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Is an epitope on keratin 17 a major target for autoreactive T lymphocytes in psoriasis?

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SUMMARY

Psoriasis is a T cell-mediated inflammatory skin disease that has been associated with infections by group A β-haemolytic streptococci. In a previous study of patients with active psoriasis we demonstrated an increased frequency of circulating Th1-like cells that responded to 20 amino acid (aa) streptococcal M-peptides sharing sequences with human keratin. These cells disappeared after ultraviolet B (UVB)-induced clinical remission. Using T cells from the blood of 17 psoriatic patients and 17 healthy controls we have now compared the numbers of interferon-gamma (IFN-γ)-producing cells induced by seven 18–20 aa keratin peptides and five corresponding M-peptides. The most frequent and strongest responses were observed to a peptide from keratin 17 that shares ALEEAN sequence with M-protein. The responses to this peptide were stronger than to the corresponding M-peptide containing the ALEEAN sequence. After UVB treatment T cell responses to all the M- and keratin peptides were abolished, while responses to the positive control antigen streptokinase/streptodornase (SK/SD) were not affected. These findings are consistent with the notion that aa sequences which keratin has in common with M-protein may be a major target for autoreactive T cells in psoriasis.

Keywords psoriasis autoimmunity streptococcal M-proteins keratins T cells

INTRODUCTION

Psoriasis is an inflammatory skin disease that affects about 2% of Western populations. The characteristic lesions are erythematous scaling plaques, which most frequently begin in the scalp, but extensor surfaces of elbows and knees and buttocks are also commonly affected.

Hyperproliferation of keratinocytes is a characteristic feature of psoriatic epidermis and is associated with an altered programme of keratinocyte differentiation. There is an increased expression of keratin 14 [1] and a novel suprabasal expression of the hyperproliferative keratins 6 and 16 with a corresponding reduction of keratins 1 and 10, which are expressed suprabasally in normal skin [2]. In contrast to normal skin, keratin 17 is expressed in suprabasal keratinocytes in psoriatic lesions [3].

It has been demonstrated that epidermal infiltration and activation of $CD4^+$ T cells coincides with the onset of psoriatic lesions [4] and it was postulated that T cells play a key role in the pathogenesis of the disease [5]. The evidence supporting this theory is now compelling. Thus, a marked clinical improvement has been achieved by treatment of patients with cyclosporin A [6], anti-CD4 MoAbs [7] or administration of low-dose IL-2 linked to diphtheria toxin [8]. Furthermore, activated autologous $CD4^+$ T cell lines can induce characteristic psoriatic plaques in uninvolved psoriatic skin, transplanted on mice with severe combined immunodeficiency (SCID) [9–11]. In this model the psoriatic lesions can be maintained by injection of T cells obtained from psoriatic skin but not by T cells from the blood of psoriatic patients [12]. No evidence has been reported that antibodies or immune complexes contribute significantly to the pathogenesis of psoriasis.

The onset of acute psoriasis is frequently associated with throat infections by group A β-haemolytic streptococci [13–15], but the effect of such infections on chronic psoriasis has not been adequately documented. Streptococcal M-protein consists of fibrillar α-helical coiled coil dimers that protrude from the surface of the bacteria [16]. Keratin shares this structure with M-protein and an extensive amino acid homology has been reported between protein M6 and 50-kD (K14) type I human epidermal keratin [17].

It has been postulated that cross-reactions between M-proteins and human epidermal keratin may play a role in the pathogenesis of psoriasis [18]. Epidermal cells, from psoriatic patients, stimulate

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autologous peripheral blood mononuclear cells (PBMC) [19], and recently it has been reported that dendritic cells from lesional psoriatic skin are more effective stimulators of proliferation by autologous blood T cells than dendritic cells derived from psoriatic blood or normal skin [20].

