The Mechanism of Action of the Antiinflammatory Agents Dexamethasone and Auranofin in Human Polymorphonuclear Leukocytes

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Human polymorphonuclear neutrophils (PMN) were treated with the antiinflammatory agents dexamethasone or Auranofin. PMN treated with dexamethasone in a dose range of 0.25–1 μM or Auranofin, 5–15 mM, were stimulated with 10⁻⁷M N-formyl-methionyl-leucyl-phenylalanine (FMLP). These agents were shown to inhibit the functional responses of degranulation and superoxide production in a dose-dependent manner. Similarly, the change in electrophoretic mobility, reflecting cell surface charge, was blocked. While both agents inhibited change in the fluorescence of the calcium chelate probe chlorotetraycline (CTC), the pattern of inhibition was significantly different. Dexamethasone appeared to inhibit the CTC response during its latter phases, while Auranofin inhibited all aspects of the CTC response. Auranofin was additionally shown to significantly decrease specific binding of FMLP, as well as the number of FMLP receptors. The two agents thus appear to act by different mechanisms. Dexamethasone is shown to have an effect on membrane-bound calcium release as measured by CTC, while Auranofin interferes with receptor binding.

POLYMORPHONUCLEAR NEUTROPHILS (PMN) are involved in the early phases of the response to bacterial infection as well as other states of inflammation. Corticosteroids and gold compounds have been employed in the treatment of chronic inflammation. Both have been shown to act in part by modulation of PMN function. Both have been shown to alter the release of granule enzymes, impair superoxide production, alter adherence, and block aggregation. To further explore the mechanism of action of these substances in altering PMN function, we have studied their effect on PMN activation in response to stimulation by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP).

MATERIALS AND METHODS

Reagents

Stock solutions of 10⁻⁴M Auranofin (SKF no. D-39102, Smith Kline & French Labs, Philadelphia, PA) were prepared daily in distilled water. Dexamethasone (Hexadrol phosphate, Organon, Inc., West Orange, NJ) was obtained as a 4 mg/mL solution. FMLP (Peninsula-Labs, San Carlos, CA) was dissolved in dimethyl sulfoxide (DMSO) and diluted to a final stock concentration of 10⁻⁴M. One-milliliter aliquots were kept at –70°C until time of use. In general, 10-μl aliquots were added to 2 ml of cell suspension so that the final DMSO concentration was less than 0.1%.

Granulocyte Isolation

Blood was obtained from normal volunteers in accordance with the standards of the Human Investigation Committee of this institution. Whole blood was drawn in polyethylene syringes with 0.1 ml of 1:1,000 sodium heparin per 50 ml of whole blood and allowed in sediment at room temperature after the addition of 10 cc of 6% dextran (Macroderx, Pharmacia). The leukocyte-rich plasma was layered over Ficoll-Hypaque gradients and centrifuged at 400 g for 35 min. The red blood cells were removed by hypotonic lysis. The resulting cell suspension consisted of 96% PMN. Viability, as assessed by trypan blue exclusion, was approximately 94%. Cells were kept on ice in the absence of divalent cations until immediately prior to use. Cell viability, randomly checked at the end of experiments, was greater than 90%.

Superoxide Assay

Superoxide was determined using a modification of the assay of Babior et al. After treatment with either dexamethasone, Auranofin, or control buffer, 2.5 x 10⁶ cells in 1 ml Krebs-buffered phosphate buffer were incubated for 5 min at 37°C with 0.1 mm ferricytochrome-C (type I V, Sigma Chemical Co.). FMLP was added to a final concentration of 2.5 x 10⁻⁷M. At the appropriate times, the reaction was stopped by the addition of ice-cold 1-mM N-ethyl-maleimide. The tubes were promptly centrifuged at 4°C for 10 min, and the optical density of the supernatants read at 550 nm. Identical studies were simultaneously performed in the presence of 30 μg of superoxide dismutase.

Results of triplicate samples were averaged and converted to nmole 0₂-dependent cytochrome-C reduction using the extinction coefficient of 2.1 x 10⁻⁴ M⁻¹ cm⁻¹.

