

## Apoptosis of eosinophils and lymphocytes in allergic inflammation

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A characteristic feature of apoptosis is that the cellular contents that are biologically active are always surrounded by the cell membrane throughout the entire process. Thus the apoptotic cells are eliminated calmly and quickly without evoking inflammation. In allergic inflammation activated eosinophils and lymphocytes have been known to accumulate at the site of inflammation at least in part because of their prolonged survival. Corticosteroids are the most potent anti-inflammatory agent used for treating asthma. They inhibit the prolonged survival of eosinophils and lymphocytes directly by inducing apoptosis and indirectly by suppressing the release of cytokines supporting their survival. Theophylline, a classical bronchodilator, has been reported to have anti-inflammatory effects. Surprisingly, theophylline inhibited the prolonged survival of eosinophils in the presence of IL-5 *in vitro* by means of induction of apoptosis. Lymphocytes also undergo apoptosis in the presence of theophylline. One of the mechanisms for theophylline to induce apoptosis in eosinophils and lymphocytes is to elevate intracellular cyclic adenosine monophosphate because phosphodiesterase inhibitors and adenylate cyclase activators, which increase intracellular cyclic adenosine monophosphate, can cause apoptosis in those cells. Induction of apoptosis is beneficial in allergic inflammation, and the use of corticosteroids and theophylline in combination may be appropriate to induce apoptosis in eosinophils and lymphocytes. (*J Allergy Clin Immunol* 1999;104:14-21.)

**Key words:** Apoptosis, eosinophils, lymphocytes, asthma, allergic inflammation, corticosteroids, theophylline, phosphodiesterase inhibitors, IL-5, GM-CSF

Since the development of bronchoscopy, it has become possible to perform bronchial biopsies in asthmatic patients. Histopathologic studies have disclosed that the asthmatic airway is characterized by chronic airway inflammation, consisting of desquamation of epithelial cells, thickening of the site adjacent to the basement membrane, and accumulation of various inflammatory

### Abbreviations used

|         |                                        |
|---------|----------------------------------------|
| cAMP:   | Cyclic adenosine monophosphate         |
| d-cAMP: | Dibutyl cyclic adenosine monophosphate |
| FasL:   | Fas ligand                             |
| PDE:    | Phosphodiesterase                      |
| PI:     | Propidium iodide                       |

cells at the subepithelial region, even in subjects with mild asthma.<sup>1-3</sup> Among the accumulated cells, eosinophils and lymphocytes have been found to play pivotal roles in the development of airway inflammation in asthma.<sup>4-6</sup> Corticosteroids are the most potent anti-inflammatory agent used for treating bronchial asthma and are known to induce apoptosis in eosinophils, as well as in lymphocytes. Theophylline has been used as a bronchodilator in the treatment of asthma for more than 50 years. However, it has been recently recognized that theophylline can inhibit the functions of various inflammatory cells and act as an anti-inflammatory agent.<sup>7</sup> Because apoptosis, a form of natural or physiologic cell death distinct from necrosis, can remove somatic cells calmly without causing an inflammatory response,<sup>8</sup> one of the appropriate ways to eliminate inflammatory cells from the lesion (eg, the asthmatic airway) will be the induction of apoptosis.

In this article we will review some aspects of apoptosis and provide an overview of some data, including our own, suggesting that theophylline, as well as corticosteroids, can induce apoptosis in eosinophils and lymphocytes.

### WHAT IS APOPTOSIS?

The term "apoptosis" was first used by Kerr in 1972,<sup>9</sup> when he imagined the shape of the cells undergoing apoptosis as the falling leaf, for which Hippocrates used the word "apoptosis" in Greek. Because it requires gene expression mediated by cell stimulation, apoptosis has been described as programmed cell death or activation-induced cell death.<sup>8</sup> Apoptosis plays an important role in the metamorphosis of the frog. For instance, loss of the tail in the tadpole is known to be mediated by apoptosis. In mammals apoptosis is involved in the various steps of the development of organs and tissues in the embryo (eg, the formation of the fingers).