We have previously reported that T cells from the blood of patients with active chronic psoriasis respond by interferon-gamma (IFN-γ) production to 20 aa peptides [21] from the conserved C-terminal part of the streptococcal M6 protein [22], sharing regions of 5–6 aa with keratin. T cells from healthy controls or patients with atopic dermatitis showed minimal or no responses to these peptides. The reactive T cells could no longer be detected in the blood of the psoriatic patients after ultraviolet B (UVB)-induced remission [21].

In this study we compared T cell responses of psoriatic patients and healthy controls to 18–20 aa keratin peptides and the corresponding M-peptides used in the previous study. These two types of peptides share regions of five to six aa but contain different aa that flank the shared sequences. The T cell responses of the patients were also monitored during UVB treatment.

SUBJECTS AND METHODS

Study subjects; clinical evaluation and treatment of the patients

The study was approved by the Ethical Committee of the National University Hospital. Seventeen patients with active, untreated plaque type psoriasis and 17 healthy individuals of comparable age and sex were recruited for the study. Nine of the psoriatic patients were also tested after 2 and 4 weeks of treatment, which consisted of daily UVB exposure combined with bathing in a geothermal lagoon for 2 h a day, 6 days a week for 4 weeks. This has shown to be an effective treatment for psoriasis [23]. Disease severity was evaluated by the Psoriasis Area and Severity Index (PASI) before and on a weekly basis during the treatment [24]. Initially, throat swabs were obtained from the study participants and cultured for β-haemolytic streptococci.

Antigens and identification of shared sequences

M-protein was isolated from *Streptococcus pyogenes* serotype 6 (Public Health Laboratory Services, London, UK) according to Pruksakorn *et al*. [25] as described [21]. Streptokinase/streptodornase (SK/SD) was purchased from Behringwerke AG (Marburg, Germany).

Three different five to six aa sequences from the conserved C-terminal part of M-proteins of the serotypes 5, 6, 12 and 24 (M6, M5, M12 and M24) [22,25], which are shared with epidermal keratins, had previously been identified by multiple alignment construction and analysis workbench (MACAW) program (NCBI, Bethesda, MD) [21]. The M- and keratin peptides used in this study and shown in Table 1 [21] were synthesized by the 'tea bag' method [26]. M- and keratin peptides sharing the same sequence are identified by the same numbers. An M-peptide not sharing sequences with keratins was used as a negative control (159-M, Table 1). The purity of the peptides was checked by high performance liquid chromatography (HPLC).

Isolation of T lymphocytes

PBMC were isolated from 60 ml of heparinized blood by Ficoll–Hypaque (Sigma, St Louis, MO) centrifugation [27]. Monocytes were isolated from PBMC by plastic adherence at 37°C for 1 h in tissue culture media (TCM), RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK) with 2 mM glutamine (Gibco), 100 U/ml penicillin/100 μg streptomycin (Gibco) supplemented with 4% human serum albumin (HSA; Pharmacia, Copenhagen, Denmark). The monocytes were subsequently used as antigen-presenting cells. The PBMC were incubated with carbonyl-iron at 37°C for 30 min and neutrophils removed by magnetic attraction. The lymphocytes were washed and resuspended in TCM supplemented with 0·2% HSA. T cells were isolated by rosetting with sheep erythrocytes treated with neuraminidase (Sigma). They were separated from non-rosetting cells by Ficoll–Hypaque (Sigma) centrifugation and the sheep erythrocytes lysed with sterile distilled H_2O for 35 s [28].

Table 1. Sequences of the keratin peptides and the corresponding M-peptides

Peptides*	Peptide sequences†
145-M	LRRDLDASREAKKQVEKALE
145-K17	LRRVLDELTLARTDLEMQIE
145-K10	LRRVLDELTLTKADLEMQIE
146-M	AKKQVEKALEEANSKLAALE
146-M49	AKKKVEADLAEANSKLQALE
146-K17	SYLDKVRALEEANADLEVK
146-K9	SYLDKVQALEEANNDLENKI
149-M	KLTEKEKAELQAKLEAEAKA
149-K7	QAEIDNIKNQRAKLEAAIAE
150-M	QAKLEAEAKALKEQLAKQAE
150-K7	RAKLEAAIAEAEECGELALQ
150-K18	KVKLEAEIATYRRLLEDG
159-M	MATAGVAAVVKRKEEN

*Peptides sharing sequences are identified by the same number followed by M for M-peptide and a number for the serotype or K for keratin peptide and number for the keratin type.

