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Humoral Autoimmune Responses to the Squamous Cell Carcinoma Antigen Protein Family in Psoriasis

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Substantial evidence indicates that psoriasis is a T-lymphocyte-mediated autoimmune disease. However, longstanding data also indicate IgG and complement deposition in upper epidermis of psoriasis plaques. This led us to propose that autoantigen–autoantibody interactions in the skin may also be of pathogenic importance. Here, we have confirmed the presence of IgG in upper lesional epidermis and used high-resolution two-dimensional immunoblotting of extracts from this tissue, and laser desorption mass spectrometry of tryptic peptides, to define a series of epidermal proteins that bind IgG from psoriatic serum. The most prominent of these autoantigens are homologues of the serpin, squamous cell carcinoma antigen (SCCA), the other autoantigens identified including arginase 1, enolase 1, and keratin 10. Blood levels of IgG autoantibodies that bind to SCCA proteins were significantly higher in psoriasis than healthy controls ($P=0.005$), but were not detectable in sera from patients with active atopic dermatitis. To our knowledge, SCCA proteins have not previously been described as autoantigenic in animals or humans and form complexes with IgG that are associated with complement deposition. These findings expose potentially pathogenic humoral immunologic events and thus possible therapeutic targets in psoriasis.

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INTRODUCTION

The skin lesions or plaques of psoriasis are characterized by epidermal hyperplasia and infiltrates of leukocytes including neutrophils and homing receptor-positive T lymphocytes (Gudjonsson *et al.*, 2004). The disease can be provoked by activated T cells in xenograft models (Boyman *et al.*, 2004) and therapies that target T cells are effective (Kupper, 2003), although potentially toxic in the long term. Analysis of T-cell receptor diversity indicates oligoclonal infiltrates in many, if not all psoriatic lesions, and thus an antigen-driven process (Menssen *et al.*, 1995; Vekony *et al.*, 1997). These and other findings, including association with HLA-Cw*0602 and inflammatory arthropathy, indicate that psoriasis is an autoimmune disease that occurs in genetically predisposed individuals (Gudjonsson *et al.*, 2004). Although these findings show that T-cell-mediated events are of key pathogenic importance, it was reported more than 30 years ago that there is more IgG bound *in vivo* in the upper epidermis and stratum corneum of psoriatic lesions than in a variety of other inflammatory and hyperkeratotic skin diseases (Jablonska *et al.*, 1975). Reports also describe the presence of products of complement activation in the surface stratum corneum of plaques, including C5a/C5a des arg (Schröder and Christophers, 1986) and C5-9/membrane attack complex (Takematsu and Tagami, 1992). These data suggest that antigen–antibody interactions, which provoke complement activation, are of pathogenic relevance in psoriasis.

A previous attempt to identify autoantigens in psoriatic lesions by screening cDNA expression libraries with psoriatic serum defined three putative autoantigens, including heterogeneous nuclear ribonucleoprotein-A, keratin 13 (K13), and a previously uncharacterized protein (Jones *et al.*, 2004). Assay of IgG titers was complicated by phage reactivity requiring extensive pre-clearing of serum, and these titers were no different in psoriatic and control sera. Apart from this, there have been no reports of the systematic analysis of psoriatic lesional proteins that bind IgG. The rapidly shed and readily harvested surface stratum corneum of psoriatic lesions is a useful source of biologically active molecules that occur in the underlying viable epidermis. Analyses of extracts of this material have previously allowed demonstrations of the *in vivo* production of leukotriene B_4 (Brain *et al.*, 1984), IL-8 (Schröder and Christophers, 1986; Fincham *et al.*, 1988; Gearing *et al.*, 1990), tumor necrosis factor-α (Ettehadi *et al.*, 1994), and β-defensins (Harder *et al.*, 1997, 2001). We have therefore used stratum corneum as a source of lesional proteins to define IgG-reactive autoantigens in psoriasis.

