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REGULAR ARTICLE

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Differential storage of hydroxyethyl starch (HES) in the skin: an immunoelectron-microscopical long-term study

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Abstract Hydroxyethyl starch (HES) is widely used as a plasma substitute. Serious side effects occur only rarely, whereas a high incidence of severe pruritus has been reported. Moreover, tissue storage of HES has been demonstrated in various organs. The aim of the current study has been to examine precisely the intracellular uptake and long-term storage of HES in the skin. Skin biopsies from 119 patients who received HES of various preparations and cumulative dosage were obtained 30 min to 130 months after infusion therapy. The samples were analysed by ultrastructural and immunoelectron microscopy with HES-specific monoclonal and polyclonal antibodies. A characteristic vacuolisation of perivascular histiocytes was a regular finding in all skin biopsies as early as 1 day after a single infusion of 30 g. Immunoreactivity for HES was demonstrable within the vacuoles. Generally, the size and number of vacuoles in the histiocytes increased concomitantly with the cumulative dosage. Following administration of higher HES dosages, vacuoles were demonstrable in endothelial cells of blood and lymphatic vessels, basal keratinocytes, epithelia of sweat glands and in small peripheral nerves, the last mentioned being associated with pruritus. A subsequent reduction of the vacuoles in size and number could be demonstrated within 52 months. In nerves, HES deposits persisted no longer than 17 months paralleling the cessation of pruritus. Biopsies taken after 94 months exhibited no HES deposits in the skin. The condensation and final dissolution of the vacuoles may either indicate the release and subsequent redistribution of HES into the circulation or lysosomal degradation.

Keywords Hydroxyethyl starch (HES) · Storage · Nerves · Immunoelectron microscopy · Skin · Pruritus · Human

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Introduction

Considerable interest has been shown in the therapeutic use of hydroxyethyl starch (HES), widely used as plasma substitute and for the improvement of the microcirculation (Hulse and Jacobi 1983). HES has proved to be a safe and efficient haemodilution therapy of peripheral arterial stenosis, cerebral and cardial ischaemia, hypoperfusion of the retina or the placenta and otological disorders (Kiesewetter et al. 1990). In addition, HES has been widely used during autologous blood transfusion and haemodialysis, for the prevention of postoperative thrombosis, as a sedimenting agent during leucapheresis and as a priming fluid in extracorporal units (Richter and Heldin 1983; Förster 1989). In contrast to dextran preparations, serious side effects, such as anaphylactic reactions, occur only rarely (Kraft et al. 1992), although a high incidence of severe pruritus has been reported (Parker et al. 1982; Hermann and Gall 1990; Gall et al. 1993; Jurecka et al. 1993; Metze et al. 1997). Moreover, tissue storage of HES has often been demonstrated in various organs, including the skin of rodents and humans (Parth et al. 1992; Jurecka et al. 1993; Sirtl et al. 1999). Storage vacuoles have been found in cutaneous histiocytes, endothelial cells and nerves (Jurecka et al. 1993; Metze et al. 1997). The aim of the current study has been to identify more precisely HES deposits in the various cell types of the skin and to assess the uptake and long-term storage of HES in the skin by means of poly- and monoclonal antibodies subjected to an improved immunoelectron-microscopical method.

Patients and methods

Patients

A total of 119 patients (84 men, 35 women; 14–85 years old; median: 46 years) were investigated after administration of HES. Seventy eight patients were treated for otological disorders, including sudden loss of hearing, deafness by shock wave and Menière's disease. Among this group of patients, 36 complained of pruritus. Eight patients were referred to our department because of itching after treatment of cerebral ischaemia (*n*=4), subarachnoidal bleeding (*n*=1), peripheral arterial disease (*n*=2) and hypovolemia following severe diarrhoea (*n*=1). In 32 cases, HES was given intraoperatively, two of these patients developing pruritus. In one further case, the use of HES during haemodialysis resulted in itching. The patients received HES preparations of various molecular weights (40 kDa, 200 kDa and 450 kDa) with a degree of substitution of 0.5, 0.62, and 0.7, respectively. For haemodilution therapies, HES was given in single daily infusions of 15–60 g over 10–14 days. The cumulative dosage ranged from 30 g to 5000 g (median: 362.9 g).

After informed consent was obtained by the patients, skin biopsies were taken from the buttocks under local anaesthesia. The samples were taken 2–6 days (*n*=3), 1–7 weeks (*n*=22), 2–12 months (*n*=23), 13–24 months (*n*=16) and 25–42 months (*n*=12) after the last HES administration. In 14 patients, specimens were obtained on days 8–12 during infusion therapy. Following a single infusion of 30 g (*n*=28) or 60 g (*n*=1) given intraoperatively, the patients were biopsied once (*n*=22), twice (*n*=5) or three (*n*=2) times after 30 min (*n*=4), 60 min (*n*=3), 90 min (*n*=2), 1 day (*n*=5), 2 days (*n*=1), 3 days (*n*=5), 4 days (*n*=8), 5 days (*n*=5), and 6, 7, 8, 17, or 34 days (*n*=1, each), respectively. Furthermore, two to three consecutive biopsies (*n*=23) were obtained in 16 other pruritic patients at time points of 1–12 months (*n*=4), 13–24 months (*n*=6), 25–36 months (*n*=1), 37–48 months (*n*=1), 49–60 months (*n*=1), 85–96 months (*n*=2), 97–108 months (*n*=5), 108–120 months (*n*=2) or 121–130 months (*n*=1) after infusion therapy (Table 1).

