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Inhibition of Eosinophilotaxis by Chloroquine and Corticosteroids[1] (40006)

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The presence of eosinophils is a hallmark of allergic inflammatory reactions, but the role played by these cells in the inflammatory process has not been definitely established. Corticosteroids markedly ameliorate inflammatory reactions in allergic diseases, but the mechanism of the beneficial effects of the drugs in these allergic diseases is obscure (1). Corticosteroids alter the responses of certain cells involved in inflammatory processes, for example, *in vitro* they stimulate macrophage migration (2, 3), inhibit bronchoalveolar macrophage aggregation (4), inhibit the migration of polymorphonuclear neutrophils (5), and may influence lymphocyte migration (6). The eosinopenic response induced by corticosteroids *in vivo* has been known for many years, but to our knowledge no effects of corticosteroids on eosinophils *in vitro* have been demonstrated. Chloroquine hydrochloride, another anti-inflammatory agent, has also been shown to affect migration of polymorphonuclear neutrophils (5), but its action upon eosinophils has not been investigated. In this study we have assessed the effect of a variety of agents including chloroquine and corticosteroids on the migration of eosinophils using an *in vitro* chemotactic assay.

Materials and methods. Drugs. Hydrocortisone sodium succinate and methylprednisolone sodium succinate (Upjohn Co., Kalamazoo, Mich.) and chloroquine hydrochloride (Sigma Chemical Co., St. Louis, Mo.) were diluted in: $CaCl_2$, 0.20 g/liter; KCl, 0.20 g/liter; $MgCl_2$, 0.0469 g/liter; NaCl, 8.0 g/liter; $NaH_2PO_4 \cdot H_2O$, 0.050 g/liter; glucose, 1.0 g/liter (Tyrode's solution, Grand Island Biological Co., Grand Island, N.Y.) which also contained 2% human serum albumin (Sigma Chemical Co., St. Louis, Mo.) and was denoted T-HSA. Estrone and estradiol (Sigma Chemical Co.) were dissolved in absolute ethanol and diluted in T-HSA so that the final ethanol concentration in the cell suspension was 0.5%. The final concentrations of these drugs were 0.02 (7.9×10^{-5} *M*) and 0.1 mg/ml (3.7×10^{-4} *M*), respectively. Testosterone (Sigma Chemical Co.) was dissolved in absolute ethanol to a concentration of 10^{-1} *M* and was diluted with T-HSA to a final concentration of 0.28 mg/ml (10^{-3} *M*) and a final ethanol concentration of 0.5%.

Chemotactants. Two chemotactants were used: eosinophil chemotactic factor of anaphylaxis (ECF-A) and C5a. ECF-A was prepared from human lung tissue according to Wasserman and associates (7). Briefly, normal lung tissue was obtained from patients undergoing pneumonectomy. The pleura, large vessels, and bronchi were dissected off; the tissue was minced with fine scissors and washed in Tyrode's solution until free of blood. It was then suspended in 0.15 *M* NaCl in a proportion of 1 g of wet tissue per 3 ml of saline, homogenized (Virtis homogenizer, Gardiner, N.Y. Model No. 60K) at 15,000 rpm for 1 min and centrifuged at 400*g* for 10 min. Part of the supernatant was stored in small aliquots at −70° and the remainder was lyophilized. Samples were reconstituted in distilled water and analyzed by gel filtration on a Sephadex G-25 column (50 × 1.5 cm) equilibrated with Tyrode's solution. C5a was prepared by treating guinea pig sera with zymosan (Sigma Chemical Co.) according to Kay and associates (8). Briefly, zymosan was boiled, washed twice in distilled water, and incubated for 30 min in a rotating 37° water bath with guinea pig sera using 25 mg of zymosan per ml of serum. After the zymosan had been removed by centrifugation, the supernatant was incubated at 56° for 30 min. The heated serum was stored in small aliquots at −70° and a sample was analyzed

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by gel filtration on a Sephadex G-75 column (50 × 1.5 cm) equilibrated with Tyrode's solution.

