Complement activation in hidradenitis suppurativa: a new pathway of pathogenesis?*

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Summary

Background Despite the heavy purulence observed in hidradenitis suppurativa (HS), the kinetics of complement anaphylatoxins acting to prime chemotaxis of neutrophils has not been studied.

Objectives To explore complement activation in HS.

Methods Circulating concentrations of complement factor C5a, as well as of membrane attack complex C5b-9, were determined in the plasma of 54 treatment-naive patients and of 14 healthy controls, as well as in the pus of seven patients. Results were correlated with Hurley stage and International Hidradenitis Suppurativa Severity Score. Peripheral blood mononuclear cells (PBMCs) were isolated from seven patients with Hurley stage III HS and seven healthy volunteers and stimulated in the presence of 25% of plasma for the production of tumour necrosis factor-α (TNF-α).

Results Circulating C5a and C5b-9 were significantly greater in patient than in control plasma; however, concentrations in pus were very low. Circulating C5a levels exceeding 28 ng mL⁻¹ were associated with a specificity > 90% with the occurrence of HS. Circulating levels of C5a and C5b-9 were greater in patients with more severe HS. PBMCs of patients produced high concentrations of TNF-α only when growth medium was enriched with patient plasma; this was reversed with the addition of the C5a blocker IFX-1.

Conclusions Systemic complement activation occurs in HS and may be used as a surrogate biomarker of HS. C5a stimulates overproduction of TNF-α and may be a future therapeutic target.

What’s already known about this topic?
- Complement activation has not been studied in hidradenitis suppurativa (HS).

What does this study add?
- Circulating C5a and C5b-9 are increased in HS.
- Consumption of circulating C5a is associated with disease severity.
- C5a primes the overproduction of tumour necrosis factor-α by circulating mononuclear cells.

What is the translational message?
- Inhibition of C5a may be a new treatment option in HS, based on an entirely new mode of action.
- C5a may be a new surrogate biomarker for HS.
Hidradenitis suppurativa (HS) is a chronic debilitating skin disorder affecting skin areas rich in apocrine glands. The main affected areas are axillae, breasts, groins and buttocks. HS is characterized by flare-ups and remission. During each flare-up, the affected apocrine gland becomes an inflamed nodule that spontaneously ruptures with the release of pus. Repeated cycles of these processes lead either to the formation of a fistula, with continuous drainage of pus, or to scar formation. Affected skin areas are remarkably painful. HS is a frequent disorder with a prevalence of 0.97% in white populations. This point prevalence may be up to 4% in Scandinavian countries.

The exact cause of pathogenesis of HS is unknown. Several recent findings suggest an autoimmune inflammatory mechanism of pathogenesis. These findings include defective cytokine responses from circulating monocytes, and the infiltration of affected skin lesions with monocytes, dendritic cells and lymphocytes, where an abundant expression of proinflammatory cytokines has been noted. These findings are in line with the remarkable improvement seen in patients administered the tumour necrosis factor-α (TNF-α) blocker adalimumab, as outlined by two recent randomized clinical studies.

One major, yet unanswered, question is how neutrophils are recruited to the affected skin lesion to produce pus. It is hypothesized that this process is mediated by anaphylatoxins produced during complement activation, namely C5a. Complement activation has not been studied in HS. The present study protocol, based on measurements of complement activation products in plasma of patients with HS, aimed to explore the kinetics of circulating concentrations of anaphylatoxin C5a, as well as of membrane attack complex C5b-9, in HS.

Patients and methods

Study population

A total of 54 patients with HS and 14 healthy volunteers were enrolled. Patients were being followed-up at the outpatient department of the Department of Immunology of Infectious Diseases, Attikon University Hospital, Athens, Greece. The study was approved by the hospital’s ethics committee. Written informed consent was provided by all patients.

Diagnosis of HS was based on the following criteria: (i) onset soon after puberty; (ii) presence of subcutaneous nodules in areas of skin rich in apocrine glands; and (iii) a compatible history of recurrent drainage of pus from the affected areas. In parallel, all enrolled patients had to be naïve of any treatment.

Exclusion criteria were: (i) neutropenia, defined as any neutrophil count < 1000 neutrophils per mm³; (ii) HIV-1 infection; and (iii) any disease-directed treatment in the last 6 months.

