Synthesis of Complement Components C3 and Factor B in Human Keratinocytes is Differentially Regulated by Cytokines

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The complement system plays an important part in host defense and inflammation. Locally synthesized complement may perform these functions at tissue and organ level. In skin the keratinocyte is the major cell type, it is known to produce two soluble complement components, C3 and factor B. In this study we investigated the regulation of synthesis of these components in foreskin keratinocytes by cytokines. Human keratinocytes were cultured in the presence of supernatant of activated peripheral blood mononuclear cells, interleukin-1α, interleukin-2, interleukin-6, transforming growth factor-β1, tumor necrosis factor-α, or interferon-γ. C3 and factor B proteins were measured in culture supernatant by enzyme-linked immunosorbent assay and C3 and factor B transcripts in harvested cells by reverse transcriptase–polymerase chain reaction. Cultured keratinocytes constitutively produced C3 and factor B. Supernatant of activated mononuclear cells upregulated C3 and factor B production by 27- and 15-fold, respectively. interleukin-1α, interferon-γ, and tumor necrosis factor-α upregulated C3 synthesis by 7-, 8-, and 22-fold, and interleukin-1α, interleukin-6, and interferon-γ upregulated factor B synthesis by 3-, 3-, and 34-fold, respectively. Tumor necrosis factor-α induced production of C3 and interferon-γ induced production of factor B were inhibited by cycloheximide. Cytokine induced upregulation of C3 and factor B proteins was always associated with the upregulation of levels of C3 and factor B mRNA. This indicated that, as expected, cytokine-induced enhancement in C3 and factor B levels was due to an increase in synthesis rather than their possible release from intracellular stores. In conclusion, synthesis of C3 and factor B in keratinocytes is regulated by some cytokines, known to be produced by inflammatory cells and keratinocytes. ***Key words: C3/complement/cytokines/enzyme-linked immunosorbent assay/factor B/interferon-γ/interleukin-1α/interleukin-2/interleukin-6/keratinocytes/reverse transcription–polymerase chain reaction/transforming growth factor-β/tumor necrosis-α. J Invest Dermatol 114:78–82, 2000***

The complement system (C) is comprised of a large number of proteins which include components of classical and alternative pathways (Liszewski *et al*, 1996; Asghar and Pasch, 1998). Complement components C3 and factor B occupy the central position in the alternative pathway as, following activation, they become constituents of the C5-convertase ($C3b_n$.Bb). C3 is also central to the classical pathway as it provides the catalytic subunit of classical pathway C5-convertase (C4b.C2a.C3b). C5-convertases of both pathways can eventually generate the membrane attack complex (C5b–9; MAC). Generation of MAC on foreign cells, such as microbes, can lead to their killing, but under certain circumstances its generation on self cell can lead to effects such as release of inflammatory mediators and cell-proliferation (Morgan, 1992; Asghar and Pasch, 1998).

Liver is the primary source of plasma complement. Complement is also produced by cells of other organs and this locally produced complement is believed to perform some important functions at organ level (Lappin *et al*, 1986; Brooimans *et al*, 1990; Legoedec *et al*, 1995; Volanakis, 1995; Watanabe *et al*, 1995; Morgan and Gasque, 1996). Human keratinocytes, the most abundant cell-type in epidermis, have been studied for the biosynthesis of two complement components, C3 and factor B. Biosynthetic labeling and pulse chase studies with human keratinocytes and a human keratinocyte cell line A431 have shown that these cells synthesize a 195 kDa pro-C3 (Basset-Seguin *et al*, 1990) and a 100 kDa pro-factor B (Yancey *et al*, 1992) molecule. These precursor molecules are processed intracellularly to a mature C3 molecule consisting of disulfide-linked 120- and 75 kDa C3 α and β chains and to a 105 kDa mature factor B molecule. Secreted mature C3 and factor B were biologically active. Northern blot analysis from human keratinocytes and A431 revealed the presence of a 5.1 kb C3 mRNA and 2.8 kb factor B mRNA in these cells. These results convincingly demonstrated that human keratinocytes are a local source of C3 and factor B.

Demonstration of constitutive synthesis of C3 and factor B led to the question that under what conditions or biologic stimuli, synthesis of C3 and factor B in keratinocytes is regulated? Terui *et al* (1997) recently studied a large number of cytokines and growth factors and showed that tumor necrosis factor (TNF)-α and interferon (IFN)-γ are the main cytokines involved in regulation of production of C3 in human keratinocytes, although interleukin (IL)-1α and IL-1β also upregulated C3 production to some extent.

