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Augmentation of Apoptosis in Bronchial Exuded Rat Eosinophils by Cyclosporin A

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The effect of cyclosporin A on apoptosis in eosinophils was examined to clarify the inhibitory mechanisms of cyclosporin A on allergen-induced eosinophilia in the airway. Eosinophils in bronchoalveolar lavage fluid from sensitized rats after inhaling an allergen were used. More than 50% of the eosinophils that died spontaneously by apoptosis within 24 hour incubation in RPMI 1640 medium contained 10% fetal calf serum. The addition of cyclosporin A or dexamethasone significantly enhanced the eosinophils apoptosis at concentrations of more than 0.1 μM. Apoptosis in eosinophils was considerably suppressed in the presence of culture supernatant of activated splenocytes as a source of various cytokines. Even in the presence of culture supernatant of activated splenocytes, cyclosporin A or dexamethasone facilitated apoptosis in eosinophils. These results suggest that apoptotic death of activated eosinophils is augmented with cyclosporin A and that accelerated apoptosis in eosinophils of the airway may account for the inhibitory effect of cyclosporin A on eosinophilia.

The death of eosinophils exhibits typical apoptosis features which are characterized by cell shrinkage and DNA fragmentation (1). Cytokines such as interleukin-5 (IL-5) have been reported to regulate eosinophil functions, especially rescuing them from apoptotic death and consequently prolonging their life in inflammatory sites (2–4). The cancellation of eosinophil apoptosis may cause exacerbation of inflammation in bronchial asthma (5). Thus, it is of particular interest to examine whether or not anti-inflammatory drugs modulate apoptosis of eosinophils in progressive inflammatory sites.

It has been demonstrated that, following an allergen challenge, eosinophilia was inhibited by treatment with cyclosporin A (CyA) in rodents (6,7). Furthermore, CyA is effective in alleviating severe asthma for which patients are dependent on steroid therapy (8). Because of the immunosuppressive nature of CyA, the reduction of cytokine(s) production may be a main mechanism for the inhibitory effect of CyA on eosinophilia. Interleukin-5 (IL-5) plays a key role in eosinophilia in asthma (9), thus CyA may suppress eosinophilia through the inhibition of IL-5 production. However, the inhibitory effect of CyA on IL-5 production is relatively weak in comparison with its effect on IL-2 (10).

The present study was performed to clarify the modulatory effect of CyA on eosinophil apoptosis, which may account for the effectiveness of CyA on bronchial asthma.

MATERIALS AND METHODS

Animals and purification of bronchial exuded eosinophils. Male, 8-week-old Brown-Norway rats (Charles River) were injected with 10 μg of ovalbumin (OVA, Sigma) together with 10 mg of aluminum hydroxide subcutaneously and a 5 billion organism pertussis vaccine (Wako Pure Chemical) intraperitonealy on day 0, 13 and 20. On day 27, the rats were exposed to an aerosolized OVA for 60 min at a concentration of 10 mg/ml generated by a nebulizer (Hospitak). On day 29, the rats were sacrificed by the intraperitoneal injection of 150 mg/kg pentobarbital sodium. Bronchoalveolar lavage fluid was obtained by washing three times with 4 ml of calcium- and magnesium-free Hank's balanced salt solution (Gibco)

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supplemented with 10 mM EDTA and 1 mM HEPES. The eosinophils were enriched by a discontinuous Percoll (Pharmacia) gradient from bronchoalveolar lavage fluid. A cell smear was prepared from each interface by Cytospin III (Shandon). After staining with Diff-Quik (Kokusai Shinyaku), cell compositions were determined by 500 cells/smear under a microscope. The purity of the eosinophils was more than 90%. The viability of the cells assessed by trypan blue stain was more than 98%.

Preparation of cultured supernatant of activated splenocyte (CSS). Splenocytes obtained from the sensitized Brown-Norway rats were cultured with 100 μg/ml of OVA in culture medium (RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES) for 24 hours. The cells were placed in a humidified atmosphere containing 5% CO_2 at 37°C.

