

Epidermal Cells Enhance Interleukin 4 and Immunoglobulin E Production After Stimulation with Protein Allergen

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Exposure to certain allergens via epithelial tissues is the primary route for the induction of immunoglobulin E-dependent allergies of the immediate type associated with atopic diseases. In order to address the question whether and how epithelial cells might contribute to the induction or increase of T_{H2} -dependent IgE production, we performed co-culture experiments of syngeneic epidermal cells and cells from the associated lymphoid tissue or spleen (responder cells) of BALB/c mice primed with ovalbumin *in vivo*. In the presence of ovalbumin *in vitro*, immunoglobulin E but not immunoglobulin G_{2a} production was significantly enhanced by the addition of epidermal cells, and separation of epidermal cells from responder cells by a membrane that prevented cellular contacts or addition of antibodies against intercellular adhesion molecule-1 reduced the enhancement of immunoglobulin E production induced by epidermal cells. Depletion of major histocompatibility complex

class II⁺ antigen presenting Langerhans cells from the epidermal cells prior to co-culture also reduced the enhancement of immunoglobulin E production induced by epidermal cells. The enhanced immunoglobulin E production was dependent on the induction of T_{H2} cell-derived interleukin-4 detected in co-cultures because it was completely inhibited after addition of anti-interleukin-4 antibodies that also lead to increased immunoglobulin G_{2a} production. Whereas interleukin-4 was not produced by epidermal cells, interleukin-10 seemed to be one important mediator contributed by epidermal cells. Interleukin-10 skewed the response toward a T_{H2} -mediated IgE response because antibodies against interleukin-10 inhibited interleukin-4 and immunoglobulin E production, whereas they enhanced interferon- γ and immunoglobulin G_{2a} production. **Key words:** epidermis/cytokines/IL-10/ovalbumin. *J Invest Dermatol* 107:582-588, 1996

Epithelial tissues may provide favorable conditions for the induction, development, and maintenance of T_{H2} cells, which produce interleukin-4 (IL-4) and provide help for production of immunoglobulin E (IgE) (Benner and Savelkoul, 1991; Del Prete *et al*, 1991; Romagnani, 1992; Vercelli, 1993) leading to allergies of the immediate type associated with atopic diseases (Del Prete, 1992; Muller *et al*, 1993). This assumption is supported by the fact that allergen-specific IgE production and allergies of the immediate type can be induced experimentally via epithelial tissues without the help of adjuvants (McMenamin *et al*, 1991; Renz *et al*, 1992; McMenamin and Holt, 1993; Saloga *et al*, 1994). Also, atopic diseases manifest in epithelial tissues as T_{H2} -dominated "allergic inflammation" (van der Heijden *et al*, 1991; Sager *et al*, 1992). These tissues are also exposed to allergens implicated in the pathogenesis of these diseases.

Recently, we were able to demonstrate that ovalbumin (OVA)-specific IgE responses and other manifestations of immediate hypersensitivity can be induced through the skin in a mouse model (Saloga *et al*, 1994). Therefore, and because exposure to this allergen can be controlled easily in mice fed an OVA-free diet, we chose this allergen to explore the influence of epithelial cells on induction or enhancement of allergen-specific IgE responses. We employed an *in vitro* surrogate designed to mirror the *in vivo* situation as well to allow manipulation. *In vivo*, cytokines and other factors produced by epidermal cells (mainly keratinocytes) may prime migrating epidermal cells or may reach the local draining lymph nodes via the afferent lymph stream, especially under inflammatory conditions. Migrating epidermal cells such as Langerhans cells reach the local draining lymph nodes and can deliver additional signals, including contact signals (Kripke *et al*, 1990). We therefore co-cultured epidermal cells and "responder cells," i.e., lymph node (LN) or spleen cells, in order to analyze the potential influence of different cell populations as well as the importance of contact and soluble signals that may be involved in the induction and reactivation of T_{H2} -type cells (IL-4 production) and IgE responses. We demonstrate in this study that epidermal cells preferentially enhance allergen-driven IL-4 and IgE production in the context of a protein allergen like OVA.

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Abbreviations: DC, dendritic cells; EC, epidermal cells; EU, ELISA units; LC, Langerhans cells; OVA, ovalbumin; RC, responder cells; DIG, dioxigenin.

MATERIALS AND METHODS

Experimental Animals Female BALB/c mice of 8–10 wk of age originally obtained from the Zentralinstitut für Versuchstierforschung (Hannover, Germany) were used in all experiments.

Priming Procedure Mice receiving an OVA-free diet were primed by intraperitoneal injection of 100 μ g (unless indicated otherwise) of OVA Grade IV (Sigma, Deisenhofen, Germany) adsorbed to 100 μ g of aluminum hydroxide (Merck, Darmstadt, Germany) diluted in 0.9% NaCl. Ten days later these mice were boosted with 100 μ g (unless otherwise indicated) of OVA intraperitoneally. Control groups received NaCl alone. Sera were collected at day 14 and stored at -20°C until determination of OVA-specific IgE and IgG titers.

Determination of OVA-Specific Antibody Titers in the Serum Anti-OVA IgE and IgG antibody concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (Saloga *et al*, 1994). Readings were compared to OVA-specific standards generated in the laboratory as described previously (Renz *et al*, 1992) and arbitrarily assigned 1000 ELISA units (EU per ml).

Cell Preparation Spleen and local draining LN cells (responder cells, RC) were prepared from OVA-primed mice or control animals under sterile conditions 15 d after the first injection. The tissue was dispersed through stainless steel mesh, and lymph node single cell suspensions were washed twice with tissue culture medium (see cell culture). Splenic cells were purified by mouse Lympholyte (CedarLane, Hornby, Ontario, Canada) density centrifugation to obtain mononuclear cells and washed two times with tissue culture medium.

