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ENHANCEMENT OF LECTIN-INDUCED CAP FORMATION IN HUMAN NEUTROPHILS BY CYCLOSPORIN A

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Cyclosporin A, a potent immunosuppressive agent, has been extensively studied for its immunomodulatory effects on T-cells. Recently Cyclosporin A has been shown to cause renal damage, which correlates with increased glomerular neutrophil migration. The precise role of Cyclosporin A on nuetrophil function has not been established. In this study we investigated the role of Cyclosporin A in the regulation of cap formation in human neutrophils. Our results show an increased concanavalin A-induced cap formation in human neutrophils pretreated with Cyclosporin A, implicating a possible role of Cyclosporin A in human neutrophil activation. The correlation between enhanced capping, renal nephrotoxicity and neutrophil migration remains to be studied.

Cyclosporin A (CSA), a fungal polypeptide with potent immunosuppressive properties (1,2), has been associated with several forms of nephrotoxicity. Most of the research directed at CSA has focused on its effects on T-cells as primary targets (1-3). Cyclosporin A has been shown to inhibit proliferation of helper T-cells but does not affect suppressor T-cell activation or the lytic activity of the mature cytotoxic T-cell (4-6).

It is generally agreed that CSA affects immature T-cells preferentially and that it somehow interferes with the early stages of lymphocyte activation (2,7). On the other hand, CSA is also shown to have an effect on the other cells participating in the immune response. It has been shown to reduce interleukin-1 elaboration (8) and to increase prostaglandin E production (9). These mediators play a major role in influencing the macrophage-related immune responses. Accessory functions of macrophage (10) and antigen-specific T-cell activation is also shown to be affected by CSA (11,12).

In a recent study, CSA-treated animals demonstrated significantly higher levels of blood urea nitrogen and serum creatinine, greater tubular toxicity

ABBREVIATIONS
CSA = Cyclosporin A; HBSS = Hank's balanced salt solution; Con A = concanavalin A; FITC = fluoroscein isothiocyanate conjugate; BSA - bovine serum albumin; PMN = polymorpholeukocyte

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and higher glomerular polymorpholeukocyte (PMN) infiltration, as compared to control animals (13).

To evaluate the effects of CSA on PMN function, we studied lectin-induced cap formation in human neutrophils. Treatment of lymphocytes and neutrophils with concanavalin A (Con A) leads to the aggregation of the lectin into one pole of the cell, forming a so-called cap. The understanding of the consequence of capping in relation to cell function remains poor (14); however, it is generally recognized that clustering of receptors by interaction with cross-linked ligands are early signals required for cell activation (15,16). Thus, clustering of neutrophil receptors by interaction with Con A may lead to the early signal required for neutrophil activation. Our studies show an increased Con A-induced cap formation in human neutrophils pretreated with CSA, implicating a possible role for CSA in human neutrophil activation.

METHODS

Isolation of neutrophils. Neutrophils were isolated as described earlier (15). Briefly, one-half volume of 4.5% dextran T-500 in 0.85% NaCl was added to heparinized (10 U/ml) whole blood obtained from normal, healthy volunteers (17). The mixture was allowed to stand for 30 min at room temperature. The supernatant containing leukocytes was removed and layered on Ficoll-Hypaque (63.5 g of Ficoll and 100 g of Hypaque in 1 L of water). The mixture was centrifuged at 500 g for 15 min at room temperature. The pellet predominantly contained erythrocytes and neutrophils. The erythrocytes were lysed by the addition of 9 ml of water for 30 sec, followed by the addition of 1 ml 10X Hank's balanced salt solution (HBSS), mixed well, centrifuged at 250 g for 15 min and resuspended in modified HBSS (containing 1 mg glucose/ml, 1 mg BSA and 10 mM Hepes, pH 7.4). The cell preparation contained >98% neutrophils. Neutrophil viability was routinely check by trypan blue and found to be >98%.

Cyclosporin A pretreatment of neutrophils. Cyclosporin A was dissolved in 70% ethanol at 4 mg/ml and kept at -20°C as a stock solution. Whole-cell treatment with CSA was carried out by incubating the neutrophils (1×10^7 cells/ml) with 0.01 to 10 µg CSA/ml at 37°C for 15-120 min in modified HBSS. The cells were then centrifuged at 250 g for 15 min and resuspended in modified HBSS and assayred for cap formation. The control for the whole-cell treatment consisted of treatment with buffer alone under identical incubation conditions and was included in every assay.

