INVESTIGATIVE REPORT

Granular C3 Dermatosis

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There has been no previous systematic study of bullous skin diseases with granular basement membrane zone deposition exclusively of C3. In this study we collected 20 such patients, none of whom showed cutaneous vasculitis histopathologically. Oral dapsone and topical steroids were effective. Various serological tests detected no auto-antibodies or autoantigens. Direct immunofluorescence for various complement components revealed deposition only of C3 and C5–C9, indicating that no known complement pathways were involved. Studies of *in situ* hybridization and micro-dissection with quantitative RT-PCR revealed a slight reduction in expression of C3 in patient epidermis. These patients may represent a new disease entity, for which we propose the term "granular C3 dermatosis". The mechanism for granular C3 deposition in these patients is unknown, but it is possible that the condition is caused by autoantibodies to skin or aberrant C3 expression in epidermal keratinocytes. *Key words: basement membrane zone; bullous disease; C3; direct immunofluorescence; granular.*

As one of the centres for diagnosis of autoimmune bullous diseases (AIBDs) in Japan, we have collected sera, frozen skin samples and data for more than 5,000 patients for whom there was difficulty in diagnosis over a period of 20 years. In this large cohort, we found that 20 patients, some of whom showed dermatitis herpetiformis (DH)-like clinical features, showed granular deposition of C3, but not immunoglobulin (Ig)A, IgG or IgM, in the epidermal basement membrane zone (BMZ) by direct immunofluorescence (IF).

Clinically, DH shows pruritic papulo-vesicular skin lesions preferentially on the elbows, knees and buttocks; histopathologically, neutrophilic microabscesses are present in papillary dermis; and immunologically, granular deposition of IgA with or without C3 is present in papillary dermis (1–4). DH is usually associated with coeliac disease in Caucasians (5). DH shows circulating IgA antibodies to various antigens, including gliadin and endomysium (EMA) (1). However, recent studies have shown that patients with DH have pathogenic IgA antibodies to epidermal transglutaminase (eTG, also called as TG3), as well as IgA antibodies to tissue transglutaminase (tTG) (6–10).

There are 3 distinct pathways in activation of complement cascade; i.e. the classical, alternative and lectin pathways (11, 12). The classical pathway is activated mainly by binding of IgG and IgM antibodies and involves all complement components from C1 to C5–C9. The alternative pathway is activated mainly by various microbes or IgA antibodies, and involves factor B and C5–C9. The lectin pathway is activated mainly by various bacteria, dying tissues and mannose-containing sugar chains, and involves mannose-binding lectin, ficolins and C5–C9. Thus, IgG autoantibodies in various subepidermal AIBDs may activate complement via either the classical or the alternative pathway, while IgA antibodies in either DH or linear IgA bullous dermatosis may activate complement mainly via the alternative pathway (11, 12).

In this study, we clinically, histopathologically and immunologically characterized 20 patients with granular BMZ deposition exclusively of C3, with particular comparison with DH.

The 20 patients showed several common findings in terms of clinical and histopathological features, as well as a pattern of deposition of complement components and production of C3 in epidermis. These findings were different from those found in any known AIBDs or other inflammatory skin diseases. Therefore, we propose the term granular C3 dermatosis (GCD) as a possible new disease entity for this condition.

MATERIALS AND METHODS (for complete details see Appendix S1[1])

Immunofluorescence studies

Indirect IF and complement-fixing IF studies were performed as described previously (13, 14). IF studies for various complement components were also performed.

Immunoblot and enzyme-linked immunoassay (ELISA) studies for non-dermatitis herpetiformis diseases

Immunoblot analyses of 6 antigen sources were performed as described previously (13, 15–20). ELISAs of BP180, BP230, Dsg1 and Dsg3 were performed using commercial kits (MBL, Nagoya, Japan). IgA ELISA of longer RP of BP180 ectodomain was also performed (21).