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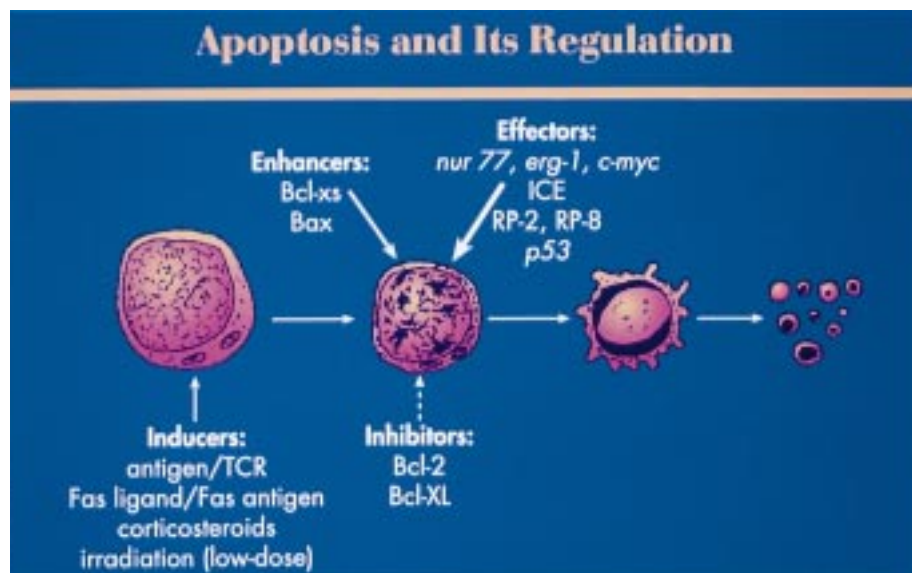


**FIG 1.** Apoptosis and its regulation. Morphologic changes from normal cell to apoptotic body in lymphocytes<sup>10</sup> are shown, with regulatory factors for apoptosis.

When we look closely at the cells undergoing apoptosis, we find that they have characteristic findings, such as cell shrinkage, chromatin condensation, nuclear segmentation, cellular disintegration, and formation of an apoptotic body (Fig 1).<sup>10,11</sup> The morphologic changes are accompanied by fragmentation of DNA into 180 to 200 bp, which corresponds to the length of a unit of the nucleosome repeat.<sup>12</sup> In contrast, necrosis is a kind of accidental cell death caused by, for example, cytotoxic agents, heat, unphysiologic osmotic pressure, and antibody-complement interactions. The cells undergoing necrosis are expanded to be broken down because of the influx of water with  $\text{Ca}^{++}$  and  $\text{Na}^{+}$  into the cells and the suppression of adenosine triphosphate formation after the injury of the cellular membrane. Of note, inflammation is caused by the leakage of biologically active cellular contents into the extracellular space during necrosis.

## REGULATION OF APOPTOSIS

The pathway of apoptosis is controlled by various factors classified as inducers, enhancers, effectors, and inhibitors. The representative inducer is the Fas-Fas ligand system, the enhancer is Bax, the effector is p53, and the inhibitor is Bcl-2, which will be mentioned later with *c-myc* (Fig 1). Cancer cells irradiated at a lower dose will undergo apoptosis by means of p53, and those at a higher dose will be necrotic. Apoptosis is also induced in the T cells stimulated with a sensitized antigen through T-cell receptors by means of *nur-77*, *erg-1*, and *c-myc* and in those treated with corticosteroids by means of RP-2 and RP-8. A wide variety of cells are known to become apoptotic after engagement of a certain kind of cell surface receptors, the so-called death receptors, such as TNF receptor 1, Fas/apoptosis-inducing protein 1, and IL-

**TABLE I.** Inhibitory effect of theophylline on eosinophil survival with IL-5

| Culture             | n  | Survival rate<br>(mean $\pm$ SEM %) |
|---------------------|----|-------------------------------------|
| IL-5 only           | 11 | 100.0 $\pm$ 0.0*                    |
| IL-5 + theophylline | 11 | 63.1 $\pm$ 3.4†                     |
| Theophylline only   | 4  | 34.3 $\pm$ 7.3‡                     |
| Medium alone        | 5  | 32.2 $\pm$ 11.8§                    |

\* $P < .005$ , comparing IL-5 only with IL-5 + theophylline.

