ULTRASTRUCTURAL LOCALIZATION OF IN VIVO-BOUND COMPLEMENT IN BULLOUS PEMPHIGOID

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Antihuman complement component C3 labeled with horseradish peroxidase was used to reveal the ultrastructural localization of complement in two cases of bullous pemphigoid. The complement deposits were shown to be exclusively located in the space between the plasma membrane of the basal cells and the basal lamina. This corresponds exactly to the ultrastructural localization of immunoglobulins in bullous pemphigoid.

Chorzelski and Cormane [1], using fluorescein-labeled antihuman complement, have shown that complement is bound in vivo to the basement membrane of the skin in patients with bullous pemphigoid. These authors stated that the pattern of fluorescence obtained with conjugated antihuman IgG was used. Moreover, further studies have shown that 75% of the patients with bullous pemphigoid have circulating, complement-fixing IgG antibodies [2]. These investigations suggest that both IgG and complement are located at the same site. However, the low resolution obtained with light microscopy utilizing fluorescent reagents is not adequate to show the exact localization of complement and immunoglobulins. In this study, horseradish peroxidase was coupled to antihuman complement and employed to determine the ultrastructural localization of complement.

MATERIALS AND METHODS

Preparation of the conjugate. Antihuman complement component C3 was conjugated to highly purified horseradish peroxidase by the method of Avrameas [3]. The specificity of antihuman-C3 was proved by double-diffusion tests in agar gel against fresh human serum and against purified complement component C3. One hundred milligrams of horseradish peroxidase (HRP, Sigma Chemical Company, No. P9225) were dissolved in 20 ml of 0.05 M phosphate buffer, pH 7.2, and centrifuged at 40,000 × g for 45 min at 4°C. Forty milligrams of lyophilized goat-antihuman complement (Cappel Laboratories) were reconstituted to 2.5 ml. The supernatants of the peroxidase solution and of the antihuman complement solution were combined, and 4.0 ml of 0.1% glutaraldehyde solution was added dropwise with gentle stirring at room temperature. The mixture was left standing for 2 hr at room temperature and was then dialyzed against 5 liters of PO₄-buffered 0.9% NaCl (PBS), pH 7.4, overnight at 4°C. The conjugate was centrifuged at 40,000 × g to remove impurities, and used in a concentration of 3 mg per ml for light microscopy and in a concentration of 1 mg per ml for electron microscopy.

Tissue processing. In 2 patients with bullous pemphigoid without circulating antibase membrane antibodies, 4-mm punch biopsies were taken from erythematous skin close to a blister after local anesthesia with 1% lidocaine. The tissue was sliced and fixed for 24 hr in a buffered solution of ammonium sulfate and subsequently rinsed in citrate buffer as described by Michel et al [4] for the same time period, frozen in liquid nitrogen, and stored at -25°C.

Light microscopy. Six-micron cryostat sections were made with an International Harris cryostat, Model CTD, placed on microscope slides, and incubated for 30 min in the antihuman-C3 peroxidase conjugate (3 mg/ml). The sections were rinsed in PBS for 30 min, subsequently fixed in 5% glutaraldehyde in 0.1 M PO₄ buffer, pH 7.2, rinsed again in PBS for 30 min, and incubated for 30 min in 3,3-diaminobenzidine (DAB) as described by Graham and Karnovsky [5]. After rinsing in PBS for 30 min, the sections were postfixed with 1% OsO₄ in 0.1 M PO₄ buffer, pH 7.2, and rinsed in PBS for 30 min. Finally, the sections were mounted and examined microscopically.

Electron microscopy. Thirty-two-micron sections were placed in PBS and rinsed for 1 hr at 4°C. Subsequently, the sections were incubated in the antihuman-C3 peroxidase conjugate (1 mg/ml) for 14 hr at 15°C. After rinsing in PBS for 3 hr, the sections were fixed for 2 hr in 5% glutaraldehyde in 0.1 M PO₄ buffer, pH 7.2. The sections were rinsed again in PBS for 30 min and incubated for 30 min in DAB to reveal the location of the complement-conjugate complexes [5]. Thereafter, the sections were rinsed in 0.1 M PO₄ buffer, pH 7.2, for 15 min and

Fig. 1. Light microscopy. Linear staining of the basement-membrane zone (arrows). E = epidermis (× 300).
Fig. 2. a: Electron-dense precipitates (arrowheads) located in the space between the plasma membrane (arrows) of a basal cell (BC) and the basal lamina (asterisks), on the epidermal site of the basal lamina and on the outer leaflet of the plasma membrane of the basal cell. C with arrows = collagen (× 30,000). b: Fine granular deposits (arrowheads) located on the anchoring filaments. Asterisks = basal lamina; BC = basal cell (× 20,000).