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These workers did not study transcriptional regulation of C3 by the above cytokines. They also did not include the regulation of synthesis of factor B in their studies. In the current study we investigated the cytokine regulation of C3 and factor B production both at protein and mRNA levels.

MATERIALS AND METHODS

Chemicals and reagents Human recombinant cytokines IFN-γ, IL-1α, IL-2, IL-6, transforming growth factor (TGF) -β1 and TNF-α were purchased from Boehringer Mannheim (Mannheim, Germany). Normal rabbit IgG, neutralizing rabbit antibodies to IFN-γ and cycloheximide were purchased from Sigma (St Louis, MO). Supernatant of activated mononuclear cells was prepared from stimulated peripheral blood mononuclear cells as described (Miltenburg *et al*, 1988). Briefly, peripheral blood mononuclear cells (50×10^6 per ml) from normal donors were stimulated for 2 h at 37°C with 0.1 μg per ml phorbol myristate acetate in Iscove's modified Dulbecco's modified Eagles medium (supplemented with 10% heat-inactivated fetal bovine serum, 100 U penicillin per ml, and 100 μg streptomycin per ml). Phorbol myristate acetate-treated cells were washed extensively and cultured (50×10^6 per ml) for 48 h with 15 μg per ml concanavalin A in supplemented Iscove's modified Dulbecco's modified Eagles medium. Concanavalin A was neutralized by addition of 50 mM α-methyl-mannoside for 30 min at 37°C. Supernatant was obtained by centrifugation. Cytokines, supernatant of activated mononuclear cells and neutralizing antibodies were aliquoted in small portions and stored at −20°C and diluted in keratinocyte serum-free medium (keratinocyte SFM; GibcoBRL, Breda, The Netherlands) just before use. The sources of other chemicals and reagents are indicated below.

Keratinocyte culture Human keratinocytes were isolated from foreskin obtained by circumcision of children (<5 y). Foreskin was incubated with thermolysin (0.50 mg per ml, Sigma) at 4°C for 16 h and subsequently trypsinized (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by an excess volume of heat inactivated fetal calf serum (GibcoBRL, Breda, The Netherlands). Cells were separated from debris by filtering through a nylon mesh, centrifuged, and resuspended in keratinocyte SFM supplemented with penicillin/streptomycin (100 IU per ml, 100 μg per ml; GibcoBRL). The keratinocytes were plated on to 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in humidified, 5% CO_2, tissue culture incubator. Medium was changed every 2–3 d, and at 70% confluence cultures were split after a 5 min exposure to 0.025% trypsin, 1.5 mM ethylenediamine tetraacetic acid and recultured.

Stimulation of keratinocytes For stimulation of keratinocytes with cytokines, cells were seeded at a density of 100,000 per well in 2000 μl of medium. When cultures reached 60–80% confluence, medium was removed and monolayers were washed twice with phosphate-buffered saline. Keratinocyte SFMs containing different cytokines was added to the cells. The concentrations and ranges of concentrations of cytokines used in different experiments are mentioned in *Results*. After stimulation with cytokines for a predetermined suitable period of 72 h (unless indicated otherwise), culture supernatants were collected for C3 and factor B analysis by enzyme-linked immunosorbent assay (ELISA). As controls, supernatants were collected from keratinocytes cultured in keratinocyte SFM without cytokines. Cells in representative wells were counted by hematocytometer before the experiment and the cells in all wells were counted after finishing the experiment. Cells in passages 2–5 were used.

In some experiments supernatant of activated mononuclear cells or lipopolysaccharide, at concentrations indicated in the *Results*, were used instead of cytokines.