The patients’ clinical characteristics were recorded and included demographics, involved areas, type of lesions, frequency of flare-ups, Hurley severity stage, physician global assessment (PGA) and Sartorius score. For all patients the International Hidradenitis Suppurativa Severity Score (iHS4) proposed by the European Hidradenitis Suppurativa Foundation was retrospectively assessed. During visits patients were also asked to grade the degree of purulence from the lesions as absent (grade 0), mild (grade 1), moderate (grade 2) or intense (grade 3). Blood (4 mL) was collected, under aseptic conditions, after venepuncture of one forearm vein and collected in heparin-coated tubes (Vacutainer; Becton Dickinson, Cockeysville, MD, U.S.A.). After several gentle inversions, tubes were immediately centrifuged for 10 min at 1800 g at room temperature. The resultant plasma was collected and stored at −70 °C until assayed.

Pus was collected from the lesions of seven patients. Lesion area was cleaned with sterile gauze moistened with normal saline 0-9% before the collection of pus. After prudent palpation of a representative draining fistula, a plastic 20 G needle was put inside the fistula and 100 μL pus was aspirated into an insulin syringe applied at the end of the needle. Pus was diluted with 900 μL water for injection (dilution 1 : 10). A final volume of 1 mL was poured into a sterile Eppendorf tube and kept at −70 °C until measurement.

Measurement of complement activation

Concentrations of complement activation products C5a and membrane attack complex C5b-9 were measured in the InflaRx laboratory (Jena, Germany). C5b-9 concentration was determined using the BD OptEIA™ Human C5b-9 ELISA Set (BD Biosciences, Heidelberg, Germany). C5a concentration was measured using one C5a enzyme-linked immunosorbent assay (ELISA) established and validated by InflaRx.

Modulation of the function of peripheral blood mononuclear cells after C5a inhibition

Peripheral blood mononuclear cells (PBMCs) were isolated from seven patients with Hurley stage III HS and from seven healthy volunteers. The day of isolation was selected to be a visit on which patients were self-admitted to the outpatient department because of a flare-up. On the same day, blood was sampled from a healthy volunteer of the same age and sex. A total of 15 mL heparinized venous blood was sampled after venepuncture of one forearm vein, under aseptic conditions. PBMCs were isolated after gradient centrifugation of whole blood over Ficoll (Merck, Darmstadt, Germany). Following three serial washings with phosphate-buffered saline (pH 7.2) and trypan blue staining for the exclusion of dead cells, PBMCs were plated at a density of 5 x 10⁶ cells mL⁻¹ into wells of a 96-well plate in RPMI 1640 enriched with 2 mmol L⁻¹ glutamine (Merck) without/with 5 x 10⁵ colony-forming units mL⁻¹ of one heat-killed isolate of Staphylococcus aureus from the pus of one patient with HS. Experiments were run in duplicate and wells were supplemented with 25% plasma from the same individuals with/without 2 μg/mL IFX-1 (InflaRx®, Jena, Germany). IFX-1 is a chimeric monoclonal IgG4 kappa antibody that specifically binds to the soluble...
human complement split product C5a. The concentration of 2 μg mL\(^{-1}\) that was added into wells is equal to the trough concentration of IFX-1 achieved in healthy volunteers after intravenous administration of 800 mg InflaRx (data on file).

After 24 h incubation at 37 °C in 5% CO\(_2\), plates were centrifuged and supernatants stored at −80 °C. Concentrations of TNF-α were measured in duplicate by an ELISA (eBiosciences, San Diego, CA, U.S.A.). The lowest limit of detection was 20 pg mL\(^{-1}\).

**Statistical analysis**

Concentrations of C5a and C5b-9 are expressed as median and 95% confidence interval (CIs) as they followed a non-normal distribution, as defined by Kolmogorov–Smirnov statistics; TNF-α concentrations are expressed as mean ± SE. Comparisons were done with the Mann–Whitney U-test. A receiver–operator characteristic (ROC) curve was designed to define a cut-off with specificity > 90% that can distinguish patients from healthy controls. The odds ratio (OR) and 95% CI of developing HS with values above the determined cut-off were calculated with Mantel–Haenszel statistics. Based on these cut-offs patients were divided into those with low complement activation and into those with high complement activation. Correlations between C5a and C5b-9 levels with clinical variables of HS were done according to Spearman’s rank of order. Any P-value < 0.05 after adjustment for multiple comparisons was considered significant.

