

## Involvement of Cyclic AMP and Nitric Oxide in Immunoglobulin E–dependent Activation of Fc $\epsilon$ RII/CD23<sup>+</sup> Normal Human Keratinocytes

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### Abstract

Epidermal keratinocytes (EK) are exposed to multiple inflammatory stimuli and paracrine factors secreted by various dermal cells (lymphocytes, mast cells, macrophages, fibroblasts) during wounding, cutaneous allergy, and infections. We have previously demonstrated that after stimulation with interleukin 4 or interferon- $\gamma$ , human EK express the low-affinity receptor for IgE (Fc $\epsilon$ RII/CD23) on their surface. In the present study, we showed that the ligation of CD23 by IgE/anti-IgE immune complexes or specific monoclonal antibody induces a dose-dependent release of interleukin 6 and tumor necrosis factor- $\alpha$  from EK. CD23-ligation activates the nitric oxide–dependent pathway, as demonstrated by the high levels of nitrites released in cell supernatants, and the accumulation of intracellular cyclic nucleotides in EK. These second messengers are required for IgE-dependent stimulation of cytokine production by these cells, inasmuch as this is completely abolished by the use of cAMP or nitric oxide synthase antagonists. Human epithelial keratinocytes may thus participate in IgE-mediated immune responses, through their ability to express functional CD23 antigen. (*J. Clin. Invest.* 1994. 2275–2279.) **Key words:** human keratinocytes • CD23 • nitric oxide • immunoglobulin E • cyclic adenosine monophosphate

### Introduction

Epidermal keratinocytes (EK),<sup>1</sup> which have long been considered as simply a physical barrier, are an important source of proinflammatory cytokines including IL-1, IL-3, IL-6, IL-7, GM-CSF, IFN- $\alpha$  and IFN- $\beta$ , TNF- $\alpha$ , TGF- $\beta$ , and PDGF (1, 2). Most of these factors are not constitutively produced, but can be induced by a variety of nonspecific stimuli (1), includ-

ing chemicals that induce irritant dermatitis, ultraviolet irradiation, tumor-promoting agents, epithelial trauma, and other types of injury. EK may thus be involved in many different inflammatory and immunological skin reactions.

Recently, we have shown that EK (3), like their tumoral counterparts (4), are the only nonhematopoietic cells able to express Fc $\epsilon$ RII/CD23. This antigen is a 45-kD type 2 glycoprotein exhibiting substantial homology with several C-type animal lectins, and is expressed on a variety of hematopoietic cells, including T and B lymphocytes, epidermal Langerhans cells, monocytes/macrophages, eosinophils, and platelets (5). Human CD23 have two isoforms, a and b, differing in their cytoplasmic amino-terminal tail, whereas only CD23a is detected in mice (6). The ligation of CD23a, expressed only in B lymphocytes, triggers progression of B cells in the cell cycle through an intracellular Ca<sup>++</sup> increase (5). CD23b isoform, expressed on B and other hematopoietic cells, was shown to induce an intracellular accumulation of cAMP in human monocytes/macrophages (7). CD23b is the form expressed by EK, after their activation by IL-4 and IFN- $\gamma$  (3), two cytokines secreted during the early phases of the immune response (8).

In the present study, we have assayed the role of CD23 expression in EK and the intracellular signals induced after the ligation of this antigen by IgE/anti-IgE immune complexes or a specific mAb. We evidenced a role for CD23 in IgE-mediated activation of EK-derived IL-6 and TNF- $\alpha$  release. We also showed the involvement of cyclic nucleotides and nitric oxide intracellular transduction pathways in this phenomenon.

### Methods

**Cells.** Human EK were obtained from neonatal foreskins, expanded by *ex vivo* cultures as detailed elsewhere (3). Cytokine production by EK required special culture conditions. The cells must be grown to confluence, and thus be in a low proliferative state. They were therefore incubated in DME medium (Gibco BRL, Cergy-Pontoise, France) for CD23 induction and cytokine release. In fact, we had previously shown that EGF and hydrocortisone downregulated CD23 transcription (3). EGF was also shown to inhibit nitric oxide production by EK (9), an important mediator for cytokine production (see below).

