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ULTRASTRUCTURAL LOCALIZATION OF PEMPHIGUS AUTOANTIBODIES*

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Electron microscopic studies of skin biopsies from the cases of pemphigus gave controversial results regarding primary ultrastructural changes in the formation of acantholytic bullae. Wilgram *et al* (1–3) suggested that acantholysis is preceded by destruction of the desmosome-tonofilament system. In sections of apparently normal skin with positive Nikolski phenomenon however, Braun-Falco and Vogell (4) showed that the earliest changes occur in the intercellular substance of the epidermis.

By means of indirect immunofluorescent (IF) staining, Beutner *et al* (5, 6) and Chorzelski *et al* (7–10) demonstrated the presence of circulating autoantibodies reacting specifically with the intercellular substance of squamous cells. The specificity of these antibodies has been further confirmed by repeated examinations in a large number of patients, and a correlation of the antibody titres with the severity of the disease has been found.

It would be tempting to assume that these results in some way correspond to the findings of Braun-Falco and Vogell (4). The purpose of our present study was to establish the localization of the pemphigus antibodies with electron microscopy using ferritin-labelled immunoglobulins from a patient with pemphigus.

MATERIALS AND METHODS

Investigations were carried out by means of direct staining with ferritin†-labelled globulins of pemphigus serum. The antibody titre of this serum, determined by means of indirect IF staining before labelling, was 1:600.

Labelling with ferritin was performed by the method of Singer and Schick (11), using toluene 2,4, diisocyanate as a coupling agent.

The immunological activity of the resultant conjugate was controlled by indirect immunofluorescent procedure (Fig. 1). The loss of activity due to labelling was insignificant (one-fold dilution).

The immunological reaction was performed using rabbit oral mucosa immediately frozen in liquid nitrogen and cut in a cryostat at −20° C into 20 micron sections. Part of the fresh tissue was cut with a razor blade into pieces measuring approximately one mm^3.

The sections were subsequently incubated at room temperature for 30 minutes in the conjugate, diluted with phosphate buffered saline (pH 7.5)

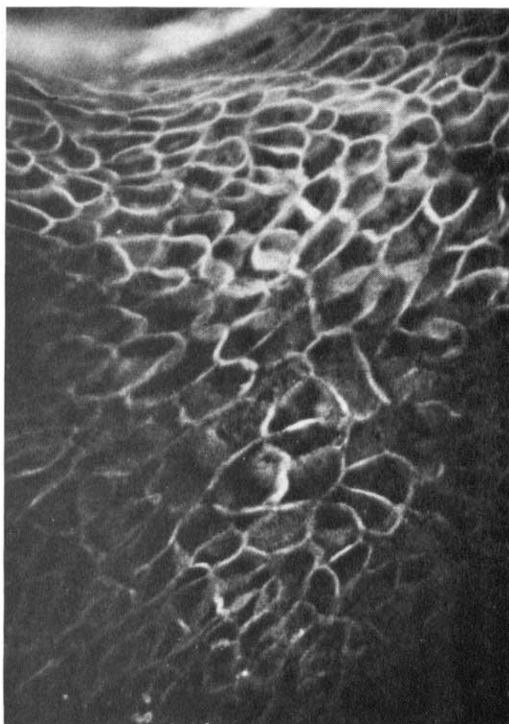

FIG. 1. Indirect immunofluorescent staining of the ferritin conjugate. Note specific fluorescence of the intercellular substance of rabbit epithelium.

to a concentration corresponding to a 1:200 solution of the original serum. After incubation the sections were washed in the refrigerator three times for 5 minutes in phosphate buffered saline cooled to 4° C.

Controls

1. Sections were incubated in 1.5% solution of ferritin in phosphate buffered saline pH 7.5
2. Sections were incubated in a solution of un-

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† Ferritin Horse Spleen 2x cryst. Mann Research Lab. Inc., New York.