Serological tests for dermatitis herpetiformis

IgA anti-EMA antibodies were examined as described previously (22). ELISAs were performed for IgA anti-eTG and anti-tTG antibodies (6), IgA anti-eTG antibodies (23), IgA anti-tTG and anti-gliadin derived peptides (DGP) antibodies (24), IgA anti-gliadin antibodies (25) and IgA anti-F-actin antibodies (26). The result was evaluated as positive or negative using the cut-off value for each ELISA (Table SI[1]).

In situ *hybridization*

Specificity and sensitivity of probes used for *in situ* hybridization were confirmed as described previously (27). Immunohistochemical *in situ* hybridization for C3 was performed as described previously (28, 29). 28S rRNA probe was used as positive control (30).

Micro-dissection and semi-quantitative RT-PCR (qPCR)

Micro-dissection and qPCR were performed as described previously (31). Statistical analysis was performed using unpaired *t*-test.

RESULTS

Clinical and histopathological features

Mean age at onset of skin lesions for the 20 patients, 10 females and 10 males, was 61.2 years, ranging from 8 to 83 years. There were no particular or significant findings in past histories, complications and given drugs. Because there were no gastrointestinal symptoms suggesting coeliac disease, no patients underwent endoscopic studies for either upper or lower intestinal tracts.

The overall appearance of skin lesions was assessed, in particular for the presence of blisters, erythemas and eczematous changes (Table SII[1]). Approximately half of the 20 patients showed clinical features, which in general mimicked DH; i.e. annular or nummular exudative erythemas, vesicles on peripheries of erythemas and eczematous lesions (Fig. 1 a–c), However, some patients showed bullous pemphigoid (BP)-like tense bullae (Fig. 1 d, e), prurigo-like papular lesions (Fig. 1f) or annular erythemas without any blisters (Fig. 1 g, h). Seven patients showed no apparent blister formation. Seventeen patients had severe pruritus. Because of the extreme heterogeneity in the skin lesions, times when the diagnoses of AIBDs were suspected were variable.

Regarding treatments, oral administrations of various corticosteroids, dapsone and combination of minocycline and nicotinamide were used in addition to topical corticosteroids. All therapies perfectly or partially controlled skin lesions. In general, dapsone could suppress the disease completely, although discontinuation of dapsone led to recurrence. No patients died during follow-up, indicating good prognosis in this condition. However, we could not obtain final information about therapies and outcome for some patients who had consulted dermatologists in other institutes.

Histopathology and various immunofluorescence studies

Histopathologically, most patients showed either subepidermal blisters (Fig. 1 i, j) or liquefaction degeneration/oedema in papillary dermis (Fig. 1 k, l) with inflammatory infiltration of lymphocytes, eosinophils and/or neutrophils. In particular, 9 patients showed no apparent subepidermal blister, although inflammatory infiltrates were seen in dermis and around dilated blood vessels (Fig. 1 m, n). Spongiosis was seen in 12 patients. No patient showed changes suggesting the presence of cutaneous vasculitis, such as leukocytoclasis and fibrinoid deposition.

Direct IF for the 20 patients showed granular deposition of C3 in and just below the BMZ, while no depositions of IgG, IgA and IgM were detected (Fig. 2a). No patient showed C3 deposition in blood vessel walls, excluding the presence of cutaneous vasculitis. Control BP skin biopsies showed strong linear BMZ deposition of C3 and/or IgG (Fig. 2a).

No patients showed positive results in indirect IF of either normal human skin or 1M NaCl-split normal human skin (data not shown). Complement-fixing IF of both normal human skin and 1M NaCl-split normal human skin also showed negative results (data not shown).