† $P < .01$ , comparing IL-5 + theophylline with theophylline only.

‡Not statistically significant when comparing theophylline only with medium alone.

§ $P < .01$ , comparing IL-5 + theophylline with medium alone.

1 $\beta$ -converting enzyme.<sup>13-15</sup> Both TNF receptor 1 and Fas are members of the TNFR superfamily and are activated by TNF and Fas ligand (FasL), respectively. Apoptosis induced by the Fas-FasL system is regulated by the rate of expression of FasL, and anti-Fas antibodies can mimic FasL and therefore induce apoptosis efficiently with Fas on the cell surface.<sup>13</sup> An inhibitor, Bcl-2, downregulates apoptosis mediated by p53 and *c-myc*.<sup>16</sup> An enhancer, Bax, upregulates the cell death by binding to Bcl-2 to suppress the inhibitory effect of Bcl-2 on apoptosis.<sup>17</sup> More genes or gene products seem to be involved in regulating apoptosis, but further investigation is required to clarify the whole scheme of the regulatory system of apoptosis.

## EOSINOPHILS AND LYMPHOCYTES IN ALLERGIC INFLAMMATION

One of the characteristic features of allergic inflammation is the accumulation of activated eosinophils at the site of inflammation.<sup>18</sup> The eosinophil accumulation

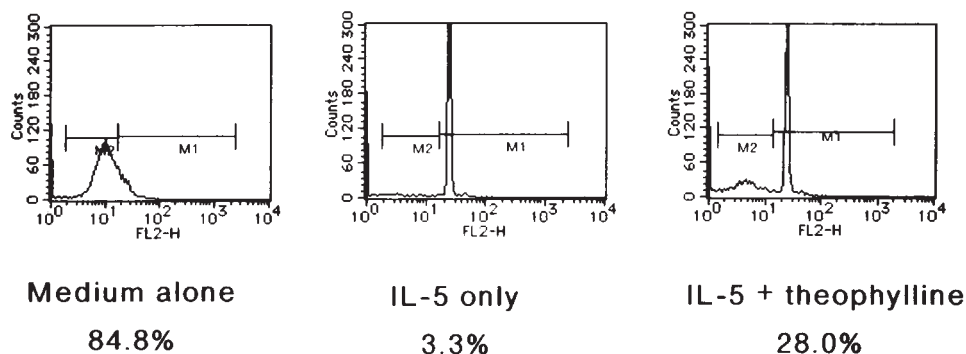


**FIG 2.** Detection of apoptosis in eosinophils induced with IL-5 plus theophylline by PI staining. Eosinophils were collected after a 96-hour culture period and stained with hypotonic PI-fluorochrome solution to detect DNA fragmentation by flow cytometry.<sup>32</sup> A representative result out of 10 experiments is shown. Percentages indicate the percentage of apoptotic cell nuclei/total cell nuclei. *IL-5 only*, Only cultured with IL-5 10 ng/mL; *IL-5 + theophylline*, cultured with theophylline  $10^{-4}$  mol/L in addition to IL-5 10 ng/mL.