Electrophoretic Mobility

Electrophoretic mobility measurements were made in a modified Northrop-Kunitz cell (Arthur Thomas Scientific, Philadelphia, PA) mounted in the vertical plane. Observation of cell movement was made with a monocular microscope coupled to a video camera and recorder (Panasonic model WV-1300). All measurements were made at room temperature.
PMN were suspended in phosphate-buffered saline at 10^7 cells/ml. Stock calcium and magnesium ions were added to final concentrations of 1 mM and 1.5 mM, respectively, just prior to starting each experiment. After 10-min incubation with the stimulus, the cell suspension was transferred into a saline-ice bath and then centrifuged at 400 g for 5 min; the resulting pellet was washed twice in 5% sorbitol buffer (4 parts 5% sorbitol, 1 part 1/15 M sorbitol buffer, pH 7.2).21 Electrophoretic mobility measurements were made in the sorbitol/Sorenson's buffer. The buffer was made from stock solutions each day. The conductivity of the buffer was measured before use and showed little day-to-day variability.

The electrophoretic mobility measurements are expressed as microns per second per volt-cm. Each cell was measured in two directions after reversing the polarity of the electric field. The mean time to travel 50 microns was then determined by the formula:

\[ t = \frac{2a(b - a)}{b} \]

where \( t \) is the actual migration time; \( a \) the migration time in one direction; and \( b \) is the total migration time in both directions.22,23

Each velocity measurement represents the mean of at least 10 cells. The standard error used to detect difference from control is derived from measurement of at least 3 samples. Each experiment was repeated on at least 3 different days.

**Degranulation**

After stimulation, as noted for electrophoretic mobility experiments, supernatants were assayed for the presence of the granule enzyme, lysozyme, and the specific granule protein, lactoferrin, as well as the cytoplasmic enzyme lactate dehydrogenase.12,24-26 Lactic dehydrogenase release from the cell with all stimuli and inhibitors did not exceed 7% when randomly measured. Total cellular enzyme activity released after sonic disruption was measured to determine percent release. Total cellular lactoferrin content was similarly measured.12,27

**Chlorotetracycline Fluorescence**

All fluorescence measurements were made on a Perkin-Elmer model MPF-44B fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The spectrophotometer is equipped with a four-sample thermostatically controlled sample chamber and magnetic stirring device. Corrected spectra were obtained with reference to the quantum counter Rhodamine-B using a Perkin-Elmer DCSU-2 differential corrected spectra unit. Data were recorded with a Perkin-Elmer model 56 chart recorder or with an Apple II microcomputer (Apple Computer, Inc., Cupertino, CA) interfaced through a 12-bit analog to digital converter (Interactive Structures model Al-13, Bala Cynwyd, PA).

Chlorotetracycline hydrochloride (ICN Pharmaceuticals, Inc., Cleveland, OH) was prepared fresh daily as a 10^-4 M stock in distilled water and protected from light. Stock calcium and magnesium ions were added to the cell preparation to a final concentration of 1 mM and 1.5 mM, respectively, followed by stock CTC to a final concentration of 10^-4 M. The cells were then incubated at 37°C for 30 min, either in a water bath or in the spectrophotometer.28-30 After stabilization of baseline fluorescence occurred, the stimulus was added to the cell suspension at time 0, and the subsequent response recorded.

Fluorescence was excited at 386 nm and read at 540 nm to favor monitoring of the Ca-CTC chelate. Corrected excitation spectra indicated a peak at 355 nm for Mg-CTC and at 375 nm for Ca-CTC.

**RESULTS**

**Superoxide Production**

The effect of Auranofin on superoxide production is seen in Fig. 1. Total ablation of superoxide production was seen at 5 μM Auranofin.

Similarly, cytochalasin-B-treated PMN were incubated with 5 μM dexamethasone and then stimulated with 10^-7 M FMLP. As shown in Fig. 2, there was a dose-dependent suppression of superoxide produc-
Fig. 2. The effect of dexamethasone on superoxide production in human PMN pretreated with 5 µg/ml cytochalasin-B and stimulated with 10^{-7} M FMLP. The cells were preincubated with dexamethasone for 20 min at 37°C prior to stimulation. Studies not shown indicate no appreciable effect of either agent on the assay system itself.

