MATERIALS AND METHODS

All studies were performed following the guidelines of Medical Ethics Committee of Kurume University School of Medicine, and were conducted according to the principles of the Declaration of Helsinki. All participants provided informed consent.

Sera and skin biopsies

Sera and were obtained from the 20 patients, as well as patients with bullous pemphigoid (BP), other AIBDs and normal individuals, which were kept at –80°C for long duration and at 4°C with 0.1% sodium azide as preservative during experiments. Skin biopsies containing both lesional and perilesional skin were taken from the 20 patients and BP patients, some of which were kept at –80°C for 1–7 years. Lesional skin part was processed for histopathology, and perilesional skin part was frozen and used for IF studies.

Immunofluorescence studies

Routine direct IF for IgG, IgA, IgM and C3 was performed by standard method using frozen sections of biopsied perilesional skin. Indirect IF studies of both normal human skin and 1M NaCl-split-human skin for both IgG and IgA antibodies were performed as described previously (13). Complement fixing IF of both normal human skin and 1M NaCl-split-human skin for C3 was performed as described previously (14).

In addition, IF studies of patient skin for depositions of various complement components were performed using fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal antibodies (pAbs) specific to human C4, Factor B and C5-C9 (mAbs) specific to mannose-binding lectin A (MBL) (Abcam, Cambridge, UK) and H-, M- and L-ficolins (Hicult Biotech, Uden, Netherlands). FITC-conjugated anti-rabbit IgG or mouse IgG pAb was used as second antibody.

Immunoblot and ELISA studies for autoantigens in non-dermatitis herpetiformis diseases

Immunoblot analyses of normal human epidermal extract (13,15), BP180 NC16a domain recombinant protein (RP) (16) and BP180 C-terminal domain RP (17), concentrated culture supernatant of HaCaT cells (18), normal human dermal extract (19) and purified human laminin-332 (20) were performed as previously described.

ELISAs of BP180 NC16a domain RP and BP230 RP for both IgG and IgA antibodies were performed using commercial kits (MESACUP, Medical & Biological Laboratories, Nagoya, Japan). Cut-off values were index 15.0 for BP180 ELISA and index 9.0 for BP230 ELISA for IgG antibodies, while optical density (OD) 0.15 was set as cut-off value for IgA antibodies. Commercial ELISAs for Dsg1 and Dsg3 (Medical & Biological Laboratories) were performed only for IgG antibodies (cut-off values-index 12). Absorbance at 490 nm was measured by an ELISA reader (Nalge Nunc International Co., New York, NY, USA). IgA ELISA of RP of larger BP180 ectodomain was performed as described previously (21).

Serological tests for dermatitis herpetiformis

To detect IgA anti-EMA antibodies, both indirect IF for reactivity with the aboral part of monkey oesophagus and ELISA were performed (22). Serum IgA anti-c-TG and anti-tTG antibodies were detected by ELISAs, as described previously (6). In addition, commercial ELISA was also used for IgA anti-c-TG antibodies (23). Screening ELISA for IgA/IgG anti-tTG/DGP antibodies, and IgA and IgG ELISAs for both tTG and DGP were performed as reported previously (24). Commercial ELISAs of gliadin for both IgA and IgG antibodies were performed as described previously (25). IgA anti-F-actin antibodies were also examined by ELISA (26). The result was evaluated as positive or negative using cut-off values for each ELISA (Table S1).

In situ hybridization

Specificity and sensitivity of digoxigenin-labelled C3 oligo-DNA probe and T-T dimer-labelled 28S rRNA oligo-DNA probe were confirmed by immunodetection and dot-blot hybridization (27). Immunohistochemical in situ hybridization for C3 was performed as described previously (28). Briefly, sections, which were fixed with 4% paraformaldehyde and treated with 0.2N HCl, were first treated with 50 µg/ml proteinase K. After post-fixation with 4% paraformaldehyde, sections were immersed in 2 mg/ml glycine and kept in 20% deionized formamide until used for hybridization. Hybridization was performed for 15–17 h at 37°C with oligo-DNA probe, as described previously (29). 28S rRNA probe was used as positive control (30). Horseradish peroxidase-conjugated sheep anti-digoxigenin pAb and mouse anti-T-T dimer mAb were used for immunohistochemistry with 3,3’-diaminobenzidine tetrahydrochloride, H2O2, nickel and cobalt ions.

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

For qPCR, 10 serial frozen skin sections (8 um) were prepared for each skin sample, and the first and the tenth sections were stained with haematoxylin and eosin to identify epidermal area for needle microdissection. Epidermal tissues were dissected using 24-gauge needle under a stereomicroscope and transferred immediately to an Eppendorf tube containing buffer RLT in the RNAeasy micro kit (Qiagen, Frederick, MD, USA). Levels of mRNA encoding human C3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample were measured by qPCR using an ABI PRISM 7700 sequence detector (Applied Biosystems; Foster City, CA, USA) according to the previously reported method (31). Taqman probes and primers for C3 (Hs00163811) and GAPDH were obtained from Applied Biosystems. Primers for GAPDH were purchased from Sigma-Genosys (Hokkaido, Japan). RT-PCR condition was: 42°C for 20 min; 95°C for 5 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. The level of C3 mRNA was normalized by the results of GAPDH mRNA. Statistical analysis was performed using unpaired t-test.

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