could be induced by upregulation of the growth and differentiation of eosinophils in the bone marrow and their migration to the site of inflammation. Because the life span of eosinophils is as short as 4 days, prolonged survival at the sites where the eosinophils have migrated is essential to exert their function sufficiently. Through this whole process toward eosinophilic inflammation, 3 cytokines targeting eosinophils (ie, IL-5, IL-3, and GM-CSF) may play a pivotal role.<sup>19</sup> The 3 factors share the  $\beta$ -chain of their receptors, which is termed the common IL-3 receptor  $\beta$ -chain,<sup>20</sup> and act on eosinophils to prolong their life span<sup>21</sup> and to augment their functions, such as migration and degranulation.<sup>22</sup> Among the 3 cytokines, IL-5 has the most potent effect on eosinophil survival.<sup>23</sup> It has been reported that eosinophilia develops in transgenic mice overexpressing IL-5.<sup>24</sup> Importantly, activated T cells, which produce the eosinophil survival cytokines (ie, IL-5, IL-3, and GM-CSF), have been found at the site of allergic inflammation with eosinophilia in patients with bronchial asthma<sup>25</sup> and atopic dermatitis.<sup>26</sup> Therefore it has been hypothesized that T cells, namely  $T_H2$  cells, are first activated by some stimuli such as antigens and start producing various cytokines, including IL-5, IL-3, and GM-CSF. With these cytokines, eosinophils are activated not only to survive but also to release their cationic granular proteins, oxygen radicals, and lipid mediators (eg, leukotriene C4) and to cause tissue damage as a part of allergic inflammation. Therefore the induction of apoptosis in eosinophils, T lymphocytes, or both could resolve allergic inflammation and improve the impaired condition. In other words one of the therapeutic strategies for allergic diseases is to eliminate the activated eosinophils, lymphocytes, or both with a drug capable of promoting apoptosis in these inflammatory cells.

#### APOPTOSIS OF EOSINOPHILS WITH CORTICOSTEROIDS AND THEOPHYLLINE IN VITRO

Human eosinophils cultured in the presence of IL-5 can mostly stay alive at day 4, whereas 75% are dead by

means of apoptosis without IL-5.<sup>27,28</sup> Among the therapeutic drugs for bronchial asthma, glucocorticoids have been reported to inhibit the effects of IL-5 and GM-CSF on eosinophil survival.<sup>29</sup> The steroids can directly act on eosinophils by means of their receptors to accelerate apoptosis, although they will inhibit apoptosis in neutrophils.<sup>30</sup> The acceleration of apoptosis with steroids is overcome by increased amounts of the cytokines, which prolong eosinophil survival,<sup>29</sup> suggesting that the steroids and cytokines are antagonistic to each other. The other possible mechanism by which glucocorticoids induce apoptosis in eosinophils is to decrease the production of cytokines supporting eosinophil viability (ie, IL-5, GM-CSF, and IL-3) through the regulatory system of cytokine production and/or reduction of cytokine-producing cells, such as T cells, by means of apoptosis.

We studied whether theophylline, a kind of classical and useful agent in treating asthma, has some effect on the prolonged survival of eosinophils with IL-5.<sup>28</sup> We discovered that theophylline inhibits IL-5-dependent eosinophil survival by means of apoptosis in a dose-dependent manner. What should be emphasized is that the significant inhibition was observed at  $10^{-4}$  mol/L (approximately equal to 18  $\mu$ g/mL), which can be reached clinically (Table I). We observed ladder formation with DNA samples from the cells cultured with IL-5 plus theophylline but not with those from the cells cultured with IL-5 alone.<sup>28</sup> We confirmed DNA fragmentation in eosinophils by propidium iodide (PI) staining (Fig 2).<sup>31</sup> By adding dexamethasone or dibutyryl cyclic adenosine monophosphate (d-cAMP) to eosinophils in the presence of IL-5, we also observed ladder formation in the DNA electrophoresis in accordance with a decrease of cell viability (Fig 3),<sup>28</sup> confirming that glucocorticoids induce apoptosis in eosinophils and indicating that induction of apoptosis in activated eosinophils is a mode of their action in the treatment of asthma.<sup>32</sup> Interestingly, corticosteroids and theophylline showed some additive effect on induction of apoptosis in guinea pig peritoneal eosinophils stimulated with IL-5 in vitro,<sup>33</sup> suggesting that the 2 drugs may cooperate well with each