Electrophoretic Mobility

To further evaluate the effect of these agents on membrane activation, electrophoretic mobility was measured using whole cell electrophoresis. The cells were pretreated with Auranofin or dexamethasone for 30 min prior to addition of cytochalasin-B. The results of these in experiments are shown in Figs. 3A and 4A. Cytochalasin-B has no measurable effect on electrophoretic mobility (data not shown). The control measurements in each case were determined within 2–3 min of the stimulated measurement. As can be seen, dexamethasone and Auranofin have little effect on electrophoretic mobility themselves; however, both drugs completely block the change in electrophoretic mobility induced by the addition of 10^{-7} M FMLP. Because of day-to-day variability in the absolute magnitude of the electrophoretic mobility, the data shown are representative experiments. The standard errors represent 3 replicates within a single day. Each experiment was repeated on at least 3 separate days. Experiments were completed within 6 hr of phlebotomy.

Degranulation

The release of the specific granule constituent, lactoferrin, and the granule enzyme, lysozyme, were measured after treatment of PMN with either dexamethasone or Auranofin (Figs. 3B, 3C, 4B, 4C). The data shown represent triplicate determinations from the supernatants generated during the electrophoretic mobility experiments. After a 15-min incubation with FMLP, the cells were centrifuged and the supernatants saved for the enzyme determination. The effect of Auranofin on basal enzyme release was somewhat variable; however, in general, it resulted in no release of either lysozyme or lactoferrin. Lactoferrin release and lysozyme release were blocked at a dexamethasone concentration of 0.75 mM and an Auranofin concentration of 10 µM.
**Chlorotetracycline Fluorescence**

The time course of CTC fluorescence change, expressed as \((F_0 - F)/F_0 \times 100\) for Auranofin, is noted in Fig. 5. Decrease in fluorescence is completely blocked at a concentration of 15 \(\mu M\). The response during the first 30 sec and the later responses are inhibited to the same extent.

The response after 20-min pretreatment with dexamethasone is seen in Fig. 6. There is 3% drop followed by a further drop in intensity to approximately 15%. While the basal level absolute value of the drop varied day-to-day and among donors, the pattern of change was constant. There is no appreciable change in signal with the addition of buffer alone. There is some quenching of the absolute fluorescence signal with increasing dose of dexamethasone. There is a constant drop in fluorescence of approximately 5% regardless of the dose of dexamethasone. In untreated PMN, there is a continual decrease in fluorescence to a maximum of approximately 13%. This returns to baseline levels after 15–20 min (data not shown). In the dexamethasone-treated PMN, the continued loss in fluorescence was blocked in a dose-dependent manner.

**FMLP Binding**

As shown in Fig. 7, the binding of FMLP to the PMN surface in the presence of Auranofin is decreased to 60% at a dose of 10 \(\mu M\), the dose at which the functional responses are inhibited. The inhibition was dose-dependent between 0.0 and 250 \(\mu M\). No such effect was observed from comparable controls. The behavior of FMLP binding in the presence or absence of 15 \(\mu M\) Auranofin was examined at different concentrations of \(^3\)H-FMLP. Representative Scatchard plots...
Local efflux of PMN during inflammatory states, as well as intravascular aggregation at the time of complement activation, plays an important role in many pathologic states. Alterations of the surface properties of PMN that affect adherence and aggregation aid in localizing PMN at sites of inflammation or in the case of leukoembolization, sequestering PMN in the microvasculature. Accordingly, the ability to pharmacologically modulate these processes, both in inflammatory disease and in states of intravascular PMN activation, is of great clinical importance. In this study, we have explored the mechanisms of action of two antiinflammatory agents that directly modulate human PMN function.