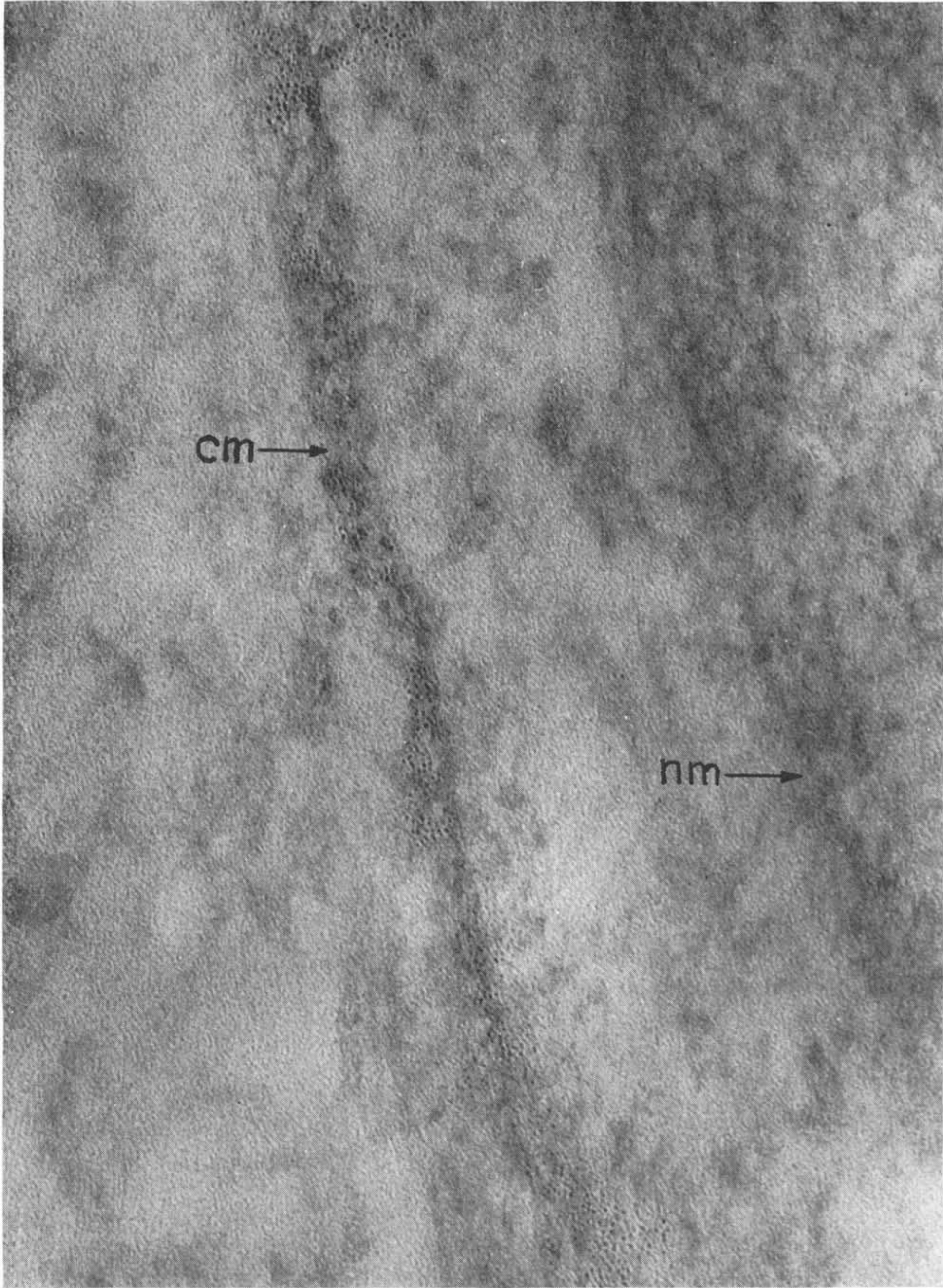

FIG. 2a. Ferritin granules in the intercellular space and within cell membrane. CM = cell membrane; NM = nuclear membrane; 160000 ×.

labelled globulins and subsequently with labelled globulins of the same patient (specific inhibition test).

After the reaction, the tissue sections were fixed in 1% OsO_4 buffered to pH 7.4 with 0.1 M phosphate buffer containing 5% sucrose, for 3 hours at 4° C.