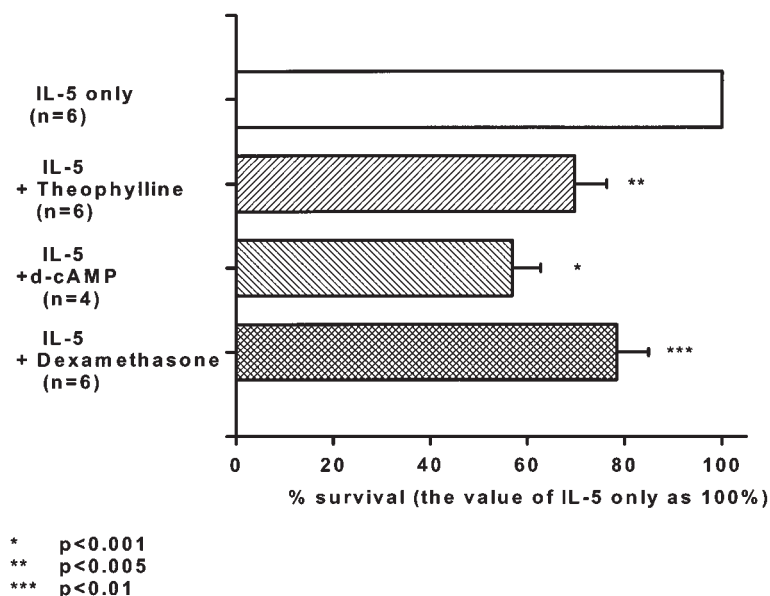


**FIG 3.** Effect of dexamethasone on eosinophil survival with IL-5. Highly purified eosinophils (more than 95% purity) were cultured in the presence of IL-5 10 ng/mL only or together with dexamethasone  $10^{-6}$  mol/L, theophylline  $10^{-4}$  mol/L, or d-cAMP  $10^{-4}$  mol/L for 4 days. Viable cells were enumerated by staining the cells with fluorescein diacetate and observing them by means of a fluorescent microscope.<sup>28</sup> The data of percent survival are expressed as means  $\pm$  SEM by defining the value of IL-5 alone as 100%. Addition of dexamethasone, as well as that of theophylline and d-cAMP, resulted in significant inhibition of prolonged survival of eosinophils cultured with IL-5. Note that the concentration of dexamethasone is 100 times less than that of either theophylline or d-cAMP ( $10^{-6}$  mol/L vs  $10^{-4}$  mol/L).

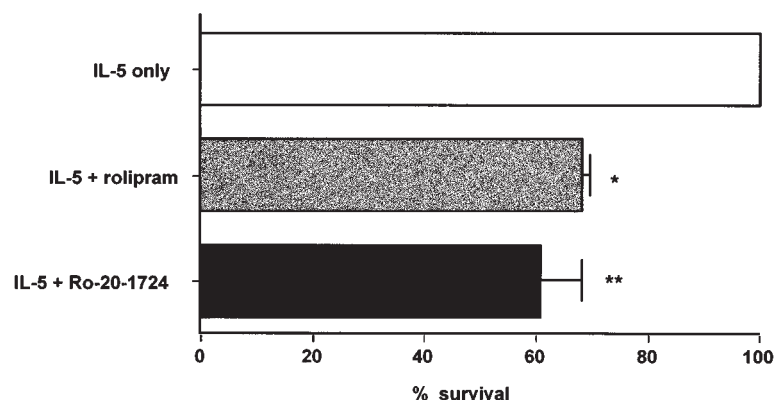


**FIG 4.** The effect of PDE IV inhibitors on eosinophil survival with IL-5. Highly purified eosinophils (more than 95% purity) were cultured with IL-5 10 ng/mL and/or  $10^{-4}$  mol/L PDE IV inhibitors (ie, rolipram and Ro-20-1724). After 96 hours, viable cells were enumerated by trypan blue dye exclusion. The value of IL-5 alone is expressed as 100%. The data are shown as means  $\pm$  SEM of 8 experiments from different donors. \* $P < .01$  and \*\* $P < .001$  compared with IL-5 alone.

other for the long-term management of bronchial asthma.