†All peptides are 20 aa long except for 146-K17 (19 aa), 150-K18 (18 aa) and the control peptide 159-M (16 aa) that does not share sequences with keratin.

T cell stimulation and ELISPOT assay

Purified T cells were washed and adjusted to 1×10^6 cells/ml in TCM with 10% heat-inactivated human AB serum and 5×10^4 autologous monocytes/ml (5%) were added. The cells were then prestimulated with optimal concentrations of M6-protein (5·0 μg/ml), SK/SD (200/50 U/ml), M-peptides or keratin peptides (2·5 μg/ml) in tubes at 37°C and humidified 5% CO_2 for 5 h. ELISPOT assay [29] was used to determine the frequency of cytokine-producing cells, essentially as described [30,31]. Nitrocellulose-bottomed 96-well Millititer HA plates (Millipore Co., Bedford, MA) were coated with 15 μg/ml of MoAbs to IFN-γ (1-D1K) (Mabtech AB, Stockholm, Sweden) at 4°C for 5 h and unbound antibodies removed by washing. The prestimulated T cells (10^5 in 150 μl per well) were transferred to the antibody-coated wells and the plates incubated at 37°C and humidified 5% CO_2 for 3 days. The cells were then removed and biotin-conjugated MoAb (Mabtech) to IFN-γ (1 μg/ml) was added and the plates were

incubated for 3 h, followed by incubation with 1 μg/ml strepavidin-alkaline phosphatase (Mabtech) for 1 h. The reaction was developed by 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate solution (BioRad Labs, Hercules, CA) for about 1 h, the plates were dried and blue spots counted using a dissection microscope, one spot representing cytokine production by a single cell. Results are expressed as number of spots per 10^5 T cells. Positive Th1-like response was defined as ≥ 10 IFN-γ spots/10^5 T cells, above the background.

Statistical analysis

Mann–Whitney rank sum test was used to compare the T cell responses of psoriatic patients and healthy controls to different antigens. Frequency of psoriatic patients and healthy controls responding to one or more of the peptides was compared with the Fisher's exact test. That test was also used to compare the frequency of patients and controls responding to one or more of the M-peptides and corresponding keratin peptides. The responses of the patients' T cells to 146-K17 were compared with the responses to 146-M, 146-K9 and 146-M49 by paired *t*-test and Wilcoxon signed rank test. The Pearson's product moment correlation test was used to analyse the relationship between responses of individual patients to different stimuli and PASI score.

RESULTS

Clinical findings

Before treatment the PASI score of the 17 patients ranged from 3·6 to 17·2 (mean 10·4). Nine patients who were also tested during and after the UVB treatment had a mean PASI score of 8·9 (range 3·6–16·4) before treatment and 2·1 (range 0–5·4) after treatment.

None of the patients had any evidence of group A streptococcal infections at the beginning of the study, but group C streptococci were isolated from one control (no. 13).

T cell responses of untreated patients and controls

T cells from the patients and the controls were stimulated with seven keratin peptides and five M-peptides containing three different shared sequences, and an M-peptide not sharing sequences with keratins was used as a negative control (Table 1).

As previously observed for the M-peptides [21], no IL-4 production was detected after stimulation with the keratin peptides, either in the patients or in the healthy controls (data not shown). Therefore, only IFN-γ-producing T cells were enumerated throughout this study. There was no significant difference between the psoriatic patients and the controls in responses to the whole M6 protein or to the positive control antigen SK/SD, which elicited much stronger responses than the other antigens (data not shown).