Epidermal cells (EC) were prepared from ears of non-primed mice by trypsinization with 0.5% trypsin (United States Biochemicals, Bad Homburg, Germany) diluted in phosphate-buffered saline (PBS) for 40 min at 37°C . The epidermis then was removed from the dermis, incubated in PBS with 3000 U of DNase (Sigma, St. Louis, MO) for 10 min, mechanically disintegrated with a 50 ml syringe, and filtered through sterile gauze. Thereafter, epidermal cells were washed twice and resuspended in tissue culture medium. For some experiments major histocompatibility complex (MHC) class II⁺ Langerhans cells were removed from the EC suspension with the anti-mouse I-A/I-E monoclonal antibody M5/114.15.2 [rat IgG_{2b}, American Type Culture Collection (ATCC), Rockville, MD] and sheep anti-rat IgG magnetic beads M-450 (Dynal, Hamburg, Germany). No MHC class II⁺ cells could be detected by flow cytometry after three cycles of magnetic cell separation.

The mouse keratinocyte line PAM 212 (kindly provided by Dr. Stuart Yuspa, NIH, Bethesda, MD) was cultivated in tissue culture medium in 75-cm² tissue culture flasks (5×10^5 cells per ml).

For preparation of dermal fibroblasts, after incubation with 0.5% trypsin for 40 min at 37°C , the dermis (see above) was incubated in PBS containing 0.2% collagenase (Boehringer Mannheim, Mannheim, Germany) for 15 min at 37°C , and dispersed through stainless steel mesh. After addition of 3000 U of DNase (Sigma) for 10 min, the resulting dermal cells were washed twice and cultivated in 75-cm² tissue culture flasks (1×10^6 cells per ml). Tissue culture medium was changed every other day after removal of non-adherent cells. On day 8 the remaining adherent fibroblasts were harvested with 0.05% trypsin, 0.02% EDTA for 3 min at 37°C , stopped with 10% fetal bovine serum, washed twice, and resuspended in tissue culture medium.

Bone marrow dendritic cells (DC) were prepared according to a modification of a protocol described previously (Inaba *et al*, 1992). Briefly, femurs and tibias of BALB/c mice of 7 wk of age were removed, and the marrow was flushed out with a syringe. The tissue was suspended in RPMI and passed through nylon mesh, and red cells were lysed with 0.15 M NH₄Cl. After washing, lymphocytes, granulocytes, and I-A⁺ cells were depleted with monoclonal antibodies (mAbs) and low-tox-M rabbit complement (CedarLane). The mAbs were anti-mouse I-A/I-E M5/114.15.2 (rat IgG_{2b}, ATCC), anti-mouse CD4 GK1.5 (rat IgG_{2b}, ATCC), anti-mouse CD8 α 53-6.72 (rat IgG_{2a}, ATCC), anti-mouse granulocyte RB6 (hamster IgG), and anti-mouse B220 RA3-3A1/6.1 (rat IgM, ATCC). Cells (1×10^6 per ml) were placed in 24-well plates in 1.5 ml of tissue culture medium supplemented with 5 ng per ml granulocyte-macrophage colony stimulating factor (R&D Systems, Abingdon, U.K.). The cultures were fed every 2 d by aspirating 1 ml of medium and adding back fresh medium with granulocyte-macrophage colony stimulating factor. On day 7 the aggregates of attached cells were dislodged of the adherent macrophages, centrifuged, and subcultured at 1×10^6 per ml in fresh medium with granulocyte-macrophage colony stimulating factor in a tissue culture petri dish. After overnight culture, clusters of dendritic cells were released.

Flow Cytometry Surface phenotyping of EC, DC, and RC was performed by staining 10^6 cells with marker-specific mAb for 30 min on ice. Antibodies used were anti-mouse CD3 ϵ 145-2.C11 (hamster IgG, ATCC), anti-mouse CD4 GK1.5 (rat IgG_{2b}, ATCC), anti-mouse CD8 α 53-6.72 (rat IgG_{2a}, ATCC), anti-mouse granulocyte RB6 (hamster IgG), anti-mouse dendritic cell subpopulation 33D1 (rat IgG_{2b}, ATCC), 4F7 (rat IgG₁; Mohamadzadeh *et al*, 1993), and N418 (hamster IgG, ATCC); NLDC145 (rat IgG_{2a}, Dianova, Hamburg, Germany); anti-mouse macrophage and dendritic cell F4/80 (rat IgG_{2b}, ATCC); anti-mouse B7-1 (rat IgG_{2a}, PharMingen, San Diego, CA), anti-mouse B7-2 (rat IgG_{2a}, PharMingen), anti-mouse I-A/I-E M5/114.15.2 (rat IgG_{2b}, ATCC), and fluorescein isothiocyanate-conjugated Ly-5 B220 (rat anti-mouse IgG_{2a}, Caltag Laboratories, South San Francisco, CA). After washing in PBS supplemented with 2% heat-inactivated fetal bovine serum (Gibco BRL, Paisley, Scotland), the cells were stained with phycoerythrin-conjugated anti-rat IgG, phycoerythrin-conjugated anti-hamster IgG (both from Southern Biotechnology, Birmingham, U.K.), or fluorescein isothiocyanate-conjugated goat anti-Syrian hamster IgG (H+L) (Jackson ImmunoResearch Laboratories, Baltimore, MD) for 15 min on ice. Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) equipped with Lysis II software.