**Results**

Patient demographics are shown in Table 1. Concentrations of C5a and C5b-9 measured in the plasma of patients were significantly greater than those measured in the controls (Fig. 1).

No patient had any sign of known systemic disorder that is accompanied by systemic complement activation, such as vasculitis, kidney disease, systemic lupus erythematosus or hypersensitivity reactions. When comparisons were done in relation to Hurley stage of severity, it was found that all stages of severity had greater concentrations of C5a and C5b-9 than healthy controls; however, concentrations were greater among patients at Hurley stage I than patients at Hurley stages II and III (Fig. 2). When grading of severity was done by the iHS score, circulating C5b-9 was greater among patients with mild HS than those with moderate and severe HS (Fig. 3).

C5a and C5b-9 were also measured in pus from the fistulae of seven patients at Hurley stage III. C5b-9 was below the limit of detection in all samples. The median level of C5a in pus was 6.20 ng mL\(^{-1}\) (range 2.24–13.86).

Concentrations of circulating C5a > 28 ng mL\(^{-1}\) had a specificity > 90% for HS (Fig. 4a). More precisely, 61.1% of patients and 7.1% of healthy controls had C5a levels > 28 ng mL\(^{-1}\). The OR for HS was 20.43 (95% CI 2.49–167.87; P = 0.005). After ROC analysis, we failed to define a
cut-off of circulating C5b-9 that can discriminate patients from controls at the level of statistical significance (data not shown). Using the defined cut-off for C5a, we divided patients into those with low complement activation and into those with high complement activation. The frequency of high complement activation was significantly greater among patients than controls, irrespective of the Hurley stage or iHS4 severity score (Fig. 4b, c).

No significant correlation was found between circulating levels of C5a and frequency of flare-ups ($r_s = +0.163; P = 0.268$), whereas a trend towards a negative significant correlation was found between circulating levels of C5a and Sartorius score (Fig. 5a). A negative correlation was found between circulating levels of C5b-9 and Sartorius score (Fig. 5b), whereas no significant correlation was found between circulating levels of C5b-9 and frequency of flare-ups ($r_s = -0.128; P = 0.386$). Concentrations of C5b-9 were greater among patients with a mild grade of purulence (Fig. 5c) and PGA score of 1–3 (Fig. 5d). Concentrations of C5a did not differ between patients with different grades of purulence and different PGA scores (data not shown).

Previous studies have shown that PBMCs of patients with HS produce fewer cytokines than healthy controls after stimulation with heat-killed S. aureus. However, when growth medium was enriched with 25% plasma, PBMCs started to overproduce TNF-α (Fig. 6). This generated the hypothesis that C5a may be the in vivo stimulus mediating high TNF-α production in patients; this is missing when PBMCs are transferred to ex vivo conditions so cells fail to overproduce TNF-α.

The addition of IFX-1, which blocks C5a, reversed the high production of TNF-α stimulated by the patients’ plasma, verifying this hypothesis.

**Discussion**

HS is a disorder characterized by flare-ups of heavy purulence from the affected skin lesions. The potential drivers of chemotaxis and of neutrophil activation causing enzyme release and oxygen radical production in the affected skin areas have not yet been identified and studied. C5a and especially C5a are traditional activation products of the complement cascade that can potentially orchestrate the
infiltration of neutrophils and strongly activate neutrophils in the affected skin areas. The results of this study indicate high systemic activation of complement in HS. Surprisingly, circulating levels of C5a seem to be greater among patients with Hurley stage I HS than among patients with Hurley stages II and III HS, whereas there is a negative correlation between C5b-9 levels and severity scores. The negative correlation of C5a with HS severity probably indicates that complement activation takes place well before HS progresses to Hurley stages II and III, leading to irreversible skin damage.

