Biochimica et Biophysica Acta, 692 (1982) 52–60
Elsevier Biomedical Press

BBA 71371

CHANGES OF MEMBRANE CONDUCTANCE AND PERMEABILITY BY CONSTITUENTS OF NORMAL AND BURNED SKIN

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(Received March 5th, 1982)

Key words: Membrane conductance; Membrane permeability; Burn toxin; Thermal injury; (Skin)

Generation and release of toxic factors from burned tissue is an important pathogenetic mechanism after severe thermal injury. In this paper the interactions between cell membranes or artificial lipid bilayer membranes and burn toxin were studied. It turned out that low concentrations of the toxic material released from dermal tissue by standardized thermal injury or mechanical homogenization create significant permeability changes. The dependence of the effect upon salt concentration, lipid composition, and voltage was investigated. High current resolution experiments did not reveal formation of defined pores. In general, the studies show that burn toxin introduces hydrophilic pathways into cell membranes as well as lipid bilayers. The effects measured in this work are likely to play a major role in the etiology of tissue edema after burns.

Introduction

Major complications of severely burned subjects are hypovolemic shock and septicemia. The hypovolemia is caused by fluid leakage from the circulation into the burn due to exudation, evaporation and sequestration. The underlaying permeability changes are only partly understood. The increased local permeability is associated with the inflammatory reaction of the burned tissue.

The mechanisms of the permeability increase are complicated but at least in a graded reaction the clinical result of a massive tissue edema is produced by the thermal trauma. The generalized permeability changes after major burns merit further study in order to identify the mediator substances produced or released in the heat-affected tissue.

Although chemical mediators have frequently been implicated in the syndrom of early and late death after severe thermal injury, generally accepted evidence for their etiological significance has so far not been adduced. Nevertheless, the concept of a non-bacterial toxemia cannot be wholly set aside, since a number of research groups have demonstrated the presence of burn toxins [1–3], pyrotoxins [4] myocardial depressants [5], antigens [6], competitins [7] and immunosuppressive agents [8] in the burned skin.

Chemical mediators generating permeability changes of cells and tissue are biologically active by virtue of interaction with plasma membranes. Although the mechanisms of this interaction are only poorly understood, it is clear that there exists a considerable diversity in the manner in which different mediators interact with the cell surface.

Planar lipid bilayer membranes have been widely used as models for biological membranes. By use of this technique it is possible to directly measure the effect of cytolytic compounds upon the electrical conductance. On the cellular level,

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cytolytic activity of the products isolated from normal or thermally injured skin can be studied by use of red blood cells. This is an appropriate test system, as hemolysis is a well known symptom in severely burned patients.

Additional information can be obtained using isolated rat liver cells as a target system of the cytotoxic skin components. As a consequence of the permeability changes a number of cellular functions exhibit pathological alterations such as release of intracellular, intramitochondrial or lysosomal enzymes [9], disorganization of the cytoskeleton [10], reduced hormonal responsiveness [11,12], disorders in the central metabolic sequences [13], and impaired host defense mechanisms [14]. In 1965 Fox et al. reported an antisodium effect of compounds extracted from burned skin. It is very likeky that in these experiments the sodium ions were shifted to the intracellular compartment owing to a disturbance of membrane structure and function. The present studies describe specific effects of purified constituents of normal and burned skin upon membrane conductance and permeability, indicating a potential hazard of release of these substances from the skin into the systemic circulation.

Materials and Methods

Chemicals. Organic solvents and reagents used were of highest commercially available purity. Water was twice distilled an an all-quartz apparatus.

Animals. NMRI mice weighing 20–25 g and of either sex (Animal Farm Tuttlingen, F.R.G.) served as skin donors. Skin was prepared from donor mice killed by cervical dislocation. After stripping and shaving, the subcutaneous fat layer was removed.

Burn injury. Thermal energy was applied to skin pieces of about 15 cm^2 in a standard high temperature burn model similar to that described by Staedtler [15] using a copper template heated to 250°C.

Preparative procedure. The preparative procedures have in part been published elsewhere [16]. The freshly obtained and the burned murine skin was cut into small pieces, suspended in 0.1 M sucrose/phosphate buffer (pH 8.4) and homogenized for 15 min in a high-speed blendor. The milky white particulate suspension was centrifuged ($3000 \times g$, 30 min, 4°C). The fatty float and the sediment were discarded and the middle layer dialyzed against water in order to remove the sucrose and the salts, and subsequently lyophilized.

The lipid moiety of the lyophilized material was removed by sequential extraction with organic solvents. According to a procedure as described by Scanu [17] and Folch [18] the material was extracted with ethanol/diethyl ether (3:2, v:v) and methanol/chloroform (2:1, v:v). The delipidated residue was tested in the lipid bilayer experiments. Further purification of the active fraction was carried out by gel filtration.