Among the 119 patients studied, 47 suffered from pruritus of non-inflammatory skin. Overal, symptoms appeared in the first 3 weeks after the last HES administration. Ten patients first complained of itching at the end of the infusion therapy. The minimal cumulative dosage associated with pruritus was 210 g; the median cumulative dosage was 475 g. The patients with pruritus were regularly screened. The symptoms subsided spontaneously within 6–25 months (median: 15.2 months).

Tissue processing and staining procedures

All biopsies were fixed in Karnovsky's fixative, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Semithin sections were cut with glass knives on an ultramicrotome (Reichert, OMU3) and stained with toluidine blue. For electron microscopy, ultrathin sections were cut with diamond knives, mounted on copper grids and stained with uranyl acetate and lead citrate.

Immunoelectron microscopy

A polyclonal antibody and a panel of monoclonal antibodies specific for HES were subjected to a post-embedding immunogold technique. The polyclonal rabbit anti-HES serum was provided by D. Kraft (Vienna, Austria). It was raised by immunising rabbits with HES coupled to bovine serum albumin by the cyanogen bromide method (Richter and deBelder 1976). The specificity of the polyclonal serum was determined by several immunochemical methods, including reversed single radial immunodiffusion, passive haemagglutination and passive cutaneous anaphylaxis (Richter and deBelder 1976). Monoclonal anti-HES antibodies were raised from the ascites of intraperitoneally immunised Balb/c mice with HES coupled to bovine serum albumin by the cyanogen bromide method (Richter and deBelder 1976). In order to determine the specificity for HES, the antibodies were tested in inhibition studies. For this purpose, monoclonal culture supernatants were preincubated with various types of starch, amylose and bovine serum albumin and the inhibition of binding to HES was evaluated in enzyme-linked immunosorbent assays (B. Bohle et al., in preparation). Only antibodies not inhibited by starch derivatives and/or albumin were used in this study. Ultrathin sections were mounted on uncoated nickel grids, etched with 0.5 M hydrochloric acid for 10 min and then thoroughly washed with twice-distilled water. Preincubation with millipore-filtered 4% ovalbumin for 10 min was followed by overnight incubation with the primary antibody, viz. polyclonal and monoclonal antiserum diluted 1:5000 in phosphate-buffered saline (PBS). The grids were then thoroughly rinsed in PBS. The primary antibody was visualised with a protein-A/gold technique (Romano and Romano 1977) by using 15-nm gold particles (Biotrend, Cologne, Germany) diluted 1:50 in PBS. After the final PBS rinses followed by washing with twice-distilled water and short air-drying, the sections were counterstained with uranyl acetate and lead citrate. Light-microscopic investigations of the semithin sections were performed on a Zeiss-Axioplan microscope. All of the ultrathin preparations were examined in a Philips CM10 electron microscope.

Table 1 Consecutive biopsies: cumulative dosages, timepoint of biopsy after infusion therapy, and intensity of vacuolisation as found in dermal histiocytes (degree of vacuolisation: +++ strong, ++ mean, + weak, – none), *n.d.* not done

Patient	Cumulative dosage	1st biopsy	Vacuolisation	2nd biopsy	Vacuolisation	3rd biopsy	Vacuolisation
1	270 g	17 months	++	47 months	++	130 months	–
2	360 g	3 months	+++	17 months	++	110 months	–
3	1400 g	2 days	+++	17 months	++	109 months	–
4	330 g	8 months	+	108 months	–	n.d.	
5	630 g	7 months	++	23 months	+	107 months	–
6	420 g	13 months	++	16 months	+	106 months	–
7	600 g	3 weeks	+++	104 months	–	n.d.	
8	450 g	2 months	+++	104 months	–	n.d.	
9	600 g	12 months	+	94 months	–	n.d.	
10	150 g	2 weeks	+	94 months	–	n.d.	
11	1840 g	12 months	++	52 months	+	n.d.	
12	450 g	2 months	+++	5 months	+++	28 months	++
13	450 g	3 months	+++	10 months	+	17 months	+
14	360 g	4 months	++	16 months	+	n.d.	
15	5000 g	3 months	+++	8 months	++	n.d.	
16	1080 g	2 days	+++	8 months	+	n.d.	

Controls

The morphological and immunohistochemical results were compared with normal skin obtained from six healthy volunteers and 22 patients treated with dextran for otological disorders. Informed consent for the biopsies was obtained from all patients. Controls for immunohistochemistry included a dilution series, the omission of primary antisera, the preabsorption of primary antibodies with HES and the replacement of antisera by diluted non-immune sera and unrelated isotype matched monoclonal antibodies (Dianova, Hamburg, Germany). The specificity of the immunogold labeling was assessed by the application of protein-A/gold alone and by incubation with non-labeled protein-A before staining with protein-A/gold.