The histology of lesions from patients with HS clearly suggests overexpression of metalloproteinases (MMPs), namely MMP-2 and MMP-8, in the skin.11,12 MMPs are stored in neutrophil granules and they are released locally, leading to destruction of extracellular matrix and fistula formation. This may explain why C5a anaphylatoxin is found to be more elevated at Hurley stage I HS. At this stage, overproduction of C5a mediates neutrophil recruitment at the skin where MMPs are released. This leads to the skin destruction observed at Hurley stages II and III. In skin, TNF-α expression correlates with that of MMP-2 and MMP-8,12,13 and this is compatible with our findings that show C5a primes overproduction of TNF-α.

One major question is what the stimulus leading to activation of complement is. Current data cannot help us answer this question. In a mouse model of skin infection induced by tetradecanoyl phorbol acetate it was found that serine proteases such as MMP-2 and cathepsin B are activated. This was associated with both increased activation of the mannose complement pathway and increased infiltration of neutrophils, providing some clue as to how local skin inflammation can induce complement activation.14 In a mouse model of atopic dermatitis, overexpression of C5a was induced by homozygous deletion of the C5L2 receptor of C5a. This led to enhanced skin infiltration by neutrophils and exacerbated local

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**Fig 4.** Frequency of C5a activation. (a) Receiver–operator characteristic curve to define a plasma cut-off of C5a to discriminate between patients and healthy controls. (b) Frequency of patients with circulating C5a above 28 ng mL⁻¹ in relation to Hurley disease stage. (c) Frequency of patients with circulating C5a above 28 ng mL⁻¹ in relation to International Hidradenitis Suppurativa Severity (iHS4) score. The significant P-value in comparison with healthy controls is given (comparisons by Fisher’s exact test after Bonferroni correction). AUC, area under the curve; CIs, confidence intervals.
inflammation; findings were reversed upon administration of an antibody blocking C5a. 15

The concentrations of C5a and C5b-9 found in patients with HS are far greater than the circulating concentrations described for other systemic inflammatory disorders like multiple injuries, severe sepsis and multiple organ failure. 16,17 This impressive component of HS points towards a systemic inflammatory reaction as a potential step in the pathogenesis of HS.

Fig 5. Concentrations of C5a and of C5b-9 in the plasma of 54 patients with hidradenitis suppurativa in relation to disease characteristics. (a, b) Correlation of C5a and C5b-9 with Sartorius score. Spearman rank of order coefficient and significant P-values are provided. (c) C5b-9 levels in patients with different grades of purulence. The P-values of indicated comparisons are shown. (d) C5b-9 levels in patients with physician global assessment (PGA) score 1–3 and 4–5. The P-value of comparison is provided.

Fig 6. Stimulation of production of tumour necrosis factor-α (TNF-α) by heat-killed Staphylococcus aureus. Results are provided after stimulation in the presence of 25% plasma without/with the C5a inhibitor IFX-1. The P-value of the respective comparison is provided.
Irrespective of what the aetiology of HS may be, the results of the present study allow interpretation of previous findings by our group. We have described in the past that when PBMCs or purified monocytes from patients are stimulated \textit{in vivo} with bacterial ligands they fail to produce proinflammatory cytokines in primary cells isolated from healthy controls.\textsuperscript{4,10} However, in these previous studies, cells were stimulated in the presence of growth medium and in the absence of plasma from patients. In the present study, the addition of patient plasma transformed PBMCs into overproducers of TNF-\(\alpha\), attenuated by the C5a blocker IFX-1. This generates the hypothesis that C5a is a host component mandatory for priming of the production of TNF-\(\alpha\) by human monocytes in HS. Although our findings were produced using \textit{S. aureus} to stimulate TNF-\(\alpha\) production by PBMCs, it has to be noted that failed cytokine responses from patient monocytes were seen, even when cells were stimulated with purified bacterial lipopolysaccharide or with \textit{Candida albicans}.\textsuperscript{5,10} As a consequence, similar beneficial responses with IFX-1 blockade can also be expected with bacterial species other than \textit{S. aureus}. Nevertheless, findings favour the development of C5a blockers as a therapeutic strategy for HS.

Randomized clinical trials in HS with agents aiming to bind proinflammatory cytokines like TNF-\(\alpha\) and interleukin-1\(\beta\) mandates the use of easier and more accurate scores of HS when biomarkers may play a salient role. Current results suggest that complement factors in plasma should be evaluated as a surrogate marker for these studies and may also be considered as a potential future therapeutic target.

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