RESULTS

IgG deposition in the upper epidermis of psoriasis lesions

Lesional stratum corneum was obtained from an informed, consenting volunteer with extensive, untreated chronic

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Abbreviations: Ab, antibody; AP, alkaline phosphatase; K, keratin; PBS, phosphate-buffered saline; PBS/T, PBS containing 0.05% Tween 20; PBS/T/M, PBS/T containing 1% skimmed milk powder; SCCA, squamous cell carcinoma antigen

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plaque psoriasis, and aqueous extracts were prepared as described in Materials and Methods. Total proteins in supernatants of these extracts were used to coat microtiter plate wells, and the presence of IgG was demonstrated by the use of alkaline phosphatase (AP)-labeled goat anti-human IgG either in the absence of added serum or after the addition of 100-fold diluted, pooled psoriatic serum (Figure 1). IgG was shown in stratum corneum extracts, and the amount of detected IgG could not be enhanced by the prior addition of psoriatic serum, which suggested that target molecules were substantially bound by IgG *in vivo*. This supports the longstanding findings of Jablonska *et al.* (1975), who reported IgG deposition in upper lesional epidermis by the use of direct immunofluorescence, which could not be usually enhanced by prior incubation of sections with psoriatic serum.

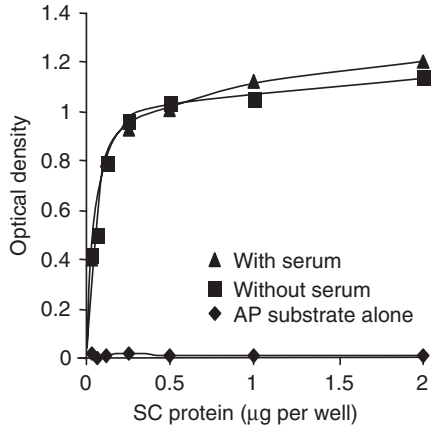

Figure 1. IgG is present in the stratum corneum (SC) of psoriasis lesions. The ELISA used AP-labeled Ab to detect IgG in aqueous stratum corneum extract, with and without prior addition of pooled, 100-fold diluted serum from eight psoriasis patients. The lowest curve shows optical readings in the presence of AP substrate alone, without added AP-labeled Ab. This indicates that there is no significant endogenous AP activity in the stratum corneum samples. Each point represents the mean of quadruplicate estimations.

Identification of IgG-reactive proteins in the stratum corneum of psoriasis lesions

Protein extracts were prepared by homogenization of psoriatic lesional stratum corneum and further solubilized by the addition of urea, thiourea, and non-ionic detergent. In these extracts, constituent proteins were successively separated by high-resolution isoelectric focusing and denaturing PAGE. Two-dimensional gels were prepared in the same electrophoresis tank and were either stained with colloidal Coomassie blue (Candiano *et al.*, 2004) (Figure 2a) or electroblotted onto nitrocellulose and incubated with pooled psoriatic serum (1:200) (Figure 2b) or pooled serum from age- and sex-matched healthy controls (Figure 2c). Bound IgG, detected with AP-labeled anti-human IgG, showed the presence of a series of IgG-reactive proteins that were more numerous on blots incubated with psoriatic sera than with control sera. Spots labeled 1–11 (Figure 2b and c) corresponded with similarly numbered proteins on the Coomassie-stained gel (Figure 2a). Appropriate Coomassie-stained protein spots were cut from the gel, subjected to trypsin digestion, and tryptic peptides analyzed by MALDI-TOF (matrix-assisted, laser desorption ionization-time of flight) mass spectrometry (Shevchenko *et al.*, 1996; Bonetto *et al.*, 1997). The proteins identified included enolase 1, squamous cell carcinoma antigen (SCCA), SCCA 1, SCCA 2, SCCA 2b, arginase 1, and K10, as indicated in the lower panel of Figure 2.