Fluoroscein isothiocyanate conjugated (FITC)-Con A-induced cap formation. Our previously described method was used to study capping reaction (15,16). Briefly, neutrophils were suspended in modified HBSS to a final concentration of 1×10^6 cells/ml. FITC-Con A (10 µg/ml) in HBSS was added to initiate the capping reaction at 37°C for the periods of time indicated in the individual experiments. The cells were fixed with 2% paraformaldehyde for 10 min at 37°C, and a wet mount was prepared by placing 10 µl of cell mixture onto a glass slide, using a no. 1 cover slip and clear nail polish to seal it. The cells were then examined with an Olympus epifluorescence microscope fitted with FITC interference filter and a ×40 or ×60 objective. Scoring was carried out in two categories with respect to the distribution of the label as "random clusters" (patched non-capped) or "capped." Typically, the capped structures showed most of the label clustered to the one pole of the cell as a protuberance or bleb from the cell membrane. A random cluster is defined as a distribution of label in small aggregates throughout the surface of the cell (and also includes cells that have barely-visible surfaces) with little local concentration of label. Incubation at 0°C in the presence of FITC-Con A was included as a control and showed no capping. A total number of 200 cells/slide were counted, and percent capping was calculated from the triplicate samples.

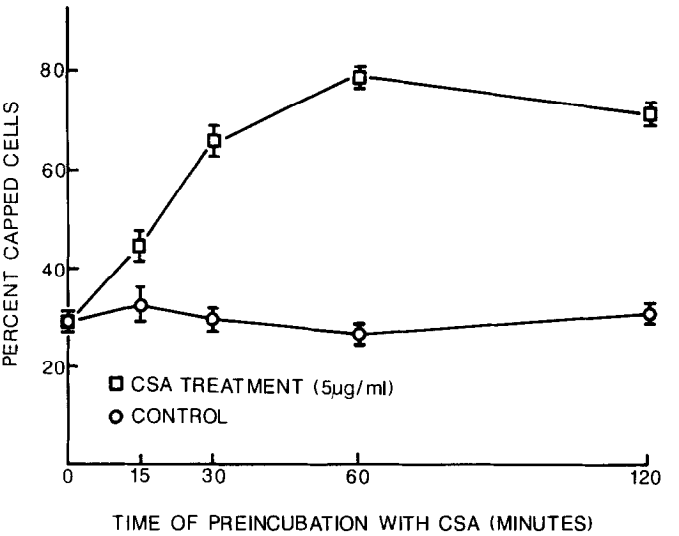

Figure 1. Effect of CSA (5 µg/ml) incubation on 10 µg/ml FITC-Con A mediated capping in human neutrophils (10^6 cells/ml) in modified HBBS. Bars indicated the S.E.M. of triplicate measurements.

RESULTS

Human neutrophil capping was noted, with concentration of the FITC-labeled Con A at one end of the cell and increased spreading and shape change. After treatment with CSA, neutrophil cap formation was substantially increased compared to buffer-treated cells, where most of the cells show patched structures. The time-course of CSA pretreatment at fixed concentration of 5 µg/ml was studied in relation to human neutrophil cap formation. The data obtained show an increased cap formation with increased CSA pretreatment time (Fig. 1). Maximum cap formation was achieved at preincubation periods of between 1 and 2 hr in the presence of CSA. The time-dependent increase in the capping may be due to the necessity of CSA entering the cell in order to exert its effects or, possibly, that interaction of CSA with the membrane component of the cell surface of human neutrophils is a slow process.

To determine the optimal dose of CSA required to achieve maximum cap formation, we studied a dose-response of CSA at a fixed preincubation time of 2 hr. A dose-dependent increase in cap formation was noted, as shown in Fig. 2, with maximum capping achieved at a concentration of CSA >1 µg/ml. These findings suggest that CSA action on neutrophil function may be directed through membrane proteins and that complete occupancy of the proteins may be required for CSA to exert its maximum effect.

To determine if CSA-mediated increase in cap formation is due to an increase in stable caps, the time-course of cap formation in control and CSA-pretreated human neutrophils were studied (Fig. 3). An increase in cap formation with CSA pretreatment was noted. As in the buffer-treated human

