Antioxidant Enzymes in Psoriatic Fibroblasts and Erythrocytes

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Antioxidant enzyme activities in fibroblasts and erythrocytes prepared from normal and psoriatic patients were measured and compared. The most significant differences were noted in superoxide dismutase (SOD) activities. A dramatic (5.2-fold) increase in Mn-SOD activity along with a lesser (1.8-fold) increase in CuZn-SOD activity was observed in fibroblasts from lesional and nonlesional psoriatic skin. The increase of Mn-SOD activity was correlated with an increase of both protein and mRNA. A slight (1.2-fold) increase in CuZn-SOD activity was also found in psoriatic as compared to normal red blood cells, while Mn-SOD activity was not present in these cells. In contrast, both glutathione peroxidase and catalase activities were only slightly (1.3-fold) increased in psoriatic fibroblasts, with no appreciable change noted in psoriatic erythrocytes. Likewise, glutathione levels were observed to be similar in normal and psoriatic cells. The increases in SOD activities did not appear to correlate with the severity of the disease as expressed by the Psoriatic Area Severity Index score or with plasma inflammatory markers. These results demonstrate that antioxidant enzyme activities, particularly Mn-SOD in fibroblasts and CuZn-SOD in erythrocytes, are significantly elevated in cells from psoriatic patients. ***Key words: psoriasis/superoxide dismutases/free radicals/fibroblasts/erythrocytes. J Invest Dermatol 106:1325–1328, 1996***

Reactive oxygen species such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) have been linked to skin cancers, cutaneous aging, and many inflammatory disorders (Cross *et al*, 1987; Niwa *et al*, 1987). Reactive oxygen species are generated by fibroblasts in response to stimulation by various hormones and cytokines (Meier *et al*, 1989) and have been reported to act as second messengers (Schreck and Baeuerle, 1991). In turn, the oxidative modification of several key proteins including protein kinases involved in cellular signal transduction has been described (Whisler *et al*, 1995). Previously, we have established that cAMP-dependent protein kinase activity as well as 8-azido-[^{32}P]cyclic AMP binding to the RI and RII regulatory subunits are decreased in cells from psoriatic patients compared to cells from normal subjects (Evain-Brion *et al*, 1986; Raynaud *et al*, 1989). The ability of retinoic acid treatment of intact cells to rapidly reverse the oxidatively modified state of protein kinase C (Gundimala *et al*, 1993) as well as to reverse the altered state of protein kinase A noted in psoriatic cells (Raynaud *et al*, 1987, 1993), coupled with the inflammatory evolution of psoriasis, suggested the possibility that there may be changes in the oxidative state of psoriatic cells. The cellular oxidative status is related to a balance between the production of oxygen-derived free radicals and their destruction by enzymes that metabolize oxygen-reduction products (antioxidant enzymes). Thus, we carried out studies to determine whether the activities of enzymes involved in modulating the redox state of the cell, such as superoxide dismutases (SOD), catalase, and glutathione peroxidase, might be altered in fibroblasts and erythrocytes from psoriatic patients. The results indicate a selective increase in SOD activity in these psoriatic cells.

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Abbreviations: SOD, superoxide dismutase; EDTA, ethylenediamine tetraacetic acid; INT, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; HPLC, high-performance liquid chromatography; GSH, reduced form of glutathione; GPX, glutathione peroxidase; IPS, Involved Psoriatic Skin; UIPS, Uninvolved Psoriatic Skin.

MATERIALS AND METHODS

Cells Human fibroblasts were isolated from eight normal and eight untreated adult psoriatic patients (*Psoriasis vulgaris* in flare for approximately 3 months) by enzymatic digestion of lesional and nonlesional buttock and abdominal skin punch biopsies (4 mm) as previously described (Raynaud *et al*, 1995). These white subjects were age- and sex-matched. This study was approved by the ethical committee of the Hôpital Cochin (Paris). Cells were grown as previously described (Raynaud *et al*, 1995) and were used subconfluently (10^6 cells/dish) at the fifth passage. All data were obtained with cells propagated for the same number of passages (fifth passage) under identical conditions (culture medium, serum, etc). For each donor the different assays were performed on the same cell extracts prepared from cells arising from the same cell seeding. Erythrocytes were isolated from healthy subjects and from age- and sex-matched untreated patients as previously described (Raynaud *et al*, 1993).

Preparation of Cellular Fractions Fibroblasts were washed three times with ice-cold phosphate-buffered saline and harvested by scraping into ice-cold buffer [0.25 M sucrose, 20 mM Tris (pH 7.4), 1 mM $MgCl_2$] with a cell scraper. The cells were recovered by centrifugation at $1000 \times g$ for 5 min and the cell pellet was then frozen at −80°C. For antioxidant enzyme activity measurements, the control and psoriatic cell pellets were disrupted

Table I. Modulation of CuZn-SOD, Glutathione Peroxidase, and Catalase Activities in Erythrocytes From Normal and Psoriatic Subjects[a]