The sections were then dehydrated with increasing concentrations of ethanol, and embedded in Epon. Tissue blocks were cut in a Porter Blum

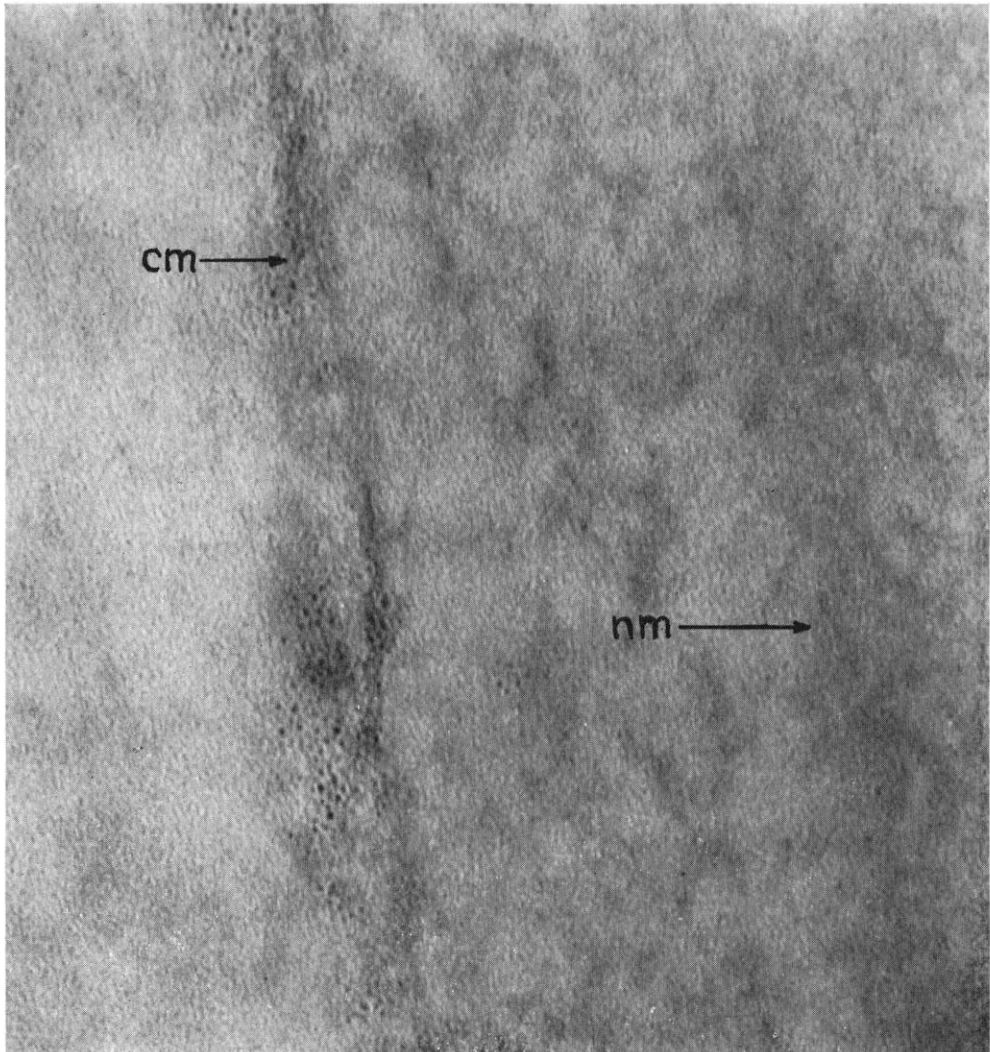

FIG. 2b. 240000 ×

M-2 ultramicrotome and examined under an electron microscope (JEM 7).

The ultrasections were not stained.

RESULTS

The structure of epidermal cells in the cryostat sections was considerably damaged and its interpretation was rather difficult. Only the cell and nuclear membranes were relatively well preserved. The cytoplasm was homogenous and the respective organells were indistinguishable. Numerous minute granules corresponding to ferritin were found in the vicinity of the preserved cell membranes, particularly in the region of the intercellular substance.

