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# Surface Distribution of Pemphigus Antibody-Binding Substance(s) on Isolated Guinea Pig Epidermal Cells. An Immunoferritin Electron Microscopic Study

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**The surface distribution of pemphigus antibody-binding substance(s) on trypsinized, isolated guinea pig epidermal cells was studied by means of immunoferritin electron microscopy. Both fresh and 4% paraformaldehyde-fixed cells were incubated with pemphigus serum at 4°C for 15 min and then labeled with ferritin-conjugated goat antihuman IgG at 4°C or 37°C for 15 min, respectively. In these cells clusters of ferritin particles were distributed randomly on the surface of nondesmosomal membranes and hemidesmosomes. Fresh basal and lower spinous cells showed clusters of 10 to 20 ferritin particles at 4°C while they displayed various sizes of clusters of 10 to more than 50 particles, some of which were interpreted as patches, at 37°C. Fresh upper spinous and granular cells showed clusters of 10 to 20 particles at both 4°C and 37°C. Fixed cells displayed clusters of no more than 20 ferritin particles irrespective of the layers from which they derived and of labeling conditions. Occurrence of patches implied that aggregation of binding substance(s) was induced by pemphigus antibodies. It was suggested that pemphigus antibody-binding substance(s) were movable in the membrane of basal and lower spinous cells while they were less mobile or immobile in upper spinous and granular cells.**

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Abbreviations:
BS: binding substance(s)
PA: pemphigus antibodies
PBS: phosphate-buffered saline

Immunofluorescence and immunoelectron microscopy have shown an intercellular deposit of immunoglobulin in the lesion and the adjoining region of the epidermis in pemphigus [1–4]. Sera from patients with pemphigus contain antibodies that react with the stratified squamous epithelium of human, monkeys and guinea pigs [1]. Intercellular binding substance(s) (BS) for pemphigus antibodies (PA) have been isolated from