Antigen detection studies for non-dermatitis herpetiformis autoimmune bullous diseases

There were no positive results in immunoblot analyses for either IgG or IgA antibodies (data not shown). All patients also showed negative results in IgG and IgA ELISAs for BP230 and BP180 and IgG ELISAs for Dsg1 and Dsg3. In additional IgA ELISA for larger BP180 ectodomain, none of 19 patients examined showed definitely positive results, except for borderline positive reactivity in 2 patients (Table SI[1], right-hand column).

Fig. 1. Dermatitis herpetiformis-like lesions on (a) the buttock and (b) the knees. (c) Vesicles and eczematous lesions on the shins. Bullous pemphigoid-like tense blisters on (d) the abdomen and (e) palm. (f) Pruriginous lichenification on the back, and annular erythemas without apparent blisters on (g) the thigh and (h) waist. Histopathological features (haematoxylin-eosin staining). (i, j) Subepidermal blister with inflammatory infiltration. (k, l) Oedema in the upper dermis with inflammatory infiltration. (m, n) Perivascular inflammatory infiltration without apparent change in the epidermis. Original magnification: (i, m, n) ×100; (j–l) ×200.

Serological tests for dermatitis herpetiformis

To exclude the diagnosis of DH, various serological tests for DH were also performed for IgA and/or IgG antibodies for 19 of the 20 patients (Table SI[1]). In general, the 20 patients showed negative results in these studies, although a few patients showed relatively weak positive results in several tests.

Thus, both IF of monkey oesophagus and ELISA did not detect IgA anti-EMA antibodies in any patients. Commercial ELISA for IgA anti-eTG antibodies showed positive results in 3 patients. However, its significance is obscure, because all sera were negative in another home-made ELISA for eTG. IgA and IgG ELISAs for tTG, tTG/DGP and DGP and IgA ELISAs for gliadin and F-actin in general showed negative results, except for sporadic sera with positive reactivity with low titre. Although IgG ELISA for gliadin showed positive results in 4 patients, the significance was unknown.

Immunofluorescence study for various complement components

Next, to determine the complement activation pathway in the patient skin, we performed IF for various complement components using frozen skins from 6 patients. Control skin biopsies were also obtained from 6 BP patients and 6 normal volunteers. These studies used antibodies specific to IgG and C4 for the classical activation pathway, Factor B for the alternative pathway, MBL and ficolins for the lectin pathway, and C5–C9 for final stage.

All 6 skin biopsies from the patients showed negative results for all complement components, except for positive results for C5–C9 (Fig. 2b, Table SIII[1]). In contrast, all control BP skins showed linear BMZ deposition of C4 and several BP skin showed minimum linear deposition of factor B, while neither MBL nor ficolins deposited in any BP skins. Normal skin biopsies showed no positive reactivity.

In situ hybridization and qPCR studies for C3 production in epidermis

From the results of immunofluorescence for various complement components, none of classical, alternative and lectin pathways were considered to be activated in the patient skin. Therefore, we hypothesized that C3 deposition is caused by over-production of C3 in the epidermis of patient skin. In order to confirm this speculation, *in situ* hybridization and qPCR studies were performed for C3 in 4 selected patient skins, which were kept at −80°C for 1–4 years.

In situ hybridization with 28S rRNA as positive control confirmed that the procedure worked well (Fig. 3a). Lower expression of C3 mRNA was constantly seen in patient epidermis compared with normal control epidermis (Fig. 3a). Sense probe for C3 used as negative control showed no staining in adjacent sections (Fig. 3a).