Table 2. T cell responses of untreated patients and controls to streptococcal M-peptides and keratin peptides sharing sequences*†

	145-M	145-K17	145-K10	146-M	146-K17	146-K9	146-M49	149-M	149-K7	150-M	150-K7	150-K18	159-M
Patients													
1	−	−	−	+++	++++	−	−	−	−	−	−	−	−
2	+	−	++	−	++	−	−	−	−	−	+	−	−
3	−	−	++	+	−	−	−	−	−	−	−	−	−
4	−	++	−	−	+	−	−	+	−	−	−	−	−
5	−	−	−	−	++++	−	−	++	+	−	+	−	++
6	−	−	−	+	++	+	−	−	−	−	−	−	−
7	−	−	−	++	++	−	−	−	−	−	+	−	−
8	−	−	−	+	++++	−	+	−	−	−	−	−	−
9	++++	+	+	−	++	+	+	+++	−	−	−	−	−
10	−	−	+	−	+	−	−	−	−	−	−	−	+
14	−	+	−	−	+	+	−	−	+	−	−	−	−
15	−	−	+	−	++++	−	−	−	−	−	−	−	−
16	−	++++	−	+	+	++++	−	−	+	−	−	++++	+
17	−	−	−	−	++	−	−	−	−	−	−	−	−
Controls													
1	−	−	−	−	−	+	−	−	+	−	−	−	−
2	++	−	−	−	−	−	−	−	−	−	−	−	−
3	+	+	−	−	−	−	−	−	−	−	−	−	−
7	−	−	+++	−	−	−	−	−	++	−	−	−	−
9	++	−	++++	−	++++	−	−	−	−	−	−	−	−
10	++	−	++	−	++++	+	−	+++	−	−	−	−	−
13	−	−	++	−	++	−	−	−	−	−	−	−	−
15	−	−	++	+	−	−	−	−	−	−	++	−	−
17	−	−	−	−	+	−	−	−	−	−	++	−	−

*Positive response was defined as ≥ 10 IFN-γ^+ cells/10^5 cells: −, < 10 IFN-γ^+ cells; +, 10–19 IFN-γ^+ cells; ++, 20–29 IFN-γ^+ cells; +++, 30–39 IFN-γ^+ cells; ++++, ≥ 40 IFN-γ^+ cells.

†The three patients and eight controls who showed no responses are omitted from the table.