**Induction of CD23 expression in EK by IL-4.** *Ex vivo* expanded EK were transferred to eight-chamber glass slides (Nunc, Roskilde, DK), at 10<sup>5</sup> cells/ml (400  $\mu$ l/well). Half of the wells were treated with 25 ng/ml rhIL-4 (recombinant human IL-4; Immugenex, Los Angeles, CA) for 48 h. The cells were then fixed with acetone at room temperature before staining with the following monoclonal antibodies: anti-cytokeratin (Immunotech, Marseille, France); anti-vimentin (Amersham, Les Ulis, France); and anti-CD23 mAb (clone 135, Ciba-Geigy, Basel, Switzerland). Reactivity of these mAbs was visualized by the

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1. *Abbreviations used in this paper:* EK, epidermal keratinocyte; LNMA, N<sup>G</sup>-monomethyl-L-arginine; NOS, nitric oxide synthase; SNP, sodium nitroprusside.

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immunoperoxidase method (Vector Laboratories, Burlingame, CA) and a subsequent Mayer's hemalum solution staining.

**In situ hybridization for CD23 mRNA.** EK cultures were trypsinized, suspended, and cytocentrifuged at  $5 \times 10^5$  per slide. The slides were then stained with a  $^{35}\text{S}$ -labeled cDNA probe as previously detailed (3). They were also stained with Giemsa thereafter for better cell definition, and examined by light microscopy. As a negative control, EK were also hybridized with the murine IL-3 probe.

**Cytokine production by EK after CD23 ligation.** Confluent cultures of EK were trypsinized and transferred to 24-well plates (Becton Dickinson, Plymouth, UK), at  $8 \times 10^4$  cells/ml per well. They were stimulated by rIL-4 (25 ng/ml), for 48 h. CD23<sup>+</sup> EK were then treated with the anti-CD23 mAb (0–25  $\mu\text{g}/\text{ml}$ ). EK cultures were also supplemented with IgE for 1 h (10  $\mu\text{g}/\text{ml}$ , Stallergene, Paris, France), then human anti-IgE (5–50  $\mu\text{g}/\text{ml}$ , Nordic, Tilburg, The Netherlands) in the presence or the absence of Fab fragments of anti-CD23 mAb (clone 135, 10  $\mu\text{g}/\text{ml}$ ; Ciba-Geigy), a cAMP analogue (dibutyrate-cAMP, 1 mM, Bioblock, Illkirch, France) or a cAMP antagonist (Rp-cAMP, 0.2 mM, Bioblock). Cell supernatants were collected 48 h later and IL-6 and TNF- $\alpha$  levels assayed by specific ELISA (Medgenix, Fleurus, Belgium).

**Determination of cAMP and cGMP content in EK.** CD23<sup>+</sup> EK (previously treated with 25 ng/ml IL-4 for 48 h) were incubated in Eppendorf microtubes ( $4 \times 10^5$  cells/tube) in the presence of IgE (10  $\mu\text{g}/\text{ml}$ ) for 1 h, followed by anti-IgE (50  $\mu\text{g}/\text{ml}$ ), or with anti-CD23 mAb (20  $\mu\text{g}/\text{ml}$ ). The reaction was stopped at different times (0–20 min) by cooling the tubes at  $-170^\circ\text{C}$  for 5 min, and then incubating them 3 min at  $100^\circ\text{C}$ . The tubes were spun at 10,000 g for 5 min, and the supernatants were kept frozen at  $-80^\circ\text{C}$  until cAMP and cGMP measurement by radioimmunoassay (RIA kit, Amersham).

**Nitric oxide synthase (NOS) pathway.** CD23<sup>+</sup> cells were cultured with/without NOS antagonist, *N*<sup>G</sup>-monomethyl-L-arginine (1 mM, LNMMA, Sigma Chemical Co., St. Louis, MO), or with NOS substrate, L-arginine hydrochloride (2 mM, L-arg, Sigma Chemical Co.) for 1 h before treatment with IgE/anti-IgE. The levels of IL-6 and TNF- $\alpha$  were determined in 48 h cell supernatants by ELISA as mentioned above. Nitrites were determined in the same supernatants by the Griess reaction as described elsewhere (10). EK were also stimulated with Sodium nitroprusside (SNP, Sigma Chemical Co.), a NO donor, used at 100  $\mu\text{M}$  final dilution, and the subsequent secretion of cytokines and nitrites was then evaluated.