Results

Patients

After intravenous administration of HES, a characteristic vacuolisation of perivascular histiocytes was a regular finding. In semithin sections, clear vacuoles with a distinct marginal bluish content were invariably visible in the cytoplasm of histiocytes (Fig. 1). At the ultrastructural level, the vacuoles were lined by an unit cell membrane, appeared to be electron-lucent and contained some electron-dense amorphous material at the periphery (Fig. 2a). Strong immunoreactivity for HES was demonstrable along the inner side of the vacuoles and in the amorphous material with both the polyclonal and monoclonal antibody as visualized by the post-embedding immunogold technique (Figs. 2a, 3a, 4b). The staining pattern of the polyclonal antibody was nearly congruent with that of the monoclonal antibodies, yet, the signal to noise ratio of the immunoreaction seemed to be higher using the former (Figs. 3a, 4b).

Initial intracellular deposits of HES were detectable in skin biopsies taken one day after a single intraoperative infusion of 30 g HES. At the ultrastructural level only, storage vacuoles could be found in histiocytes surrounding the superficial vessel plexus. The vacuoles were small and expressed peripheral amorphous material reactive for the anti-HES antibodies (Fig. 2a). Biopsies taken 30 or 60 min after a single HES-infusion exhibited no signs of HES deposits. Interestingly, in biopsies taken 90 min after HES administration, single small vacuoles that contained granular material throughout the vacuole and that were poorly reactive for the anti-HES antibodies were found (Fig. 2b). Larger vacuoles with typical marginal electron-dense material were demonstrable in biopsies taken on the 8th day after a 30-g infusion and were detectable in semithin sections by light microscopy. In these biopsies, almost all of the cutaneous histiocytes showed vacuolisation. After the 8th day of a 30-g infusion, a progressive increase in size of the vacuoles was evident.

In addition, the size and number of the HES-reactive vacuoles in histiocytes increased concomitantly with the cumulative dosage of HES (Fig. 5a) and timepoint of biopsy (Fig. 5b). The various HES preparations of different molecular weight and degree of substitution revealed no obvious difference in size and number of vacuoles, apart from the fact that HES infusion of 40 kDa was associated with mostly smaller vacuoles.

Following the administration of higher HES dosages, similar HES-reactive vacuoles with marginal electron-dense material were demonstrable in endothelial cells of blood and lymphatic vessels (51 patients), in basal keratinocytes (26 patients), epithelia of sweat glands (15 patients) and small peripheral nerves (47 patients;

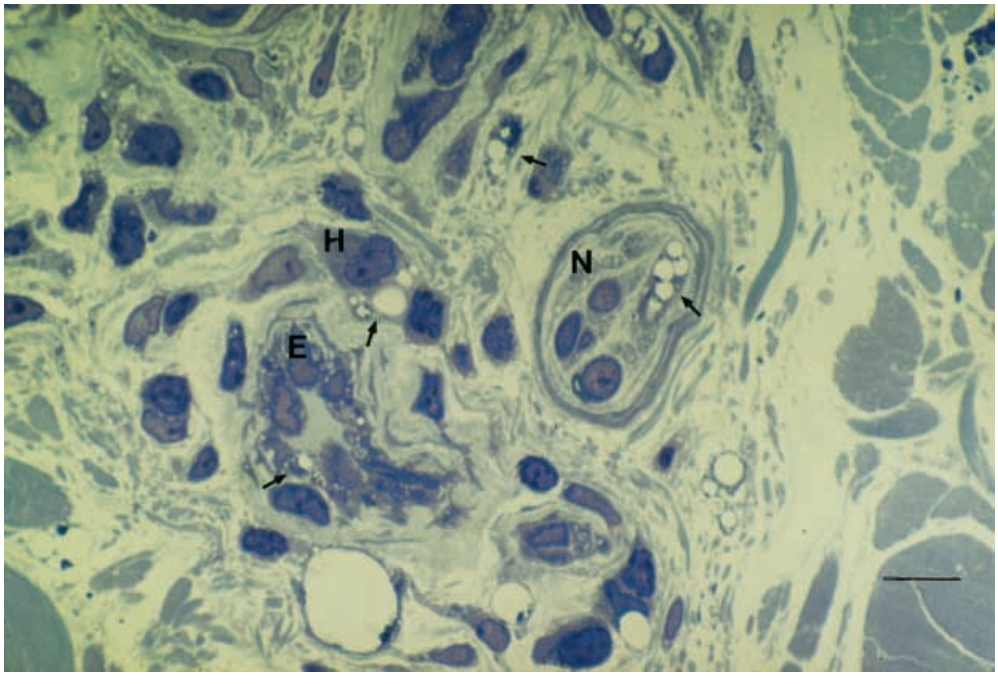

Fig. 1 Perivascular histiocytes (*H*), endothelial cells (*E*), and nerve fibres (*N*) contain clear vacuoles with distinct bluish content (*arrows*). Skin biopsy taken immediately after eight daily infusions of 240 g HES. Semithin section stained with toluidine blue. *Bar* 16 µm

Table 2 HES deposits and storage time in various cellular compartments of the skin as correlated to infused amounts of HES and number of studied patients (*ND* no HES deposits detectable)