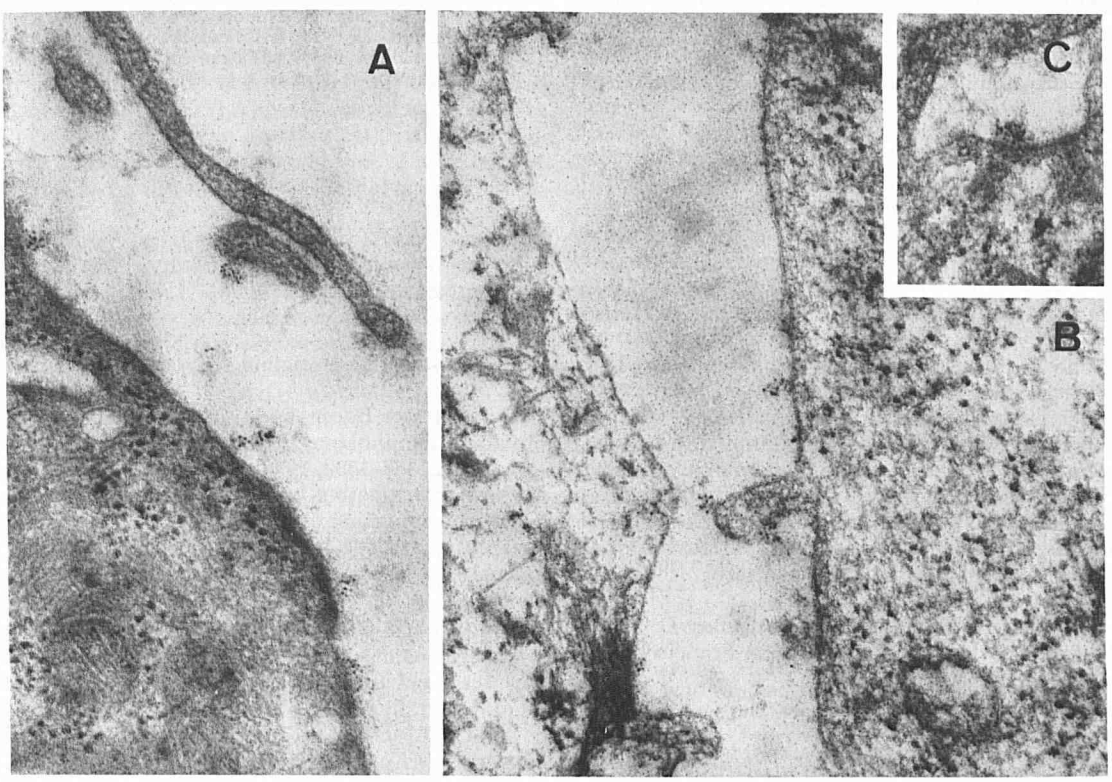


FIG 1. A basal and lower spinous (*A*) and 2 upper spinous (*B*) cells labeled at 4°C throughout. There were small clusters of ferritin particles. The label is also bound to the surface of a hemidesmosome (*C*) of a basal and lower spinous cell (× 60,000).