	Antioxidant activities		
Erythrocytes	CuZn-SOD (U/g Hb)	GPX (U/g Hb)	Catalase (U/mg protein)
Normal	790 ± 30.94 (508–1216) (n = 28)	27.49 ± 1.28 (19.33–52.33) (n = 28)	9.21 ± 1.26 (5.6–16.8) (n = 8)
Psoriatic	983.77 ± 29.59 (754–1236) (n = 25)	32.22 ± 1.68 (18.5–46.62) (n = 22)	8.11 ± 0.52 (6.5–10.7) (n = 7)

[a] The antioxidant enzymatic activities were assayed as described under *Materials and Methods*. CuZn-SOD and GPX activities are expressed as units per milligram of hemoglobin (Hb), and catalase activity is given as units per milligram of membrane protein. Data are expressed as the mean ± SEM of triplicate determinations carried out with cells from each donor with the range given below in parentheses. n = number of individual donors included in the study.

by sonication in 500 μl of 10 mM sodium phosphate buffer, pH 7, and used in the same experiment. Erythrocyte hemolysates were prepared by suspending the cells in 50 mM sodium phosphate buffer, pH 7.2, containing 1 mM ethylenediamine tetraacetic acid (EDTA), and then freezing at −80°C. Hemoglobin levels were determined for each sample by the method of Drabkin and Austin (1955).

Erythrocyte membranes (ghosts) were prepared by suspending erythrocytes into 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA for 30 min on ice and then harvesting by centrifugation at 17,000 × *g* for 30 min. Cell membranes were then washed three times (hemoglobin free) in the same buffer prior to freezing at −80°C.

SOD Activity Xanthine-xanthine oxidase was utilized to generate a $O^{\cdot}2$ flux and the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to red formazan by $O^{\cdot}2$ was followed at 505 nm at 30°C. The rate of INT reduction in the absence of samples was used as the reference rate (0.02 ± 0.005 absorbance/min). The data were plotted as percentage inhibition of INT reduction *versus* protein concentration. One unit of activity was defined as the amount of protein necessary to reduce the rate of INT reduction to 50% of maximum inhibition. Each assay tube contained 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 10.2, to determine CuZn-SOD activity (Bannister *et al*, 1987) or 50 mM phosphate buffer, pH 7.8, to determine total SOD activity (O'Neill *et al*, 1988), along with 1 mM EDTA, 25 μM INT, 50 μM xanthine, 1 U/ml catalase, 0.05 mM bathocuproine disulfonate disodium salt, 0.13 mg/ml bovine serum albumine, and sufficient xanthine oxidase to achieve the required 100% level of noninhibition. Bathocuproine disulfonate disodium salt and bovine serum albumine were added to inhibit nonenzymatic scavenging of $O^{\cdot}2$ (Spitz and Oberley, 1989). Mn-SOD activity levels, measured by adding cyanide (2 mM) to the assay mixture at pH 7.8 to inhibit any CuZn-SOD activity or determined by substracting the CuZn-SOD activity determined at pH 10.2 from the total SOD activity measured at pH 7.8, were found to be similar (data not shown). All data were expressed in units of SOD activity per milligram of protein for fibroblasts or hemoglobin for erythrocytes.

Catalase Activity Catalase activity was measured as described by Johansson and Borg (1988). Catalase is able to decompose H_2O_2 by two types of reaction. Both reactions include a first step of formation of an intermediate consisting of the enzyme and H_2O_2. In a second step the catalase activity was measured by reaction of this intermediate with a hydrogen donor other than H_2O_2. Methanol was used as the hydrogen donor, and we measured the production of formaldehyde spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald Sigma, St. Quentin Fallavier, France) as a chromogen. Interferences due to oxidation of methanol by other enzyme activities were not observed for the present method.

Glutathione Determination Cellular levels of the reduced form of glutathione (GSH) were determined by ion-pairing reversed-phase high-performance liquid chromatography (HPLC) coupled to a Coulometric electrochemical detector. A 200-μl aliquot of the cell suspensions was mixed with 200 μl of cold 10% trichloroacetic acid. The solution was kept on ice for 15 min, and precipitated proteins were then removed by centrifugation. Fifty microliters of the supernatant was then analyzed by HPLC. A Spherisorb C-18 reversed-phase ODS-2 column (5 μm; 250 × 4.6 mm) was used. Isocratic elution was performed employing the HPLC solvent 10 mM NaH_2PO_4 adjusted to pH 2.7 with 85% H_3PO_4 containing 0.05 mM of the ion-pairing reagent octylsulfate and 2% acetonitrile (v/v). Separations were performed at room temperature with a flow rate of 1.0 ml/min. GSH was detected following isolation by HPLC with a Coulochem detector equipped with a model 5010 dual analytical cell and a model 5020 guard cell. The applied electrode potentials for detector 1, detector 2, and guard cell working electrodes were set at +0.6, +1.2, and +1.4 volts, respectively. A standard GSH was prepared to produce a standard curve within the biologic concentration range. The standard curve was derived from dilutions of known concentrations of glutathione (1–20 μmol/liter).