Cumulative HES dosage	30g-180 g	210g-300 g	330g-630 g	>630 g	Storage time
Patients (*n*=119)	36[a]	40[a]	34[a]	9[a]	
Histiocytes	36	40	34	9	52 months
Endothelial cells	ND	20	25	6	16 months
Nerves	ND	17	24	6	17 months
Keratinocytes	ND	ND	20	6	18 months
Sweat gland epithelia	ND	ND	13	2	18 months

[a] Only first biopsy is considered

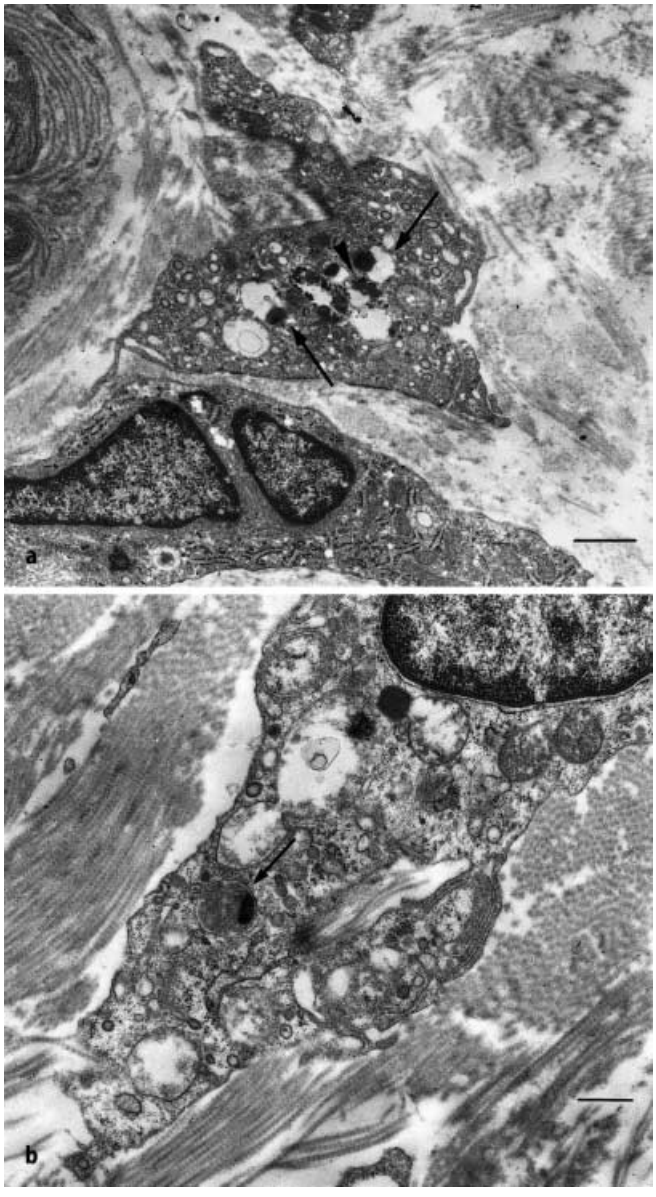

Fig. 2 a Initial intracellular deposits of HES in histiocytes surrounding the superficial vessel plexus in skin biopsies taken one day after a single intraoperative infusion of 30 g HES (*arrows*). The vacuoles expressed the characteristic peripheral amorphous material reactive for the anti-HES antibody (*arrowhead*). *Bar* 1 µm. **b** Biopsy taken 90 min after a single infusion of 30 g HES. Intracytoplasmic vacuole with intravacuolar granular material (*arrow*) reactive for anti-HES antibody, possibly indicating the beginning of HES storage. Polyclonal HES-specific antibody; section subjected to a post-embedding immunogold technique and counterstained with uranyl acetate and lead citrate. *Bar* 560 nm

Fig. 3a, b). A minimal dosage of 210 g HES resulted in the vacuolisation of endothelial cells. Following infusion of 330 g HES, vacuoles could be demonstrated in keratinocytes and sweat gland epithelia (Table 2). The epithelia of sebaceous glands and hair follicles showed no storage phenomenon. Again, the number of the HES-reactive vacuoles correlated with the cumulative dosage of HES, whereas the size of the vacuoles remained mainly small; only a few large vacuoles could be found in endothelial cells. Characteristic HES deposits were also found in perineural, endoneural and Schwann cells of cutaneous nerves, following a minimal infusion of 210 g HES. It is noteworthy that all of the patients with neural HES deposits suffered from pruritus. In general, no other pathological changes were found at either the light or ultrastructural levels. In particular, inflammatory cells were absent and mast cells showed no signs of degranulation.

A subsequent reduction of the HES-reactive vacuoles in size and number within 52 months could be demonstrated by consecutive control biopsies of single patients (Fig. 5b, Table 1). After 52 months, the vacuolization of histiocytes was hardly detectable at the light-microscopical level. However, immunoelectron microscopy still showed the persistence of tiny vacuoles containing amorphous material strongly reactive for HES within histiocytes (Fig. 4a, b), but not within endothelial, neural or epithelial cells. HES deposits in endothelial cells persisted no longer than 16 months. In nerves, HES deposits persisted up to 17 months in parallel with the cessation of pruritus. Keratinocytes and epithelial cells of sweat glands revealed immunoreactive vacuoles no longer than 18 months (Table 2).