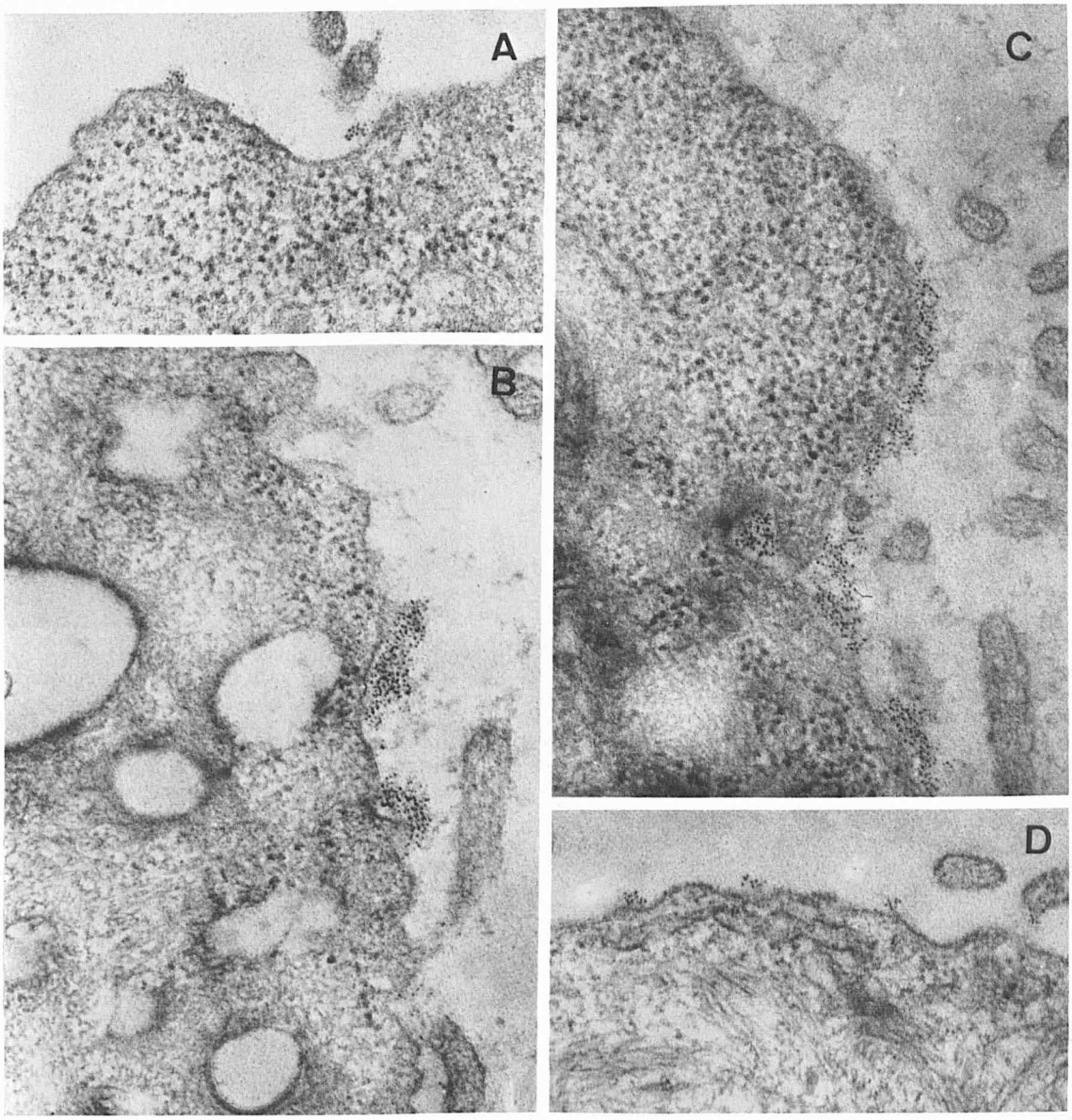


FIG 2. Cells reacted with pemphigus antibodies at 4°C and then labeled with the ferritin-conjugate at 37°C. Basal and lower spinous cells show small clusters (*A*), large aggregates (*B*) and patches (*C*) of ferritin particles. A upper spinous cell shows small clusters (*D*) (× 60,000).

the epithelial tissue of several mammalian species [5–7]. In guinea pig skin, BS is a protein containing RNA and hexose, having a molecular weight of 18,000 [7]. Thus, PA are directed towards what may be defined as tissue-specific but not species-specific antigens of epidermal cells.

Recently membrane immunofluorescence has shown deposition of PA on the surface of enzymatically isolated, guinea pig epidermal cells, suggesting the presence of PA-BS on the cell membrane [8,9]. In the present study immunoferritin electron microscopy was used to reveal ultrastructural localization of PA-BS on isolated epidermal cells.

## MATERIALS AND METHODS

### *Epidermal Cell Suspension*

Isolation of epidermal cells of guinea pigs (Hartley strain) was performed using trypsin as previously described [9]. More than 95% of the cells were viable when tested by trypan blue exclusion.

### *Paraformaldehyde Fixation*

Washed epidermal cells were fixed in 4% paraformaldehyde-0.85% saline at a concentration of $10^6$/ml at 4°C for 15 min. Following fixation the cells were washed 3 times with phosphate-buffered saline (PBS).

### *Pemphigus Sera*

Sera were obtained from 3 patients with pemphigus vulgaris (2 males and 1 female; age range 32–65). Antibody titers on frozen oral mucosa of guinea pigs were 40, 80 and 320, respectively. After absorption once with guinea pig red blood cells and lymphoid cells, sera were stored at −20°C.

### *Ferritin-Conjugated Antihuman IgG*

Horse spleen ferritin (twice crystallized, Nutritional Biological Co.) was further purified by cadmium sulfate recrystallization and ultracentrifugation. The IgG fraction of goat antihuman IgG was purchased from Miles Laboratories, Ill. Conjugation of ferritin to goat antibody was performed by the method of Kishida et al [10] with some modification. To 35 mg of glutaraldehyde-activated ferritin in 4 ml of 0.05 M phosphate buffer (pH 7.5) was added 10 mg of goat antibody in 3 ml of 0.1 M carbonate-bicarbonate buffer (pH 9.1). The mixture was dialyzed against the carbonate-bicarbonate buffer at 4°C for 36 hr. The reaction was stopped by addition of sodium borohydride (3 mg/ml). After dialysis against PBS the mixture was centrifuged on a discontinuous sucrose gradient for 3 hr at 35,000 rpm to remove any unconjugated goat IgG [11]. The concentration of goat IgG in the conjugate was determined by passive hemagglutination assay using human IgG-tagged sheep red blood cells as indicator cells.