Drugs. The drugs used were CyA (Sandoz Pharma Ltd., Switzerland) and dexamethasone (Sigma). The original solution of each drug was dissolved in dimethyl sulfoxide (10,000 times more concentrated than the final concentrations) and subsequently diluted in culture medium. The control group with vehicle contained the highest concentration of dimethyl sulfoxide corresponding to the drugs prepared.

Culture of eosinophil and drug treatment. The eosinophil suspension was adjusted to a concentration of 4×10^6 cells/ml. Each drug was then immediately added to get the indicated concentrations. The suspension was composed as follow; the eosinophil suspension, drug contained- medium, and the culture medium or CSS as their ratio of 1:1:2. They were cultured in 24 well flat plate (Corning) at a final volume of 0.5 ml for morphological assessment and flow cytometric analysis. For collection of DNA, 60 mm dish (Corning) at a final volume of 5 ml was used in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell morphology. The cell smear, prepared 24 hours after a culture by Cytospin III and stained with Diff-Quik, was examined under light microscopy to assess the apoptotic changes in cell morphology. Five hundred cells/smear were examined.

Flow cytometric analysis. Flow cytometric analysis was performed to identify apoptotic cells according to a method reported elsewhere (11). Briefly, the cells were fixed in 70% ethanol for 1 hour on ice. The cells were incubated with 100 μg/ml propidium iodine and 100 μg/ml RNAase at 37°C for 1 hour and then cell cycle was examined by an EPICS-ELITE (Coulter Corporation). It was confirmed in the preliminary study that the cells which displayed a "hypodiploid" peak as shown in Figure 1 stood for the apoptotic cells which showed DNA condensation and fragmentation. Eosinophil apoptosis was expressed as proportion (%) of the cell number displaying a "hypodiploid" peak in total cells.

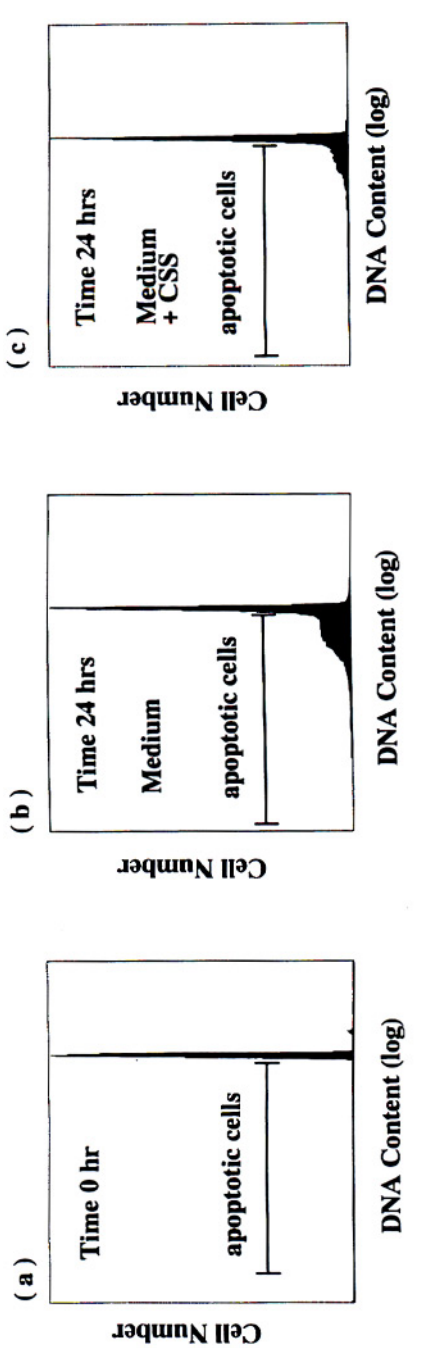
DNA extraction and electrophoresis. The DNA cleavage pattern was analyzed by agarose gel electrophoresis according to a method reported elsewhere (12). Briefly, cells (5×10^6 cells) were lysed in a 1 ml lysis buffer (10 mM Tris-HCl (Sigma), 0.1 M EDTA and 0.5%SDS (Wako)) and incubated with 100 μg/ml proteinase K (Merck) at 37°C overnight. After extraction in phenol and precipitation in ethanol, a 1.5 μg sample treated with 2 μg/ml pancreatic RNAase (Sigma) in each lane were placed in electrophoresis on a 1.0 % agarose at 80 V for 3 hours. DNA was stained with ethidium bromide.

