Charge-selective Permeability of Dermo-epidermal Junction: Tracer Studies with Cationic and Anionic Ferritins

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To investigate quantitatively the charge-selective permeability of the basement membrane (BM) of the dermo-epidermal junction (DEJ), tracer experiments using anionic and cationic ferritins were performed on an epidermal sheet, whose lamina densa was exposed on the dermal surface; its dermis was removed with forceps after the treatment of newborn rat skin with 10 mM dithiothreitol. The lamina densa and epidermal components of the sheets were electron microscopically well preserved, and anionic sites were ultrastructurally demonstrated on both the dermal and epidermal aspects of the lamina densa in the DEJ as clusters of cationic ferritins (CF) [isoelectric point (pI) > 9.5] or polyethyleneimine particles, indicating that the epidermal sheets were suitable for study of permeability.

In tracer experiments, a large number of CF (pI 8.0–9.4) passed the lamina densa and formed clusters on both aspects of the lamina densa and in the intercellular space. The number of native anionic ferritins (NF) (pI 4.1–4.6) passing it was apparently much smaller than that of CF. When the epidermal sheets were pre-treated with protamine sulfate to neutralize the negative charges in the tissue, the number of NF penetrating the lamina densa was significantly larger than the number of those in the untreated sheet.

These results indicate that the BM of the DEJ plays a role in a charge-selective filtration, although it is not as selective a barrier as the glomerular basement membrane. *J Invest Dermatol 91:560–565, 1988*

Studies of the glomerular basement membrane (GBM) have shown that there are not only size-selective but also charge-selective properties in its permeability [1,2], and that anionic sites composed of heparan sulfate are thought to play an important role in the latter property [3]. In the skin, the basement membrane (BM) in the dermo-epidermal junction (DEJ) is thought to be a semipermeable filter like the GBM for various components in tissue fluid [4]; it may control the exchanges of macromolecules through the DEJ. Anionic sites sensitive to a specific enzyme, which digests heparan sulfate proteoglycans, were also demonstrated to be localized along the BM of the DEJ (DEJ-BM) [5], suggesting that the DEJ-BM as well as the GBM has a charge-selective permeability. Although the size-selective permeability function of the DEJ has been studied by some investigators using tracers [6–8], the charge-selective one has not been studied. The GBM is in contact directly with blood circulation, whereas the DEJ is disconnected from it by a thick connective tissue. Therefore, if one uses the whole skin tissue, it seems very difficult to establish and control the experimental conditions in the study of the permeability; hydrostatic pressures or concentrations of tracers around the DEJ-BM are not strictly controlled as in the GBM experiment [1,3].

Because an epidermal sheet having exposed lamina densa [9] solves these problems, it can be utilized for the study of the permeability of the DEJ [10]. Therefore, the charge-selective permeability of the DEJ was examined on the epidermal sheets using countable tracers with similar molecular sizes such as cationic ferritins (CF) and native anionic ferritins (NF) in the present study.

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Abbreviations:
BM: basement membrane
CF: cationic ferritins
DEJ: dermo-epidermal junction
DEJ-BM: basement membrane of dermo-epidermal junction
DTT: dithiothreitol
GBM: glomerular basement membrane
NF: native anionic ferritins
PEI: polyethyleneimine
pI: isoelectric point
PS: protamine sulfate

MATERIALS AND METHODS

Epidermal Sheet Pregnant Wistar rats were purchased from Doken (Shimodate, Japan). According to the method by Epstein et al [9], epidermal sheets were obtained; the skin specimens obtained from newborn rats were immersed in a RPMI 1640 medium containing 10 mM dithiothreitol (DTT) for 30 min at 37°C in an atmosphere of 95% air and 5% carbon dioxides. Intact epidermal sheets were separated from dermis with forceps and then washed three times for 30 min each in a RMPI 1640 medium under the same conditions. Two epidermal sheets obtained from different newborn rats were used in each experiment described below.