## Results

**CD23 ligation induces cytokine production by CD23<sup>+</sup> EK.** To induce surface expression of CD23, EK were first treated with rIL-4. Fig. 1 A demonstrates the absence of vimentin-positive cells (fibroblasts) in these cultures, whereas all the cells were cytokeratin positive with diffuse labeling (Fig. 1 B). Fig. 1 also shows that surface CD23 (C and D) and intracellular CD23-mRNA (E and F) are present only in EK treated with IL-4 (D and F). To assay the function of CD23, both CD23<sup>+</sup> and CD23<sup>-</sup> EK were then treated with IgE/anti-IgE or anti-CD23 mAbs and their culture supernatants assayed for TNF- $\alpha$  and IL-6 levels. Data in Fig. 2 clearly show that the ligation of CD23 induces a dose-dependent increase in cytokine production by EK. The IgE-mediated cytokine release is specific of the CD23 ligation, as no cytokine induction is observed in CD23<sup>-</sup> cells. In addition, preincubation of EK with Fab fragments of anti-CD23 mAb, able to block IgE-binding domain of CD23 (11), 1 h before EK stimulation by IgE/anti-IgE, strongly abolishes cytokine release. IgE is then able to activate EK through the ligation of surface CD23.

**Involvement of cyclic nucleotides in CD23-mediated stimulation of cytokine release in EK.** As CD23b ligation induced the

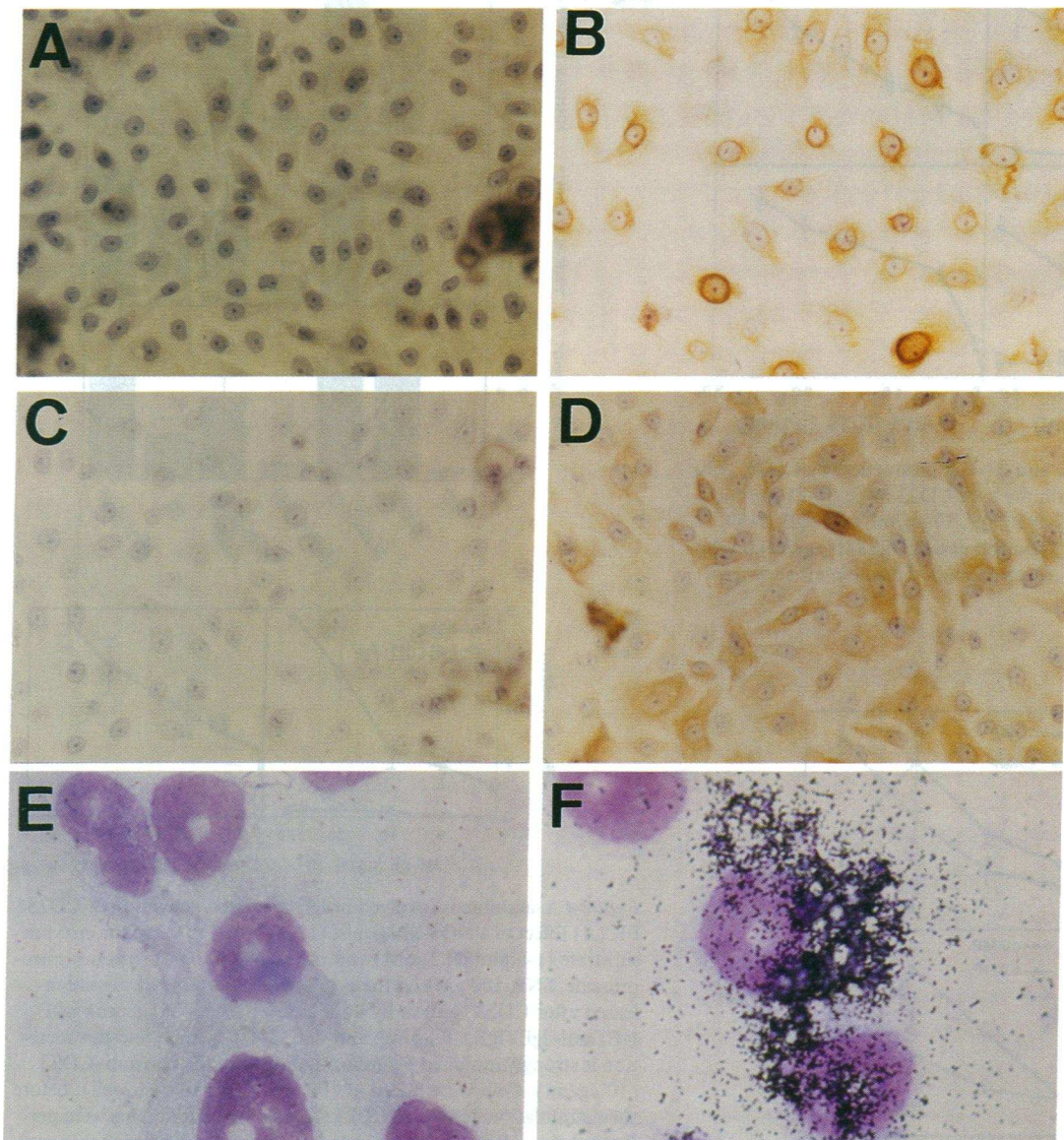
generation of cyclic nucleotides in monocytes (7), we asked whether CD23 could transduce such signal in EK. After CD23 ligation by anti-CD23 mAb or IgE/anti-IgE, a substantial accumulation of cAMP and cGMP was observed (Fig. 3). While cGMP levels peaked at 10 min and then decreased, cAMP levels still increased at 20 min in both culture conditions (Fig. 3 A). To investigate the role of cAMP in cytokine production by EK, we have treated them with Rp-cAMP and But-cAMP, respectively inhibitor and active analogue of cAMP, before CD23 ligation. Data in Fig. 3 B show that Rp-cAMP significantly inhibited cytokine production by IgE activated cells, whereas pretreatment of EK with But-cAMP increased the cytokine levels. On the other hand, But-cAMP alone induced cytokine production by IL-4-primed EK. These data together indicate that cyclic nucleotides are involved in CD23-mediated activation of EK functions.