In qPCR, C3 mRNA expression was also slightly lower in patient skins, although statistical significance was not achieved (Fig. 3b).
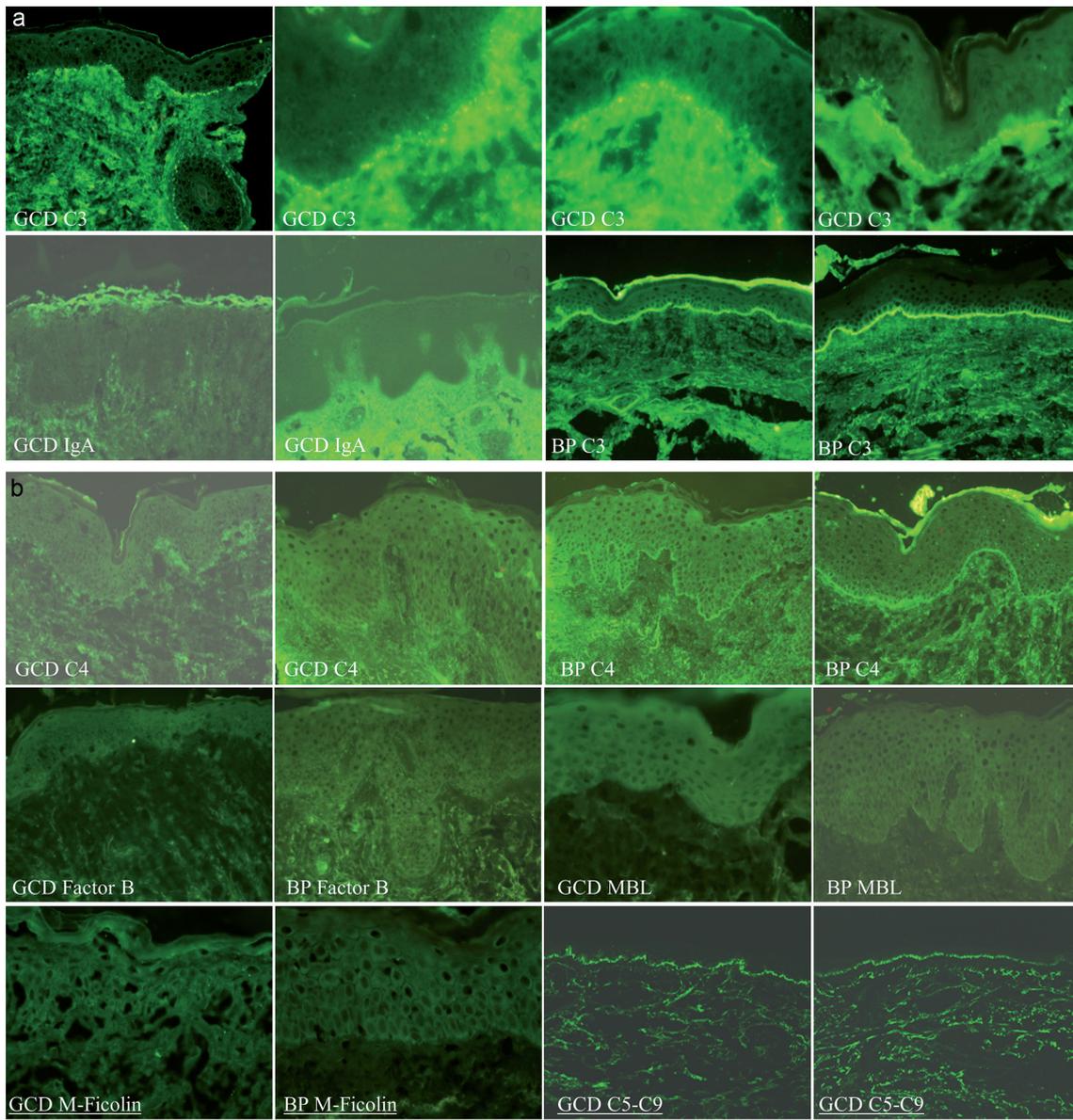
Fig. 2. Immunofluorescence (IF) studies. (a) Results of initial direct IF examinations. Original magnification: ×100 (upper-left); ×200 (others). (b) Results of IF studies of various complement components to clarify involvement of three known complement activating pathways. Original magnification: ×200 (M-ficolin); ×100 (others). GCD: granular C3 dermatosis; BP: bullous pemphigoid; MBL: mannose-binding lectin A.
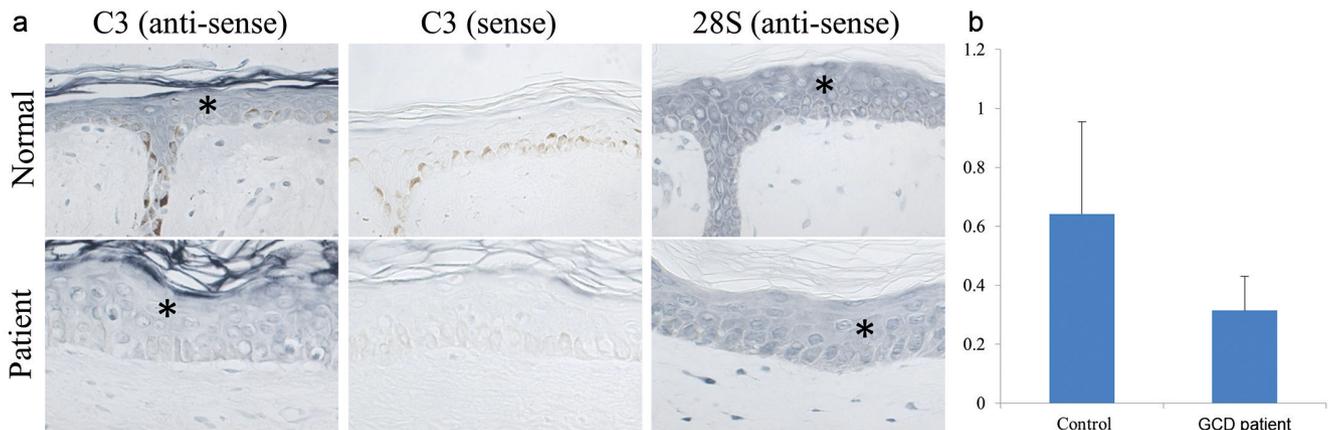