Anionic Sites of the Epidermal Sheet In order to examine the anionic sites in the epidermal sheet, the sheets were stained with polyethyleneimine (PEI) (MW 1,800, Polysciences Inc., Warrington, PA) by the previous method [11], or incubated in a RPMI 1640 medium containing CF [isoelectric point (pI) > 9.5] at the concentration of 0.2 mg/ml for 1 h under the same conditions as described above. The CF had been produced from NF by modification of the

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charges by the method of Danon et al [12] and was kindly presented by Dr. Oite, Department of Immunology, Institute of Nephrology, Niigata University School of Medicine.

Tracer Experiments The epidermal sheets were similarly incubated in media containing NF (United State Biochemical Co., Cleveland, OH) at the concentrations of 1.0, 3.0, and 10.0 mg/ml or in a medium containing CF (Miles-Yeda LTD., Israel) at the concentration of 1.0 mg/ml for 2 h at 37°C.

To study the effects of neutralization of the negative charges in the epidermal sheet on the permeability function [13], some epidermal sheets were pre-incubated in a 0.15 M NaCl solution containing 0.2% protamine sulfate (PS) (Wako Pure Chemical Industries Inc., Osaka) or in a saline solution alone for 5 min and then washed in a saline solution three times for 10 min each. They were incubated in a medium containing NF at the concentration of 3 mg/ml or CF at the concentration of 1 mg/ml under the same conditions as described above.

The epidermal sheets were fixed in a glutaraldehyde solution without washing after these treatments, and processed for electron microscopy.

The pI of CF purchased was determined by analytical isoelectric focusing on column gels of 3.8% acrylamide and 0.2% bis-acrylamide containing 1.2% Ampholine of pH range from 7 to 10 and 0.8% Ampholine of pH range from 3.5 to 10; it was 8.0–9.4. The pI of NF is 4.1–4.6 [14].

Electron Microscopy All the epidermal sheets treated as above were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4), postfixed in 2% osmium tetroxide in the same buffer solution, dehydrated in ethanol solutions and n-butyl glycidyl ether, and embedded in epoxy resin.

Ultrathin sections were cut in a Sorvall MT-5000 ultramicrotome with a diamond knife and examined with a JEM 100S transmission electron microscope with or without electron staining.

Analysis of Permeability For the statistical analysis of the effects of neutralization of negative charges of the epidermal sheets on the permeability, the epidermal sheet obtained from each rat was cut