**Involvement of the NOS pathway in the IgE stimulation of CD23<sup>+</sup> EK.** The rapid increase in cGMP after CD23-ligation led us to investigate the involvement of NOS transduction pathway in EK. In fact, this pathway leads to an accumulation of cGMP, and EK were previously reported to possess NOS (9). Data in Fig. 4 suggest the involvement of a NOS pathway in IgE-mediated EK activation, as pretreatment of CD23<sup>+</sup> EK with 1 mmol LNMMA, an antagonist of NOS, strongly inhibited the IgE-induced cytokine production by these cells. L-arginine, the substrate of NOS, was able to overcome this inhibitory effect (Fig. 4 A). We did not overcome the inhibitory effect of LNMMA by D-arginine (not shown). The addition of SNP, a NO generating chemical, alone to EK, induced the release of significant levels of cytokines, which further supports the role of NO in the above phenomenon (Fig. 4 A). In addition, we have quantified the effect of CD23 ligation on the release of nitrites by EK. Data on Fig. 4 B demonstrate the increased nitrites release from IgE/anti-IgE-stimulated EK, their inhibition by the Fab anti-CD23, and further confirm the involvement of NOS pathway in CD23-mediated EK activation.

**Relationship between cAMP accumulation and NOS pathway.** Involvement of both mediators during IgE-induced cytokine release from EK, led us to investigate the relationship between these two signals. Fig. 5 indicates that these two mediators are interdependent, as inhibitors of cAMP (Rp-cAMP) and of NO (LNMMA), respectively, decrease the levels of nitrites and cAMP generated in IgE-activated cells. In addition, But-cAMP induces nitrite production in EK, while SNP increases intracellular levels of cAMP in these cells. Meanwhile, these levels remain lower than when the cells are directly stimulated by IgE/anti-IgE. Together these data suggest an amplifying role of each signal in the increase of the other one.