Cell preparation. Cells obtained by peritoneal lavage of guinea pigs with normal sterile saline (9) were centrifuged for 5 min at 100*g*, washed in Tyrode's solution, and resuspended in T-HSA at a concentration of 10^7 cells/mm^3. The final preparation contained approximately 30% eosinophils, the remaining cells being mononuclear in type.

Chemotactic assay. Chemotaxis was tested using a modified Boyden chamber (10), especially adapted for analysis of small volumes (Neuroprobe Company, Bethesda, Md.). The chemotactants, in a volume of 0.030 ml, were placed in the bottom chamber, and the cell suspension was placed on the top. The chambers were separated by an 8-μm Millipore membrane (Millipore Corporation, Bedford, Mass.) (11). For study of the capacity of drugs to alter chemotaxis, 0.05 ml of a solution of drug and 0.05 ml of the cell suspension were added to the top chamber. After the chambers had been incubated for 3 hr at 37° the filters were removed and stained with Carrazi's hematoxylin and Chromotrope 2R (11). Migration of the eosinophils was measured using the "in filter count" method (12). In this method an arbitrary point 90 μm from the top of the stained membrane was chosen and counts were made there and at every 5 μm until the bottom of the filter was reached. All the eosinophils with distinct nuclei were counted at each level. The chemotactic counts were expressed as the mean cell count of five high-power fields (11). The chambers were processed in duplicate. In some experiments the test drug was placed in the lower compartment with the same volume of chemotactant, in order to determine whether the action of the drug was directly on the cells or on the chemotactant. Because of reports of cells dropping off the top of the filter and interfering with the final chemotactic count (13), the liquid remaining in the lower compartment after the 3-hr incubation period was pelleted on a slide using a cytocentrifuge and was stained with Wright's stain. No cells were found.

Viability assay. The effects of hydrocortisone sodium succinate, methylprednisolone sodium succinate, and chloroquine hydrochloride on the viability of the eosinophils were investigated by trypan blue exlusion. After the cells were incubated at 37° for 3 hr with the different concentrations of the drugs, the cells were diluted in 0.5% trypan blue in saline and examined microscopically after 10 min. The number of stained cells per 100 cells was taken as the number of killed cells. The results were compared with a control consisting of cells in T-HSA.

Results. Gel filtration of the chemotactants. As shown in Fig. 1, gel filtration of the lung tissue extract on the Sephadex G-25 column showed chemotactic activity in fractions eluting at volumes roughly corresponding to glycyl-L-tyrosine, indicating that the chemotactic substance had a molecular weight approximately the same as that of ECF-A (mol wt 360–390) (14). Fractions obtained by gel filtration of zymosan-treated guinea pig sera were tested for chemotactic activity as shown in Fig. 2. The activity emerged from the column slightly before cytochrome *C* (mol wt 12,000) and well before bacitracin (mol wt 1400), indicating that the molecular weight of the chemotactant is approximately the same as that of guinea pig C5a (mol wt 15,000) (8).

Effect of corticosteroids on chemotaxis. Corticosteroids produced a dose-related inhibition of chemotaxis induced by both ECF-A and C5a with concentrations ranging from 0.15 to 2 mg/ml (examples shown in Figs. 3 and 4) corresponding to 1.4×10^{-4} to 4.1×10^{-3} *M*, respectively. Methylprednisolone appeared to be more inhibiting than hydrocortisone. In five assays marked variation in response was noted

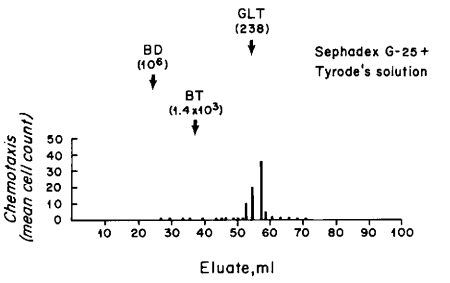

FIG. 1. Analysis of ECF-A chemotactic activity by gel filtration of the lung extract. Markers are blue dextran (BD), bacitracin (BT), and glycyl-L-tyrosine (GLT).