Quantification of SCCA- and K10-reactive IgG in blood samples from psoriasis patients and controls

In repeated two-dimensional immunoblots of separated stratum corneum proteins, incubated with sera from

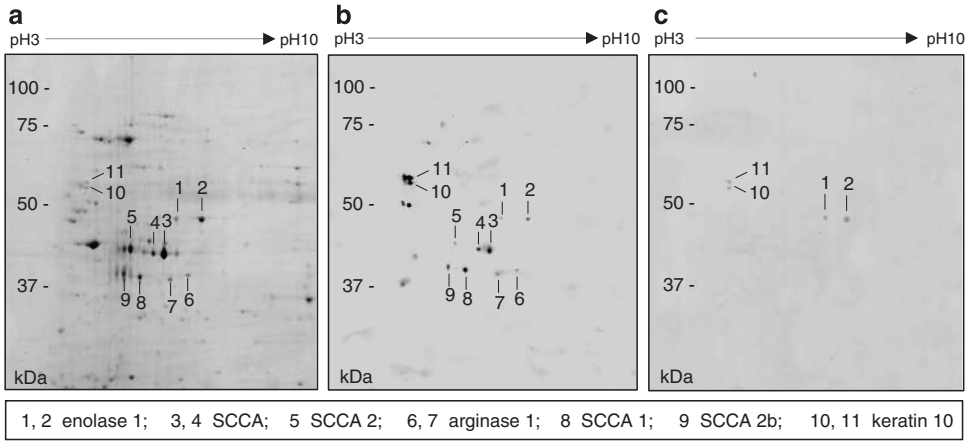

Figure 2. IgGs in pooled serum from psoriasis patients recognize a greater range of proteins in lesional stratum corneum extract than pooled serum from age- and sex-matched controls. (**a**) Coomassie-stained two-dimensional gel showing separated proteins. Blots of 2-dimensional gels run in parallel were incubated with pooled serum (1:200 dilution) from eight patients with psoriasis (**b**) and eight age- and sex-matched healthy controls (**c**), and IgG-binding proteins localized with AP-labeled anti-IgG. IgG-reactive proteins that corresponded with Coomassie-stained spots in panel a were identified by MALDI-TOF mass spectrometry and are labeled 1–11. The identities of these proteins are indicated in the lower panel. Similar results to those shown in panels a–c were obtained in a second experiment with different stratum corneum extract and pooled serum samples.

individual patients with chronic plaque psoriasis, serum IgGs reactive with SCCA proteins (spots 3, 4, 5, 8 and 9 in Figure 2) were consistently observed to be the most abundant. The measurement of titers of SCCA-reactive IgG in individual sera from patients with chronic plaque psoriasis and controls was therefore attempted by ELISA. Initial work showed that the use of ELISA with *Escherichia coli*-derived recombinant proteins as substrates gave false-positive readings (data not shown). This was likely to be a result of reactivity of serum IgGs with trace amounts of vector contaminants, a problem that has been recently recognized as leading to falsely high autoantibody titers in such ELISAs (Schmetzer *et al.*, 2005). Attempts to eliminate vector contaminants from the ELISA by anchoring recombinant SCCA substrate to microtiter plate wells coated with murine monoclonal SCCA antibody (Ab) were also unsuccessful, because a significant percentage of human sera contained IgG that was reactive with the anchoring murine Ab (data not shown).

Large numbers of high-resolution two-dimensional blots of lesional stratum corneum proteins, which provided the separations shown (Figure 2), were therefore incubated with individual serum or plasma samples from patients with chronic plaque psoriasis and controls (1:200 dilutions). Bound IgGs were detected with AP-labeled goat anti-human IgG Ab, and the intensities of the labeled spots co-migrating with individual SCCA proteins were determined by densitometry. The intensity values for each SCCA spot were normalized to a standard, as described in Materials and Methods. Levels of SCCA-reactive IgG, expressed in arbitrary units (Figure 3a), were significantly higher in blood samples from patients with chronic plaque psoriasis (42.1 ± 7.7, $n = 34$) than in samples from healthy controls (18.8 ± 3.9, $n = 28$; means ± SEM, $P = 0.005$, Mann–Whitney test). There was no correlation between SCCA-specific IgG levels and the clinical severity of psoriasis as determined by the psoriasis area and severity index (Fredricksson and Pettersson, 1978), nor any significant difference between the levels in patients who were HLA-Cw*0602-positive or -negative (data not shown). Levels of K10-reactive IgG were also quantified, in view of the preliminary evidence that these levels might be higher in blood samples from patients than controls (Figure 2b and c). However, densitometric analysis of large numbers of two-dimensional immunoblots showed that blood levels of K10-reactive IgG in patients (23.1 ± 9.1 AU (arbitrary units), $n = 33$) were not significantly different from those in controls (15.1 ± 4.7, $n = 23$; means ± SEM, $P = 0.85$, Mann–Whitney test; Figure 3b).