Glutathione Peroxidase (GPX) Activity GPX was assayed by an adaptation of the method of Paglia and Valentine, 1967 with cumene hydroperoxide as substrate. Both sample and reference tubes contained 0.05 M phosphate buffer, pH 7.2, 4.3 mM EDTA, 0.28 mM NADPH, 0.5 U of glutathione reductase, 4 mM glutathione, and the appropriate amount of red blood cell hemolysates or of fibroblasts. The oxidation of NADPH by cumene hydroperoxide (0.18 mM) added to the sample tube was followed spectrophotometrically at 340 nm at 30°C. An additional blank, containing all components except the sample, was used to correct for nonenzymatic oxidation of GSH and NADPH by cumene hydroperoxide. One unit of GPX was defined as 1 μM substrate converted in 1 min.

Western Blot Analysis Cell pellets were prepared as described above. After measurement of protein levels, lysates were heated for 5 min at 95°C in Laemmli sample buffer and analyzed by electrophoresis on 10% polyacrylamide gels (50 μg/sample). Proteins were electrotransferred to nitrocellulose and western blots were performed as described in the ECL kit (Amersham, Les Ulis, France). Proteins were detected with a monoclonal antibody raised against human Mn-SOD, diluted 100-fold (Bender, Vienna, Austria).

Slot Blot Analysis of the Mn-SOD mRNA Total RNA was extracted from normal (N), lesional (IPS, Involved Psoriatic Skin), and nonlesional (UIPS, UnInvolved Psoriatic Skin) cultured fibroblasts (Chomczynski and Sacchi, 1987). Decreasing amounts (20, 10, 5, 2.5 μg) of total RNA were blotted onto a nylon sheet (Amersham, les Ulis, France) and were hybridized with a ^{32}P-labeled human Mn-SOD oligonucleotide antisense probe (5′-GAACTTCAGTGCAGGCTGA)(Frankenne *et al*, 1992) and with GAPDH (glyceraldhehyde-3-phosphate dehydrogenase) as control. The specificity of the Mn-SOD oligonucleotide probe was assessed by northern blot analysis. Two transcripts of 4.0 and 1.5 kb were detected (data not shown).

Plasmatic Inflammatory Markers Orosomucoid and ceruloplasmin measurements were performed using a nephelometric method with the Behring Nephelometer Analyzer (BNA; Behringwerke, AG, Marburg, Germany) as described by the manufacturer.

Analysis of Statistical Significance Data were analyzed by Student's t test method. Data from the lesional and nonlesional skin of psoriatic patients were analyzed in a paired Student's t test.

RESULTS

Psoriatic Erythrocytes: Increased Antioxidant Enzymes The activities of the three major antioxidant enzymes were measured in red blood cells from normal and psoriatic subjects. In these cells the only active SOD is the CuZn-SOD (Nakano *et al*, 1990). As presented in **Table I** a significant increase (1.2-fold) ($p < 0.001$) in CuZn-SOD activity was observed in psoriatic red blood cells as compared to normal cells. No correlation was found between CuZn-SOD activity and the age of the normal and psoriatic donors. Glutathione peroxidase activity was also somewhat increased (1.2-fold) ($p < 0.05$), whereas no significant differences were observed in catalase activity between normal and psoriatic erythrocytes.

Psoriatic Fibroblasts: Antioxidant Enzymatic Activities and Glutathione Levels The results presented in **Table II** show that in normal fibroblasts antioxidant enzyme activities varied over a

Table II. CuZn- and Mn-SOD Activities, GPX, and Catalase Activities in Cultured Fibroblasts Prepared From Skin of Normal Subjects and From Lesional and Nonlesional Skin of Psoriatic Patients[a]

Fibroblasts	Antioxidant activities				
	CuZn-SOD (mU/mg prot)	Mn-SOD (mU/mg prot)	GPX (mU/mg prot)	Catalase (U/mg prot)	GSH (nmol/mg prot)
Normal	210 ± 38 (88–367)	57.6 ± 8.8 (22–90)	5.98 ± 0.51 (3.8–8)	4.49 ± 0.41 (2.7–5.9)	16.25 ± 0.99 (12.5–21.3)
Psoriatic Nonlesional	387 ± 38 (275–601)	297 ± 42 (149–503)	9.0 ± 1.16 (4–13)	6.42 ± 1.11 (4.2–10.3)	14.08 ± 1.20 (12–17.5)
Psoriatic Lesional	372 ± 25 (295–478)	243 ± 21 (139–345)	7.63 ± 0.84 (2.3–9.8)	6.20 ± 0.46 (5.2–7.8)	14.30 ± 1.15 (12.1–16)

[a] Primary fibroblast cultures prepared from eight normal and eight age- and sex-matched psoriatic patients were assayed for the antioxidant enzymatic activities indicated, as well as for reduced glutathione (GSH) levels, as described under *Materials and Methods*. Data are expressed as mean ± SEM of triplicate determinations and as the range in parentheses of triplicate determinations in the cell extracts from eight fibroblast primary cultures.

wide range with the different donors. No correlation was found between the variable activities and the ages of the subjects (data not shown). In psoriatic subjects no significant differences were observed for any of the antioxidant enzyme activities determined with fibroblasts prepared from lesional and nonlesional skin of the same patient. GPX activity was not significantly different between psoriatic fibroblasts cultured from lesional skin and normal fibroblasts and was found to be slightly increased (1.5-fold) ($p < 0.05$) in fibroblasts from nonlesional skin when compared to normal fibroblasts. Catalase activity was also slightly enhanced (1.4-fold) ($p < 0.05$) in psoriatic fibroblasts from lesional and nonlesional skin as compared to normal cells, while glutathione levels were similar in normal and psoriatic cells.