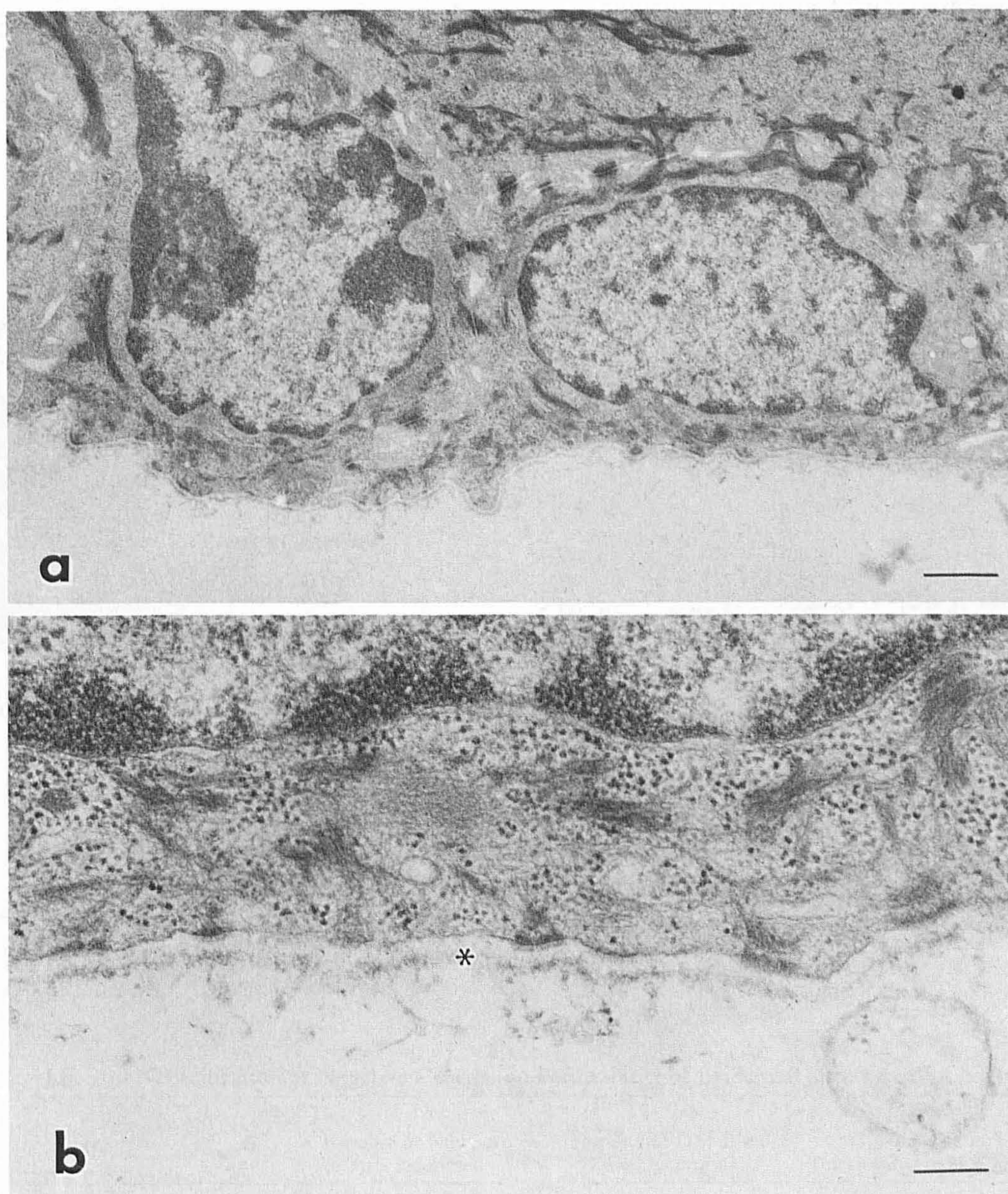

Figure 1. Ultrastructure of the epidermal sheet. *a*: The lamina densa continues along the dermal surfaces of the basal cells (*bar:* 1 μm). *b*: The dermo-epidermal junction is ultrastructurally preserved, and the lamina densa is 40–50 nm in thickness (*bar:* 0.2 μm). Uranyl acetate-lead citrate staining. *Asterisk:* lamina densa.

into two pieces. One of two pieces of each sheet was treated in a saline solution with PS and the other was without PS for the tracer studies. Ten parts of 2-μm-long DEJ-BM zone of each piece of the sheet examined were randomly selected and photographed; the numbers of NF in the laminae lucidae of twenty parts on each experiment were counted. The results were expressed as the mean value ± SD and analyzed by student's t-test.

RESULTS

Electron microscopically, the DTT separated epidermal sheets examined always demonstrated well-preserved structures; the lamina densa was 40–50 nm in thickness and had a normal electron density, and epidermal cells showed normal ultrastructures (Figs 1*a*,*b*). The regular anionic sites on the lamina densa were shown as electron dense particles of PEI (Fig 2*a*) or as clusters of CF molecules when incubated in a medium containing CF at the concentration of 0.2 mg/ml (Fig 2*b*); the intervals between the anionic sites were 50–70 nm in length. Both PEI particles and CF clusters were also observed on anchoring fibrils (Figs 2*a*,*b*).

The depositions of NF at the dermal side of lamina densa and the number of NF in the lamina lucida were increased when the concentrations of NF in the media were increased from 1 to 10 mg/ml (Figs 3*a*–*c*). However, the number of ferritin molecules in the lamina lucida incubated in the medium containing 1 mg/ml of CF (Fig 4*a*) was apparently much larger than that in 10 mg/ml of NF (Fig 3*c*). Thick layers of CF were located on both the lamina lucida and the dermal surface of the lamina densa (Fig 4*a*). CF were also seen as clusters in the intercellular space, especially on the surfaces of the keratinocytes in the lower epidermis (Fig 4*b*).