Cell Culture Culture medium consisted of RPMI 1640 (Gibco BRL) with L-glutamine (2 mM) supplemented with streptomycin (100 μ g per ml), penicillin (100 U per ml), 2-mercaptoethanol (5 mM), HEPES buffer (20 mM), Na-pyruvate (1 mM), Na-hydrogencarbonate (24 mM), and 5% heat-inactivated fetal bovine serum (Gibco BRL). Incubator conditions were 37°C and 5% CO₂.

For some experiments monoclonal antibodies were added to the cell cultures: anti-mouse intercellular adhesion molecule-1 YN1/1.7 (rat IgG_{2b}, ATCC), anti-mouse B7-1 and B7-2 (human CTLA4-Ig, kindly provided by Dr. P. Lane, Institute for Immunology, Basel, Switzerland), anti-mouse IL-4 (11B11, rat IgG₁, ATCC), and anti-mouse IL-10 (JES5-2A5, rat IgG₁, PharMingen). Murine rIL-12 was kindly provided by Dr. Maurice K. Gately (Hoffmann-La Roche, Nutley, NJ).

For *in vitro* cytokine and immunoglobulin production assay, 1×10^6 lymph node or spleen cells were incubated in 24-well tissue-culture plates at a final volume of 1 ml per well in co-culture with none, 1×10^3 , 1×10^4 , or 1×10^5 epidermal cells. OVA was added at a final concentration of 50 μ g per ml and pokeweed mitogen as positive control at a final concentration of 4 μ g per ml. For determination of cytokine production, 300 μ l of cell-free culture supernatant was harvested at day 4 of the culture period and stored at -70°C until analysis; at the same time 300 μ l of fresh medium was added back to the culture. After 10 additional days of culture, remaining supernatants were harvested and stored at -20°C for determination of immunoglobulin production.

For proliferation assays, 2×10^5 LN or spleen cells were incubated in 96-well, flat-bottom, tissue culture plates in 200 μ l per well in triplicate for 4 d. OVA was added at a final concentration of 50 μ g per ml and concanavalin A as positive control at a final concentration of 4 μ g per ml.

Determination of Proliferation One microcurie of [³H]thymidine (ICN, Irvine, CA; 5 mCi per ml) was added to the culture for 6–8 h. Cells were harvested with a Skatron AS cell harvester (Skatron, Norway), and [³H]thymidine incorporation was measured with a liquid scintillation counter 1205 Betaplate (LKB Wallac, Turku, Finland) after addition of scintillation liquid (Roth, Karlsruhe, Germany).

Determination of Cytokine Contents in Tissue Culture Supernatants Mouse IL-4, IL-10, and interferon- γ (IFN- γ) protein concentrations were measured by ELISA of the employed pairs of antibodies according to instructions of the manufacturer (PharMingen). The limit of detection was 0.5 pg per ml for IL-4, 10 U per ml for IL-10, and 10 U per ml for IFN- γ .

Determination of Immunoglobulin Contents in Tissue Culture Supernatants The concentration of total IgE and IgG was measured by ELISA. Maxisorp Nunc-Immunoplates (Nunc GmbH, Wiesbaden, Germany) were coated with 45 μ g anti-mouse IgE per ml (purified sheep IgE-IgG polyclonal Ab; The Binding Site, Birmingham, England) or 5 μ g anti-mouse IgG (H+L) per ml (purified rat polyclonal Ab, Jackson ImmunoResearch) diluted in 0.1 M NaHCO₃, pH 9.6 overnight at 4°C . The next day the plates were washed twice with PBS and wells were blocked with 0.2% (wt/vol) gelatin-Tris(hydroxymethyl)aminomethane buffer (0.1 M, pH 8.2) containing 0.05% Tween-20 for 2 h at 37°C . Serial dilutions of supernatants and mouse IgG or IgE standard (PharMingen) were incubated overnight at 4°C . After plates were washed twice, they were incubated with alkaline phosphatase-conjugated rat anti-mouse IgE, IgG₁, or IgG_{2a} mAbs (PharMingen) for 2 h at 37°C . After development with phosphatase substrate (Sigma), plates were read in a microplate ELISA reader model 450