Lipid bilayer experiments. Black lipid bilayer membranes were formed in the ususal way [19] from two different lipids dissolved in *n*-decane. The cell used for bilayer formation was made from Teflon. The circular hole in the well between both aqueous compartments had an area between 0.1 mm^2 (for measurements with high current resolution) and 2 mm^2 (for macroscopic conductance measurements). Most of the experiments were carried out at 25°C, a small number at 37°C. In the experiments a variety of alkali chlorides was used. The aqueous solutions were buffered with 5 mM Tris-HCl and pH adjusted to 7.5, although pH values between 5 and 8.5 did not influence the results measured. The protein was added to the aqueous solution from the stock solutions either previous to membrane formation or after the membrane had turned completely black. In order to prevent bacterial growth and protein damage the solutions were prepared immediately before use.

Egg phosphatidyl choline was isolated and purified according to standard methods [20], oxidized cholesterol was prepared as described earlier. Ag|AgCl electrodes were inserted in the aqueous compartment on both sides of the membrane. The stationary conductance measurements were performed using a voltage source and a Keithley 610 C electrometer. For the measurements with high current resolution a Keithley 427 current amplifier was used. The amplifier signal was monitored with a Tektronix 5115/7A22 storage oscilloscope and recorded with a strip chart

recorder. The zero-current membrane potentials were performed as described previously [21] using a Keithley 610 C electrometer and Calomel electrodes with salt bridges instead of Ag|AgCl electrodes.

Isolation of hepatocytes. Hepatocytes were isolated according to the method described by Berry and Friend [22]. The rat liver was perfused with a calcium containing Hanks' buffer supplied with 0.04% bacterial collagenase (20 min, 37°C) in a recirculating system. Viability of the cells was tested using Trypan blue (100 μl 0.5% Trypan blue + 100 μl cell suspension) staining as a measure. The freshly isolated hepatocytes were shown to have a 95% viability. Stimulation of respiratory oxygen consumption was used for assessment of damaging effects of the toxin on the cellular membrane of the hepatocytes.

Results

Macroscopic conductance measurements

A strong conductance increase was observed on lipid bilayer membranes if the delipidated burn toxin or its analog from native skin were present in the aqueous phase prior to membrane formation or after the membrane had turned completely black. An even larger effect was observed if the toxins were incubated prior to use with a 0.1% (w:v) solution of sodium cholate or sodium dodecyl sulfate (SDS). In addition, the increase in conductance in the latter case was more smooth and conductance jumps were only rarely observed. An example where a sample of burn toxin treated with 0.1% (w/v) SDS was added to a black membrane is presented in Fig. 1. The membrane was formed in 1 M KCl/5mM Tris-HCl (pH 7.5) from oxidized cholesterol dissolved in *n*-decane. 10 min after blackening the toxin was added to both sides of the membrane to a final concentration of 0.5 μg/ml toxin and 10^{-5}% SDS under stirring (see arrow in Fig. 1). As can be seen from Fig. 1, a strong increase in conductance occurs about 2–3 min after addition of the protein. This delay is presumably caused by the diffusion of the toxin through the unstirred layers adjacent to both membrane surfaces.

Under the conditions of Fig. 1, a conductance increase of about four orders of magnitude was

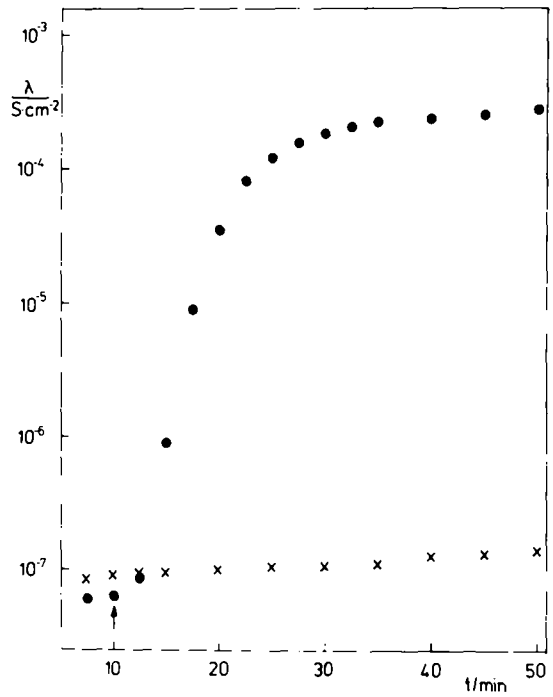

Fig. 1. Specific membrane conductance as a function of the time, t, after the addition (arrow) of 0.5 μg/ml burn toxin, 0.1 μg/ml SDS (●) or of 0.1 μg/ml SDS (control, ×) to membranes from oxidized cholesterol/*n*-decane. The aqueous phase contained 1 M KCl/5 mM Tris, (pH 7.5); $t = 25°C$, $V_m = 10$ mV. For experiments of the same type the reproducibility of $\lambda(t)$ was about 50%.