*Labeling Conditions*

In all experiments, pemphigus sera and normal human serum (as a control) were used at a dilution of 1:10. All 3 pemphigus sera gave comparable results. Ferritin-goat antibody conjugate was employed at a concentration of 150 $\mu$g of goat antibody/ml. Either fresh or fixed cells (3–5 $\times$ $10^6$ cells) were first incubated with 0.5 ml of pemphigus or normal human serum at 4°C for 15 min. After washing the cells with PBS, aliquots of cells were incubated with 0.5 ml of the ferritin-conjugate for 15 min at either 4°C or 37°C. Following washing the cells with PBS, cell pellets were fixed in phosphate-buffered 2.5% glutaraldehyde and then 1% osmium tetroxide for 2 hr at 4°C, respectively, dehydrated in graded acetones and embedded in Epon 812. Thin sections were cut by glass knives on a Porter Blum MT-2B ultramicrotome. Only sections of 60 to 80 $\mu$m thickness, as determined by interference color, were collected on grids coated by carbonized Formvar, stained with methanolic uranyl acetate and examined in a Hitachi HS-9 electron microscope.

*Analysis of Labeling Density*

In order to compare the density of ferritin particles in each cluster on the surface of different cell membranes, black dots representing individual ferritin particles were counted on electron micrographs at a final magnification of 60,000. Only perpendicularly sectioned membranes were examined. At least 10 to 20 cells were counted in each.

## RESULTS

The trypsinization technique used in this study resulted in a heterogenous mixture of cell populations. Isolated cells were classified as follows. Cells showing both keratohyalin granules and membrane-coating granules were described as granular cells. Those showing only membrane-coating granules were termed upper spinous cells. Those showing none of the above mentioned organelles were described as lower spinous and basal cells since by the available morphological criteria it was difficult to distinguish lower spinous from basal cells. The cell suspension was composed mostly of basal, spinous and granular cells with a small number of fibroblasts. Since cornified cells, melanocytes and Langerhans cells were not always observed in the specimens, the description of these cells is omitted in the results.

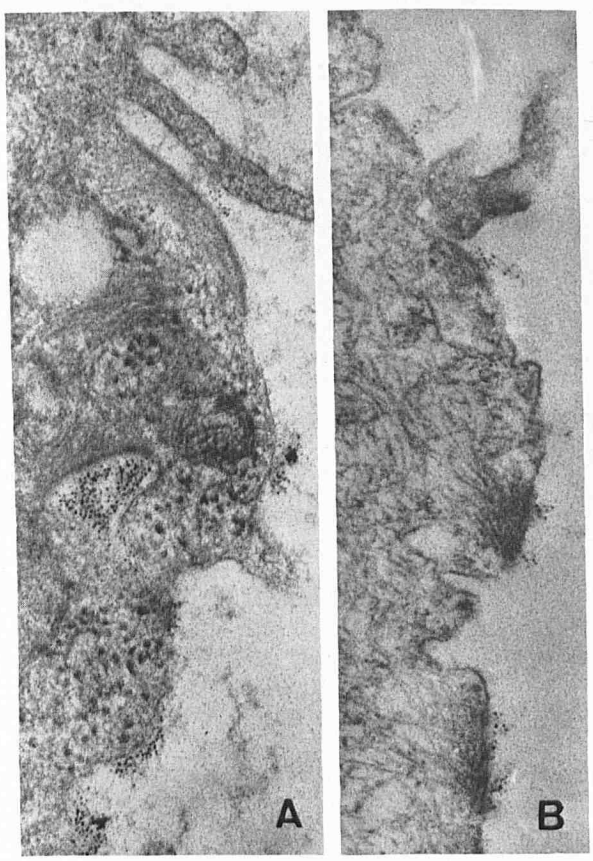


FIG 3. Cells incubated with PA at 4°C and then labeled with the ferritin-conjugate at 37°C. A basal and lower spinous cell shows a pinocytic vesicle containing a labeled hemidesmosome (*A*). An upper spinous cell displays labeled hemidesmosomes on the cell surface (*B*) (× 60,000).