Fig. 3. Studies of expression of C3 mRNA using skin biopsies from 4 patients. (a) The results of *in situ* hybridization using anti-sense probe, and sense probe (negative control) for C3 and positive control probe for 28S rRNA. Positive reaction: blue colour (*). (b) Results of qPCR for expression of C3 in the epidermis for 4 normal controls and 4 granular C3 dermatosis (GCD) patients. Error bars = SD.

DISCUSSION

This study revealed that the 20 GCD patients had several common features; i.e. (*i*) DH-like clinical features, (*ii*) histopathological features of subepidermal blister/oedema and liquefaction degeneration with infiltrations of lymphocytes, eosinophils and neutrophils in various combinations, (*iii*) benign disease course with good response to oral dapsone and topical steroids, (*iv*) granular deposition of C3 and C5–C9, but not other complement components, and (*v*) slightly reduced expression of C3 in epidermis.

Clearly, we must differentiate GCD from several other skin diseases, including various subepidermal AIBDs (DH, linear IgA bullous dermatosis, BP and epidermolysis bullosa acquisita), cutaneous leukocyto-clastic vasculitis, porphyria, polymorphous light eruption, pruritic urticarial papules or plaques of pregnancy (PUPPP), insect bite and various eczematous diseases.

DH and linear IgA bullous dermatosis show positive IgA deposition in BMZ in granular and linear patterns, respectively, in direct IF (32). BP and epidermolysis bullosa acquisita show IgG autoantibodies to BP230/BP180 and type VII collagen, respectively, in various serological tests (32). Cutaneous leukocytoclastic vasculitis shows characteristic histopathological and direct IF finding in blood vessels in dermis. Porphyria and polymorphous light eruption show skin lesions on sun-exposed sites with photosensitivity (33). PUPPP should occur in pregnant females (34). However, both insect bite and various eczematous diseases may show GCD-like features and cannot easily be excluded.