Because theophylline is known to increase intracellular cyclic adenosine monophosphate (cAMP) by inhibiting phosphodiesterase (PDE)<sup>34</sup> and because d-cAMP induced apoptosis in our experiments, it is conceivable that theophylline can induce apoptosis by functioning as a PDE inhibitor on activated eosinophils to increase intracellular cAMP. As we expected, the PDE IV

inhibitors we used (ie, rolipram and Ro-20-1724) down-regulated eosinophil survival with IL-5 (Fig 4). Moreover, an adenylate cyclase activator, forskolin, which increases intracellular cAMP, also inhibited eosinophil survival, as in the cases of the PDE IV inhibitors and dibutyryl cAMP (Fig 5).<sup>35</sup> Similarly, by using GM-CSF instead of IL-5, the prolonged survival of eosinophils can be inhibited with either d-cAMP or cholera toxin, another

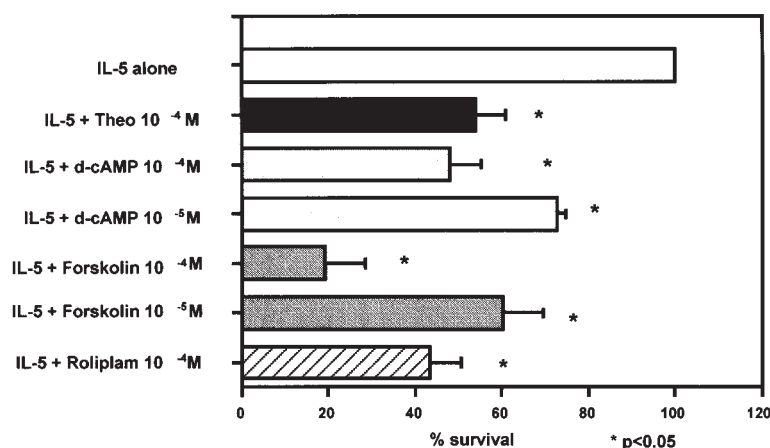


**FIG 5.** The effect of agents increasing intracellular cAMP on eosinophil survival. Highly purified eosinophils (more than 95% purity) were incubated with IL-5 10 ng/mL, an agent that increases intracellular cAMP, or both. After 96 hours, viable cells were enumerated by trypan blue dye exclusion. \* $P < .05$  compared with IL-5 only.

er adenylate cyclase activator.<sup>36</sup> These data support our hypothesis that the elevation of intracellular cAMP can induce apoptosis in eosinophils surviving with some cytokines, such as IL-5 and GM-CSF. We do not know the reason, but one report after our experiments with theophylline has concluded that the inhibitory effect of theophylline on granulocyte survival is not mediated by the elevation of intracellular cAMP.<sup>37</sup>

In addition to its direct induction of apoptosis by means of PDE inhibition, theophylline may induce apoptosis indirectly through the inhibition of cytokine production, as has been reported in corticosteroids. For instance, theophylline decreases GM-CSF biosynthesis by eosinophils stimulated with secretory IgA at a therapeutic concentration. As noted, GM-CSF could be acting on the cytokine-producing eosinophils to prolong their own survival, suggesting that theophylline, and presumably PDE IV inhibitors, indirectly induces apoptosis by inhibiting IL-5 and GM-CSF secretion in the autocrine system of eosinophils.<sup>38</sup>

### APOPTOSIS OF LYMPHOCYTES WITH CORTICOSTEROIDS AND THEOPHYLLINE IN VITRO

T lymphocytes can survive in the presence of IL-2, as in the case of eosinophils in the presence of IL-5.<sup>12</sup> Corticosteroids inhibit the survival of T lymphocytes directly by inducing apoptosis<sup>39</sup> and indirectly by suppressing IL-2 formation.<sup>40</sup> The inhibitory effect of the steroids on T-cell survival could be attenuated by adding a combination of IL-1, IL-6, and IFN- $\gamma$  to the culture.<sup>41</sup> Interestingly, T cells obtained from patients with glucocorticoid-resistant asthma (or steroid-insensitive asthma) maintain their proliferation and cytokine production in the presence of corticosteroids at least in part because of a decreased affinity of the glucocorticoid receptor caused by either a primary defect of the receptor or increased secretion of certain cytokines, such as IL-2 and IL-4.<sup>42</sup> B

cells are also sensitive to glucocorticoids. B cells in patients with chronic lymphocytic leukemia are reported to become apoptotic with the introduction of steroids, and unexpectedly, the apoptotic effect of the steroids are antagonized by IL-2, which is known to act mainly on T cells.<sup>43</sup>