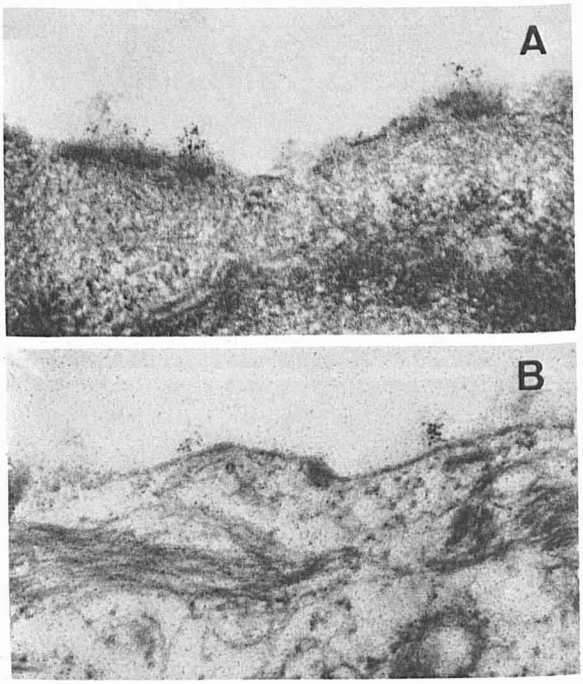


FIG 4. Paraformaldehyde-fixed basal and lower (*A*) and upper spinous (*B*) cells showing small clusters of ferritin particles (× 60,000).

*Fresh Cells Labeled at 4°C*

Labeling was similar in basal, spinous and granular cells when they were incubated with pemphigus serum and the conjugate throughout at 4°C. The cell surface showed small clusters of ferritin particles that were separated by long unlabeled regions (Fig 1*A, B*). The surface of hemidesmosomes was occasionally labeled (Fig 1*C*). There were no pinocytic vesicles.

*Fresh Cells Labeled at 37°C*

Incubation of the cells with pemphigus sera at 4°C and then with the conjugate at 37°C resulted in some noticeable changes in the labeling pattern. On basal and lower spinous cells there were clusters of various sizes (Fig 2*A, B*). Some of large aggregates (Fig 2*C*) were interpreted as patches. Pinocytic vesicles containing ferritin and ferritin-labeled hemidesmosomes were seen in these cells (Fig 3*A*). On the other hand, upper spinous and granular cells usually showed small clusters (Fig 2*D*). Most of the hemidesmosomes in these cells were labeled (Fig 3*B*). Pinocytic vesicles were found only in some of the upper spinous cells.

*Paraformaldehyde-Fixed Cells*

Fixed cells that were labeled at either 4°C or 37°C showed ferritin particles only as small clusters (Fig 4). The number of such clusters on the surface was markedly reduced when compared to those on fresh cells. Indeed, about 70% of the cells in thin sections showed no clusters. There were no pinocytic vesicles.

*Control*

No binding of ferritin was seen in controls in which the cells were incubated with normal human serum at 4°C and with the conjugate at 4°C or 37°C.