No morphologic abnormality of the BM and epidermal structures in the epidermal sheets was found by electron microscope after pre-incubation in a saline solution with (Fig 5*a*) or without PS. In the neutralization experiments of negative charges of tissue by PS, the concentration of 3.0 mg/ml of NF in the medium was employed, because some preliminary experiments with various concentrations of NF indicated that this concentration of NF allowed us to obtain the most quantitative electron microscopic findings (Fig 3*b*); the number of deposited NF molecules in the lamina lucida was neither too large nor too small to count at this concentration. The effect of neutralization of negative charges on permeability of the epidermal sheets against NF was summarized at Table I; the number of NF observed in the laminae lucidae of the control sheets (Fig 3*b*) was 36.2 ± 9.0 per 2 μm of the lamina lucida, whereas that of the sheets pre-treated with PS (Fig 5*a*) was 57.8 ± 11.2. The difference of the two values was statistically significant ($p < 0.001$). This result demonstrated that the number of NF penetrating the lamina densa was increased by neutralization of negative charges; the DEJ-BMs were more permeable to NF in the treated sheets than in the non-treated ones. The number of CF was slightly decreased on the dermal surface of the lamina densa after neutralization of the epidermal sheets, while it was increased in some parts of the lamina lucida (compare Fig 5*b* with Fig 4*a*).

Neither NF nor CF molecules were observed in horny and granular cell layers of the epidermal sheets, indicating that the molecules penetrated the lamina densa and then reached the lamina lucida and the surfaces of the lower epidermal cells; they did not derive from the medium penetrating the horny layer. Some CF (Fig 5*b*) or NF molecules were observed in some pinocytotic vesicles of the epidermal cells.