Interestingly, significantly elevated levels (1.8-fold) of the CuZn-SOD activity ($p < 0.005$ for nonlesional and $p < 0.003$ for lesional skin), and particularly of the Mn-SOD activity [$p < 0.0001$ for both nonlesional (5.2-fold) and lesional skin (4.2-fold)] were found in psoriatic fibroblasts compared to normal fibroblasts. This very high Mn-SOD activity was associated with an increase in Mn-SOD mRNA **(Fig 1)** and in protein expression as determined by western blot analysis **(Fig 2)**. Densitometric scanning of the Mn-SOD band on the autoradiograph revealed a significant increase ($p < 0.05$) in the amount of Mn-SOD protein in psoriatic fibroblasts ($n = 5$) (mean ± SEM, 1814 ± 473 and 1743 ± 548 arbitrary units, respectively, in lesional and nonlesional skin) as compared to normal fibroblasts ($n = 5$) (1107 ± 287 arbitrary units).

Plasmatic Inflammatory Markers No significant differences were found in the levels of plasmatic orosomucoid and ceruloplasmin between psoriatic patients (mean ± SEM, 0.55 ± 0.19 g/liter and 310 ± 69 g/liter, respectively) and normal donors (0.45 ± 0.13 g/liter and 356 ± 105 g/liter, respectively).

DISCUSSION

In this study we present evidence that SOD is significantly increased in cells (fibroblasts and circulating erythrocytes) prepared from psoriatic patients. SOD plays a major antioxidant role in cells by converting the superoxide ion ($O^{\cdot}_2$) into H_2O_2. In most cells, SOD includes both the soluble CuZn-SOD and the mitochondrial Mn-SOD. In fibroblasts, CuZn-SOD has been reported to be localized in the peroxisomes (Keller *et al*, 1991), where there is additional cooperation with the catalase and GPX activities. Catalase acts to neutralize high peroxisomal H_2O_2 concentrations, although some H_2O_2 may escape. This excess H_2O_2 in turn is normally neutralized by cytosolic GPX. Any non-neutralized H_2O_2 can lead to the formation of the highly reactive and damaging $OH^{\cdot}$ by the iron-catalyzed reaction of $O^{\cdot}_2$ and H_2O_2 (Weiss, 1986). Taking into account the effect of elevated levels of SOD activity on free radical production, our data suggest that both $O^{\cdot}_2$ and H_2O_2 levels might be significantly increased in cultured psoriatic fibroblasts. Direct measurement of free radicals in psoriatic fibroblasts is required to determine if highly reactive $OH^{\cdot}$ are also produced even with the slight increase in catalase activity observed.

Antioxidant enzyme activities have been reported to vary from one cell type to another. Fibroblasts have higher levels of catalase, GPX, and SOD activity than keratinocytes (Yohn *et al*, 1991). Red blood cells were found to have high levels of catalase activity (Goth, 1989). The values obtained in this study for catalase and GPX activities in normal human fibroblasts and in red blood cells are in agreement with previous reports (Moysan *et al*, 1993; Shindo *et al*, 1994), but the SOD levels determined here in fibroblasts differ from a previous report (Moysan *et al*, 1993). This is apparently due to the differences in the assays employed, as SOD values have been shown to vary significantly depending on the type of assay (Spitz and Oberley, 1989). Variations in SOD activity have also been observed in different disease states. For example, SOD activity has been reported to be decreased in various cancer tissues (Hamanaka *et al*, 1991). Previous studies that examined psoriatic epidermis reported either normal total SOD or decreased CuZn-SOD activity (Van Baar *et al*, 1987; Iizuka *et al*, 1993) and normal or reduced amounts of the Mn-SOD enzyme determined by immunochemical staining of frozen sections of skin (Kobayashi *et al*, 1991). A recent report by Löntz *et al* (1995), however, describes an increase in Mn-SOD mRNA expression as determined by reverse transcription polymerase chain reaction in frozen-involved epidermis of psoriatic patients.

Interestingly, the increase in psoriatic fibroblast SOD activity was found to be unrelated to passage number in fibroblasts cultured

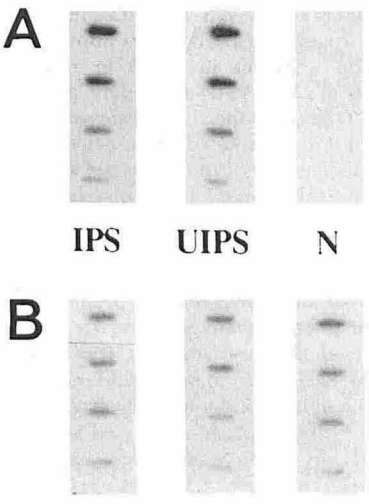

Figure 1. Increased Mn-SOD mRNA in cultured psoriatic fibroblasts. Slot blot analysis of total RNA from normal (*N*), lesional (*IPS*, Involved Psoriatic Skin) and nonlesional (*UIPS*, Uninvolved Psoriatic Skin) cultured fibroblasts hybridized with an Mn-SOD oligonucleotide antisense probe (*A*) as described under *Materials and Methods*. Hybridization was performed with a GAPDH probe as control (*B*).