Fig. 3. The coarsely agglomerated reaction product (arrowheads) fills the space between the plasma membrane (arrows) of a basal cell (BC) and the basal lamina (asterisks) entirely. C with arrows = collagen (× 37,500).

postfixed for 1 hr with 1% OsO₄ in 0.1 M PO₄ buffer, pH 7.2. The sections were stained en bloc for 1 hr in 50% ethanol containing 1% uranyl acetate before dehydration and araldite embedding. The sections were cut with a Reichert OM11 ultramicrotome, stained in uranyl acetate and lead citrate, and examined with a JEOL JEM 100 B electron microscope.

Controls. (1) Blocking reaction: Control sections were incubated in nonconjugated antihuman complement for 30 min for light microscopy and for 14 hr for electron microscopy to block the subsequent conjugate binding. The sections were rinsed in PBS for the same time periods before continuing with the already-described procedures. (2) Normal tissue of a healthy individual was processed for light microscopy and for electron microscopy in the same manner as that used for sections obtained from patients with bullous pemphigoid. (3) To exclude nonspecific staining due to endogenous peroxidase, sections were incubated in DAB without prior immunochemical treatment.

RESULTS

Light microscopy. Tissue sections prepared for light microscopy showed a brown linear staining of the basement-membrane zone (Fig. 1). This staining is due to the reaction of DAB and hydrogen

Fig. 4. Blocking reaction. There are no deposits between the plasma membrane (arrows) of a basal cell (BC) and the basal lamina (asterisks). C with arrows = collagen (× 24,500).
peroxydase with peroxydase contained in the antihuman-C3 conjugate. The staining pattern corresponds to the homogenous and linear staining of the basement-membrane zone obtained in immunofluorescence. Control sections did not show any staining of the basement-membrane zone.

Electron microscopy. By coupling peroxydase to antihuman complement antibody, in vivo-bound complement can be localized. The reaction of peroxydase and DAB in the presence of hydrogen peroxide results in an electron-dense precipitate, which we shall refer to as the "reaction product."

Electron micrographs showed the presence of the reaction product as electron-dense, coalescent, globular deposits in the space between the basal cells and the basal lamina. In areas with less intense reaction, the electron-dense, globular deposits were located mainly on the epidermal side of the basal lamina and on the other leaflet of the trilaminar plasma membrane of the basal cells (Fig. 2a). In some areas, the fine globular deposits were found on the anchoring filaments (Fig. 2b). In areas with an intense reaction, coalescent electron-dense material filled the space between the basal cells and the basal lamina entirely (Fig. 3). The reaction product never extended beyond the basal lamina and was never observed within the basal cells. In control sections in which the immunologic reaction was blocked, normal skin was used or DAB was used without immunochemical treatment; the space between the basal cells and the basal lamina was free of electron-dense material (Fig. 4).

DISCUSSION

Previous investigators have shown that nearly 75% of all patients with bullous pemphigoid have circulating IgG antibodies able to fix complement [2]. This would be in agreement with the observation that staining of the basement-membrane zone with fluorescein-labeled antihuman IgG or fluorescein-labeled antihuman complement resulted in a similar staining pattern [1]. These investigations indicate that both IgG and complement are located at the same site. The ultrastructural localization of in vivo-bound complement corresponds exactly to the sites of immunoglobulin deposits recently described [6]. Braun-Falco and Rupec [7] as well as Wilgram [8] reported that the blister in bullous pemphigoid is formed between the basal cells and the basal lamina. Immunoglobulins and complement are located at the same site where the blister is formed and were found prior to the blister formation. Thus our results indicate that antigen-antibody complexes together with complement might initiate blister formation and tissue injury.

REFERENCES