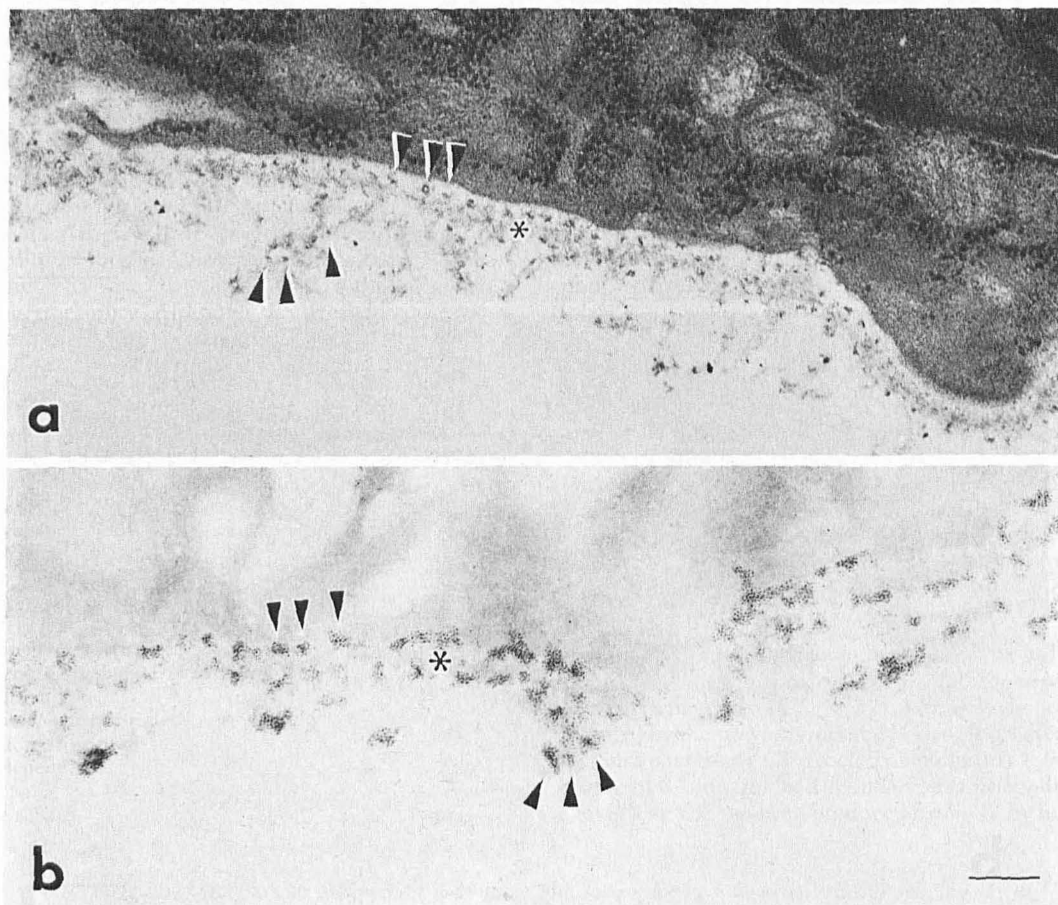

Figure 2. PEI and CF stainings. *a:* Anionic sites are demonstrated as electron-dense PEI particles *(arrowheads)* on the both aspects of the lamina densa and on the anchoring fibrils. Uranyl acetate-lead citrate staining. *b:* They are also shown as clusters of CF (pI > 9.5) *(arrowheads)*. No staining. *Asterisk:* lamina densa (*bar:* 0.2 μm).

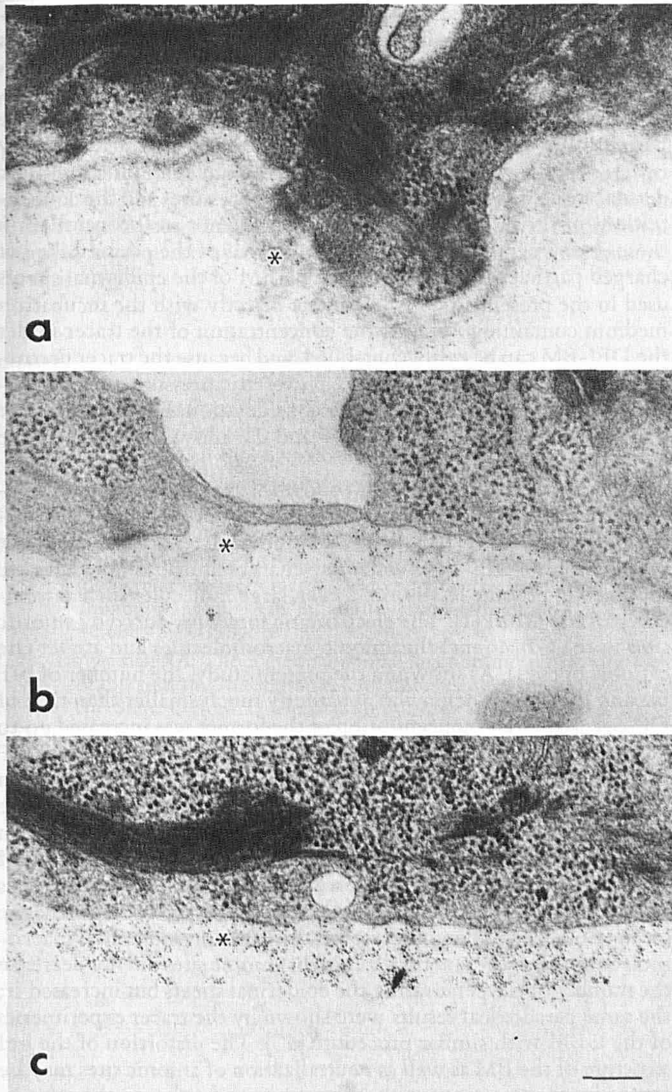

Figure 3. The epidermal sheets incubated in the media containing various concentrations of NF (pI = 4.1–4.6). *a:* 1 mg/ml; *b:* 3 mg/ml; *c:* 10 mg/ml. The number of NF molecules observed in the lamina lucida is increased in proportion to NF concentrations of the media. Lead citrate staining. *Asterisk:* lamina densa (*bar:* 0.2 μm).