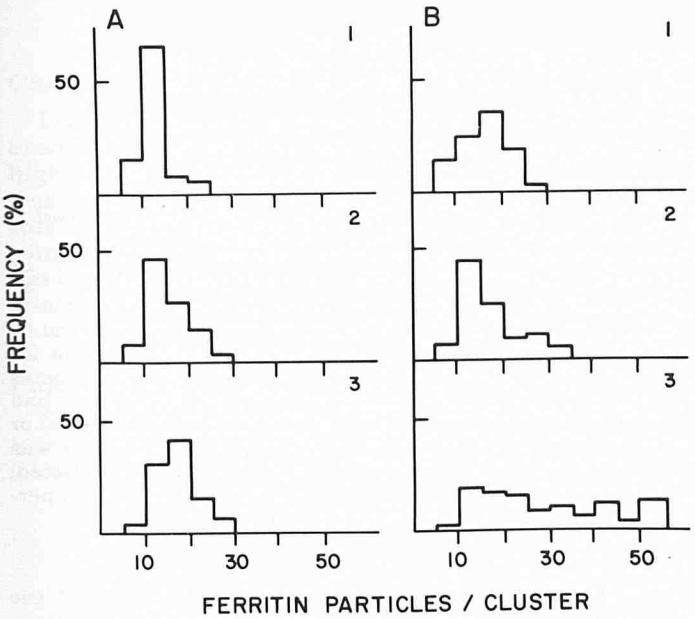


FIG 5. Histogram showing the frequency distribution of ferritin clusters on epidermal cells from different layers. *Ordinate:* frequency in percent. The scale is drawn so that the distance between the lines represents 75% of total numbers of clusters at each cell population. *Abscissa:* ferritin particles counted per cluster on the cell surface. *A*, cells labeled at 4°C throughout. *B*, cells incubated with pemphigus antibodies at 4°C and then labeled with the conjugate at 37°C. *1*, granular cells; *2*, upper spinous cells; and *3*, basal and lower spinous cells. In *B, graph 3,* more than 50 particles per cluster on the cell surface are expressed in the area above 50.

### *Analysis of Labeling Density*

Fig 5 shows a histogram summarizing measurements made of labeling density of the clusters on the nondesmosomal membranes of epidermal cells from different layers. Most of the fresh cells labeled throughout at 4°C displayed clusters composed of 10 to 20 ferritin particles (Fig 5*A*). In fresh basal and lower spinous cells labeled at 37°C, the number of ferritin particles in each cluster ranged from 10 to more than 50 (Fig 5*B-3*). On the other hand, fresh upper spinous and granular cells labeled at 37°C showed clusters composed of 10 to 20 particles (Fig 5*B-1,2*). The clusters on fixed cells, when they were found on the cell surface, were composed of less than 20 particles (data not shown).

## DISCUSSION

PA-BS were distributed randomly on the surface of enzymatically dispersed guinea pig epidermal cells. They were seen as widely separated small clusters of ferritin particles on both paraformaldehyde-fixed and fresh cells. Furthermore, fresh basal and lower spinous cells labeled at 37°C also showed larger clusters that could be interpreted as patches, presumably resulting from aggregation of smaller clusters. At both 4°C and 37°C, the surfaces of hemidesmosomes were labeled by ferritin particles. PA-BS were, thus, present on the surface of nondesmosomal membranes and within desmosomes. This finding was consistent with other studies [2–4]. Paraformaldehyde fixation reduced the reactivity of BS since the numbers of labeled cells and numbers of clusters per cell were reduced in fixed cells in comparison with fresh cells.

Ultrastructural examinations of PA-BS in tissue sections using peroxidase- and ferritin-antibody techniques have shown that PA-BS are distributed uniformly over the entire cell surface [2–4]. The difference in the distribution between the present and the other studies may be ascribed to the effect of trypsin on epidermal cells. Partial degradation of PA-BS by trypsin would result in the random localization of BS observed here. Susceptibility of PA-BS to proteolytic enzymes has been well established [6,9]. Another possibility is that clustering of PA-BS was induced by trypsin treatment, since proteolytic enzymes induce modification of membrane and junctional structures [12]. Nicolson [13] has shown that there is a marked clustering of Concanavalin A receptors on mildly trypsinized mouse 3T3 cells compared to untreated cells. Marchesi et al [14] have obtained evidence that proteolysis produces clustering of the intramembranous particles in erythrocyte ghost membranes. These considerations suggest that the localization of PA-BS seen in tissue sections most closely resembles the true in vivo situation.

Labeling of the cells at 37°C induces formation of patches in fresh basal and lower spinous cells but not in fresh upper spinous and granular cells. The occurrence of patches suggested that aggregation of BS was induced by PA. However, cells fixed with paraformaldehyde, a reagent that immobilizes membrane macromolecules [15], as well as fresh cells labeled at 4°C, mostly showed clusters containing no more than 20 particles. Thus, mobilization of PA-BS appeared to be temperature-dependent and inhibited by aldehyde fixation. These findings agree with our recent study* showing that epidermis-specific antigens are mobile in the membrane of basal and lower spinous cells, and of some upper spinous cells at physiological temperatures. The presence of pinocytic vesicles in basal and lower spinous cells at 37°C also supports this view [15]. The fact that patch formation was not found in upper spinous and granular cells may be explained by progressive decrease in membrane fluidity in these cells.

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