Interestingly, biopsies taken after 94–130 months (*n*=10) exhibited neither vacuolisation nor immunoreactivity for HES in any cells of the skin, including histiocytes (Table 1, Fig. 6). These biopsies were obtained from patients who had previously suffered from HES-associated pruritus and, therefore, were regularly screened for years. Nerves revealed no remnants of vacuoles and showed no signs of degeneration or inflammation.

Controls

Skin biopsies obtained from healthy volunteers were consistently devoid of vacuolated histiocytes, endothelial cells, epithelia or nerves. Likewise, patients who had received dextran showed neither vacuoles nor amorphous

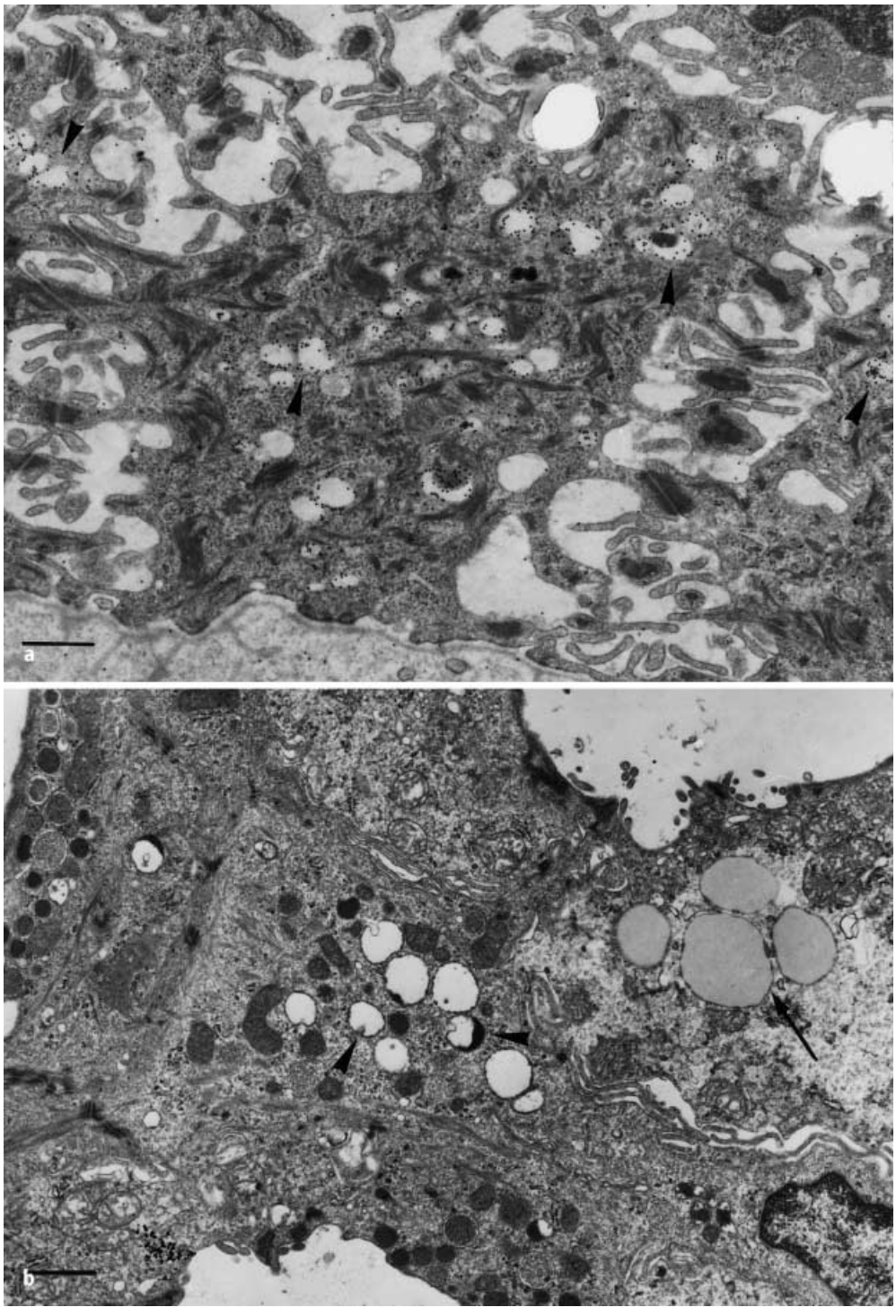

Fig. 3 **a** After long-term administration, HES deposits (*arrowheads*) can be found in the cytoplasm of a keratinocyte. Polyclonal HES-specific antibody; section subjected to a post-embedding immunogold technique and counterstained with uranyl acetate and lead citrate. *Bar* 450 nm. **b** HES vacuoles with a marginal amorphous material (*arrowheads*) were apparent in the secretory epithelia of eccrine sweat glands. Mucous granules differ markedly from HES deposits (*arrow*). Routine electron microscopy; section counterstained with uranyl acetate and lead citrate. *Bar* 1 µm

material in their histiocytes. The antibodies used were unreactive with these control specimens and, indeed, did not reveal any crossreactivity with endogenous glycogen as constitutively expressed in keratinocytes and adnexal epithelia. All other controls routinely performed to rule out unspecific binding of the primary antibodies and to establish the specificity of the detection systems were negative.