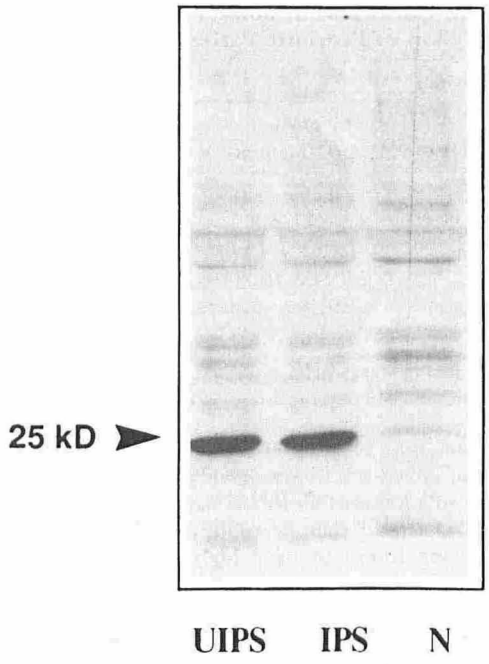

Figure 2. Immunoblot comparison of Mn-SOD protein levels in normal and psoriatic fibroblasts. Fifty micrograms of lysates from normal fibroblasts (*N*), nonlesional (*UIPS*), or lesional (*IPS*) psoriatic fibroblasts were analyzed by sodium dodecyl sulfate gel electrophoresis, transferred to nitrocellulose, and immunoblotted with a monoclonal anti-human Mn-SOD antibody. The figure shows samples with the highest difference in Mn-SOD expression between normal and psoriatic cells.

from both lesional and nonlesional skin (Gerbaud, personal data). This correlates with our previous observation that the alteration in cAMP binding to the RI and RII regulatory subunits of cAMP-dependent protein kinase is also observed in both lesional and nonlesional skin (Raynaud *et al*, 1987). This suggests that alterations observed in psoriatic fibroblasts may be independent of the inflammatory lymphocyte infiltration noted in lesional tissue. An increase in $O^{\cdot}2$ production could initiate an increase in the cellular antioxidant defense mechanism, i.e., an increase in SOD activity in psoriatic fibroblasts. Elevation of SOD activity alone, however, could result in increased levels of H_2O_2 in psoriatic fibroblasts, which might act to modify oxidatively important proteins within the cell including enzymes involved in transmembrane signal transduction such as the cAMP-dependent protein kinase system. These abnormal biologic properties of psoriatic fibroblasts might be involved in the inductive role of these cells in the appearance of psoriatic lesions (Saiag *et al*, 1985; Krueger and Jorgensen, 1990).

This increase in SOD activity also is observed in circulating cells (i.e., erythrocytes). To determine whether an increase in the inflammatory process might correlate with the increase in SOD activity observed in circulating red blood cells, we measured primary markers of the inflammatory process (orosomucoid and ceruloplasmin) in plasma obtained from the psoriatic donors. No change in the level of these markers was observed. Thus, the increase noted in SOD activities in psoriatic cells appears to be independent of the general inflammatory process of the disease and of the severity of the psoriasis expressed as determined by the Psoriatic Area Severity Index Score (Raynaud *et al*, 1987) (data not shown).

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Lack of Induction of IL-10 Expression in Human Keratinocytes

To the Editor:

We read with interest the report by Grewe *et al* [4] discussing the induction of IL-10 following ultraviolet B (UV-B) and ultraviolet A1 irradiation of human keratinocytes (KC) in primary culture. We too have performed similar UV-B experiments, and have also treated human KC and A431 epidermoid carcinoma cells with sensitizing and irritant chemicals. We were unable, however, to detect any IL-10 protein in the culture supernatants by an enzyme-linked immunosorbent assay (ELISA) with a sensitivity of 1.5 pg/ml before or after any of the above treatments. Furthermore, we could not detect IL-10 mRNA except 24 h after UV-B doses of 200 and 300 J/m^2. Generally, our results agree with those of Teunissen *et al*, who were unable to detect interleukin 10 (IL-10) mRNA (35 thermal cycles) or IL-10 protein in KC after UV-B (15-200 J/m^2), or treatment with nickel sulfate, IL-1α/β, tumor necrosis factor-α, interferon-γ, lipopolysaccharide, phorbol ester, or supernatants from phytohemagglutinin-stimulated T cells (Teunissen MBM, Koomen CW, de Waal Malefyt R, Bos JD: Human keratinocytes are unable to produce IL-10. *J Invest Dermatol* 102:632, 1994 (abstr.).