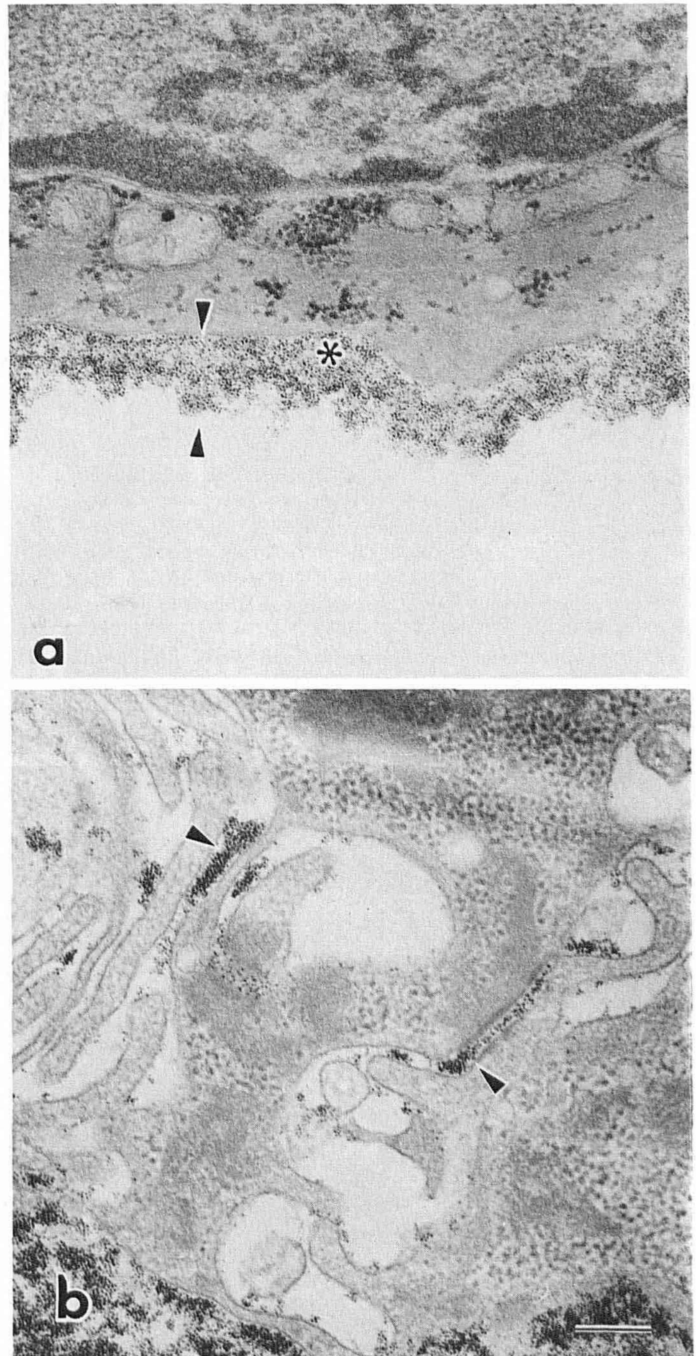

Figure 4. The epidermal sheets incubated in the medium containing CF (1 mg/ml). *a:* DEJ. A large number of CF molecules form thick layers *(arrowheads)* on both the epidermal and dermal aspects of the lamina densa *(asterisk)*. *b:* Basal cells. Clusters *(arrowheads)* are seen in the intercellular space. Lead citrate staining (*bar:* 0.2 μm).

DISCUSSION

The laminae densae of the epidermal sheets obtained in the present study were morphologically preserved and possessed anionic sites as shown by PEI staining or by incubation in a medium containing a low concentration (0.2 mg/ml) of CF; the anionic sites were never removed by treatment with DTT. Therefore, the epidermal sheet seems to be a suitable model for the study of the charge-selective permeability of the DEJ-BM dependent on anionic sites, as previously described [10]. However, the results obtained with this

Table I. Effect of Neutralization of Negative Charges on Permeability of Epidermal Sheets Against Native Ferritin

	Number of Newborn Rats Examined	Number of DEJ-BM Zones Examined	Number of Ferritin Molecules Located in Lamina Lucida (mean value ± SD/2μm-long lamina lucida)
Epidermal sheets treated with protamine sulfate	2	20	57.8 ± 11.2[a]
Control epidermal sheets	2	20	36.2 ± 9.0[a]

[a] Statistically different ($p < 0.001$)