reached 30 min after addition. A stationary conductance level, however, could not be reached in this and similar experiments and the membrane conductance usually increased continuously until membrane breakage. This behavior did not change whether the toxin was added to one or both sides of the membrane. In addition the use of the protein from native tissue or the presence or the absence of detergent was not able to change the time course considerably, although the absolute level was about a factor of 50 lower in the absence of any detergent. This detergent effect is not due to an interaction of the detergent with the membrane, because no increase in conductance was observed with 100-times larger detergent concentrations in the absence of toxin (see Fig. 1). Thus it is very likely that the detergent simply increases the concentration of dissolved toxin in the aqueous phase and does not have a direct

influence on the conductance of a membrane.

Because of the continuous increase of the membrane conductance, it was somewhat difficult to compare experiments which were carried out under different conditions. A meaningful comparison, however, could be given by comparing the conductance increase at a certain time after blackening of the membrane or after addition of the protein, say after 30 min, when most of the conductance increase is over.

Fig. 2 shows a comparison of the effect of the toxin and the protein isolated in a similar way from the native skin on membranes from the two lipids used in this study. The increase in conductance was found to be about 100-times larger for membranes from oxidized cholesterol than for membranes from egg phosphatidylcholine. However, as with membranes from oxidized cholesterol, a linear dependence of the conductance increase upon the protein concentration was found. The difference in the level of conductance observed for the two types of membrane may be caused by a different rate of insertion of the toxin and its analog into the membranes due to intrinsic properties of the membranes themselves. It is interesting to note that a similar lipid specificity has also been observed for the reconstitution of porins in lipid bilayer membranes, although the properties of a single pore have been found to be identical for membranes of different composition [23,24].

The reproducibility of the measurements was excellent when the same preparation of the toxin or its analog from unburned skin was used. Differences in the response were in the range of one order of magnitude if different preparations of the toxin material were used in the experiments. The effect of salt concentration on the specific conductance was studied by measuring the conductance 20 min after membrane formation at a given toxin concentration. Fig. 3 shows that the conductance is a linear function of the salt concentration within the limits of experimental error for a variety of different salts. This result indicated that the rate of incorporation of the toxin and its analog from native skin into the membrane was not dependent

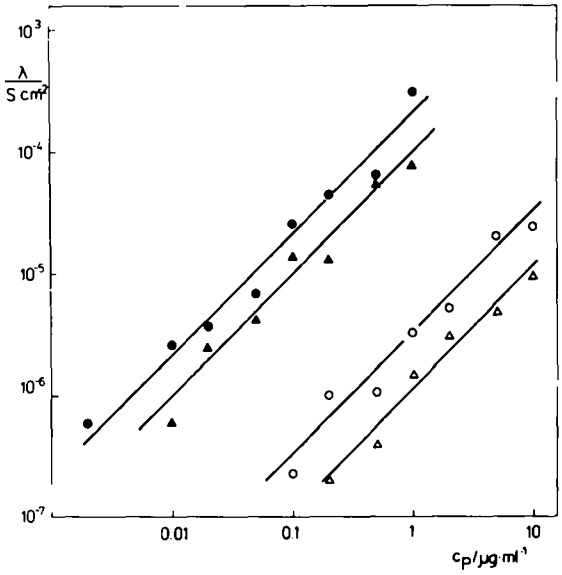

Fig. 2. Specific membrane conductance, λ, measured 30 min after addition of the toxins or after blackening of the membranes as a function of the concentration of burn toxin (●, ○), or its native analog (▲, △) in the aqueous phase. The membranes were formed either from oxidized cholesterol/*n*-decane (●, ▲) or from egg phosphatidylcholine/*n*-decane (○, △). The aqueous phase contained 1 M KCl/5 mM Tris (pH 7.5); $t = 25°C$. The applied voltage was 10 mV. Each point represents the mean value from at least three experiments.