Statistical analysis. Data are given as means ± SE. Statistical analysis of the difference among the groups was made with one-way analysis of variance and followed by unpaired two-tailed Student's *t*-tests.

RESULTS

Morphological change of bronchial exuded rat eosinophils with a histogram of DNA fluorescence is shown in Figure 1. Freshly isolated eosinophils are indicated by a sharp single peak in the histogram (Fig. 1-a). Hypodiploid cells were markedly increased in eosinophils cultured over 24 hours with the medium alone (Fig. 1-b). Decreased cell size, cytoplasmic vacuolation, nuclear condensation or extrusion, all characteristic changes of apoptosis, were also observed after 24 hours (Fig. 1-B).

The addition of CSS to the culture markedly decreased the number of hypodiploid cells and suppressed apoptotic changes in morphology (Fig. 1-c & C). On the other hand, neither 100 μg/ml of OVA itself nor splenocyte culture supernatant without OVA treatment inhibited eosinophil apoptosis (data not shown). Eosinophil apoptosis progressed in a time dependent manner (Fig. 2). The kinetics for augmentation of eosinophil apoptosis by CyA and dexamethasone at a concentration of 1 μM were examined. The eosinophil apoptosis estimated with either morphological or flow cytometory method was significantly augmented by CyA at 12 and 24 hours but not earlier period of time (Fig. 2-A). When the response was expressed as (apoptosis with drug) / (apoptosis without drug), it was maximum at 12 hours; 1.30, 2.25, 1.70 and 1.36 at 4, 12, 24 and 48 hours for morphological assessment, and 1.09, 1.70, 1.18 and 1.09 at 4, 12, 24 and 48 hours for flow cytometoric analysis, respectively. The augmentation was also seen in the presence of CSS which delayed the augmentation; maximum at 24 hours with morphological assessment (Fig. 2-B). Simi-



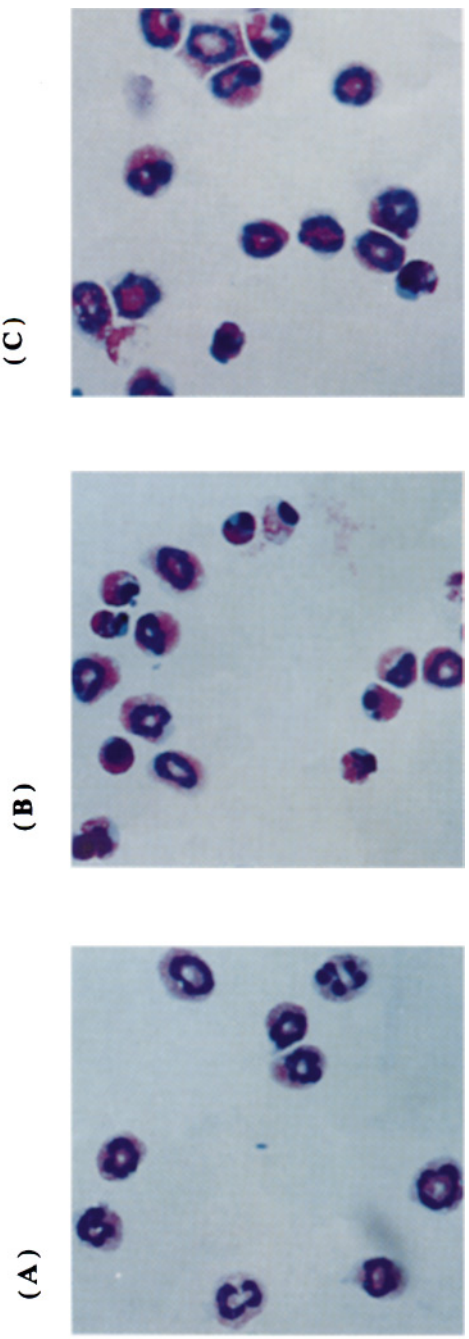


FIG. 1. Morphological changes (A, B, C) and DNA fluorescence histogram (a, b, c) of bronchial exuded rat eosinophils. A, a: freshly isolated eosinophils. B, b: eosinophils cultured in the medium alone over 24 hours; C, c: eosinophils cultured in the medium plus culture supernatant of splenocytes over 24 hours.