## Discussion

We had previously demonstrated that EK could express CD23 when stimulated with IL-4 (3). The present study indicates that Fc $\epsilon$ R2/CD23 expressed by EK is indeed functional and constitutes the first well-defined human surface antigen that directly mediates the activation of the NO-dependent transduction pathway in IL-4-primed EK. In contrast to most immunoglobulin receptors, this 45-kD glycoprotein did not belong to the Ig superfamily but to the C-type animal lectins (5). After proteolysis, soluble fragments of CD23 are produced and detected in normal human serum (5). In addition to IgE-bind-



**Figure 1.** Pure EK were obtained under previously described conditions. This was confirmed by an immunoperoxidase labeling with an anti-vimentin mAb in *A*, which exhibited no staining, and with an anti-cytokeratin mAb in *B*, which showed a diffuse staining. IL-4 induction of CD23 in EK was confirmed either by an immunoperoxidase labeling with an anti-CD23 mAb before (*C*), and after a 48-h IL-4 pretreatment (*D*), or by in situ hybridization for CD23 mRNA expression, before (*E*) and after a 48-h IL-4 pretreatment (*F*).

ing site, CD23 and sCD23 were shown to display other functional domains (11–14), including a lectin region and a site for binding of CD21 antigen (15). The transcription and surface expression of the human b isoform of CD23 are directly induced by IL-4 (8). The present study shows first evidence of a functional role of CD23 as IgE receptors in a nonhematopoietic cell lineage. The activation of EK by IgE is mediated through CD23 ligation as suggested by the following findings: (a) no cytokine induction was observed in IgE/anti-IgE-treated CD23<sup>-</sup> EK; (b) the ligation of CD23 by specific mAb induced similar cytokine levels as did IgE/anti-IgE, while such an effect was not observed with anti-CD19 mAb (isotype-matched control to anti-CD23 mAb; not shown); and (c) the Fab fragments of the anti-CD23 mAb completely inhibited IgE-mediated stimulation of cytokine release from EK. The use

of Fab fragments allows the occupancy of surface CD23 antigens without cell activation. EK are also able to produce sCD23, which has the ability to stimulate mast cell/basophil differentiation and/or histamine release by these cells (14). Therefore, in above activation conditions, CD23 expression by EK may have dual functions: (a) production of inflammatory mediators by these cells through IgE binding; and (b) induction of mast cell/basophil functions through the secretion of soluble forms.

Cyclic nucleotides and NOS are involved in CD23-mediated stimulation of EK, and given our results, these two pathways seem to collaborate in this stimulation. It is now well established that NOS transduction pathway leads to the generation of cGMP in various cellular models (16–18). In EK, cyclic GMP increase preceded the accumulation of cAMP. It is there-

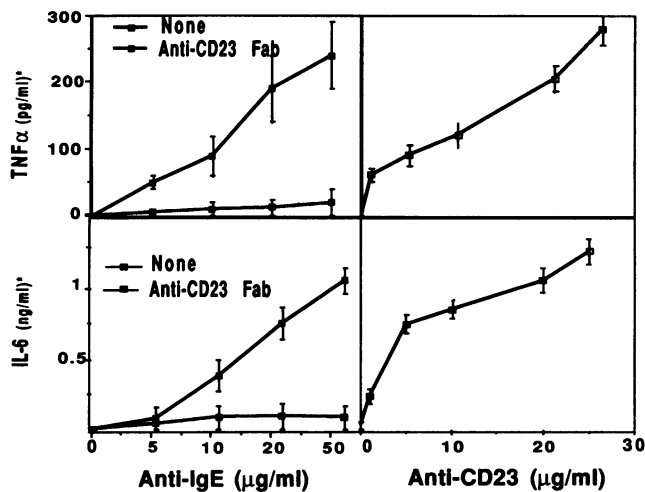


Figure 2. Stimulation of IL-6 and TNF- $\alpha$  production by CD23<sup>+</sup> EK after CD23 ligation, either with the anti-CD23 mAb (0–25  $\mu$ g/ml; left graphs), or with IgE/anti-IgE (IgE, 10  $\mu$ g/ml; anti-IgE, 5–50  $\mu$ g/ml; right graphs). Effect of the Fab anti-CD23 (10  $\mu$ g/ml) on the cytokine release (left graphs). Mean  $\pm$  standard deviation is shown for each value. \*Per  $2 \times 10^5$  cells.

