glucocorticoids clearly modify established cellular immunity to PPD in the guinea pig. While species differences may be important, our data suggest that the failure of Casey and McCall to observe an in vitro effect of glucocorticoid on MIF activity in the rabbit may have resulted from their use of a direct MIF assay and limited combinations of antigen and corticosteroids.

Data on the effect of corticosteroids on other in vitro correlates of cell-mediated immunity have also been reported. The proliferative response of human lymphocytes to phytohemagglutinin stimulation and the mixed leukocyte reaction is strongly suppressed by glucocorticoids (30, 31), while concentrations in excess of that required to suppress MIF activity in the present study produce relatively minor effects on antigen-stimulated lymphocyte proliferation (31). In addition, glucocorticoids have been reported to suppress human lymphotoxin activity assayed on L cell monolayers (32), though it is not clear whether production of this lymphokine was inhibited or whether OHC blocked the action of lymphotoxin on the target cell.

The biochemical basis of corticosteroid sensitivity has been studied using several sensitive and resistant lymphoid cell types. Glucocorticoids in sufficiently high concentrations nonspecifically suppress DNA, RNA, and protein synthesis in basal and stimulated lymphoid cell cultures (33). Munck et al. (34) found that addition of OHC to rat thymocytes induced the synthesis of a protein that inhibits membrane transport of glucose, an observation that could in part explain the catabolic and cytolytic effect of OHC seen in steroid-sensitive species. Nonetheless, an understanding of the mechanism of glucocorticoid sup-

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Rosenthal, A. S., and J. E. Balow. Unpublished observations.
pression of MIF activity will require additional knowledge of the mechanism(s) by which MIF inhibits the spontaneous migration of macrophages.

Recently, Havemann reported that MIF-rich supernatants contain protease activity, implying that inhibition of macrophage migration proceeds via enzymatic cleavage of a surface membrane polypeptide necessary for spontaneous cell movement (35). In this regard, Leu et al. have presented data suggesting that macrophages avidly bind MIF to a trypsin-sensitive surface receptor (36). Several additional studies have reported changes in macrophage membrane and cell physiology subsequent to exposure to MIF-rich fractions, including increased phagocytosis (37), increased adherence to glass (38, 39), and significant though delayed increased glucose utilization via the hexose monophosphate shunt (39).

Unfortunately, none of the alterations in macrophage function induced by MIF or known effects of glucocorticoids on cell metabolism as yet provide a wholly satisfactory explanation of the mechanism by which OHC inhibits the action of MIF on its target cell, the macrophage. Further characterization of the actions of glucocorticoid on this in vitro correlate of cellular immunity may provide insight not only into the mechanisms of steroid suppression of cellular immune phenomena, but also into the mode by which MIF itself alters macrophage migration.

SUMMARY

The ability of hydrocortisone to modify antigen-mediated inhibition of macrophage migration, an in vitro correlate of cellular immunity in the guinea pig, was investigated. Only the glucocorticoids, hydrocortisone and dexamethasone, significantly blocked migration inhibitory factor (MIF) activity in pharmacologic concentrations. Hydrocortisone had no effect on antigen “processing” by macrophages, nor on the ability of antigen-stimulated peritoneal exudate lymphocytes to produce MIF. Rather, hydrocortisone antagonized directly the inhibitory effect of MIF on the macrophage.

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