The experiments that we performed utilized KC derived from either foreskin or abdominal skin from five different donors. The sensitive reverse transcription polymerase chain reaction [5] was employed using ethidium bromide to detect amplified products resolved on 1.5% agarose gels and silver staining with a commercial kit (Bio-Rad Laboratories Ltd), or ^{33}P-labeled primers to visualize products on 12.5% polyacrylamide gels. We also carried out southern blotting of the agarose gels and hybridized IL-10 amplicons with a digoxigenin-labeled human IL-10 probe, which was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The primers were obtained from Clontech and successfully detected abundant IL-10 mRNA in RNA derived from lipopolysaccharide- or phorbol–ester-stimulated peripheral blood monocytes.

The KC from four different donors were negative for IL-10 mRNA for up to 24 h following irradiation with 100 J/m^2 of broadband UV-B, (280-320 nm). IL-10 mRNA was detected 24 h after UV-B irradiation with 200 and 300 J/m^2 only; however, we had to extend the thermal cycling to 40 cycles to detect even a weak band. In Grewe's study, 28 cycles were typically used. In contrast to the results presented in Grewe's study, 100 J/m^2 and timepoints earlier than 24 h did not induce IL-10 mRNA. Densitometry of the IL-10 southern blots probed with digoxigenin showed a 24-fold increase in IL-10 mRNA with 300 J/m^2, but no detectable IL-10 protein was found in the culture supernatants.

Although no IL-10 mRNA induction was observed, a 4.5-fold induction of IL-8 mRNA and an 8-fold increase in IL-8 protein (measured by ELISA) was noted 24 h after irradiation with 100 J/m^2 and a 30-fold induction of mRNA for the p40 subunit of IL-12 was seen 3 h after 100 J/m^2. Thus, the cells were clearly responsive to UV-B. Changing the calcium content of the medium from 0.06 mM to 2 mM for 48 h prior to irradiation or stimulating the KC with 10 ng/ml IL-1α, 200 U/ml IL-2, 0.01% sodium dodecyl sulfate, or 200 μg/ml nickel sulfate had no effect on the induction of IL-10 mRNA. Furthermore, treatment of A431 cells with irritants [sodium dodecyl sulfate (0.0001-0.01%) or nonanoic acid (0.0001-0.01%)], sensitizers [nickel sulfate (10-200 μg/ml) or diphenylcyprone (0.000003-0.003%)], or phorbol ester (10 ng/ml) were all ineffective in inducing IL-10 mRNA or protein. Phorbol ester treatment, however, did induce tumor necrosis factor-α mRNA, and irritants induced IL-8 mRNA showing that the cells were capable of responding to stimulation with cytokine gene expression.

Our observations and those of Teunissen *et al* raise several points. First, if IL-10 mRNA is induced but no protein is detected in the culture supernatant, is the IL-10 protein being sequestered, possibly by a soluble receptor, or is this another case that illustrates the pitfalls of measuring only mRNA abundance? Second, the induction of IL-10 mRNA [1] and protein [2] by sensitizers and IL-1 in murine KC could not be reproduced in human KC, either in our studies or in those of Teunissen *et al*. This suggests that IL-10 may be another example, like IL-3 in which human and murine KC express different cytokines [7]. The induction of IL-10 in murine KC and epidermis by sensitizers but not irritants has been demonstrated both *in vitro* [1,2] and *in vivo* [6]. It has been proposed that this property may be a useful means of distinguishing sensitizers from irritants *in vitro*. Clearly, this test may be inappropriate if human KC do not respond to sensitizers. Finally, is the discrepancy in results between different studies due to differences in the primers or ELISA kits used (Stratagene primers and ELISA kits from Genzyme and Laboserve in [4] and Clontech primers and ELISA kits from R & D systems in our study), or does it represent true phenotypic differences in the expression of IL-10 in human cells? In support of the latter is a recent study [3], which showed that only five out of nine KC cultures from human subjects showed constitutive IL-10 mRNA expression and that culture supernatants had to be concentrated 10-fold before low (<30 pg/ml) levels of IL-10 protein were detected, in four irradiated cultures. These questions require resolution before epidermal IL-10 expression can be effectively integrated into the skin cytokine network in human subjects.

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NOTE IN PROOF

Another report of lack of IL-10 induction in human KC has appeared (Ried C, Michel G, Beetz A, Kemeny L, Ruzicka T: Lack of induction of IL-10 in human keratinocytes by inflammatory cytokines and UVB. *J Invest Dermatol* 103:443, 1994, abstr.).

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Reply:

We read with surprise the letter by Jackson *et al*, in which the authors report on their methodological problems in detection of IL-10 mRNA and, in particular IL-10 protein production, by UV-irradiated human keratinocytes.