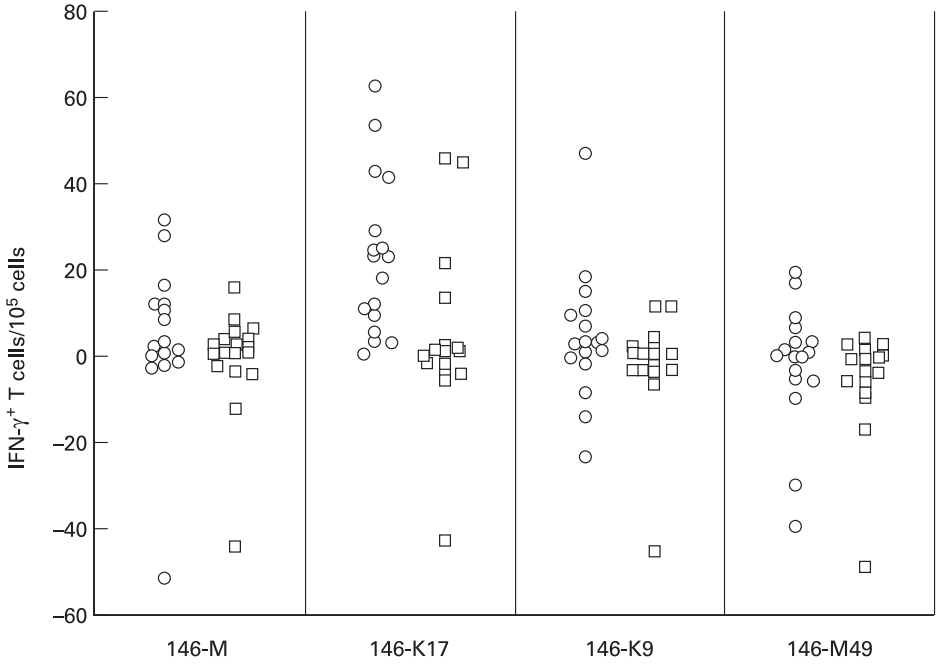

Fig. 1. Frequency of IFN-γ-producing T cells of patients (○) and controls (□) after stimulation with M- and keratin peptides sharing the ALEEAN sequence. Peptide 146-K17 was the only peptide which elicited significantly stronger responses in psoriatic patients than in healthy controls ($P = 0{\cdot}001$). The patients' responses to this peptide were significantly stronger than to the corresponding M- and keratin peptides, 146-M, 146-M49 and 146-K9 ($P = 0{\cdot}005$, 0·001 and 0·004, respectively).

Only three patients failed to respond to all the peptides compared with eight of the controls (Table 2). Peptide 146-K17 from keratin 17 induced strikingly stronger and more frequent responses than any of the other peptides. Thirteen patients responded to this peptide compared with four controls ($P = 0{\cdot}005$), and the responses were also significantly stronger in the patients ($P = 0{\cdot}001$). Furthermore, the patients' responses to 146-K17 were significantly stronger than the responses to the corresponding 146-M peptide that also contains the ALEEAN sequence ($P = 0{\cdot}001$) (Fig. 1). The 146-K17 also induced stronger responses than the keratin peptide 146-K9, which contains the ALEEAN sequence but differs in three flanking aa ($P = 0{\cdot}004$). It should be noted that only two individuals responded weakly to the 146-M49 peptide (Table 2) which does not contain the complete ALEEAN sequence, and also differs in three flanking aa from the 146-M peptide (Table 1).

Overall, more patients (10/17) than controls (3/17) showed a positive response (≥ 10 T cells/10^5 cells) to three or more peptides ($P = 0{\cdot}02$). Furthermore, eight patients responded to one M-peptide and one or more of the corresponding keratin peptides compared to three controls ($P = 0{\cdot}08$) (Table 2).

There was no significant difference between the responses of patients and controls to the other peptides, including the control peptide 159-M that does not share aa sequences with keratin.

The PASI scores of the patients correlated positively with their T cell responses to peptides 145-M, 145-K17, 146-M49 and 150-K18 ($r = 0{\cdot}49$–$0{\cdot}59$, $P = 0{\cdot}01$–$0{\cdot}05$). In contrast, a negative correlation was strikingly observed between the PASI scores and responses to 146-K17 ($r = -0{\cdot}50$, $P = 0{\cdot}04$).

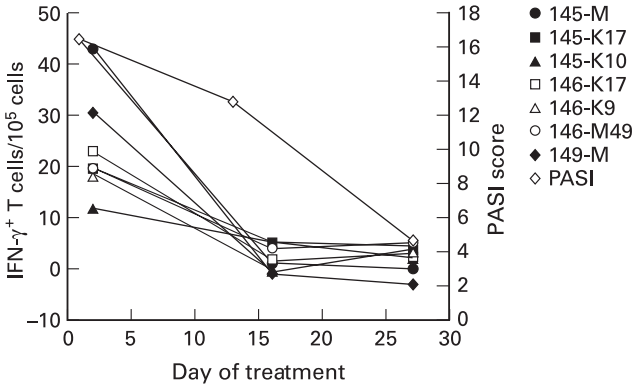

Fig. 2. Frequency of IFN-γ-producing T cells from patient 9 responding to different peptides before and after 2 and 4 weeks of treatment. The responses to all peptides had disappeared after 16 days of treatment and before the Psoriasis Area and Severity Index (PASI) score had decreased markedly.

T cell responses during and after the UVB treatment

The nine patients who responded with ≥ 15 spots/10^5 T cells to two or more of the peptides before treatment were tested after 2 and 4 weeks of treatment. For comparison, three patients who initially showed no or borderline response were tested at the end of the treatment.

After 2 weeks of treatment the PASI score was already significantly reduced and at this stage the responses to all peptides had disappeared in six of the nine patients. Figure 2 shows the responses of one representative patient to different peptides during treatment.