In the present study, no patients showed depositions of immunoglobulins and C4, excluding involvement of classical pathway for C3 activation. Furthermore, IF studies did not show depositions of factor B, mannose-binding lectin and ficolins, suggesting either the alternative or the lectin pathway was involved. Thus, none of the known complement activation pathways seemed to play a role in deposition of granular C3.

Although the true mechanism for deposition of granular C3 is unknown, we put forward the following 5 possibilities. First, that inflammation with heavy lymphocytic infiltration in uppermost dermis induced deposition of C3 from circulation. However, in contrast to deposition of all immunoglobulins and C3 seen in lupus erythematosus and other collagen diseases known as lupus band test (35), our patients showed exclusive C3 deposition. In addition, although lupus band is induced by sun-exposure, skin lesions in our patients occurred on non-sun-exposed sites.

The 2nd possibility is that immune-complex around or inside blood vessels affected by cutaneous vasculitis in upper dermis moved to and granularly deposited in BMZ by known pathway. However, previous IF study of cutaneous vasculitis showed no C3 deposition in BMZ (36). In addition, none of our patients showed apparent cutaneous vasculitis in both histopathological and direct IF studies.

The 3rd possibility is that circulating IgG or IgA autoantibodies bound to unknown autoantigen beneath BMZ and activated complements. In this case, IgG or IgA antibodies could not be detected by direct IF, either because the amount of autoantibodies was too low or because immunoglobulin deposition was masked. However, because no deposition of C1q and C4 was shown in IF studies, it was unlikely that the classical pathway was activated by binding of immunoglobulins. In addition, although IgA antibodies can activate complements via the alternative pathway, our IF study also excluded involvement of the alternative pathway. The possibility that IgG deposition disappeared more quickly than C3 deposition also cannot be completely excluded. However, repeated direct IF performed in 3 patients always showed only C3 deposition, suggesting no preceding deposition of IgG.

A 4th possibility is that complements were activated by microbes, mannose-containing sugar moieties or dead materials via the alternative or the lectin pathway. However, as mentioned above, our IF study indicated that neither the alternative nor the lectin pathway was activated.

Epidermal keratinocytes were reported to produce C3 on stimulation by various cytokines (37, 38). Therefore, the 5th and final possibility is that inflammatory cytokines upregulated C3 production in the patient skin, and over-produced C3 was secreted and deposited in the uppermost dermis. However, unexpectedly, the results in both *in situ* hybridization and q-PCR studies for C3 expression indicated a reduced amount of C3 expression in the epidermis of our patients. These results do not support the 5th possibility.

Thus, future studies are necessary to exclude the remaining possibilities, i.e. (*i*) immunoglobulin(s) and complement components for the classical pathway, such as C1q and C4, disappeared via a so-far unknown mechanism, (*ii*) over-expression of C3 by keratinocytes led to a reduction in C3 mRNA through negative feedback, and (*iii*) dysregulation of complement activation was induced by unknown complement regulators, which are reported to activate complement systems erroneously and induce abnormal condition in several disorders, including the atypical haemolytic uraemic syndrome, systemic lupus erythematosus and glomerulonephritis (39).

GCD and classical DH with granular IgA deposition show considerably similar clinical features, but different histopathological and immunological features. Therefore, it is necessary to elucidate the pathomechanisms of how GCD and DH induce similar clinical features through different histopathological and immunological changes. The granular pattern of C3 deposition, which is a common feature of both GCD and DH, may play a role in the same clinical features.

Finally, it is currently unknown whether C3 deposition plays a role in blister formation in GCD. Elucidation of the pathogenic role of C3 deposition in GCD should also provide a clue to understanding the pathophysiology of other subepidermal AIBDs, which show C3 deposition, in addition to IgG and/or IgA.

For confirmation of the identity of GCD and the pathogenic role of granular C3 deposition on BMZ, further clinical studies with a large sample of similar patients in addition to experimental disease model studies are required.

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