The failure to detect increased IL-10 mRNA expression in human keratinocytes exposed to 100 J/m^2 ultraviolet-B (UV-B) radiation may be due to lack of sensitivity of the reverse-transcriptase-polymerase chain reaction (RT-PCR) method used. Not only selection of primers, but also optimization of reverse transcription and PCR is essential for the detection of low amounts of transcripts. In our hands, reverse transcription of total RNA was optimal with the use of random hexamer primers resulting in highest amplification products for several keratinocyte-derived cytokines including IL-10. For the sake of specific reverse transcription of mRNA, the data published [1] were generated using oligo-dT_{18} primers. These findings have been corroborated in an independent study employing RT-PCR methods, demonstrating that exposure of cultured human keratinocytes to doses as low as 50 J/m^2 UVB radiation significantly induces IL-10 mRNA expression [2]. Since RT-PCR has some pitfalls, we have additionally performed Northern-blot experiments **(Fig 1)**. Increased IL-10 mRNA steady state levels could be detected in cultured normal human keratinocytes following UVB or UVA1 irradiation. Moreover, essentially identical data have been obtained by Northern blot analysis in an independent study conducted by Y. Aragane and T. Schwarz, employing cultured human keratinocytes as well as KB cells (data not shown). These *in vitro* findings are supported by recent *in vivo* studies demonstrating that UV irradiation of human skin leads to increased expression of IL-10 mRNA expression in epidermal keratinocytes [3]. In aggregate, these studies provide further proof that UVB radiation at physiologically relevant doses is capable of inducing IL-10 mRNA expression in human keratinocytes both *in vitro* and *in vivo*.

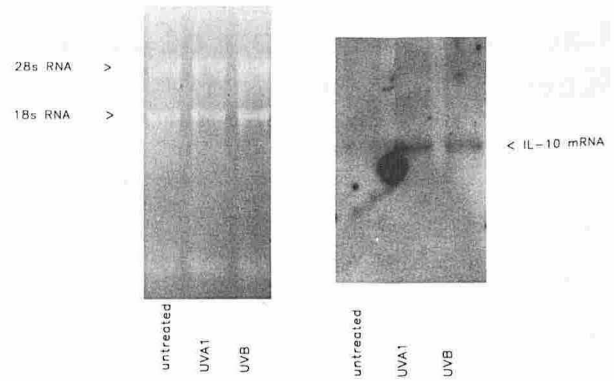

Figure 1. Northern blot analysis of IL-10 mRNA expression in UV-irradiated normal human keratinocytes. Normal human keratinocytes were left unirradiated or exposed to 100 J/m^2 UV-B and 32 kJ/cm^2 UV-A1 radiation, respectively. After 24 h, extraction of total RNA with subsequent Northern blot analysis using a digoxigenin-labeled, IL-10-specific cDNA was carried out by standard techniques. *Left panel*, ethidium bromide stain of RNA agarose gel electrophoresis; *Right panel*, IL-10 mRNA-specific signal.

We have previously reported that UVB-irradiated normal human keratinocytes secrete significant amounts of IL-10 protein into culture supernatants [1]. Parallel to their failure to detect increased IL-10 mRNA expression, Jackson *et al*, by employing enzyme-linked immunosorbent assay (ELISA) techniques, also could not measure increased IL-10 protein secretion by human keratinocytes. In our study, IL-10 protein secretion could be detected by means of two different ELISAs, both of which differed from that employed by Jackson *et al* [1]. One ELISA was based on a polyclonal anti-human-IL-10 antiserum, whereas the other used a non-neutralizing anti-IL-10 antibody. Since none of the ELISAs successfully used by us was based on neutralizing antibodies, it may be that IL-10 released into supernatants of stimulated human keratinocytes binds to a carrier protein, e.g., soluble IL-10 receptors, which may compete with binding of neutralizing anti-IL-10 antibodies and thereby result in lack of IL-10 protein detection. Our results have again been corroborated and extended in an independent study, in which IL-10 proteins could be measured by ELISA after crude supernatants were concentrated 10-fold [2]. In our hands, concentration of culture supernatants was not necessary to detect sufficient amounts of IL-10 protein. It should be noted, however, that our culture conditions were optimal in reducing the volume of supernatants to 1.5 ml/5 $\times$ 10^6 cells in order to avoid dilution of IL-10.

Essentially identical data were recently obtained in independent experiments conducted by M. Duvic and S. Ullrich **(Fig 2)**. In these experiments, UV-B radiation dose-dependent IL-10 protein release into supernatants of human skin equivalents [4] was assessed by employing an ELISA different from those used in our study [1]. In these experiments, culture supernatants did not have to be concentrated for detection of IL-10 protein. In addition, irradiation with UV-B induced IL-10 protein secretion to a level similar to that observed in our study [1]. It could also be elegantly demonstrated that increased IL-10 protein secretion of UV-B-irradiated human skin equivalents was keratinocyte-derived, since immunohistochemical studies revealed strong IL-10 protein expression within the cytoplasm of keratinocytes following UV-B radiation exposure of skin equivalents **(Fig 3)**.

In summary, we and others have independently provided definitive proof both at the mRNA and protein level that human keratinocytes are capable of synthesizing and releasing increased amounts of IL-10 upon exposure to UV-B radiation. Negative experimental results, as described by Jackson *et al*, need to be interpreted with great care in order to avoid neglect of keratinocyte functions important for the cytokine network in human skin.