Discussion

The storage of plasma substitutes in tissues is a common phenomenon and has been also shown for HES by various methods (Thompson et al. 1970; Parth et al. 1992). Active phagocytosis of HES has been demonstrated in isolated monocytes of the blood and in cultured normal human keratinocytes (Szépfalusi et al. 1993). According-

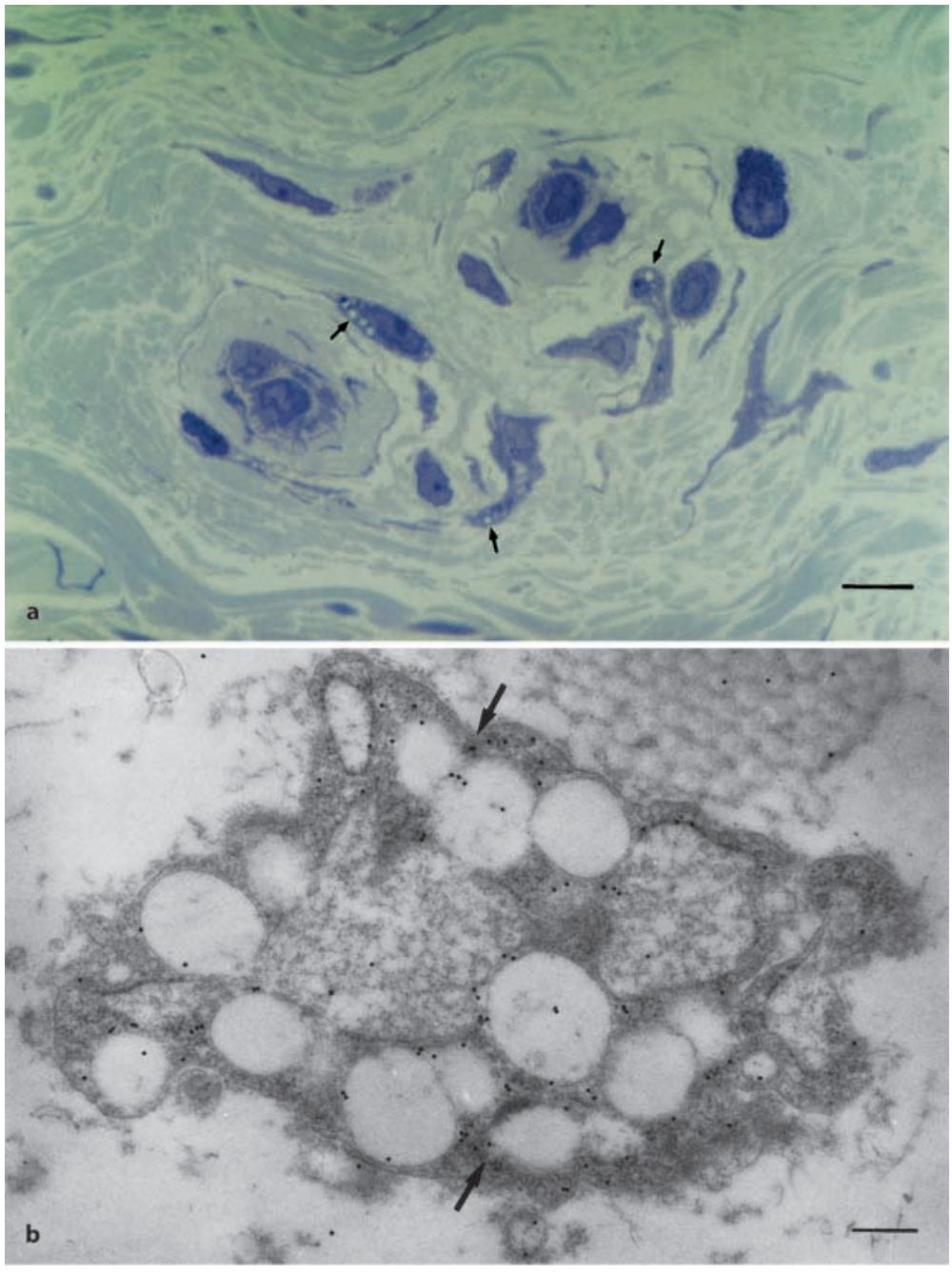

Fig. 4a,b Control biopsy taken 3 years after HES therapy verifies small vacuoles (*arrows*) in histiocytes immunoreactive for HES. **a** Semithin section stain-ed with toluidine blue. *Bar* 23 µm. **b** Monoclonal HES-specific antibody; section subjected to a post-embedding immunogold technique, and counterstained with uranyl acetate and lead citrate. *Bar* 320 nm

ly, the cutaneous deposition of HES has been shown ultrastructurally and immunohistochemically in various organs of rodents and humans (Parth et al. 1992; Jurecka et al. 1993; Metze et al. 1997). The present study of 119 patients clearly demonstrates that the skin is regularly involved in the long-term storage of this colloidal plasma substitute.