lar kinetics for the augmentation was observed with dexamethasone. There was difference in the estimated apoptosis between morphological assessment and flow cytometric analysis. Flow cytometer may give more sensitive measurement of apoptosis, the stage of which was relatively early. On the other hand, it may not count cells of apoptosis, the stage of which was progressed to an extreme extent with extinguished component of cells.

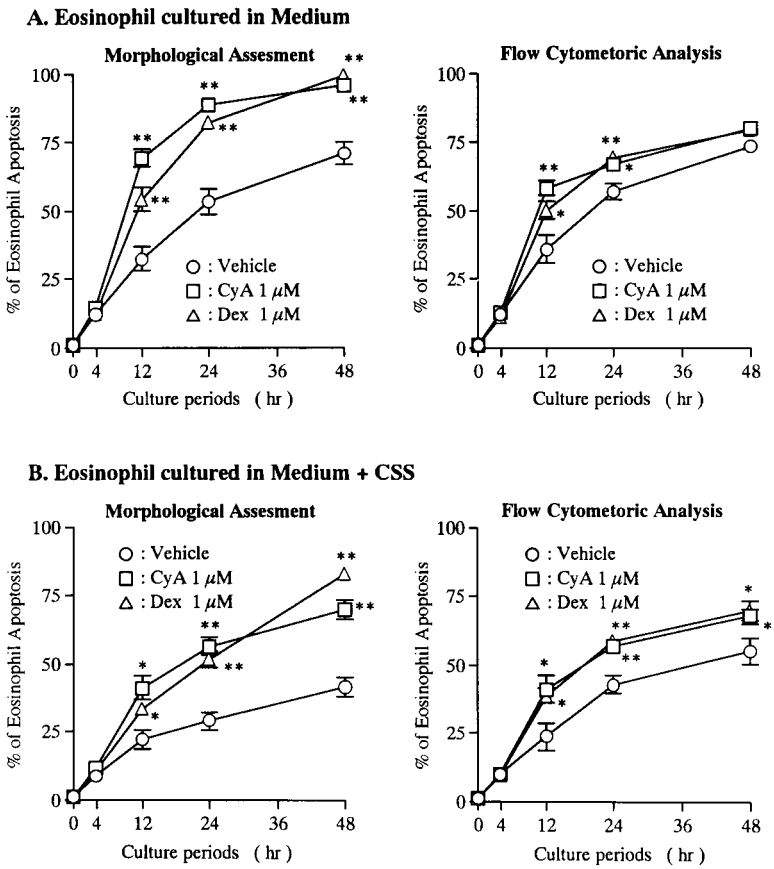


FIG. 2. (A) Kinetics for augmentation of apoptosis by cyclosporin A and dexamethasone in bronchial exuded rat eosinophils in the medium alone. *Significantly different from values in the treatment with vehicle, $p < 0.05$; **$p < 0.01$. (B) Kinetics for augmentation of apoptosis by cyclosporin A and dexamethasone in bronchial exuded rat eosinophils survived by the addition of culture splenocyte supernatant (CSS). *Significantly different from values in the treatment with vehicle plus CSS, $p < 0.05$; **$p < 0.01$. Each point consists of 5 independent experiments.

The effects of CyA and dexamethasone on apoptosis in the eosinophils were examined over 24 hours. CyA at concentrations of 0.1 and 1 μM facilitated apoptosis in the medium alone depending on its concentration (Fig. 3-A). Dexamethasone also significantly augmented apoptosis in eosinophils at concentrations of 0.01 to 1 μM. When the survival of the eosinophils was prolonged by the addition of CSS, the apoptosis was also augmented significantly by CyA and dexamethasone at 1 μM (Fig. 3-B). Augmented apoptosis in eosinophils by both agents was lower in the presence of CSS than in the absence.