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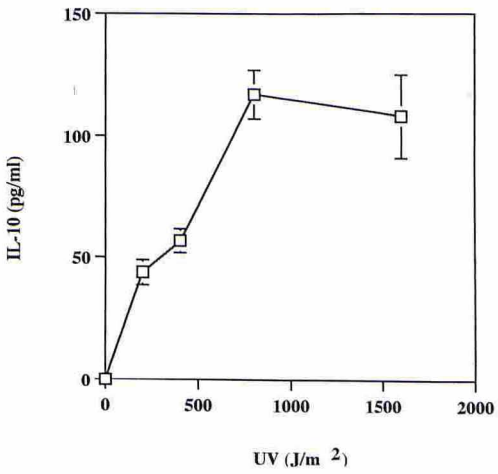

Figure 2. IL-10 protein secretion into the supernatant of human skin equivalents. Skin equivalents, constructed by plating 5×10^5 human foreskin keratinocytes over type I collagen gels containing human foreskin fibroblasts, were raised to the air interface and grown for 14 days in FAD media as described [4]. The skin equivalents were then irradiated with UV radiation supplied by a singek unfiltered FS-40 sunlamp (0–1600 J/m^2) as measured with a IL-700 research radiometer. The culture fluid was collected and the IL-10 concentration determined by ELISA using reagents supplied by PharMingen Inc. (San Diego, CA). For each treatment, the mean value of triplicate samples was determined. Error bars, SD. A representative experiment is shown.

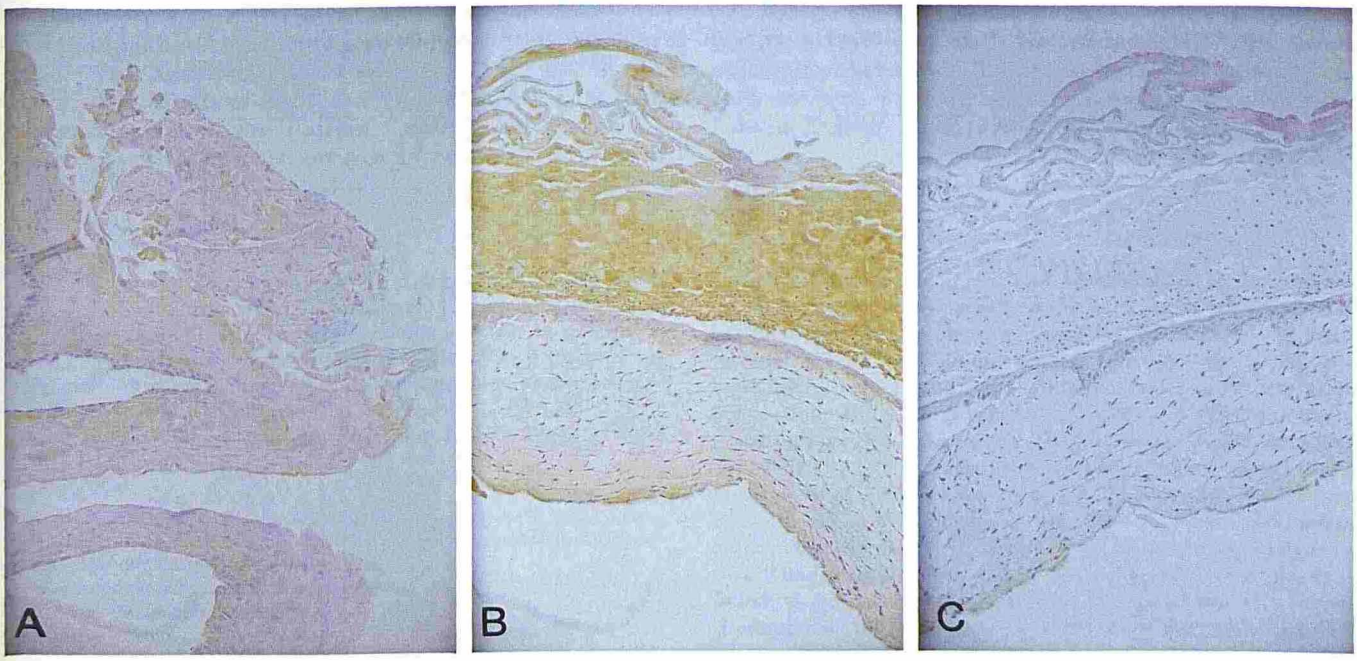

Figure 3. Keratinocyte IL-10 protein expression after UVB irradiation of human skin equivalents. The skin equivalents were treated with a UV light at varying amounts and 24 h later the SE were harvested and fixed for immunohistochemistry [4]. Anti-human IL-10 antibody (rat IgG1, clone JES3-9D7) was used at 1:10 dilution and detected with DAKO LSAB 2° antibody and counterstained with hematoxylin and eosin. *A*, non-irradiated skin equivalent—basal layer (*arrow*) (40×); *B*, skin equivalent receiving 1600 J/m^2—dermis below (40×); *C*, negative control of SE in *B* without primary IL-10 antibody (40×).

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