ELISA for measurement of C3 and factor B For quantitation of C3, a previously described sandwich ELISA was used (Pasch *et al*, 1998). Briefly, wells of 96 well flat-bottom microtiter plates were coated with 0.7 μg polyclonal goat IgG anti-human C3 (Cappel, Boxtel, The Netherlands) per ml in 100 μl carbonate buffer (pH 9.6) overnight at 4°C. After thorough washing, the wells were blocked for 1 h at room temperature with phosphate-buffered saline containing 2% bovine serum albumin (Sigma) and 1 mM ethylenediamine tetraacetic acid. Washing was repeated and wells were incubated with 100 μl of sample diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37°C. The wells were then washed and incubated with 100 μl peroxidase-labeled goat anti-human C3 IgG (0.05 μg per ml) (Cappel) for 1 h at 37°C. After washing, the wells were incubated with 100 μl 3,3′,5,5′tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany) -citrate buffer for 10 min. Reaction was stopped with 100 μl H_2SO_4 (2 M). Optical density was measured at 450 nm. The detection limit of this ELISA was 1 ng per ml of C3.

Factor B was assayed by a previously described sandwich ELISA (Pasch *et al*, 1998). Briefly, wells were coated overnight at 4°C with 3 μg polyclonal goat-anti-human factor B IgG (ATAB, Stillwater, MN) per ml in carbonate buffer. After thorough washing, wells were blocked with phosphate-buffered saline/milk powder (2%) (Nutricia, Zoetermeer, The Netherlands) for 1 h at room temperature, and then washed again. Wells were then incubated for 2 h at 37°C with 100 μl sample, diluted in same buffer that was used for blocking, and washed. They were then incubated with biotinylated goat anti-human factor B IgG (1.25 μg per ml) for 1 h at 37°C. After washing, the wells were incubated for another hour at 37°C with peroxidase conjugated polystreptavidin (1:1000; DAKO, Glostrup, Denmark). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of optical density was carried out as described for C3. The detection limit of this ELISA was 100 pg per ml. Standard curves for both ELISA were made using Human Complement Calibrator CA1 (ATAB). The batches of fetal bovine serum, supernatant of activated mononuclear cells, and keratinocyte medium did not show C3 or factor B reactivity in the respective ELISA assays.

Isolation of RNA and semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) Total RNA was isolated from human keratinocytes grown in 100 mm Petri dishes in medium alone or medium supplemented with IL-1α (200 U per ml), IL-6 (1000 U per ml), IFN-γ (100 U per ml), or TNF-α (750 U per ml) for 6 h and 24 h using Trizol (Life Technologies, Paisley, U.K.). The RNA pellet was dissolved in formamide and the amount of RNA was determined by a spectrophotometer at 260 nm and 280 nm.

RT–PCR was carried out as described (Teunissen *et al*, 1998), with some minor modifications. Briefly, 5 μg of the extracted total cellular RNA was reverse transcribed in a reaction volume of 20 μl and 1 μl of the resulting cDNA solution was used to amplify cDNA by C3- or factor B-specific PCR. The PCR were performed in 50 μl per well in polyethylene reaction tubes, applying cycles consisting of denaturation step at 94°C for 30 s, annealing for 1 min at 59°C, and extension for 1 min at 72°C. The PCR incubation mixture, in a total volume of 50 μl, contained 50 mM KCl, 10 mM Tris–HCl pH 8.1, 2.0 mM $MgCl_2$, 0.01% gelatin, 1.25 unit Taq polymerase (Gibco), 250 μM dNTP mix (Pharmacia, Uppsala, Sweden), and 140 ng of the sense and anti-sense primer each. The following specific primer sets were synthesized in our laboratory by an oligo-synthesizer: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5′-CGAGATCCCTCCAAAATCAA-3′ (nt 298–317); and GAPDH reverse primer 5′-AGGTCAGGTCCACCACTGAC-3′ (nt 799–780) (Teunissen *et al*, 1998); factor B forward primer 5′-CAACAGAAGCGGAAGATCGTC-3′ (nt 766–786); and factor B reverse primer 5′-TATCTCCAGGTCCCGCTTCTC-3′ (nt 1630–1650) (Gordon *et al*, 1992); and C3 forward primer 5′-TCGGATGAC-AAGGTCACCCT-3′ (nt 4627–4646); and C3 reverse primer 5′-GACAACCATGCTCTCGGTGA-3′ (nt 5015–5034) (Timmmerman *et al*, 1996). Each PCR product (12.5 μl) was mixed with 5 μl stop layer mix and run on a 1.7% agarose gel in Tris/borate/ethylenediamine tetraacetic acid buffer. After electrophoresis the gel was scanned by an Eagle Eye imager (Stratagene Europe, Amsterdam, The Netherlands) and the signal strength was integrated to obtain a densitometric value for each amplification product. To enable semiquantitative analysis, the number of PCR cycles was chosen in such a way that a linear relationship was achieved between PCR product formation (plotted on a log scale) and cycle number (28–34 cycles for C3 and factor B and 26–32 cycles for GAPDH), without having reached saturation of the product formation.