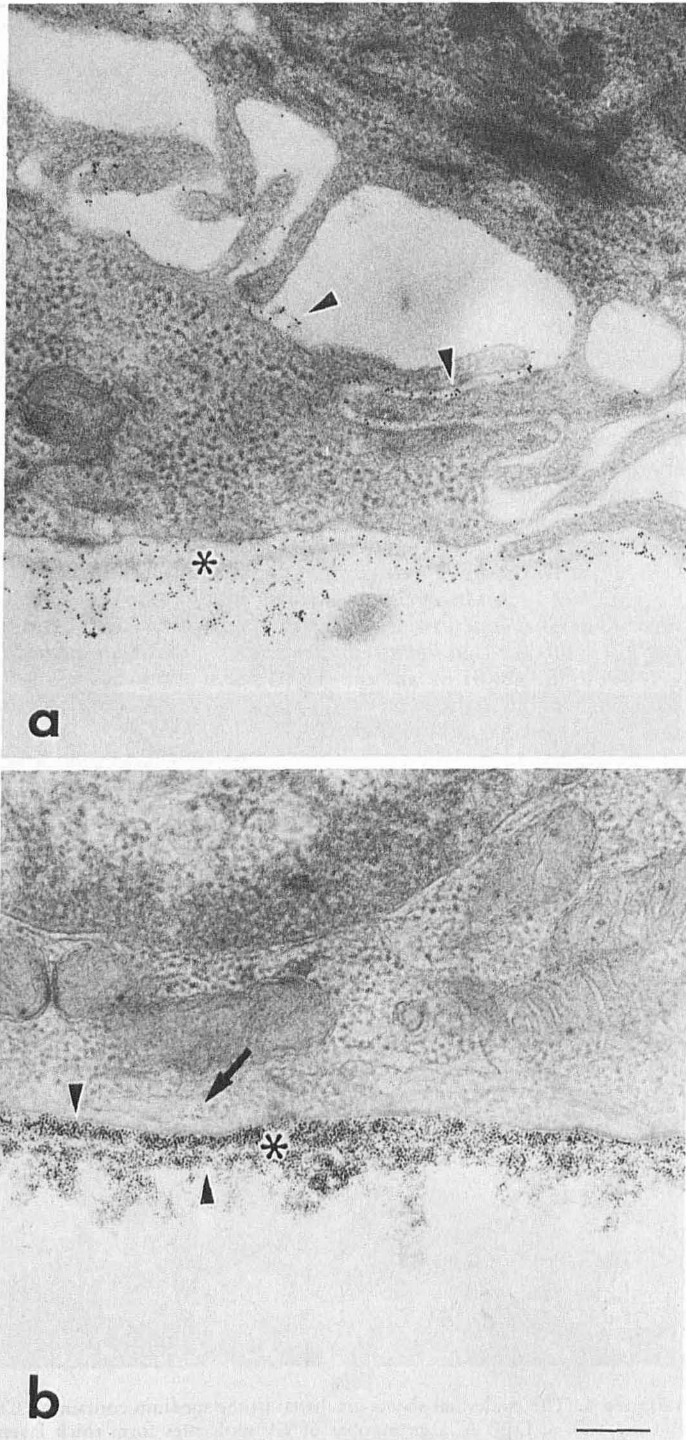

Figure 5. Neutralization of anionic sites by treatment with protamine sulfate. *a*: NF staining of the epidermal sheet. The number of NF particles observed in the lamina lucida is shown to be significantly larger than that of the control (see Fig 3*b*) by a statistical analysis. Some molecules *(arrowheads)* present on the surface of the basal cells. *b*: CF staining of epidermal sheet. The number of CF particles on the dermal side of the lamina densa *(asterisk)* seems to be decreased, while that of the particles present in the lamina lucida appears to be increased, when compared with the control (Fig. 4*a*). Some CF particles are also observed in the pinocytotic vesicles *(arrow)*. Lead citrate staining (*bar:* 0.2 μm).

model depend on the assumption that the permeability characteristics of the DEJ are unaltered by DTT treatment. Although this is supported by ultrastructural studies of the DEJ, it is not yet confirmed with functional studies.

Some tracer experiments have been performed to investigate the permeability of the DEJ [6–8]. Since tracers were intracutaneously injected [6,7] or a diaphragm composed of both epidermis and thick dermal tissue was used [8], the hydrostatic pressures and the concentrations of the tracers around the DEJ were not well controlled in these experiments. The quantitative analysis of the permeability of charged particles is not complete. The BM of the epidermal sheets used in the present study is in contact directly with the incubation medium containing a tracer; the concentration of the tracer under the DEJ-BM can be easily controlled, and because the tracer permeates the sheets only by diffusion, hydrostatic pressure on the DEJ-BM does not need to be taken into consideration. Furthermore, the use of countable tracers such as NF and CF allows us to investigate the permeability quantitatively.