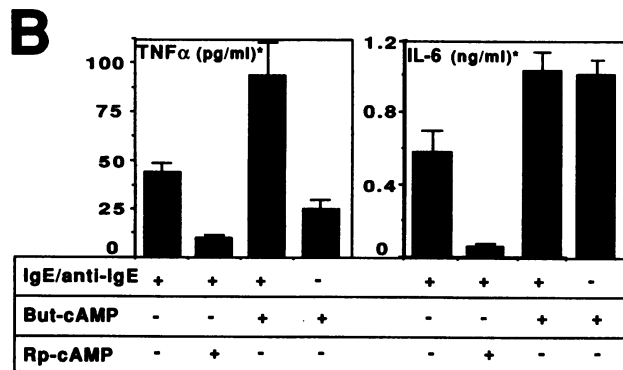
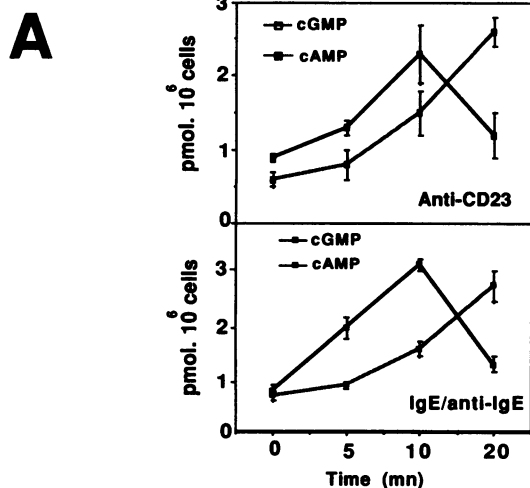


Figure 3. (A) Involvement of cGMP and cAMP in the CD23-mediated activation of CD23<sup>+</sup> EK. (B) Effect of a cAMP antagonist (Rp-cAMP; 0,2 mM) or of a cAMP analogue (dibutyrate-cAMP, But-cAMP, 1 mM) on the cytokine release by these cells. \*Per  $2 \times 10^5$  cells.

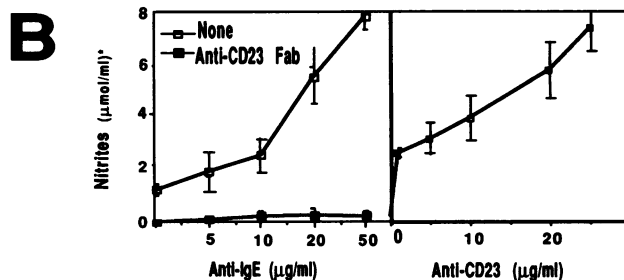
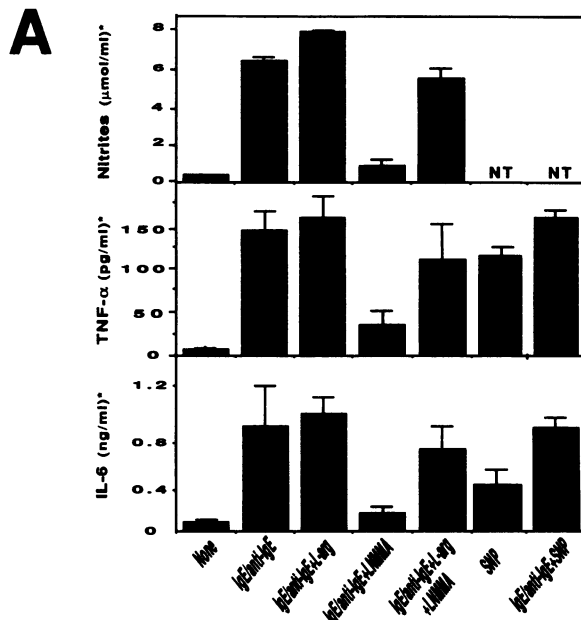
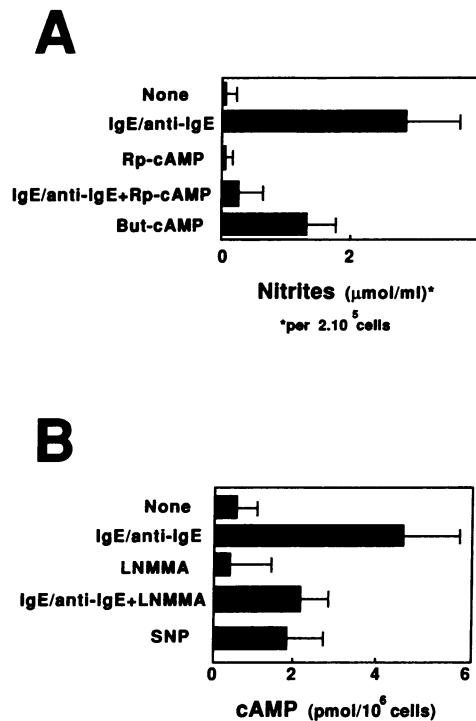