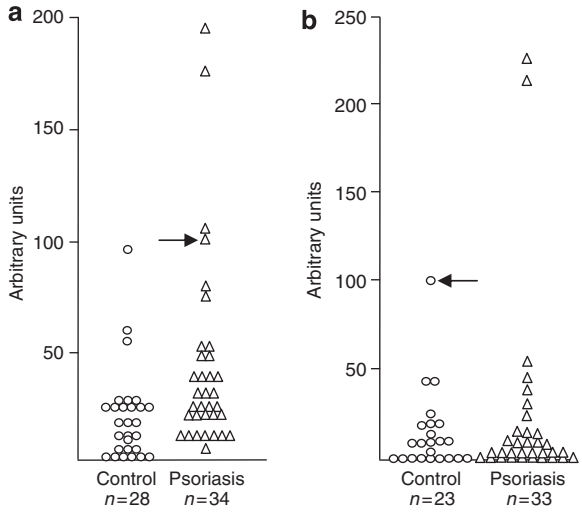

Figure 3. Levels of SCCA-reactive IgG but not keratin 10-reactive IgG are higher in blood samples from patients with chronic plaque psoriasis than in matched healthy controls. The two sets of values for SCCA-reactive IgG (**a**) derived by densitometry after two-dimensional immunoblotting with serum or plasma samples from psoriasis patients ($n = 34$) and matched healthy controls ($n = 28$) were significantly different ($P = 0.005$). In contrast (**b**), levels of keratin 10-reactive IgG in blood samples from psoriasis patients ($n = 33$) and matched controls ($n = 23$) were not significantly different ($P = 0.85$). The arrowed values show the results obtained with the standard serum sample that was included in each batch of six analyses and used to normalize data, as described in Materials and Methods.

Association of SCCA–IgG interaction with complement deposition and specificity of SCCA autoantibodies to psoriasis

To explore the potential pathogenicity of SCCA–IgG interactions, a two-dimensional blot of lesional stratum corneum extract was incubated with serum (1:20 dilution) from a psoriasis patient containing a high level of SCCA-binding IgG and deposition of the complement component C3 demonstrated with C3c Ab and autoradiography (Figure 4a). The blot was then stripped, re-incubated with the same serum (1:200 dilution), and bound IgG detected with horseradish peroxidase-labeled anti-IgG and further autoradiography (Figure 4b). This showed deposition of C3 that colocalized with IgG binding at SCCA spots. Two-dimensional immunoblots of proteins from psoriatic stratum corneum were also incubated with pooled serum from five patients with active atopic dermatitis. As shown in Figure 4c, sera from these patients did not contain detectable SCCA-reactive IgG.

DISCUSSION

In this study, we confirmed the *in vivo* deposition of IgG in the upper epidermis of the chronic plaques of psoriasis. We then defined a series of IgG-binding autoantigens in psoriatic epidermis, the most prominent of which were the SCCA family. SCCA 1 is a member of the ovalbumin-serpin group of protease inhibitors and is a truncated version of SCCA. Both are cysteine protease inhibitors, whereas their homologue SCCA 2 inhibits serine proteases (Masumoto *et al.*, 2003). SCCA 1 and 2 are expressed by keratinocytes *in vitro* (Hamada *et al.*, 2001), and SCCA protein is both overexpressed in lesional epidermis as shown by immunohistochemistry, *in situ* hybridization, and immunoelectron-microscopy (Takeda *et al.*, 2002) and is present in increased levels in the serum of patients with psoriasis (Duk *et al.*, 1989; Takeda *et al.*, 2002). Microarray analysis has also shown remarkably upregulated expression of the SCCA 1 and 2 genes (SERPINB3 and SERPINB4) in psoriatic lesional versus non-lesional skin (Haider *et al.*, 2006). SCCA 2b is another homologue of SCCA 1 (Suminami *et al.*, 2001), but its biologic properties have not been described nor has it been