Statistical analysis Statistical analysis was performed using the Student's t test for unpaired samples and a $p < 0.05$ was considered significant.

RESULTS

Human keratinocytes constitutively produce C3 and factor B Keratinocytes produce small amounts of C3 and factor B in culture as determined by ELISA (**Fig 1**). When keratinocytes were cultured up to passage 6, and the production of C3 and factor B was monitored in the culture supernatant of each passage, a significant increase of both components with increasing number of passages was observed in cultures derived from three different foreskins (C3 $p < 0.002$, and factor B $p < 0.02$; passage 6 compared with passage 1;

Fig 1). Because of these differences we used only cultures from passages 2–5 in subsequent studies.

Mediators released from activated mononuclear cells upregulate the production of C3 and factor B from keratinocytes Keratinocytes cultured in absence of supernatant of activated mononuclear cells produced 2.1 ± 0.4 ng C3 and 0.4 ± 0.07 ng factor B per 10^6 cells per 24 h. In the presence of 10% supernatant, production of C3 was 56.4 ± 20.7 and of factor B was 6.5 ± 1.4 ng per 10^6 cells per 24 h. In all cultures, supernatant of activated mononuclear cells induced a significant increase in production of both C3 ($p < 0.005$) and factor B ($p < 0.001$) in a concentration-dependent fashion.

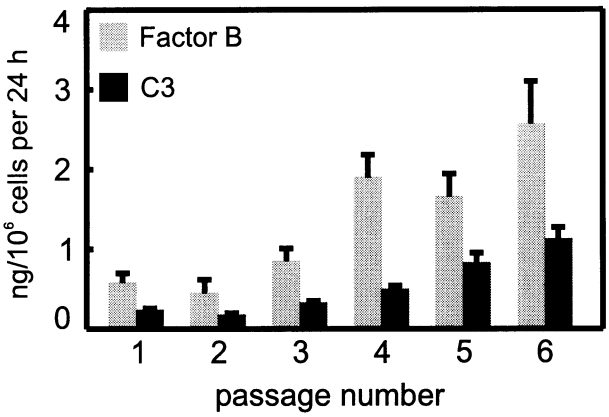

Figure 1. Constitutive release of C3 and factor B by keratinocytes is passage dependent. Subconfluent keratinocytes were cultured for 72 h. Supernatants were collected and cells were rinsed, harvested, and counted (passage 1). They were again passaged to subconfluence, fresh medium was added (at time 0) and the whole process was repeated (passage 2) until passage 6. Supernatants collected after each passage were analyzed for C3 and factor B by ELISA. Three independent experiments with cultures derived from different foreskins were carried out. Values are the mean ± SD for triplicate determinations of one representative culture.

Cytokines differentially regulate the production of C3 and factor B from keratinocytes The supernatant of activated mononuclear cells is known to contain a number of cytokines, which include IL-1α, IL-2, IL-6, TGF-β, TNF-α, and IFN-γ (van den Dobbelsteen *et al*, 1994; Pasch *et al*, 1999). We tested recombinant forms of these individual cytokines to find if one ore more of these mimics the effects seen with supernatant of activated mononuclear cells.

IL-2 (0–1000 U per ml), TGF-β1 (0–10 ng per ml), and IL-6 (0–1000 U per ml) had no effect on the production of C3 (data not shown). IL-1α, IFN-γ, and TNF-α showed a concentration-dependent upregulation of C3 production (**Fig 2**). These cytokines at doses of 100 U per ml, 50 U per ml, and 750 U per ml, respectively, upregulated C3 production 7-, 8-, and 22-fold, compared with the basal production. Further increase in dose did not cause an additional increase in response.