DNA integrity of the treated cells was analyzed by agarose gel electrophoresis. The electrophoresis of DNA from the eosinophils cultured in medium alone revealed a “ladder” pattern brighter than that from the eosinophils survived by the addition of CSS. CyA and dexamethasone at 1 μM with or without CSS elicited DNA fragmentation more clearly than vehicle (Fig. 4).

DISCUSSION

Significant facilitation of apoptosis in eosinophils was brought about by CyA at concentrations of 0.1 μM or more, as shown in the DNA fragmentation and the morphological changes with cell shrinkage and nuclear condensation. Either did dexamethasone facilitate apoptosis in eosinophils which were isolated from bronchoalveolar lavage fluid in rats of experimental asthma. Both CyA

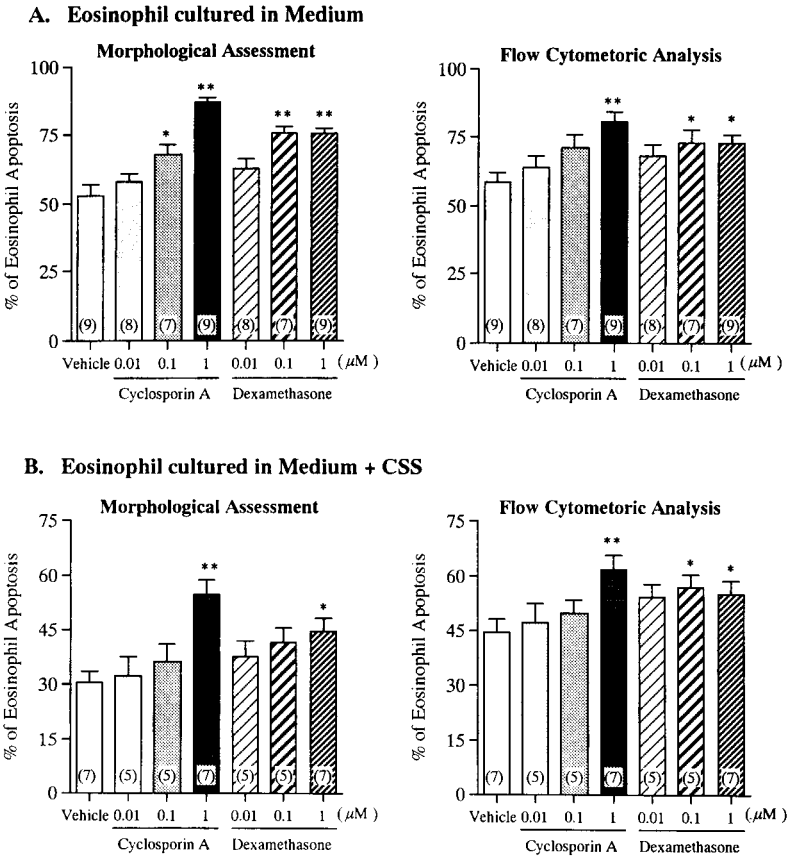


FIG. 3. (A) Augmentation of apoptosis by cyclosporin A and dexamethasone in bronchial exuded rat eosinophils in the medium alone over 24 hours. *Significantly different from values in the treatment with vehicle, $p < 0.05$; **$p < 0.01$. (B) Augmentation of apoptosis by cyclosporin A and dexamethasone in bronchial exuded rat eosinophils survived by the addition of culture splenocyte supernatant (CSS) over 24 hours. *Significantly different from values in the treatment with vehicle plus CSS, $p < 0.05$; **$p < 0.01$. Numbers in parentheses indicate the numbers of independent experiments.

and glucocorticoids actually elicited efficacious effects in asthma patients (8, 13). The mechanisms of these agents for anti-asthma effects have not been clearly elucidated yet, although main mechanism is assumed with suppression of T cells and consequent inhibition of cytokine production.