Although studies of the GBM show that there are not only size-selective properties but also charge-selective properties in the permeability of the BM [1,2], little attention has been paid to the latter property of the DEJ-BM. When the charges of macromolecules are modified to become less anionic, even large molecules such as ferritins pass the GBM [1]. The electrostatic forces produced by anionic sites in the GBM repel the anionic macromolecules and attract the cationic ones [1]. As shown in the present study, the number of NF passing the lamina densa was apparently much smaller than that of CF, even when the concentration of the former was increased up to 10 times higher than that of the latter. Because the molecules of CF and NF are very similar in shape and size [1], the difference in permeability between these macromolecules is considered to be caused by the difference in their charges. In addition, neutralization by treatment with PS increased the permeability of the DEJ-BM against NF. These findings indicate that the epidermal BM also has a charge-selective permeability, which is caused by a charge-charge interaction between anionic sites in the DEJ and interstitial macromolecules. However, neutralization of anionic sites did not decrease the number of CF penetrating the epidermal sheets but increased it; the same paradoxical results were shown by the tracer experiments of the GBM with similar procedure [13]. The distortion of the gel structure of the BM as well as neutralization of anionic sites may be caused by the treatment with polycations and enhance the permeation of CF through the BM [13].

NF particles pass the DEJ-BM of the epidermal sheets and their number in the lamina lucida increases in proportion to NF concentration in the media as shown in the present study, whereas they do not penetrate the lamina densa of the GBM even when the concentration of NF perfused into the kidney was increased up to 50 mg/ml [1]. NF injected into the normal human skin also pass the DEJ [15]. These findings indicate that although the DEJ-BM has a charge-selective barrier function, it is not so selective compared with that of the GBM. By examination of the salt concentrations required to remove ruthenium red from the anionic sites, Charonis and Wissig [16] have demonstrated that there is a difference in strength of negative charges of the BMs between fenestrated and non-fenestrated capillaries, and suggested that higher negative charges on the BM prevent more anionic macromolecules from crossing the BM and expedite the exchange of cationic ones more effectively. The DEJ-BM was shown to be less negatively charged than both the capillary BM and the GBM [17]; this may explain the incomplete prevention of NF from passing the DEJ-BM by anionic sites.

The lamina densa is also considered to be a limiting barrier against macromolecules in filtration [2]. The lamina densa of the DEJ-BM is about 40 nm in thickness, while that of the GBM is about 80 nm [18]. It remains to be investigated whether the structural differences between the GBM and the DEJ-BM may also cause the difference in permeability between the two types of BMs against NF.

The negatively charged sites, rich in heparan sulfate [3], are essential to the integrity of the charge-selective barrier of the GBM

[2]. The similar anionic sites are localized on both epidermal and dermal surfaces of the lamina densa of the DEJ [5,11]. The charge-selective permeability function of the DEJ-BM against macromolecules in the dermis seems to be localized on the level of the lamina densa-dermal interface. Cationized immune complexes injected in vivo deposit at the DEJ, while anionic ones only deposit at the BM around capillaries [19]. Although the charge-selective permeability of the DEJ-BM is not so selective compared with that of the GBM as shown in the present study, it seems to play an important role in the regulation of macromolecular passages between the dermis and epidermis.

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PREDICTION OF PERCUTANEOUS PENETRATION METHODS MEASUREMENTS MODELLING: INTERNATIONAL CONFERENCE

The conference will be held April 4-6, 1989 at The University of Manchester Institute of Science and Technology, Manchester, United Kingdom. The purpose of this Conference is to provide the latest information on techniques for the prediction of chemical penetration through the skin. It will be of particular relevance to scientists in the pharmaceutical and agrochemical industries, toxicologists, universities and government (eg, EPA, FDA, MAFF, HSE). Carefully selected, internationally respected speakers will address topics relating to their own expertise. The ability to predict absorption will be a central theme.

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