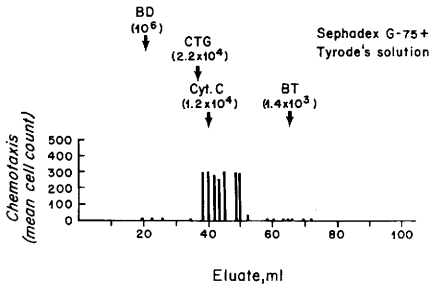

FIG. 2. Analysis of C5a chemotactic activity by gel filtration of the zymosan-treated guinea pig serum. Markers are blue dextran (BD), chymotrypsinogen (CTG), cytochrome *C* (Cyt. C), and bacitracin (BT).

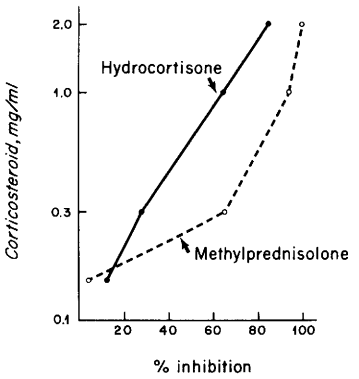

FIG. 3. Inhibition of ECF-A-mediated eosinophil migration by hydrocortisone sodium succinate and methylprednisolone sodium succinate.

with cells from different guinea pigs, but a dose-related inhibition was always present. When hydrocortisone, in concentrations varying from 0.1 to 2 mg/ml, was mixed with ECF-A in the lower compartment, no inhibition of the eosinophil migration was seen. Also, hydrocortisone together with C5a in concentrations up to 0.5 mg/ml caused no inhibition of chemotaxis. Concentrations of 1 and 2 mg/ml in the chemotactant compartment caused 13% inhibition of eosinophil migration, whereas 65 and 80% inhibition, respectively, were caused by the same doses when placed in the cell compartment. These results suggest that the corticosteroid action is directly on the cell and not on the chemotactant. In order to determine whether inhibitory action was restricted to glucocorticosteroids, three other steroids, estrone, estradiol, and testosterone, were analyzed for their eosinophil chemotactic inhibitory activity using ECF-A as the chemotactant. As a control, the diluent alone, 0.5% ethanol, was employed. In the studies by Ward (5) this concentration of ethanol was not inhibitory for the chemotaxis of polymorphonuclear neutrophils *in vitro*. However, in two assays performed with eosinophils, 20 and 40% inhibition was observed when 0.5% ethanol was mixed with cells as compared to the control without ethanol. When comparing the results for steroids to the 0.5% ethanol control, there was either no difference or the steroids were only slightly more inhibitory (approximately 10%).

Effect of chloroquine hydrochloride on chemotaxis. Chloroquine hydrochloride was tested for inhibition of eosinophil chemotaxis in doses ranging from 10^{-6} to 10^{-1} *M*. As shown in Fig. 5 an inhibitory effect was observed with lower concentrations than in the case of the corticosteroids. When placed in the bottom chamber with C5a, chloroquine, in doses ranging from 10^{-6} to 10^{-4} *M*, caused no inhibition and, at a concentra-

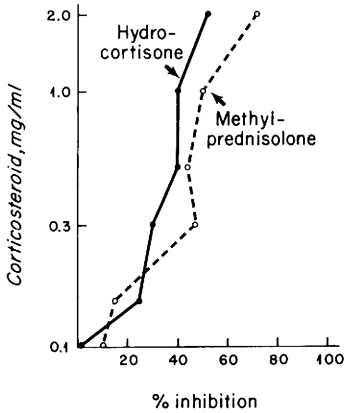

FIG. 4. Inhibition of C5a-mediated eosinophil migration by hydrocortisone and methylprednisolone.

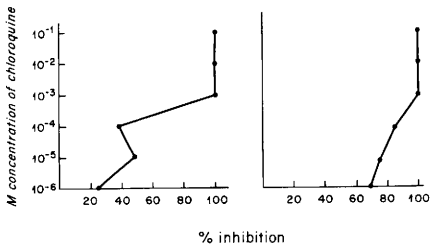

FIG. 5. Inhibition of ECF-A and C5a activity by chloroquine.

tion of 10^{-3} *M*, caused 15% inhibition compared to 100% when placed with the cells, thus suggesting a direct action of this drug on the eosinophils.