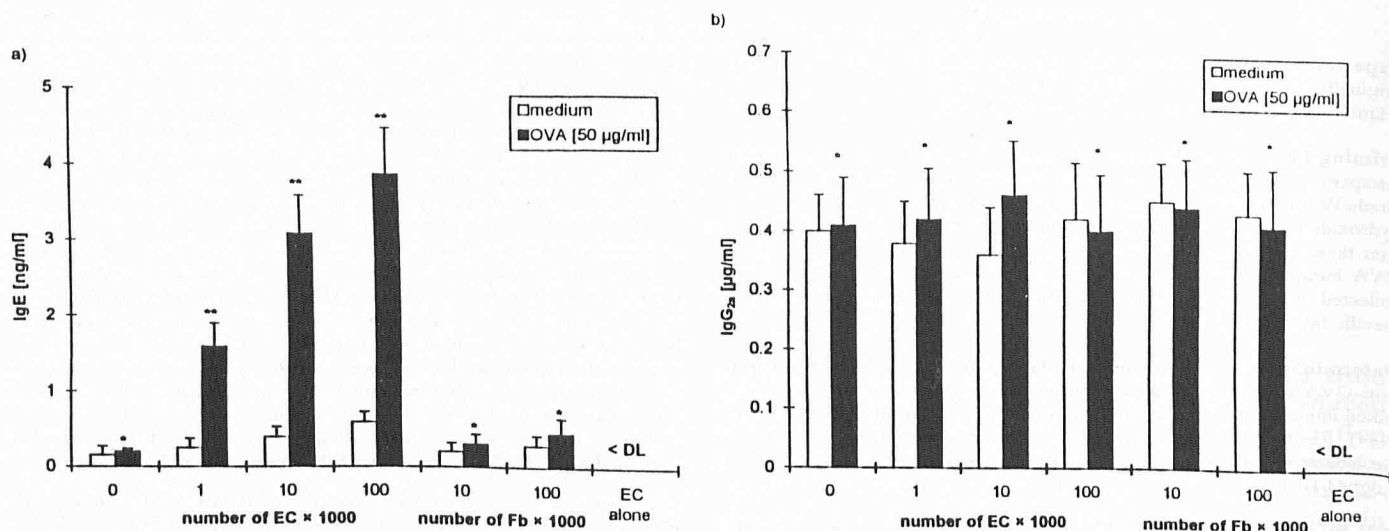


Figure 1. Concentration-dependent preferential enhancement of IgE production induced by epidermal cells in the context of OVA. 10^6 spleen cells from mice primed with 100 μ g of OVA were prepared and incubated in co-culture with none, 1×10^3 , 1×10^4 , or 1×10^5 EC or 1×10^4 or 1×10^5 dermal fibroblasts (Fb). After 14 d of culture with or without OVA stimulation (50 μ g per ml) supernatants were harvested and (a) IgE and (b) IgG_{2a} were measured by ELISA as described in *Materials and Methods*. The data represent the means $\pm$ SD from 20 experiments. * indicates results that do not differ from spontaneous Ig production of spleen cells alone, and ** indicates results that differ significantly ($p < 0.05$).

(Bio-Rad Laboratories GmbH, München, Germany) at 405 nm, and amounts of immunoglobulin were calculated according to the standard curves. The limit of detection was 1.25 ng per ml for IgG₁, 5 ng per ml for IgG_{2a}, and 300 pg per ml for IgE.

Reverse Transcriptase-Polymerase Chain Reaction (PCR) and Southern Blot Hybridization for IL-10 For total cellular ribonucleic acid (RNA) extraction, 5×10^6 RC together with 5×10^5 EC, 5×10^6 EC alone, or 5×10^6 MHC class II-depleted EC alone were incubated in 24-well tissue culture plates at a final volume of 1 ml per well. OVA was added at a final concentration of 50 μ g per ml. After 1–24 h cells were harvested and total cellular RNA was extracted with RNAzol B (Paesel & Lorei, Frankfurt, Germany) according to the manufacturer's instructions; 100 ng of the extracted RNA was reverse transcribed and the complementary deoxyribonucleic acid was amplified by 25 cycles using the Perkin-Elmer reverse transcriptase-PCR kit (Perkin-Elmer, Überlingen, Germany) in a GeneAmp PCR System 9600 machine (Perkin-Elmer). The cycling conditions chosen were 1 min at 95°C (denaturation), 1.5 min at 57°C (annealing), and 2 min at 72°C (extension). The primer sequences for IL-10 were as follows: 3' CAT CTA TGC TTC TAT GCA GTT G and 5' CCC AGA AAT CAA GGA GCA TTT G. In each experiment, amplifications were performed without addition of RNA and primers as a negative control. PCR products were separated for 1 h by agarose gel electrophoresis (1.5%) and blotted on a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) by a Southern transfer technique using $20 \times$ saline-sodium citrate buffer. After immobilization of the DNA, a non-isotopic Southern blot hybridization was performed with a specific internal digoxigenin (DIG)-labeled probe (int GCT GGA AGA CCA CTA CAA CCC) to confirm the origin of the PCR products using the DIG oligonucleotide tailing kit (Boehringer Mannheim). The DIG-labeled nucleic acids were detected by enzyme-linked immunoassay using an anti-DIG alkaline phosphatase conjugate, a luminescence substrate (DIG luminescent detection kit, Boehringer Mannheim), and subsequent exposure to x-ray film (Amersham, Buckinghamshire, U.K.).

Statistics The Student's *t* test was employed to test statistical significance of results ($p < 0.05$).

RESULTS

Priming with High Doses of OVA Leads to Sensitization to OVA with Very Limited Allergen-Specific IgE Production *In Vivo* Without priming of animals with allergen *in vivo*, no allergen-specific immune responses were observed *in vitro* (data not shown). As it was the aim of this study to analyze the effects of epithelial cells on the induction/activation of T_{H2} cells leading to IgE production, spontaneous allergen-specific IgE production

should be limited in allergen-primed animals and responder cells obtained from them. Therefore, we chose a priming protocol using high doses of OVA, i.e., intraperitoneal injections of 100 μ g. This led to sensitization of primed mice to OVA as assessed by increased allergen-induced proliferative responses [2351 ± 258 cpm *versus* 8498 ± 1541 cpm (mean $\pm$ SD) in naive *versus* OVA-primed mice) and OVA-specific IgG production [61 ± 26 ELISA units (EU) per ml *versus* 1140 ± 396 EU per ml] detected in the sera of primed mice by ELISA, whereas OVA-specific IgE levels remained low (53 ± 24 EU per ml *versus* 410 ± 112 EU per ml in naive *versus* OVA-primed mice) compared to animals primed with 10 μ g of OVA (1052 ± 316 EU per ml).

EC Preferentially Enhance Allergen-Driven IgE Production of RC from Primed Animals *In Vitro* in the Context of OVA RC from OVA-primed mice spontaneously produced substantial amounts of IgG_{2a} *in vitro* whereas allergen-driven IgE production was very limited (Fig 1a,b). This was in accordance with the titers of allergen-specific IgG and IgE measured in the sera of donor mice as described above.