Since eosinophil produce several kinds of cytokines which are known as eosinophil survival factors like IL-5 (14), eosinophil apoptosis may be augmented by inhibition of autocrine production of these cytokines. Although CyA inhibits IL-5 production from murine Th2 cell line, the inhibitory effect of CyA on IL-5 production is relatively weak in comparison with its effect on IL-2 (10). In addition, it has been demonstrated that CyA reduces allergen-induced increases of eosinophil in the airway of mice, while IL-5 released in brochoalveolar lavage fluid were slightly suppressed (15). Thus, inhibition of IL-5 production only may not be attributed to augmentation of eosinophil apoptosis by CyA, consequently to CyA-induced inhibition of eosinophilia in the airway.

There is likelihood for involvement of transforming growth factor (TGF) β1 on augmentation of apoptosis in eosinophils by CyA. TGF β1 induces apoptosis in eosinophils (16). In addition, eosinophils can also produce TGF β1 (17) and CyA is a potent inducer for TGF β1 (18). Therefore, it is postulated that the induction of TGF β1 by triggered CyA may facilitate apoptosis in eosinophils.

CyA induced apoptosis in eosinophils even in the presence of CSS as sources of various kind of

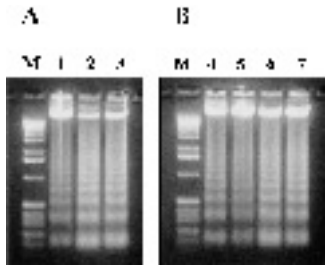


FIG. 4. Agarose gel electrophoresis of DNA from eosinophil. (A): Eosinophils cultured in the medium alone over 24 hours. M: 1kb bp DNA ladder maker (Gibco) (1), (2) and (3): Eosinophils treated with the vehicle, 1 μM of cyclosporin A and 1 μM of dexamethasone, respectively. (B): Eosinophils cultured in the medium plus culture splenocyte supernatant (CSS) in 24 hours. (4), (5), (6) and (7): Eosinophils without CSS, eosinophils treated with the vehicle, 1 μM of cyclosporin A and 1 μM of dexamethasone, respectively.

the cytokines. This finding suggests that CyA acts on the receptors of the membrane or subcellular pathways. Protein tyrosine phosphorylation regulates apoptosis of eosinophil (19). CyA may act on the intracellular signal transduction after the binding of cytokines to their receptors on eosinophil.

On the contrary to the finding in eosinophils, it has been demonstrated that CyA inhibits T cell receptor-induced apoptosis in T cell hybridomas and thymocytes (20). The inhibition of soluble Fas ligand release accounts for the inhibitory mechanism of CyA (21). Although it remains to be determined whether eosinophil apoptosis is mediated by interaction between Fas and Fas ligand, eosinophil might not release Fas ligand. This may be one of the possible explanations of the opposite effects of CyA on apoptosis between eosinophils and T cells.

Dexamethasone at concentrations more than of 0.01 μM also started to augment apoptosis in eosinophils in 12 hours. Wallen and his colleagues have demonstrated that glucocorticoids inhibit cytokine-mediated eosinophil survival, and that the inhibition takes at least 2 day's exposure of eosinophils (22). When recombinant cytokines are increased, the inhibitory effect of glucocorticoids is reduced. Therefore, critical time for eosinophil apoptosis seems to be determined on the balance of the survival factors and glucocorticoids. In the present study showing earlier apoptosis with glucocorticoid, there was no marked difference in the optimal time between cyclosporin A and dexamethasone. With CSS, the apoptosis augmented by either cyclosporin A or dexamethasone took place later than without it actually. In contrast with CyA, glucocorticoids potent strong inhibitory effects on IL-5 production (10). Therefore, dexamethasone-induced augmentation of eosinophil apoptosis may be mediated by inhibition of the release of IL-5 and/or other cytokines for cell survival. Glucocorticoids interact with glucocorticoid receptor complex and consequently bind to transcription factors like AP-1 with resultant antagonism against survival-prolonging effect of cytokines (23). Eosinophil may be survived longer by suppression of the final common pathway of programmed cell death. It is postulated that CyA prevents this suppression and facilitates apoptosis. What is involved in apoptotic augmentation of the eosinophils by cyclosporin A and dexamethasone remains to determine.

In conclusion, CyA facilitates apoptosis in rat eosinophils exuded in the airway following inhalation of allergen. This effect may account for the effectiveness of CyA on bronchial asthma.

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