Only a few studies dealing with apoptosis of lymphocytes with theophylline have been reported so far. Survival of B cells from patients with chronic lymphocytic leukemia was significantly inhibited with theophylline in a dose-dependent manner,<sup>44</sup> and at the same time, the expression of Bcl-2 protein, one of the factors inhibiting apoptosis (Fig 1), was downregulated, whereas the *c-myc* gene was overexpressed.<sup>45</sup> The changes of Bcl-2 and *c-myc* are directed toward induction of apoptosis in the cells,<sup>16</sup> and these changes could be involved also in the case of eosinophils.<sup>37</sup> Moreover, the elevation of intracellular cAMP brought about by forskolin, as well as by theophylline and PDE inhibitors, has been reported to induce apoptosis in lymphocytes in a similar fashion as that reported in eosinophils,<sup>46-49</sup> suggesting that eosinophils and lymphocytes may share the same pathway to apoptosis. Within the T-cell lineage, mature T cells in the peripheral blood appear to be less sensitive than CD4<sup>+</sup>CD8<sup>+</sup> (double positive) thymocytes to the induction of apoptosis by cAMP.<sup>50</sup> In fact, we observed that induction of apoptosis in IL-2-dependent T-cell blasts required higher concentrations of theophylline than those found in eosinophils stimulated with IL-5 (data not shown).

### CLINICAL RELEVANCE OF APOPTOSIS IN EOSINOPHILS AND LYMPHOCYTES

Inasmuch as the apoptotic cells are eliminated calmly without releasing the harmful cellular contents, the induction of apoptosis in the inflammatory cells could terminate allergic inflammation. In fact, Tsuyuki et al<sup>51</sup> demonstrated that anti-Fas mAb given intranasally in

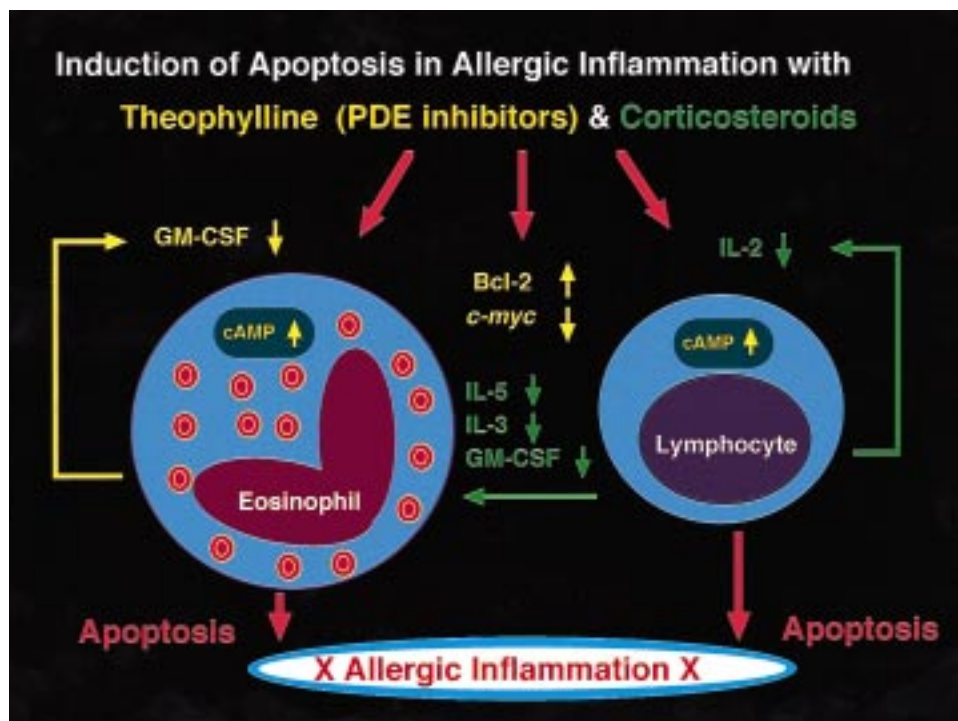


**FIG 6.** The hypothetical action of theophylline and corticosteroids in the induction of apoptosis in eosinophils and lymphocytes, resulting in suppression of allergic inflammation as a part of their therapeutic effects.