Addition of unseparated epidermal cells (keratinocytes including 2–5% I-A⁺ Langerhans cells and 1–2% γ/δ dendritic epidermal T cells) resulted in a concentration-dependent 10-fold enhancement of allergen-driven IgE production of RC [containing 30–40% T cells (20–25% CD4⁺, 10–15% CD8⁺) and 50–60% B cells], but had little effect on IgG_{2a} production (Fig 1a,b). Addition of the same number of dermal fibroblasts to RC cultures did not enhance IgE or IgG_{2a} production (Fig 1a,b). Kinetic studies revealed that enhanced IgE production induced by EC was detectable after day 10 (data not shown).

MHC Class II⁺ Langerhans Cells Play a Key Role for the Enhancement of IgE Production by EC, but Dendritic Cells Without Exposure to Keratinocytes Do Not Have the Same Bias to Support Only an IgE Response In order to analyze which cells among unseparated EC are important for the enhanced IgE production by EC, we depleted subpopulations of EC prior to co-culture with RC. Depletion of I-A⁺ dendritic Langerhans cells by magnetic beads led to a reduction of 70% in EC induced allergen-driven IgE production compared to unseparated EC, but IgE production was still significantly higher than without EC (Fig 2). Depletion of I-A⁺ cells from the RC did not significantly impact

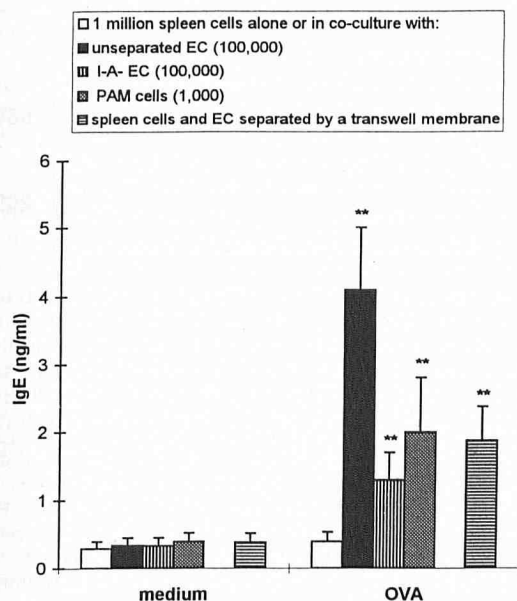


Figure 2. Importance of certain cell types and direct co-culture for induction of IgE production by EC. In a second set of experiments MHC class II⁺ Langerhans cells were depleted from EC with magnetic beads prior to co-culture. In a third set of experiments EC were replaced by 1×10^5 cells of the mouse keratinocyte line PAM 212. In a fourth set of experiments EC and spleen cells were separated by a transwell membrane by placing 1×10^6 spleen cells in the lower chamber and 1×10^5 EC in the upper chamber. Supernatants from these different approaches were harvested at day 14 and analyzed for IgE production with or without allergen stimulation. Shown are the means $\pm$ SD from five experiments. ** indicates results that differ significantly ($p < 0.05$) from spontaneous IgE production of spleen cells alone.

IgE production of RC when EC preparations containing I-A⁺ cells were used (data not shown). When EC were replaced by the PAM 212 mouse keratinocyte line, enhancement of IgE production was also much less than with unseparated EC, but still significantly higher than without EC or PAM cells (Fig 2).

In order to investigate whether predominant induction of IgE responses is primarily an intrinsic property of dendritic cells, at least in the context of a protein allergen such as OVA, or whether keratinocytes contribute to this phenomenon, we used bone marrow dendritic cells (DC) instead of EC in the same experiment. As determined by flow cytometry, DC preparations were 95% pure and free of B-cells (B220), T-cells (CD3), granulocytes (RB6), and expressed markers specific for DC (subpopulation) such as 33D1-, 4F7-, NLDC145-, N418-antigen, B7-1 and 2, and MHC class II. When these DC were used instead of EC, production of both IgE and IgG_{2a} was induced in the presence of OVA (Fig 3).

Cellular Contact Is Important for the Enhancement of IgE Production Induced by EC (Including Migrating Langerhans Cells) To determine whether direct cellular contact between RC and EC is necessary for the enhancement of IgE production, we separated EC and RC by a transwell membrane that prevented cellular contact but allowed diffusion of soluble mediators. The enhancement of IgE production induced by epidermal cells was reduced to about 50% under these conditions (RC were placed in the lower chamber and EC in the upper chamber) but was still significant compared to IgE production without EC (Fig 2).

To analyze further which contact signals might be involved in the enhancement of IgE production by EC, we returned to our direct co-culture system and added antibodies against the adhesion molecule intercellular adhesion molecule-1 or the fusion protein CTLA4-Ig, which binds to the co-stimulatory molecules B7-1 and B7-2, to the cultures at the beginning of the experiment. The fusion

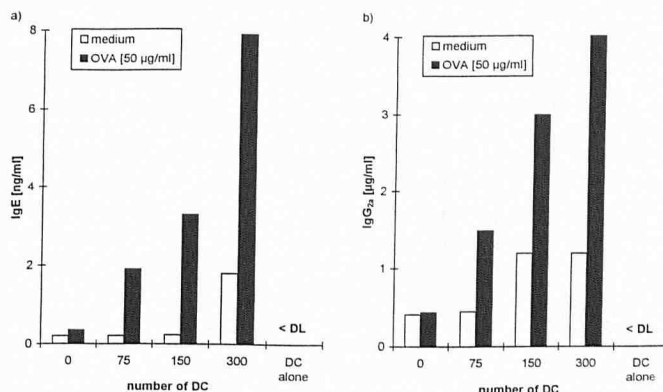


Figure 3. Induction of both IgE and IgG_{2a} production by DC from the bone marrow. Spleen cells (10^6) from mice primed with 100 μ g of OVA were prepared and incubated in co-culture with none, 75, 150, or 300 bone marrow-derived dendritic cells generated as described in *Materials and Methods*. After 14 d of culture with or without OVA stimulation (50 μ g per ml), supernatants were harvested and (a) IgE and (b) IgG_{2a} were measured by ELISA as described in *Materials and Methods*. The data represent one of three experiments.

protein against co-stimulatory molecules inhibited EC-induced IgE production only up to 40% and only at high concentrations (>10 μ g per ml, data not shown). Antibodies against intercellular adhesion molecule-1, however, inhibited IgE production almost completely already at concentrations of 10 μ g per ml (data not shown). The addition of isotype control antibodies did not affect the enhanced IgE production.