Figure 4. L-arginine is involved in IgE-mediated activation of CD23<sup>+</sup> EK (A) Effect of a NOS antagonist (LNMMA; 1 mM) of the enzyme substrate (L-arginine; 2 mM) and of the NO donor (sodium nitroprusside, SNP, 100  $\mu$ M) on the cytokine release. (B) Nitrites are released after CD23 ligation by both anti-CD23 (0–25  $\mu$ g/ml) and IgE/anti-IgE (IgE, 10  $\mu$ g/ml; anti-IgE, 5–50  $\mu$ g/ml), and this secretion is strongly inhibited by preincubation with the Fab anti-CD23 (10  $\mu$ g/ml). Cells were plated at  $10^5$ /well (1 ml in each well), before their stimulation with IL-4. CD23 stimulation experiments were performed thereafter. The mean number of cells present after this stimulation was near  $2 \times 10^5$  per well. \*Per  $2 \times 10^5$  cells.

fore possible that cAMP accumulation is a result of phosphodiesterase consumption by excess cGMP (19–22). This might explain the decrease of cAMP accumulation in EK after LNMMA addition to EK cultures. Conversely, cAMP seems to be required for the increase of nitrite production. It might be due to the ability of cAMP to stimulate synthesis of inducible NOS enzyme (23). Whatever the mechanism, cAMP, cGMP, and NOS were all reported to be involved in the induction of TNF- $\alpha$  transcription in hematopoietic cells (16, 17, 24). More recently, evidence for inducible NOS has also been presented in patients receiving immunotherapy or during sepsis (25, 26). Implication of CD23 ligation and its related transduction signals may thus explain in vivo persistence of inflammatory mediators in the skin, whereas the NO-dependent pathway could be initiated during the development of CD23-mediated processes.

In human monocytes/macrophages, early reports indicated that CD23 ligation by IgE/anti-IgE induced an accumulation of intracellular cGMP and cAMP (7); on the other hand,



**Figure 5.** Interactions between the NO and the cAMP pathways. (A) Nitrites are released after stimulation of EK with But-cAMP alone, but less than with IgE/anti-IgE alone. Rp-cAMP partially inhibits the release of nitrites by IgE/anti-IgE-stimulated EK. (B) There is an intracellular accumulation of cAMP after stimulation of EK by SNP alone, but less than with IgE/anti-IgE alone. LNMMA partially inhibits this intracellular increase of cAMP in IgE/anti-IgE-stimulated EK.

cGMP analogues directly stimulate TNF- $\alpha$  production by these cells. After CD23 ligation, monocytes produced higher TNF- $\alpha$  levels (17), but comparable IL-6 amounts (7). The similarities between the effect of IgE on monocytes (27, 28) and on EK suggest that CD23b-related transduction signals are identical in these two cell lineages.

Our study also underlines the role of IL-4 as an important early mediator in immune responses, in that this cytokine induces both CD23 and its biological ligand, IgE (8). Subsequent ligation of CD23 by IgE or other potential ligands can thus stimulate the release of inflammation cytokines from EK or other CD23<sup>+</sup> cells. This hypothesis is supported by the simultaneous *in vivo* increase of CD23 expression, serum IgE levels, and IL-4 in allergic patients (7). IgE and IL-4 are also detected in a variety of anti-microbial and anti-tumoral responses (7, 8). It will thus be of interest to investigate the *in situ* CD23 expression and its role in inflamed epithelial cells encountered in these diseases.

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