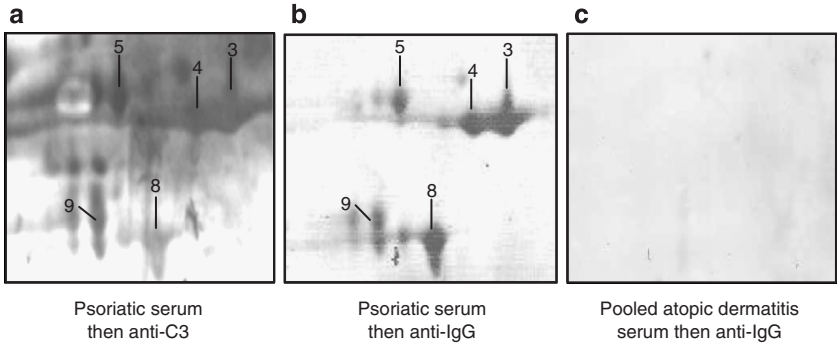

Figure 4. Complement deposition is associated with SCCA–IgG interaction, and SCCA-reactive IgG is absent in sera from patients with active atopic dermatitis. (**a**) A blot of psoriatic stratum corneum-derived SCCA proteins separated by two-dimensional electrophoresis was incubated with a 20-fold dilution of a psoriatic serum sample containing a high level of SCCA-reactive IgG. C3 deposition was shown by autoradiography after incubation with C3c Ab. (**b**) The same blot was stripped, re-incubated with a 200-fold dilution of the same serum, and IgG deposition shown with horseradish peroxidase-labeled anti-IgG and further autoradiography. The pattern of C3 deposition on SCCA proteins corresponds with the pattern of IgG binding. (**c**) A blot of psoriatic stratum corneum-derived SCCA proteins separated by high-resolution two-dimensional electrophoresis and incubated successively with a 200-fold dilution of pooled serum from five patients with active atopic dermatitis and AP-labeled anti-IgG shows no IgG-reactive proteins. Each blot (**a**–**c**) is enlarged from original size by 150%, and the area occupied by SCCA proteins illustrated.

previously reported as a product of epidermis. Importantly, we have not found any previous descriptions of autoantibodies directed against the SCCA family in any setting, either in humans or animals.

Arginase 1, which catalyzes the metabolism of arginine to urea and ornithine, is also overexpressed in the epidermis of psoriatic lesions (Bruch-Gerharz *et al.*, 2003), but we have found only one report of autoantibodies against this enzyme, that is, in human autoimmune hepatitis (Kimura *et al.*, 2000). The glycolytic enzyme enolase 1 has been described as an autoantigen in several settings including Behcet's disease (Lee *et al.*, 2003) and systemic lupus erythematosus (Kemp *et al.*, 2002), but to our knowledge not previously in psoriasis. Autoantibodies against mixed epidermal keratins are well recognized both in normal and psoriatic sera (Aoki *et al.*, 1989), but to our knowledge, a pathogenic role for these Abs has not been established and autoantibodies that specifically recognize K10 have not been reported previously. We have shown that K10 autoantibodies are detectable in normal and psoriatic blood samples, but the levels in samples from the two sources are not significantly different. It was of interest that no IgG-binding microbial proteins were identified in this study, particularly because organisms such as *Staphylococcus aureus* are known sometimes to colonize the surface of psoriasis lesions (Payne, 1967).

The measurement of SCCA Abs in human serum was not a straightforward procedure in this study in view of the described nonspecific serum IgG reactivity, when a recombinant form of SCCA was used as substrate in ELISAs either in the presence or absence of anchoring murine SCCA Ab. We therefore proceeded to analyze the intensities of Abs reactive with human epidermal SCCA proteins in large numbers of two-dimensional high-resolution immunoblots. This strategy was supported by the knowledge that there are numerous potential glycosylation and phosphorylation sites in SCCA proteins and that recombinant forms may therefore not possess the same IgG reactivity as human proteins produced *in vivo*. However, the disadvantage of our strategy was the need to use denatured human proteins in two-dimensional immunoblots.