After 4 weeks when clinical remission had generally been obtained, eight of the nine patients did not respond to any of the

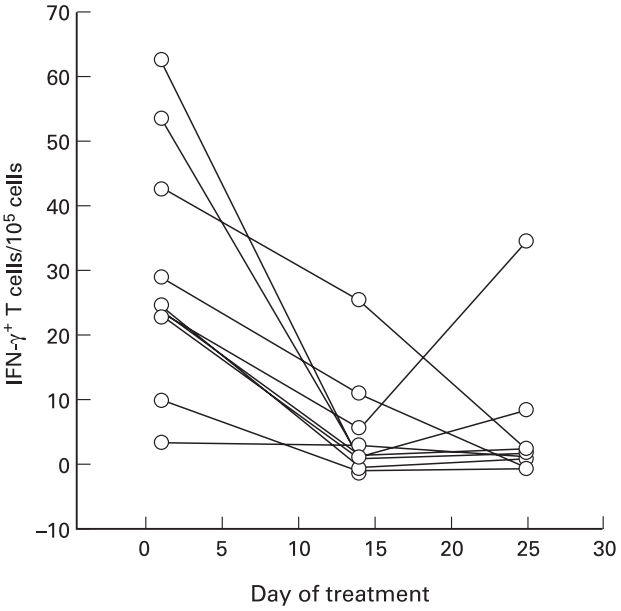

Fig. 3. Changes in T cell responses to 146-K17 during treatment. Before treatment 8/9 patients responded to this peptide and there was a significant decrease in responses after about 2 weeks ($P = 0{\cdot}004$). The only patient who responded at the end of treatment had a fairly high Psoriasis Area and Severity Index (PASI) score (5·4) and was therefore treated for one additional week.

peptides. The patient who still showed responses to several peptides, in particular to 146-K17 (Fig. 3), still had a fairly high PASI score (5·4) and was therefore treated for 1 additional week. The two patients who did not respond to any peptides before treatment were still unresponsive after 4 weeks. One patient who responded weakly to two peptides (145-K10 and 146-K17) before treatment still responded to the same peptides after 4 weeks of treatment. The T cell responses to SK/SD were not affected by treatment (data not shown).

DISCUSSION

We have previously demonstrated that patients with active psoriasis have increased frequency of circulating Th1-like cells that respond to 20 aa streptococcal M-peptides sharing sequences with human epidermal keratin. We now report that T cells from psoriatic patients respond more frequently and strongly to corresponding keratin peptides containing the same shared sequences but different flanking aa residues. This was particularly pronounced for peptide 146-K17, which is a part of keratins 14 and 17. Keratin 17, which is not expressed in normal skin except for hair follicles, sweat and sebaceous glands and basal cells of the interfollicular epidermis in the scalp, is over-expressed in psoriatic epidermis [3,32]. It is interesting in this context that the scalp is the first site to be affected in the majority of patients (Gudjonsson *et al*., unpublished).

Injection of IFN-γ can induce psoriatic lesions [33] and this cytokine is believed to play a key role in the pathogenesis of psoriasis [34,35]. It has moreover been demonstrated that the expression of keratin 17 can be induced *in vitro* by IFN-γ [36,37] and this is the only keratin so far reported to be induced by this cytokine [36,38]. The expression of K17 can also be induced by IL-6 [39], which is abundant in psoriatic lesions [40].

Keratin 14 is expressed together with keratin 5 in normal epidermis, but is over-expressed in psoriasis [1] and in other hyperproliferative conditions such as wound healing, which is also associated with epidermal infiltration of T cells [41]. Interestingly, psoriatic lesions preferentially appear at sites of wound healing (the Koebner phenomenon).

As keratin is a cytoskeletal protein it may not be obvious how it could act as an autoantigen. However, it has been observed in a carefully conducted electromicroscopical study of psoriatic epidermis that cytoplasmic processes of Langerhans cells can extend into the cytoplasm of adjacent keratinocytes with frequent absence of intervening plasma membranes at the apices of these processes [42]. Antigen-presenting cells therefore seem to have direct access to keratin in psoriatic epidermis, and CD8 T cells might also recognize keratin peptides in the groves of keratinocyte MHC I molecules.