For the precise identification of cutaneous HES deposits, an improved immunoelectron-microscopical method was applied. Epon-embedded samples routinely fixed in Karnovsky's solution and osmium tetroxide were subjected to a panel of monoclonal and polyclonal HES antibodies. Since the antigenic epitopes, as recognized by the antibodies, consist of hydroxyethylated polysaccharides (Richter and deBelder 1976), they are not degraded or masked by this fixation procedure. Consequently, the preservation of lipid-rich unit membranes allows the clear identification of cell organelles and storage vacuoles in combination with strong and distinct immunoreactivity. The sensitivity of the applied immunohistochemical method seemed to be high, since HES deposits could be detected in all of the patients, in contrast to previous studies by Sirtl et al. (1999) who used a different tissue-embedding method. Compared with the monoclonal antibodies, the polyclonal antibody revealed stronger immunoreactivity possibly because of the detec-

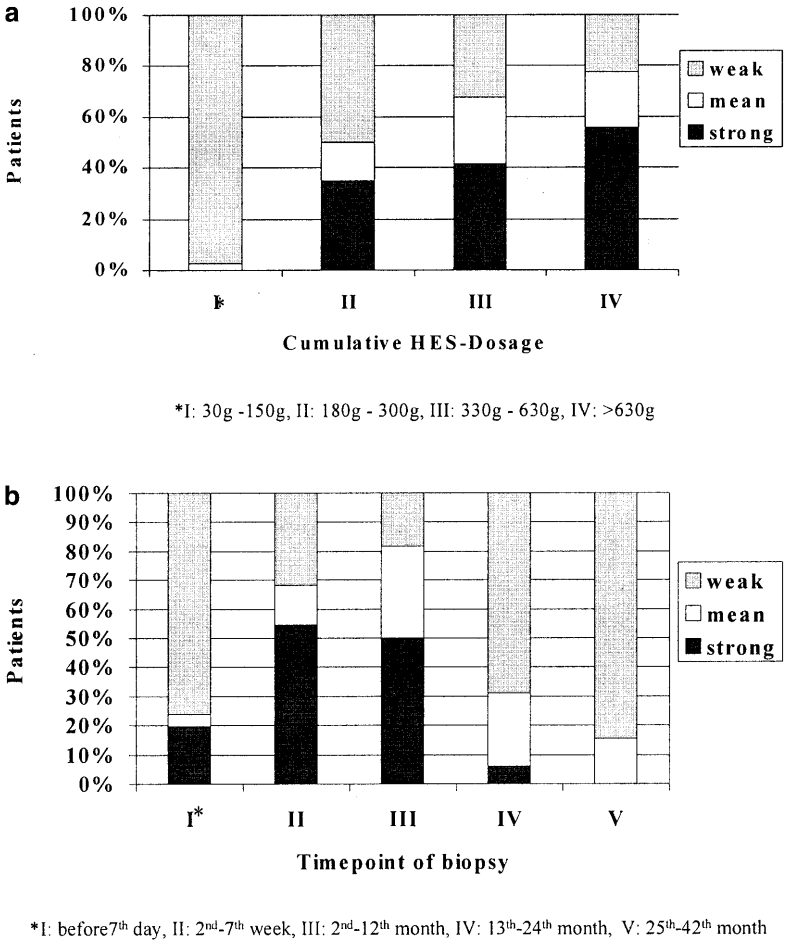

Fig. 5 **a** Vacuolisation of histiocytes. Correlation of degree of vacuolisation (*weak*, *mean*, *strong*) to cumulative HES dosage. **b** Vacuolisation of histiocytes. Correlation of degree of vacuolisation (*weak*, *mean*, *strong*) to timepoint of first biopsy