IL-4 Production Is Induced by EC and Plays a Critical Role for the Induction of IgE Production Because IL-4 is the most important inducer and enhancer of IgE production (Vercelli and Geha, 1989; Vercelli *et al*, 1989), we investigated the induction of IL-4 in our culture system. Maximal IL-4 production was observed after 4 d of culture, when a concentration-dependent induction of IL-4 production was observed after addition of EC in response to allergen in primed mice (Fig 4a). No induction of IL-4 production by EC was observed in control animals (data not shown), and omission of OVA *in vitro* also resulted in lack of IL-4 induction by added EC (Fig 4a). EC alone did not produce IL-4. In parallel with IL-4, we also measured IFN- γ in the culture supernatants under different experimental conditions but could only detect this cytokine in response to the positive control pokeweed mitogen (data not shown).

In order to investigate whether IL-4 is critical for EC-induced enhanced IgE production, we performed blocking experiments with an anti-IL-4 antibody. The addition of anti-IL-4 mAb resulted in a concentration-dependent inhibition of IgE production measured by ELISA in supernatants of 14 d cultures (Fig 4b), whereas addition of the same amount of isotype control antibody had no effect. IgG_{2a} production was increased in the presence of anti-IL-4 antibodies (data not shown).

IL-10 mRNA Is Produced by EC and IL-10 Protein Contributes to Preferential IL-4 and IgE Induction by EC As production of IL-10 by murine keratinocytes has been described previously (Enk and Katz, 1992a), we addressed the question whether this cytokine may be involved in the preferential induction of IL-4 and IgE by EC. Addition of blocking anti-IL-10 antibodies to our co-culture system with EC and RC resulted in decreased IgE production (Fig 4b) and increased IgG_{2a} production measured after 14 d of culture (Fig 5b). It also resulted in decreased IL-4 production (Fig 4a) and increased IFN- γ production measured on day 4 of cell culture (Fig 5a). Addition of isotype control antibodies did not affect immunoglobulin or cytokine production (Fig 4b).

Although IL-10 could not be detected by ELISA (data not

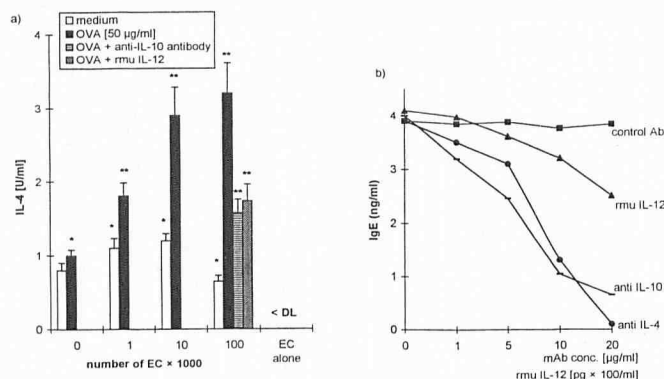


Figure 4. Importance of different soluble factors for induction of IgE production. (a) At day 4 of co-culture of 1×10^6 spleen cells together with none, 1×10^3 , 1×10^4 , or 1×10^5 EC supernatants were harvested for determination of IL-4 production with or without OVA stimulation. In separate sets of experiments anti-IL-10 antibodies (10 µg per ml) or murine rIL-12 (1 ng per ml) were added to OVA stimulated cultures from the beginning. Error bars, SD. (b) IgE production was measured in 14 d supernatants after addition of antibodies against IL-4 or IL-10 or after addition of murine rIL-12 to OVA stimulated co-cultures. The results represent the means of three experiments. * indicates results that do not differ significantly from baseline IL-4 production of OVA-primed spleen cells ($p > 0.05$), and ** indicates results that differ significantly ($p < 0.05$).

shown), the effects of the anti-IL-10 antibody indicated that this cytokine was present in the cultures. To increase sensitivity and to demonstrate active production we employed reverse transcriptase-PCR to detect IL-10 mRNA in cellular extracts of cultured EC and RC. IL-10 mRNA was detectable after 1 h in co-cultures as well as in cultures of EC alone and even in MHC class II-depleted EC after stimulation with OVA *in vitro* (Fig 6), indicating that this cytokine is produced by EC (keratinocytes) early on under these experimental conditions. The increase of IL-10 RNA expression after stimulation with OVA was not observed, however, in MHC class II-depleted EC cultures (Fig 6).