IL-2 (0–1000 U per ml), TNF-α (0–1000 U per ml), and TGF-β1 (0–10 ng per ml) did not show any significant effect on the production of factor B. IL-1α, IL-6, and IFN-γ caused concentration-dependent upregulation of factor B. IL-1α and IL-6 upregulated factor B production to about 3-fold of the basal release at 200 U per ml and 1000 U per ml, respectively, and IFN-γ

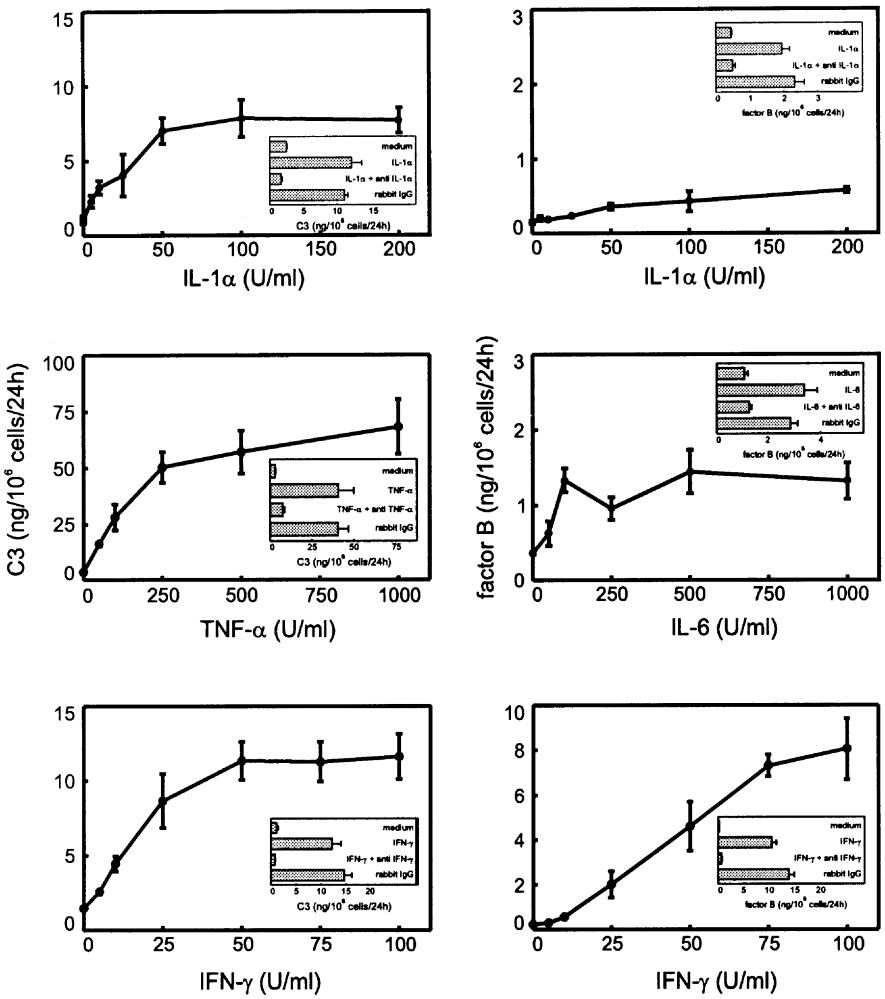

Figure 2. Release of C3 and factor B by keratinocytes is differentially regulated by cytokines. Keratinocytes were cultured for 72 h in the presence of indicated concentrations of cytokines and supernatants were collected and assessed for C3 and factor B concentrations by ELISA. Insets show the effects of cytokine (indicated) specific neutralizing rabbit antibodies on cytokine (indicated) induced C3 and factor B release by keratinocytes. Keratinocytes were cultured with an optimal concentration of indicated cytokine alone, cytokine plus specific neutralizing antibodies, or control IgG. After 72 h, supernatant were collected and assessed for C3 and factor B. The data in main figures and insets are expressed as the mean ± SD of duplicate measurements of three cultures.