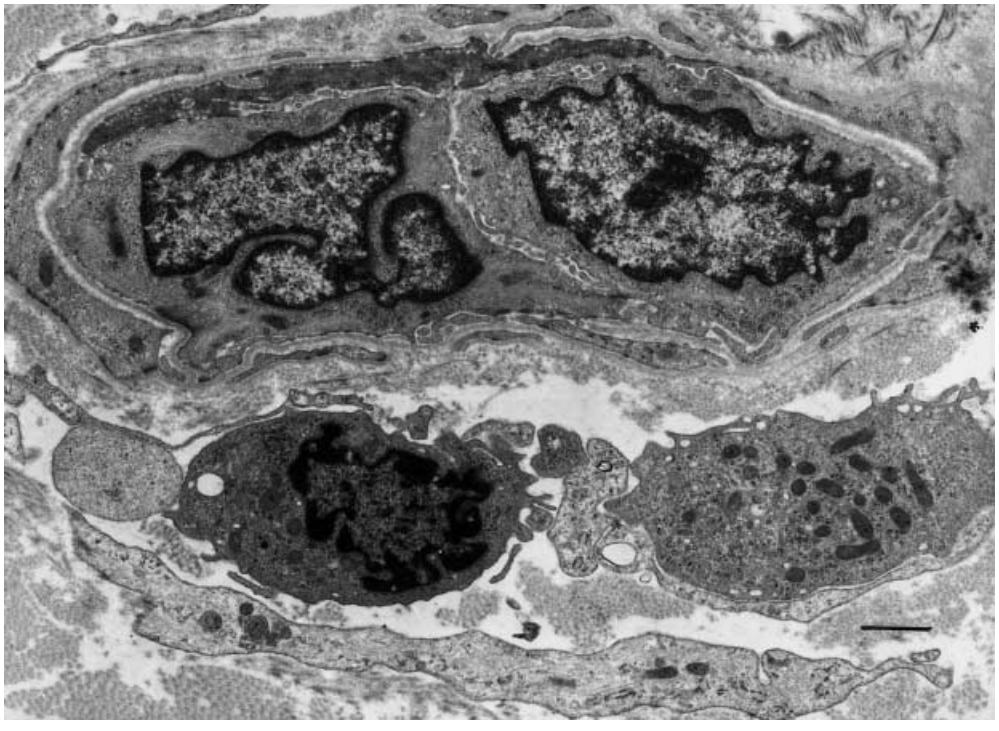

Fig. 6 Consecutive biopsy taken 94 months after an infusion therapy of 600 g HES. Histiocytes are devoid of any HES-reactive vacuoles. Routine electron microscopy; section counterstained with uranyl acetate and lead citrate. *Bar* 1.3 µm

tion of multiple epitopes of the hydroxyethylated glucose units (Richter and deBelder 1976). The absence of staining by the HES antibody in biopsies taken from patients who had not received HES clearly underlined the specificity of the immunohistochemical method, as did multiple controls.

Marginal immunoreactivity for HES along the lining wall of the lysosomes and in the amorphous material seems to be of particular interest. HES colloids represent a polydispersal solution with regard to molecular weight, structure and degree of substitution and probably contain fractions that resist enzymatic degradation (Banks et al. 1973). Following single infusion, vacuoles initially appear to be diffusely filled with HES-reactive granular material 90 min after infusion therapy. The high water-binding capacity of HES probably induces an influx of water and enlarges the vacuoles. Large parts of the vacuoles appear empty and unreactive presumably because most of the water-soluble HES is washed out during tissue processing. Hence, the marginal amorphous material that is formed as early as one day after infusion therapy and persists for years may be attributed to insoluble metabolites of unknown molecular weight and chemical composition.

Since cutaneous HES deposits are invariably present following HES infusion, regular metabolism of HES can be assumed in the skin. Ingestion of HES by the resident histiocytes was first reliably detectable one day after a single infusion of no more than 30 g HES. Interestingly, immunoreactive vacuoles containing granular material as found in biopsies taken 90 min after HES administration indicate incipient HES storage. Likewise, Jesch et al. (1979) have found single intracellular vacuoles in the liver 30 min after a single infusion of 60 g HES. Furthermore, human monocytes in culture are able to ingest HES within 120 min (Szépfalusi et al. 1993). After long-term infusion therapy, the degree of vacuolisation is strictly correlated to the cumulative dosage and time-point of biopsy, suggesting an intracellular accumulation of HES. Of particular interest, prolonged HES infusion results in a consecutive uptake in other cellular compartments of the skin, such as endothelial cells, epithelia of the epidermis or sweat glands, and perineural, endoneural and Schwann cells of cutaneous nerves. The last-mentioned deposits have been demonstrated to be associated with pruritus (Metze et al. 1997). The reason for this time- and dosage-dependent differential processing of HES remains unclear but may be attributed in part to the lower capacity of non-histiocytic cells to internalize HES compared with histiocytic cells (Szépfalusi et al. 1993). The smaller size of the vacuoles as observed after therapy with HES of low molecular weight may arise because of differences in their physicochemical properties (Banks et al. 1973).

Skin samples taken years after the last HES administration and, in particular, consecutive biopsies as obtained from single patients over many years have revealed a subsequent reduction in size and number of the HES-reactive vacuoles. Cutaneous nerves, endothelial and epithelial cells show HES deposits for no longer than 18 months, whereas, irrespective of the cumulative dosage, HES deposits persist in histiocytes for at least 52 months. Interestingly, neural devacuolisation is associated with the remission of pruritus, thus confirming the causative role of HES deposits in the pathophysiology of this side-effect. As described previously, HES-associated pruritus may be directly caused by the vacuolisation of Schwann cells independently of inflammation, mast cell degranulation and histocytic uptake of HES (Metze et al. 1997).

However, after 94 months, HES is no longer detectable in cutaneous histiocytes, as demonstrated in ten patients despite their having received higher cumulative HES dosages (mean: 520 g). The condensation and final dissolution of the vacuoles may indicate the release and subsequent redistribution of HES into the circulation, an occurrence that possibly contributes to the persisting plasma fraction (Förster 1989). These results are consistent with previous pharmacokinetic studies on rodents that have shown decreasing ^{14}C-HES concentrations within months (Hulse and Jacobi 1983; Thompson et al. 1970). In addition, a lysosomal degradation of HES within the vacuoles is feasible. Indeed, the reduced activity of lysosomal acid alpha-glycosidase in a patient heterozygous for Pompe's disease resulted in an enormous cutaneous accumulation of HES associated with swelling of the skin (Kiehl et al. 1998).

In view of the regular cutaneous metabolism and the possible neural deposition of HES, the appropriate dosage and infusion regimen of this otherwise safe plasma substitute should be re-evaluated. Alternatively, chemical modification of starch colloids could improve their metabolic degradation and thereby might reduce the high incidence of HES-induced pruritus.

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