Corticosteroids have been shown to affect phagocytic function both in vitro and in vivo. Several mechanisms of action have been demonstrated including direct alteration of membrane fusion, alteration of membrane fluidity, induction of protein inhibitors of phospholipase A2 activity, alteration of PMN membrane lipids, and direct inhibition of chemotactic peptide receptor number and function.

The present data confirm the findings of others that dexamethasone inhibits lysosomal enzyme release and prevents the decrease in electrophoretic mobility accompanying PMN activation. Since enzyme release and superoxide production are dependent on an initial ligand receptor perturbation and are related to subsequent changes in membrane calcium, we examined these relationships in more detail.

The fluorescent probe, CTC, has been shown in pancreatic acinar cells, rabbit PMN, and human PMN to reflect membrane-bound "trigger" calcium. Furthermore, in isolated membrane preparations, CTC fluorescence has been correlated with calcium concentration, as measured by $^{45}$Ca. While CTC fluorescence is enhanced by both calcium and magnesium, the differences in the excitation and emission spectra allow selective monitoring of the calcium chelate. CTC binding requires calcium and probably binds near the phospholipid head groups in the membrane. Although dexamethasone is known to alter the specific binding of FMLP to human PMN at doses in the range employed in our study, the decreased binding would be minimal and probably plays no major role in the altered PMN functional responses observed in this study. Our data showing that the initial phase of the CTC response was unaffected by dexamethasone treatment despite a profound effect on the recovery phase of the CTC response, as well as upon superoxide and lactoferrin release, provide further evidence that receptor inhibition is playing a lesser role. Based on recent data, there is a linear relation between specific binding of FMLP and functional response in the human neutrophil. One would expect approximately 30% decrease in response (degranulation) from...
a 30% decrease in specific binding if all receptor sites are not saturated. The degree of specific binding inhibition at 0.75 mM dexamethasone does not exceed 20%, yet the functional responses of superoxide production, degranulation and change in electrophoretic mobility are almost completely abolished. In contrast to the response after the first minute, the CTC response in the first 45 sec after addition of FMLP is not dose dependant. Thus, we conclude that dexamethasone exerts its effect on a later phase of PMN activation, and this effect is directed to calcium-related functional events of the cell.

In contrast to dexamethasone, Auranofin blocked both the initial and later CTC response. It also decreased the specific binding as well as the number of FMLP receptors. The effect of Auranofin on specific binding of FMLP was studied by Davis et al. They found a 15% decrease in specific binding at an Auranofin dose of 2.5 μg/ml (3.6 μM). This is in close agreement with our own data. Similar agreement is present in Auranofin’s ability to inhibit superoxide release. In our data and the data of Davis et al., there is a discrepancy in the degree of inhibition of receptor binding and the inhibition of functional response, suggesting a mechanism in addition to the inhibition of specific binding. Since all phases of the CTC response were equally affected, any possible effect of the drug on calcium translocation cannot be separated from its effect on the membrane.

Both agents are useful clinically in the treatment of inflammatory disease. While the concentrations of dexamethasone used in this study would only be encountered in cases of direct injection into a joint or site of inflammation, serum gold levels in the range of 2.6–5.5 μM may be obtained clinically.

Although both drugs inhibit PMN function, their mechanisms of action appear different. Dexamethasone affects primarily calcium-related responses of the cell monitored by CTC fluorescence, whereas Auranofin additionally interferes with ligand receptor coupling. These drugs may serve as useful probes to dissect the events of receptor-stimulus coupling.

**SUMMARY**

These studies have confirmed previous data that demonstrate that the inflammatory agents, dexamethasone and Auranofin, are capable of blocking superoxide production and lysosomal enzyme release in human PMN stimulated by FMLP. The cellular basis for the altered PMN responses obtained by administration of dexamethasone appears to be on the later phases of trigger calcium release. This effect overshadows the drug’s known slight effect on displacement of FMLP receptors. On the other hand, Auranofin blocks early events on PMN leukocyte activation, including changes in chlorotetracycline fluorescence and electrophoretic mobility. This effect may be due to its profound effects on both the number of FMLP receptors, as well as the affinity of these receptors for the ligand.

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