Densitometry of immunoblots showed that blood levels of SCCA Abs were significantly higher in patients with chronic plaque psoriasis than in controls (Figure 3a). Some healthy control subjects also showed detectable levels of these Abs, which allows the speculation that interactions between serum IgG and SCCA, and possibly some of the other autoantigens identified, may be an early event in disease pathogenesis once local homeostasis is altered in predisposed individuals. The effects of autoantibody binding on the function of SCCA proteins have not yet been explored. Whether this binding may interfere with their protease inhibitory function and thus enhance inflammation by allowing uncontrolled protease activity is, to our knowledge, currently not known. However, we have demonstrated complement deposition in association with SCCA–IgG interaction, which suggests that the interaction is pathogenic.

The specificity of anti-SCCA IgG autoantibodies to psoriasis has only been investigated to a limited extent in this study. We showed that these Abs were not detectable in pooled sera of patients with active atopic dermatitis, in spite of the fact that elevated levels of SCCA protein have been found in the affected skin and serum of patients with this skin disease (Mitsuishi *et al.*, 2005). Further study is required to determine the specificity of SCCA IgG Abs to psoriasis. SCCA protein is expressed by squamous cell carcinomas, and serum levels are widely used as a tumor marker (Kato, 1996); and it would be of particular interest if SCCA proteins are shown to be relevant tumor antigens in these cancers.

In conclusion, to our knowledge, these findings define a potentially pathogenic immune response directed against SCCA proteins, which have not been reported previously. Whether this is an early pathogenic event in psoriasis or one that occurs as a consequence of the exposure of a normally sequestered protein during plaque development and thus enhances the inflammatory response as a secondary phenomenon remains to be determined. The findings also suggest

that therapeutic blockade of complement activation in psoriasis is worthy of further study.

MATERIALS AND METHODS

Subjects and samples

This study was approved by the local research ethics committee and was conducted in accordance with the Declaration of Helsinki Principles. Written informed consent was obtained from each donor. Patients with chronic plaque psoriasis and atopic dermatitis met standard diagnostic criteria, had active, moderate-to-severe skin disease, and had not received systemic therapy or UV irradiation for their skin disease for at least 2 months before venesection. Patients with psoriasis included 15 females and 19 males aged 22–66 years, with a mean psoriasis area and severity index (Fredricksson and Pettersson, 1978) of 13.9 ± 6.8 (SD). Control subjects were healthy volunteers aged 23–66 years, including 14 females and 14 males. Venous blood samples were obtained from each subject, allowed to clot in glass test tubes, and serum was removed and centrifuged to deplete cells. In some cases, venous blood was mixed with heparin and plasma was obtained after centrifugation. Serum and plasma samples were stored at −80°C in aliquots. Desquamating stratum corneum was removed by gentle abrasion with a scalpel blade from the surface of skin lesions of a 60-year-old male volunteer with moderately severe psoriasis, which had not received topical treatment for at least 2 weeks and had never been treated with systemic agents. Recovered tissue was stored at −20°C in aliquots.

Tissue extraction, ELISA, and electrophoresis

Stratum corneum samples were fragmented in ice-cold phosphate-buffered saline (PBS) with 0.5 mM phenylmethylsulfonyl fluoride and 1 mM EDTA in a ground glass homogenizer. In experiments to demonstrate IgG in these extracts, homogenate was centrifuged at 12,000 *g* for 5 minutes, and supernatant added to wells of Maxisorb microtiter plates (Nunc, Roskilde, Denmark) in varying amounts up to a maximum of 2 μg total protein per well. Coated wells were washed five times with PBS containing 0.05% Tween 20 (PBS/T), blocked with 5% skimmed milk powder in PBS for 2 hours at 37°C, and then again washed five times with PBS/T. Some coated wells were also treated with pooled, diluted serum from eight patients with psoriasis. The pooled serum was diluted 100-fold in PBS containing 1% milk powder and left in wells at 37 °C for 2 hours before washing with PBS/T as above. AP-labeled goat anti-human IgG (product code A 3188; Sigma, Poole, UK) diluted 1:4,000 with 1% skimmed milk powder in PBS/T (PBS/T/M) was then added for 90 minutes at 37 °C, wells were again washed five times with PBS/T, substrate p-nitrophenyl phosphate (Sigma) added according to the supplier's instructions, and optical densities read at 405 nm. Before two-dimensional electrophoresis, aliquots of stratum corneum homogenate prepared as described above were solubilized with equal volumes of buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) kept at room temperature for 20 minutes with occasional agitation. After centrifugation at 12,000 *g* for 5 minutes, 50 μl aliquots were applied to Pierce spin desalting minicolumns (product code 89866; Perbio, Cramlington, UK) in accordance with the manufacturer's instructions. Eluates were mixed with rehydration buffer containing the above concentrations of urea, thiourea, and CHAPS, 2% ampholytes (Bio-Rad, Hemel Hempstead, UK), and 100 mM DeStreak reagent (Amersham, Little Chalfont, UK) to a final protein concentration of 1 $\mu g\,\mu l^{-1}$. Batches of proteins (300 μg) were separated on 17 cm (pH 3–10) ReadyStrip IPG strips in a Protean isoelectrofocusing cell (Bio-Rad). Strips were equilibrated in accordance with the manufacturer's instructions and laid on up to six 20 cm × 20 cm × 1 mm 12% acrylamide gels, which were run in parallel in an Ettan DALT*six* electrophoresis unit (Amersham) under denaturing conditions.