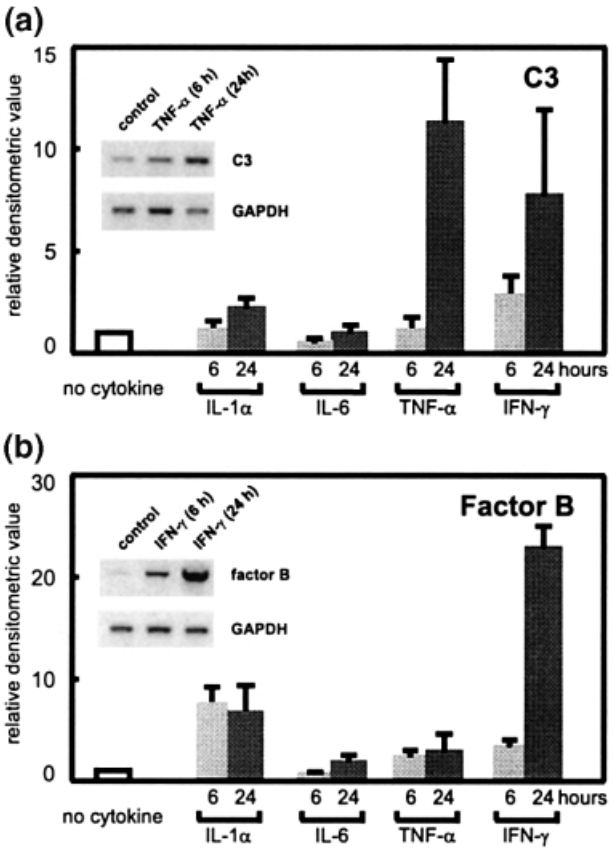

Figure 3. Expression of C3 and factor B in keratinocytes is differentially regulated by cytokines at mRNA level. RNA was isolated from keratinocytes at 0 h, 6 h, and 24 h after stimulation with indicated cytokines at concentrations given below, and subjected to semiquantitative C3- (*a*) or factor B (*b*) specific RT–PCR as described in the text. After electrophoresis, the densitometric values of the products were determined and corrected for the value of GAPDH, a household gene. The relative densitometric value obtained with untreated keratinocytes (0 h) was arbitrarily set at 1 and were related to those of cytokine-treated keratinocytes (6 h; 24 h). Inset in (*a*) shows upregulation of C3 transcripts by TNF-α (representative example; effects of other cytokines are not shown). RNA was isolated from keratinocytes after 0 h, 6 h, or 24 h of stimulation and subjected to a C3 RT–PCR. After electrophoresis the products were visualized in ethidium bromide solution. Inset in (*b*) shows upregulation of factor B transcripts by IFN-γ (representative example; effects of other cytokines are not shown) under the conditions identical to those as for C3 (*a*). Concentrations of cytokines: IL-1α, 200 U per ml; IL-6, 1000 U per ml; TNF-α, 750 U per ml; IFN-γ, 100 U per ml.

about 34-fold at 75 U per ml (**Fig 2**). A further increase in dose did not cause a further increase in factor B production.

To confirm that the regulation of C3 or factor B production by normal human keratinocytes was a specific property of the above-mentioned cytokines, antibody blocking experiments were performed. Keratinocytes were cultured in medium alone or in medium containing optimal concentration of individual cytokines in the presence and absence of neutralizing antibodies or control rabbit or mouse IgG (**Fig 2**). The results showed that neutralization of IL-1α with anti-IL-1α, IFN-γ with anti-IFN-γ, or TNF-α with anti-TNF-α abolished the induction of C3 production. Neutralization of IL-1α with anti-IL-1α, IFN-γ with anti-IFN-γ, and IL-6 with anti-IL-6, abolished the induction of factor B production.

Upregulation of production of C3 and factor B is inhibited by cycloheximide TNF-α and IFN-γ which were the strongest upregulators of C3 and factor B production from keratinocytes, respectively, were selected for further studies to find out whether they caused enhanced production by inducing *de novo* synthesis. This was investigated by observing the effect of cycloheximide on TNF-α and IFN-γ mediated upregulation of C3 and factor B, respectively. Keratinocytes were grown in medium alone, medium containing IFN-γ (100 U per ml) or TNF-α (750 U per ml) with and without 2.0 μg per ml cycloheximide. Higher concentrations of cycloheximide could not be used because of irreversible toxic effects on the cells. At definite time intervals, supernatants were harvested for C3 and factor B analysis by ELISA. These experiments revealed that cycloheximide significantly inhibited the TNF-α induced C3 and the IFN-γ induced factor B production after 24 h and 48 h (data not shown). This indicated that increased production of C3 in response to TNF-α, and of factor B in response of IFN-γ were due to increased *de novo* synthesis. Removal of cycloheximide by washing the cells and again culturing them in the medium containing TNF-α and IFN-γ restored C3 and factor B production, respectively.