Viability assays. The mechanism of action of the corticosteroids on the cells remains to be explained. That they do not kill the eosinophils was demonstrated by the trypan blue exclusion test. In two assays the number of killed cells was much lower than the control (Table I), suggesting that the effects of the drugs were to improve the viability of the eosinophils. When eosinophils were incubated with chloroquine in the trypan blue exclusion test, slightly fewer dead cells than in the control were obtained at chloroquine concentrations up to 10^{-4} *M*. Above this concentration, trypan blue precipitated and no conclusions could be drawn.

Discussion. The results obtained in this study indicate that two corticosteroids, hydrocortisone sodium succinate and methylprednisolone sodium succinate, and chloroquine hydrochloride cause a drug-related inhibition of eosinophil migration *in vitro*, and these results are in agreement with the results of Goetzl and his associates who reported that hydrocortisone and hydroxychloroquine suppress directed migration of eosinophils (15). In both studies the doses of corticosteroids necessary to obtain this effect were much above physiological blood concentrations of hydrocortisone and even above high pharmacological blood levels. For example, intravenous administration of 100 mg of hydrocortisone yields plasma levels of up to 0.0018 mg/ml (16). In the treatment of patients with asthma 200 mg of hydrocortisone might be administered as a single intravenous injection, followed by a continuous intravenous infusion of 500 mg and finally another intravenous injection of 200 mg a few hours later. In this situation the plasma levels of hydrocortisone might be very high, conceivably as high as 0.01 mg/ml. However, as shown in Fig. 3, these levels would be 20-fold lower than those used in our experiments; our lowest level was about 0.2 mg/ml. We do not know whether corticosteroids or their derivatives might be concentrated in cells to render them more potent physiologically. The direct action of these drugs on the cells was confirmed by placing them with the chemotactant in the lower compartment. High concentrations caused minimal inhibition compared with much more pronounced inhibition seen when drugs were placed directly with the cell suspensions. The minimal inhibition seen with the drugs in the chemotactant compartment may be explained by drug diffusion into the upper compartment. Cytotoxic experiments demonstrated that neither corticosteroids nor chloroquine killed the eosinophils. The mechanisms of actions of these drugs are unclear. Although chloroquine affects the morphology of eosinophil granules in man and the rat (17, 18), there is no evidence that this effect alters the ability of the cell to show directed migration. Lysosomal membrane stabilization could be important (8, 19, 20). In the chemotaxis of neutrophils serine esterases are activated (21), but these have not been studied in relation to eosinophilotaxis, nor have the effects of corticosteroids on serine esterases been reported.

TABLE I. EOSINOPHIL VIABILITY

	Staining by trypan blue (%)	
	Expt 1	Expt 2
Eosinophils in Tyrode's 2% human serum albumin	15	24
Hydrocortisone		
0.2 mg/ml	0	–[a]
1 mg/ml	1	12
2 mg/ml	4	7
4 mg/ml	2	4
8 mg/ml	4	3
Methylprednisolone		
0.2 mg/ml	5	–[a]
1 mg/ml	3	1
2 mg/ml	5	0
4 mg/ml	10	4
Chloroquine hydrochloride		
10^{-6} *M*		30
10^{-5} *M*		18
10^{-4} *M*		26
10^{-3} *M*		–[b]
10^{-2} *M*		–[b]

[a] Not determined.
[b] Trypan blue precipitated.

Summary. In an *in vitro* chemotactic assay using a modified Boyden chamber, the action of anti-inflammatory agents on eosino-

philotaxis was studied. Hydrocortisone sodium succinate, methylprednisolone sodium succinate, and chloroquine hydrochloride induced a dose-related inhibition of migration of eosinophils to ECF-A and C5a. The inhibitory action appeared to be directly on the cells and it was not caused by lysis of cells.

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