In our study the patients responded markedly better to 146-K17 than to the corresponding 146-M. This may be due to difference in aa flanking the ALEEAN sequence. It is conceivable that 146-K17 may contain a motif that binds more strongly to the human leucocyte antigen (HLA) allotypes associated with psoriasis. Furthermore, the whole sequence of 146-K17 is also found in K14. Thus the over-expression of K14 and K17 may lead to increased presentation of this epitope.

In a previous study using M-peptides, peptide 146-M containing the shared six aa ALEEAN sequence gave the strongest and the most frequent responses in patients with chronic active psoriasis [21]. This was confirmed, although the difference between patients and controls was not significant in the present study ($P = 0{\cdot}09$). This discrepancy may reflect disease heterogeneity or a seasonal variation in streptococcal infections. The previous study was carried out in mid winter when streptococcal infections are most frequent, but the current study in the early autumn when such infections are less common [43].

T cells from some of the participants of our study showed pronounced spontaneous IFN-γ production resulting in apparently negative responses (Fig. 1). This may reflect induction of apoptosis by antigenic stimulation of already activated cells [44]. It has been demonstrated that activated T cells are sensitive to anti-CD3-driven apoptosis, whereas resting cells are relatively insensitive [45].

A significant positive correlation was observed between the PASI score and the responses to several of the peptides. However, a striking exception was the response to 146-K17, which before treatment correlated negatively with the PASI score. If the K17 peptide contains a dominant epidermal epitope in psoriasis, T cells that recognize this peptide may be more actively retained and thereby delayed in the skin and consequently less readily detected in the circulation. Reduction in circulating $CD4^+$ T cells has previously been reported in patients with extensive and active psoriasis [46].

In this study a significant reduction in PASI score was observed already after 2 weeks of treatment, and clinical improvement coincided with a significant decrease in T cell responses to 146-K17 ($P = 0{\cdot}004$) (Fig. 3). Only one patient still showed positive responses to some peptides at 4 weeks, but as he still had a fairly active disease his treatment was continued for an additional week. The T cell responses to SK/SD were not affected, indicating that the treatment did not induce systemic suppression. SK/SD was

used as a positive control because it is derived from streptococci, but in contrast to M- and keratin peptide-specific T cells, it was considered unlikely that SK/SD-responsive T cells would be selectively retained in psoriatic lesions.

UVB combined with bathing in a geothermal lagoon has previously been shown to be an effective treatment for psoriasis [23]. It has been demonstrated that UVB induces IL-10 expression in human keratinocytes and dermal macrophages [47,48]. Interestingly, in psoriasis the cutaneous expression of IL-10 mRNA is low compared with other inflammatory dermatoses, and administration of IL-10 has a beneficial effects on psoriatic lesions [49].

Previous studies have shown that activated T cells disappear from the epidermis and dermis during spontaneous or treatment-induced resolution of skin lesions [50,51]. It has been confirmed that T cells disappear from the epidermis of psoriasis patients during UVB treatment and, furthermore, that T cells are 10-fold more sensitive than keratinocytes to apoptotic effects of UVB [52]. Recirculating T cells specific for the M- and keratin peptides may therefore have disappeared from the blood as a result of UVB-induced anergy or apoptosis in the skin. It is interesting in this context that UVB tends to induce more prolonged remissions of psoriasis than, for example, cyclosporin A or topical steroids (personal observation). The participants of this study had a remission that lasted over 4 months on average (range 0·5 to > 14 months), and relapses were in some instances in the form of guttate type psoriasis, which is closely associated with clinical streptococcal infections.

In conclusion, our findings are consistent with the possibility that keratin 17, which is inducible by IFN-γ and contains the ALEEAN sequence, may together with keratin 14 be a major target for autoreactive T cells in psoriasis.

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