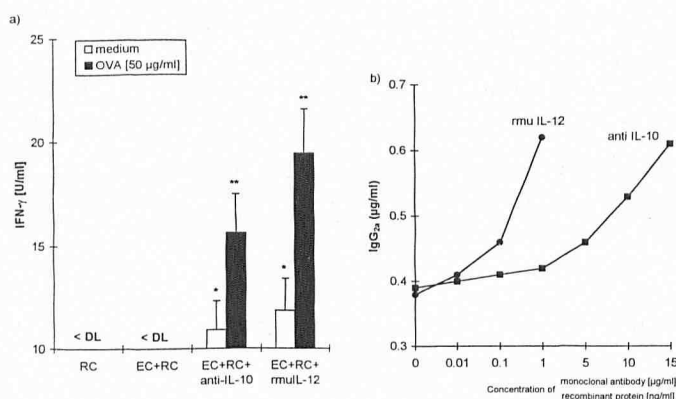


Figure 5. Addition of anti-IL-10 antibodies or IL-12 protein enhances IFN-γ and IgG_{2a} production. (a) At day 4 of the co-culture of 1×10^6 spleen cells together with none, 1×10^3 , 1×10^4 , or 1×10^5 EC supernatants were harvested for determination of IFN-γ production with or without OVA stimulation. In separate sets of experiments anti-IL-10 antibodies (10 µg per ml) or murine rIL-12 (1 ng per ml) were added to the co-culture system. (b) IgG_{2a} production was measured by ELISA in 14-d supernatants after addition of antibodies against IL-10 or after addition of murine rIL-12 to OVA stimulated co-cultures. Shown are the means ± SD from four experiments. * indicates results that do not differ significantly from IFN-γ production of OVA-primed spleen cells ($p > 0.05$), and ** indicates results that differ significantly ($p < 0.05$).

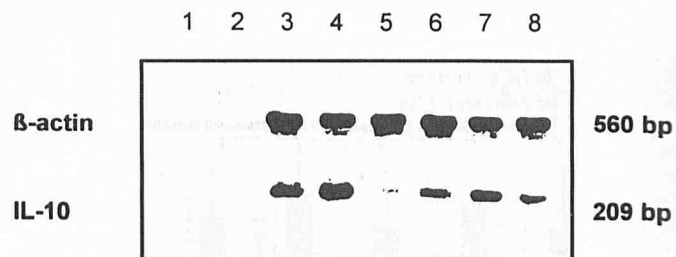


Figure 6. IL-10 mRNA production by spleen cells together with EC, EC alone, and MHC class II-depleted EC alone. Total cellular RNA was extracted 1 h after culture of 5×10^6 spleen cells together with 5×10^5 EC, 5×10^6 EC alone, or 5×10^6 MHC class II-depleted EC with or without OVA stimulation (50 µg per ml). Reverse transcriptase-PCR and Southern blot hybridization were performed as described in Materials and Methods. Shown are the signals for β-actin (560-bp template) and IL-10 (209-bp template) of the same RNA probes. Lane 1, negative control (RNA without primer); lane 2, negative control (primer without RNA); lane 3, spleen cells + EC without OVA stimulation; lane 4, spleen cells + EC with OVA stimulation; lane 5, EC alone without OVA stimulation; lane 6, EC alone with OVA stimulation; lane 7, MHC class II-depleted EC without OVA stimulation; lane 8, MHC class II-depleted EC with OVA stimulation.

Addition of Exogenous IL-12 Inhibits IL-4 and IgE Production and Enhances IFN-γ and IgG_{2a} Production In order to investigate the effect of IL-12, which is known to favor the development of T_{H1}-responses (Brunda, 1994; Modlin, 1994; Morris *et al*, 1994), in our co-culture system, we added exogenous recombinant murine IL-12 at the beginning of the culture period. Addition of recombinant murine IL-12 resulted in diminished IL-4 production (Fig 4a) and enhanced IFN-γ production (Fig 5a) at day 4 of culture. Accordingly, addition of IL-12 also led to a concentration-dependent inhibition of IgE production (Fig 4b) and increased IgG_{2a} (Fig 5b) measured by ELISA in supernatants of 14 d cultures.

DISCUSSION

Our data indicate that ECs strongly enhance T_{H2}-dependent allergen-driven IgE production in the context of a protein allergen, OVA. In conjunction with tissue-specific homing of T cells (Picker *et al*, 1993; Picker, 1994), preferential induction of T_{H2}-type cells in epithelial tissues during initial encounter of antigen (often aero-allergens) may result in long-lasting, repeated return of such T_{H2} cells to particular epithelial sites sustaining "allergic inflammation," suggesting that this mechanism may be of importance in the understanding of atopic diseases.

EC strongly induced IgE production only when the specific allergen OVA was also present *in vitro*, indicating that IgE production is at least allergen-driven if not OVA-specific. Measurement of OVA-specific IgE production was not possible *in vitro*, as the allergen had to be present *in vitro* to induce and sustain IgE production also occupying the OVA-specific binding sites of IgE produced, preventing measurement of OVA-specific IgE levels. In contrast to EC, dermal cells were unable to induce IgE with or without OVA in the culture, indicating that the enhancement of IgE production was a specific capacity of the epithelium and not the underlying tissue. EC alone produced no Ig, excluding passive contamination of EC with Ig carried over into the co-cultures.

Especially striking was the ability of EC to induce IgE but not IgG_{2a}. IgG_{2a} production by RC was not maximal to begin with and could be further enhanced, because addition of antibodies against IL-10, exogenous murine rIL-12, or bone marrow-derived dendritic cells increased IgG_{2a} production strongly, and also induced IFN-γ, which was previously undetectable. In addition to IgE, the T_{H2}-cytokine IL-4 was detected by ELISA in the cell cultures. Taken together, the data indicate that T_{H2}-type cells are induced or activated to produce IL-4 and induce or increase IgE production in an allergen-specific way, whereas T_{H1}-type cells were not induced

or activated after the addition of EC in the context of the protein allergen OVA.