Cytokines regulate the production of C3 and factor B at pretranslational level C3 and factor B mRNA transcripts obtained from cytokine treated and untreated cells were analyzed by RT–PCR. Keratinocytes were incubated for 6 h and 24 h in medium containing IFN-γ (100 U per ml), IL-1α (200 U per ml), IL-6 (1000 U per ml), or TNF-α (750 U per ml). After RT–PCR and gel electrophoresis as described in *Materials and Methods* the Eagle Eye analysis revealed 2-, 8-, and 11-fold increase of the ratio C3/GAPDH message after stimulation with IL-1α, IFN-γ, and TNF-α, respectively (**Fig 3**). The ratios of factor B/GAPDH were increased 7-, 4-, and 23-fold, by stimulation of keratinocytes with IL-1α, IL-6, and IFN-γ, respectively (**Fig 3**). These semiquantitative data suggest that the upregulation of C3 synthesis by IFN-γ, IL-1α, and TNF-α and of factor B by IL-1α, IL-6, and IFN-γ was at a pretranslational level.

DISCUSSION

In this study we investigated the regulation of synthesis of C3 and factor B by the supernatant of activated mononuclear cells, a source of mediators derived from inflammatory cells, and by recombinant forms of several cytokines known to be present in this supernatant. Constitutive production of C3 was greatly upregulated by the supernatant of activated mononuclear cells and TNF-α but also appreciably by IL-1α and IFN-γ (**Fig 2**). IL-2, IL-6, and TGF-β had no effect. Constitutive production of factor B was greatly upregulated by the supernatant of activated mononuclear cells and IFN-γ but also appreciably by IL-1α and IL-6 (**Fig 2**). IL-2, TNF-α, and TGF-β had no effect. These results show differential effects of some cytokines on the production of C3 and factor B by human keratinocytes.

As several cell types such as neutrophils store large amounts of some complement components and secrete them upon stimulation without stimulating their synthesis (Hogasen *et al*, 1995) we investigated whether the cytokine induced release of C3 and factor B was due to their export from intracellular reserves or was associated with an increase in their synthesis. The possibility of export of intracellular reserves was ruled out by the facts that: (i) cycloheximide inhibited cytokine induced enhancement of C3 and factor B production, and (ii) IL-1α, TNF-α, and IFN-γ mediated enhancement of production of C3 and IL-1α, IL-6, and IFN-γ mediated enhancement of production of factor B was associated with the increased expression of C3 and factor B transcripts as seen by semiquantitative RT–PCR (**Fig 3**). Lipopolysaccharide was also tested for its ability to induce the synthesis of C3 and factor B by keratinocytes but was found to have no effects at 100 ng per ml, a concentration 10–100 times higher than that needed to affect complement synthesis in human fibroblasts (Katz *et al*, 1988).

The upregulation of C3 synthesis in keratinocytes by IL-1α and IL-6 does not appear to be mediated by an autocrine release of TNF-α by keratinocytes in culture medium. Most likely TNF-α released in the medium is diluted to concentrations insufficient to induce the synthesis of C3 and factor B by keratinocytes. The support for this postulation comes from the following observation.

In our laboratory, the concentration of TNF-α in culture medium of stimulated keratinocytes was found to be very low (< 1 U per ml) (Pasch *et al*, 1998) whereas that required for upregulation of C3 in our system in this study was much higher (**Fig 2**). Similarly, the enhancement of factor B synthesis in response to IL-1α and IL-6 appears not to have been caused by any other cytokine released in autocrine manner from keratinocytes.

IL-1α and IL-6 had comparatively weak stimulating effects on the synthesis of C3 and factor B, respectively, by human keratinocytes. This was in contrast to the findings of Katz *et al* (1989) and Katz and Strunk (1989) in human skin fibroblasts, who showed that IL-1α and IL-6 play an important part in the regulation of both C3 and factor B synthesis. In glomerular epithelial cells IFN-γ does not increase C3 gene expression (Sheerin *et al*, 1997), which is also in sharp contrast to the strong upregulation of C3 caused by IFN-γ in keratinocytes. Comparison of our results with those obtained with other cell types confirmed the widely held view that regulation of complement synthesis by cytokines is highly cell type specific.

In conclusion, we provide data which confirm that keratinocytes constitutively produce low levels of C3 and factor B. TNF-α, IFN-γ, and IL-1α regulate the synthesis of C3 and IFN-γ, IL-1α, and IL-6 that of factor B. Local complement protein synthesis may contribute to local immunologic defenses. Other possible roles of local synthesis of complement by keratinocytes have yet to be investigated.

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