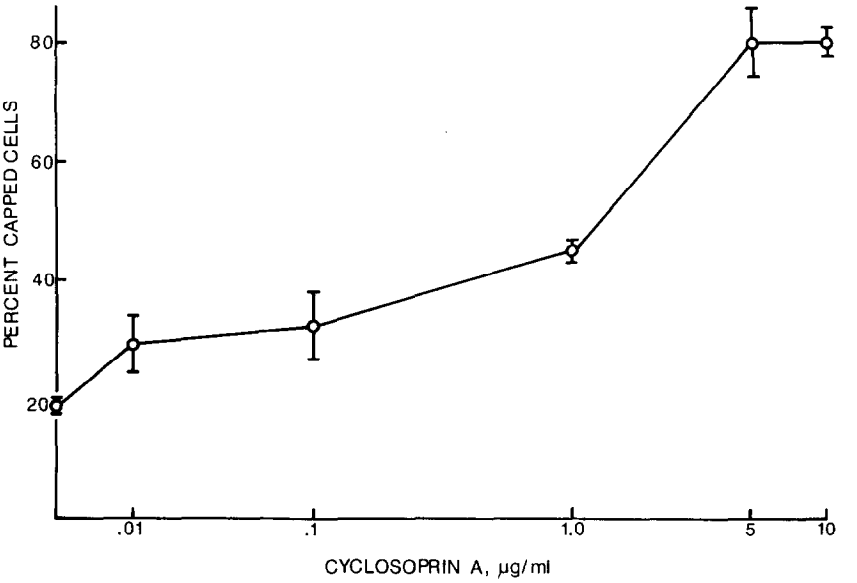

Figure 2. Dose-response of CSA (0.01 to 10 µg/ml) to FITC-Con A (10 µg/ml) mediated capping in human neutrophils. Bars indicate the S.E.M. of triplicate measurements.

neutrophils, CSA-treated neutrophils showed maximum cap formation at 10 min. Caps in both cases were stable in the time range studied, suggesting that CSA mediates an increase in stable cap formation. In several such experiments the increase noted in the CSA-treated human neutrophils was three- to four-fold.

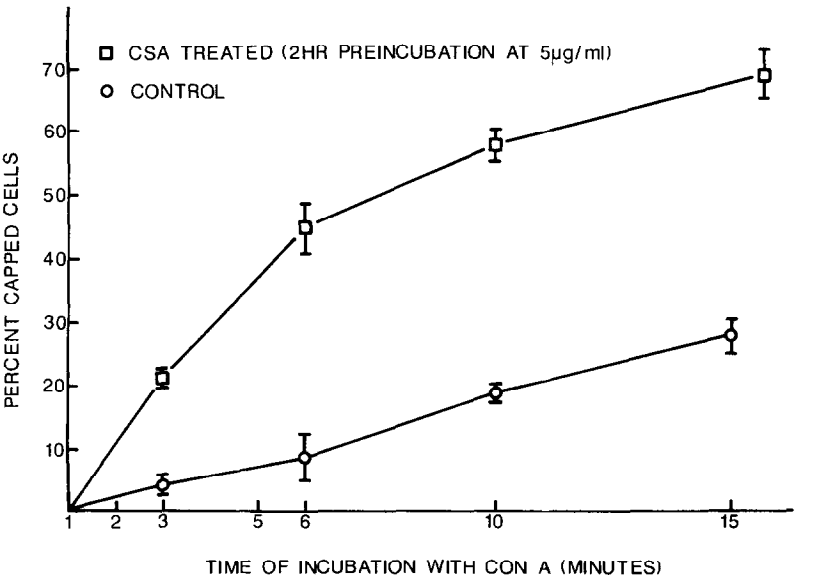

Figure 3. Time-course of FITC-Con A mediated neutrophil capping in controls (buffer alone) and CSA pretreatment (5 µg/ml for 2 hr). Bars indicate S.E.M. of triplicate measurements.

DISCUSSION

While CSA has been extensively studied for its effect on immature T-cell responses (2,7), increasing evidence suggests the involvement of CSA in the regulation of other cell functions (11,12,18-20). Recently, CSA has been shown to increase immunoglobulin cap formation in B-lymphocytes (21), thus implicating CSA as an early activator of B-cells. CSA has been shown to cause renal damage, which correlates with increased migration of neutrophils to the kidney (13); however, the precise role of CSA on neutrophil function has not been established. In the present study, we have investigated the role of CSA in the regulation of cap formation in human neutrophils. The capping reaction serves as a model for the study of the mobility of membrane proteins. The results presented here show increased cap formation in CSA-pretreated human neutrophils. The exact step(s) at which CSA acts to promote lectin-induced cap formation remains to be established, although an event relating to the membrane alteration and cytoskeleton (i.e., actin polymerization) seems likely, since cytoskeleton elements play a major role in the mobility of receptors in the membrane.

Several reviews have attempted to define the significance of the role of capping in the physiology of the cell (22,23). To date, our understanding of the consequence of capping remains unclear. It has been proposed that the first signal necessary to activate the cell is clustering of receptors by interaction with cross-linked ligands (23). If the cluster removal by capping is too slow or too fast then the activation signals might be excessive or insufficient, respectively, for proper cell activation to occur. The correlation between enhanced capping by CSA-treated neutrophils and increased neutrophil migration with CSA-induced nephrotoxicity remains to be studied.

It is possible that CSA mediates its immunomodulatory properties by enhancing capping in the B-lymphocyte and that its deleterious side-effects (viz. increased infection, nephrotoxicity) is a consequence of its enhanced capping effect on neutrophils. Further understanding of the significance of capping in the physiology of these cells is required before these questions can be answered fully.

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