Immunoblotting and protein identification

Gels run in parallel were either stained directly with Coomassie blue G250 (Candiano *et al.*, 2004) or electroblotted onto 20 × 20 cm Hybond-ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5% skimmed milk powder in PBS at 4 °C overnight, washed four times in PBS/T, and then incubated with either pooled or individual serum or plasma samples (diluted 1:200) with PBS/T/M for 2 hours at room temperature. After four washes in PBS/T, membranes were incubated with AP-labeled anti-human IgG (Sigma) (diluted 1:4,000) with PBS/T/M for 90 minutes at room temperature. After a further four washes, membranes were treated with substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride; Sigma) in accordance with the supplier's instructions. Coomassie blue-stained protein spots of interest were excised from gels, placed in Eppendorff tubes with 20 μl water and subsequently subjected to tryptic digestion, and analysis by MALDI-TOF mass spectrometry, as described previously (Shevchenko *et al.*, 1996; Bonetto *et al.*, 1997). In experiments to detect deposition of complement components, a nitrocellulose blot of two-dimensionally separated psoriatic stratum corneum proteins was incubated at room temperature for 2 hours with serum (1:20 dilution in PBS/T/M) from a psoriasis patient, followed by four washes in PBS/T, and then incubation for 90 minutes at room temperature with rabbit anti-human C3c Ab (Dako, Ely, UK) diluted 1,000-fold in PBS/T/M. After washing, the membrane was incubated for 90 minutes at room temperature with horseradish peroxidase-labeled goat anti-rabbit Ab (Sigma) diluted 4,000-fold in PBS/T/M, washed again and incubated with ECL substrate (Amersham) in accordance with the manufacturer's instructions. After autoradiography, the blot was stripped, re-incubated with the same serum diluted 1:200 in PBS/T/M, and bound IgG detected with horseradish peroxidase-conjugated anti-human IgG (Sigma) and further autoradiography.

Data analysis

For quantification of IgG levels reactive with the SCCA and K10 proteins separated by two-dimensional electrophoresis, immunoblots were prepared in sets of six from gels run in parallel in the same six-gel electrophoresis tank. Each blot was incubated with a 200-fold diluted serum or plasma sample from an individual donor, as described above, one of the sera always being a standard high titer serum from the same donor. BCIP/NBT immunoblot signals were quantified by densitometry (G5-710 instrument with Quantity One software; BioRad). Briefly, the total signal intensity inside a defined boundary drawn around each selected spot was adjusted by automated local background subtraction in accordance with the manufacturer's user guide. The adjusted intensity of each SCCA spot (numbered 3, 4, 5, 8, and 9 in Figure 2) was expressed as a percentage of the adjusted intensity of the corresponding spot

generated with the standard serum. The normalized SCCA BCIP/NBT spot intensities were then summed and a mean value obtained for each serum or plasma sample. The adjusted intensities of the two K10 spots (numbered 10 and 11 in Figure 2) were also determined as above. The derived spot intensities were expressed as arbitrary units, and the significance of differences between values obtained with serum or plasma samples from psoriasis patients and healthy controls was analyzed by the Mann–Whitney test for non-parametric data.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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