The sections from fresh tissue contained rather well preserved cytoplasmic structures. Cellular and nuclear membranes were distinct. Ultrasections from the periphery of the tissue blocks contained granular deposits of high electron density corresponding to ferritin, localized close to cell membranes, and, again, in the intercellular space (Fig. 2a & b). Large accumulation of granules which occasionally formed minute agglomerates were also observed close to cell membranes, sometimes filling the entire intercellular space. These granules were

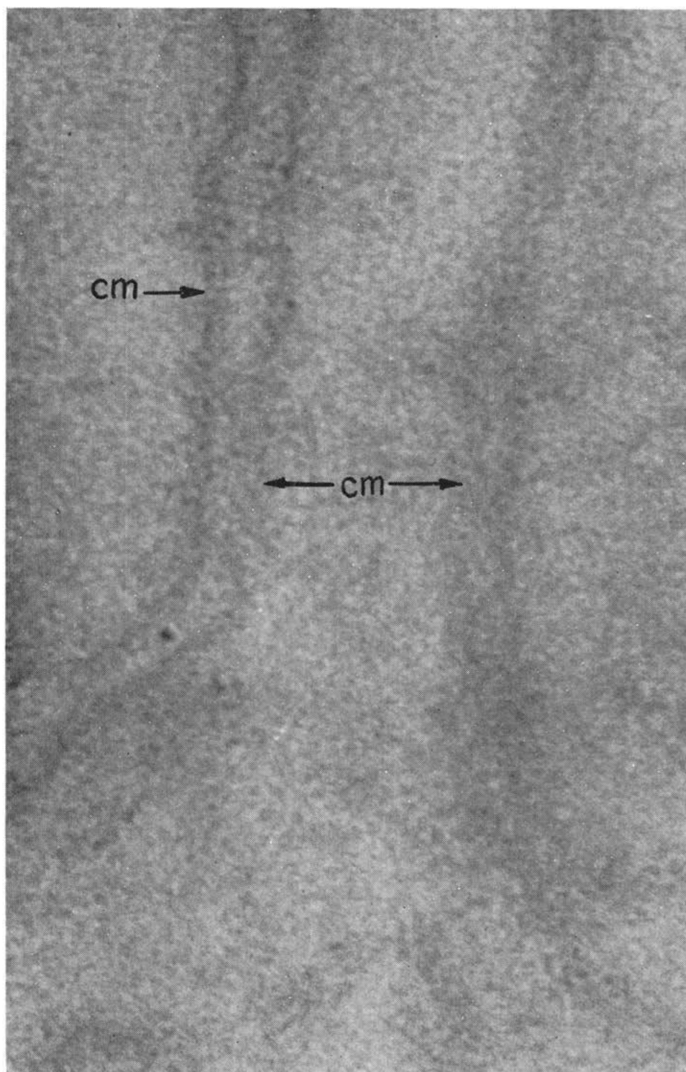

FIG. 3. Complete lack of ferritin granules in the control

not found in the deeper portions of tissue blocks.

It should be emphasized that the ultrasections from both frozen and fresh tissue did not contain ferritin granules in areas other than already described. The ferritin granules were also absent in both controls, i.e., in the sections incubated in ferritin solution alone and in the sections pretreated with a solution of unlabelled globulins prior to incubation with the ferritin conjugate (Fig. 3) (inhibition test).

DISCUSSION

The above investigations show that the ferritin labelled globulins of the pemphigus patients react in vitro with the intercellular substance of squamous epithelium. The application of electron microscopy established the binding of pemphigus autoantibodies within the cell membrane.

Negative controls, particularly with regard to sections incubated in a solution of unlabelled globulins prior to incubation with ferritin labelled globulins of the same patient, are a further proof of the specificity of this reaction. Apart from the circulating antibodies found in pemphigus patients previous experiments demonstrated the presence of gamma globulin bound in vivo in the epidermis adjacent to acantholytic bullae (6, 12). This,

however, is not sufficient proof that these are antigen-antibody complexes. Evidence suggesting the possibility of an immunological reaction has recently been provided by Cormane and Chorzelski (12), who demonstrated the concomitant binding of human complement (C'3a, C'4) at these sites.

The primary changes in the intercellular substance of the epidermis shown by Braun-Falco and Vogell (4) with electron microscopy, the presence of specific autoantibodies reacting with antigens at the same site, and the presence of gammaglobulin bound in vivo with the participation of the complement in the vicinity of acantholytic bullae, leave little doubt that an immunological process does take place in the intercellular substance and at the cell periphery, where acantholysis is known to begin. In spite of these findings, it would be premature to say that acantholysis is a direct result of an antigen-antibody reaction.

SUMMARY

Ferritin-labelled pemphigus globulins were shown by electron microscopy to combine with the intercellular substance and cell membrane of rabbit squamous epithelium.

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