mice completely suppressed eosinophilic inflammation in the airway. Importantly, the antibody was found to induce apoptosis *in vitro* in eosinophils obtained from the mice with eosinophilic inflammation, suggesting that the induction of apoptosis is associated with the resolution of eosinophilic inflammation. In terms of the *in vivo* effect of corticosteroids, eosinophilic infiltration into the jejunal mucosa in rats parasitized with *Nippostrongylus brasiliensis* completely diminished with a single injection of dexamethasone without causing any damage to the jejunal tissue.<sup>52</sup> Woolly et al<sup>53</sup> reported more convincing evidence that the treatment of asthmatic patients during exacerbations with corticosteroids for 2 weeks resulted in a decrease of activated EG-2<sup>+</sup> eosinophils and an increase of apoptotic eosinophils in their induced sputum samples as their asthmatic symptoms improved. Interestingly, macrophages recovered in the sputum samples contained eosinophil products, indicating that alveolar macrophages are scavenging apoptotic bodies of eosinophils and that induction of apoptosis is clinically relevant to asthma treatment. These results strongly suggest that induction of apoptosis in eosinophils is beneficial in the suppression of allergic inflammation and that one of the modes of action of corticosteroids in the treatment of asthma and atopic diseases is to induce apoptosis in eosinophils and, presumably, in lymphocytes.

Although it has not been directly shown that theophylline induces apoptosis *in vivo* as a part of its therapeutic effects, Sullivan et al<sup>54</sup> have reported some supportive data. After treatment with 400 mg/day slow-release theo-

phylline for 6 weeks, the number of activated EG-2<sup>+</sup> eosinophils at the bronchial mucosa after antigen challenge significantly decreased, suggesting a possibility that induction of apoptosis is involved in the decrease of activated eosinophils with theophylline treatment. T lymphocytes at the airway can also be reduced with theophylline, according to the article by Kidney et al,<sup>55</sup> who studied the effects of theophylline withdrawal in asthmatic patients receiving treatment with both theophylline (>500 mg/day) and high-dose inhaled corticosteroids (mean dose, 1548 mg/day) for at least 6 months. They observed a significant increase of CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the mucosa of the airway caused by withdrawal of theophylline. The reduction of T lymphocytes at the airway with theophylline, as well as with glucocorticoids, could be mediated by apoptosis, as has been described for eosinophils.

## CONCLUSION

Corticosteroids are known to be the most potent anti-inflammatory agents used for asthma treatment. The steroids can induce apoptosis in eosinophils and, less potently, in lymphocytes. Theophylline and PDE IV inhibitors can also induce apoptosis in eosinophils and lymphocytes by means of elevation of intracellular cAMP, which is not involved in apoptosis induced by corticosteroids. Induction of apoptosis in proinflammatory cells, including eosinophils and lymphocytes, may contribute to the anti-inflammatory activity of corticosteroids and probably theophylline in the treatment of

bronchial asthma. Moreover, corticosteroids and theophylline exert additive effects on apoptosis, suggesting that combining the 2 drugs for treating asthma is beneficial, as has been reported clinically.<sup>56,57</sup> Further investigation is required to conclude that induction of apoptosis in eosinophils and lymphocytes is relevant to the clinical effect of glucocorticoids and theophylline (Fig 6). We expect that the use of a drug capable of inducing apoptosis efficiently with high specificity for eosinophils or lymphocytes will be a new and attractive strategy for treating allergic inflammation in asthma and atopic diseases in the future.

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