The analysis of the cell types among EC important for the enhancement of IgE production revealed that MHC class II⁺ Langerhans cells play a key role, as depletion of MHC class II⁺ cells from EC prior to co-culture with RC led to a significant decrease in allergen-driven IgE but did not abrogate it. Langerhans cells are highly potent antigen-presenting cells and have been shown earlier to be able to induce T_{H2}-type responses (Bruijnzeel Koomen *et al*, 1986; Bieber *et al*, 1989; Hauser *et al*, 1989; Mudde *et al*, 1992). It cannot be excluded, however, that the development of a T_{H1} or T_{H2} immune response is influenced by contaminating keratinocytes, because it is difficult to prepare Langerhans cells completely free of keratinocytes. Enrichment of EC for Langerhans cells did not lead to a further increase in EC-induced IgE production in our co-culture system (data not shown). In order to address the question whether DC free of keratinocytes and never exposed to an epithelial micromilieu preferentially induce T_{H2}-type cells after stimulation with OVA, we used bone marrow dendritic cells cultured together with RC. This resulted in induction of both IgE and IgG_{2a} as well as of IL-4 and IFN- γ (data not shown), although, in the context of OVA, induction of IgE was stronger than induction of IgG_{2a} compared to cultures without DC. This supports the notion that DC such as Langerhans cells are potent inducers of both T_{H1} and T_{H2} immune responses and that the surrounding milieu, which is dominated by keratinocytes, strongly influences T_{H2} or T_{H1} development. This is further supported by the fact that MHC class II-depleted EC and the keratinocyte cell line PAM 212 still significantly enhance allergen-driven IgE production by RC when stimulated with OVA. Whether the induction of a T_{H1}- or T_{H2}-type immune response in the absence of epithelial cells depends on the physico-chemical properties of the allergen (hapten *versus* protein) is currently under investigation. Our data indicate that epithelial cells strongly support the polarization of an immune response to the protein allergen OVA in favor of a T_{H2} phenotype.

Further experiments performed with cultures separated by a membrane indicated that contact signals (most likely delivered by migrating Langerhans cells) are important for the enhanced IgE production induced by EC, but soluble factors alone still exert a significant effect. Blocking of B7-1 and -2 as co-stimulators had only a limited effect, indicating that this pathway is not critically involved in the enhancement of IgE production of primed responder cells. Anti-intercellular adhesion molecule 1 antibodies probably also inhibited the interaction of cells among EC and among RC like T/B cell cooperation for Ig synthesis (Vercelli and Geha, 1989; Vercelli *et al*, 1989).

The analysis of soluble mediators revealed that the EC-induced IgE production of RC also involved IL-4 as discussed above. Blocking of IL-4 activity with an antibody demonstrated the dependence of EC-induced IgE production on this mediator, as has been shown in other experimental settings (Finkelman *et al*, 1991). This also excluded the possibility that EC generated an IgE-enhancing activity that could substitute for IL-4. Another mediator that was critical for the preferential induction of IgE production by EC was IL-10, as neutralization of this cytokine by antibodies led to a shift away from allergen-driven IgE production toward IgG_{2a}. In parallel, IL-4 was decreased and IFN- γ increased. This indicated that the presence of IL-10 prevented the induction of IFN- γ production by T_{H1} cells essential for IgG_{2a} (Finkelman *et al*, 1990) but also indicated that the potential for T_{H1} development was present among RC. Unlike IL-4, IL-10 mRNA was produced by EC alone as early as 1 h after initiation of culture and was strongly upregulated after stimulation with OVA. The fact that IL-10 mRNA was produced by MHC class II-depleted EC indicated that keratinocytes were the source of this cytokine as described earlier (Enk and Katz, 1992a), but the lack of increased IL-10 RNA expression in MHC class II-depleted EC indicates that a yet-unidentified interaction of Langerhans cells and keratinocytes is involved in regulation of IL-10 production in response to OVA. Our data suggest that IL-10 is induced early in keratinocytes,

preventing a T_{H1} response and thus indirectly favoring T_{H2} development and IgE production leading to allergies of the immediate type in the context of OVA. This concept is supported by an earlier report that IL-10-treated murine Langerhans cells supported proliferation of a T_{H2} cell clone, whereas they were unable to induce proliferation in a T_{H1} cell clone (Enk *et al*, 1993). Additionally, expression of IL-10 was increased in biopsies from the skin of patients with atopic dermatitis compared to other inflammatory skin conditions, potentially contributing to the upregulation of T_{H2} and IgE responses as well as downregulation of T_{H1} responses in this atopic disease (Ohmen *et al*, 1995).

Taken together, the data indicate that EC are able to augment immune responses mostly by virtue of their potent antigen-presenting cells, the Langerhans cells, and favor the induction of T_{H2}-dependent IgE-responses to certain allergens (proteins that are typically implicated in allergies of the immediate type) due to potent regulatory cytokines like IL-10 generated by keratinocytes, which comprise the majority of the epidermal cells. Besides IL-10, neuroendocrine peptides, which also may be derived from epithelial cells, modulate human IgE synthesis *in vitro* (Aebischer *et al*, 1994). The ability to generate such mediators and preferential exposure of epithelial tissues to certain allergens may make them especially suitable for "allergic inflammation" as discussed above. In conclusion, it shall be emphasized that in the context of other stimuli like contact allergens (reactive chemicals or chemical elements) or bacteria (and their products) strong cellular T_{H1}-mediated immune responses can be initiated through and in the skin also involving signals from keratinocytes and epidermal Langerhans cells, as has been outlined in other studies (Enk and Katz, 1992b). It remains to be elucidated how compounds with different physico-chemical properties can induce different early signals in the epithelial tissues that are exposed to them before other immunologic compartments that influence the outcome of the immune response early on.

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