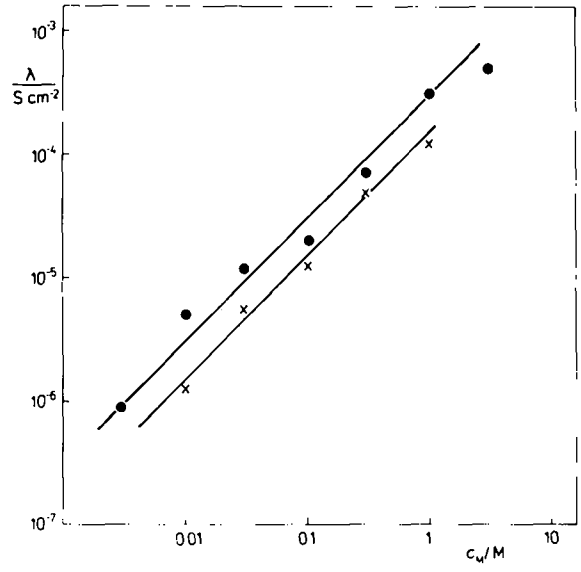

Fig. 3. Specific membrane conductance, λ, as a function of the salt concentration in the aqueous phase. The membranes were formed from oxidized cholesterol/*n*-decane. The aqueous phase contained, besides the salts, 1 μg/ml burn toxin and less than 1 μg/ml SDS; $t = 25°C$, pH 7.5. ●, KCl; ×, LiCl. Each point represents the mean value obtained from at least three membranes.

upon the aqueous salt concentration or the type of salt. This may be explained by the assumption that the interaction of the toxin and its analog is of hydrophobic nature.

Fig. 4 shows the current-voltage behavior of two membranes from oxidized cholesterol/*n*-decane in the presence of 0.2 μg/ml toxin. As can be seen, the membrane current was a linear function of the applied voltage up to at least 150 mV. This result indicates that the properties of the single conductance unit are independent on voltage and that the insertion of the toxin into the membranes is not voltage-dependent.

Experiments with high current resolution

The result of the macroscopic conductance measurements are consistent, in principle, with the expectation that the single conductance unit could be a pore. In order to test this hypothesis, experiments with high current resolution were performed with a large variety of different systems. One of these experiments is presented in Fig. 5. Burn toxin was added to a 1 M NaCl solution bathing a membrane from oxidized cholesterol/*n*-decane in a final concentration of 10 ng/ml 10 min after blackening. About 3 min after the addition of the toxin the membrane current began to increase. The current increase, however, at the microscopic level was always more or less smooth, as is clearly

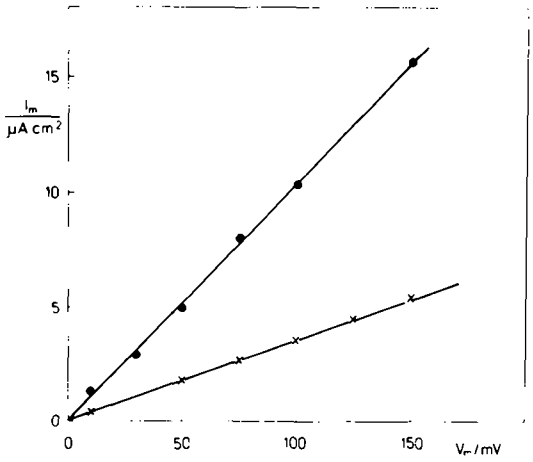

Fig. 4. Current voltage curve of a membrane from oxidized cholesterol/*n*-decane in the presence of 1 μg/ml burn toxin (●) or 1 μg/ml native analog (×) in the aqueous phase. 1 M KCl/5 mM Tris (pH 7.5); $t = 25°C$.

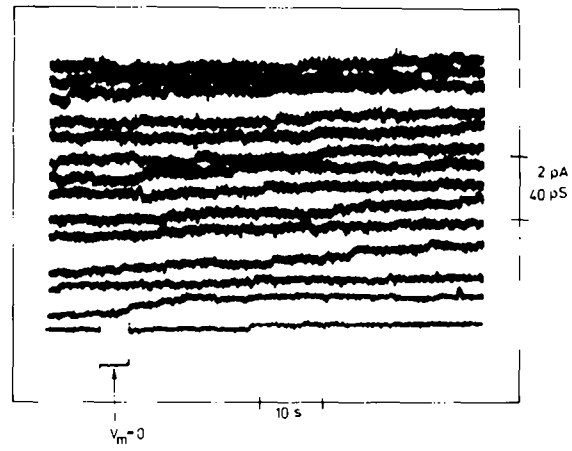

Fig. 5. Experiment with high current resolution in the presence of burn toxin (1 μg/ml) 10 min after blackening of a membrane from oxidized cholesterol/*n*-decane bathed in 1 M KCL/5 mM Tris (pH 7.5). The record began about 3 min after addition of the toxin at the lower left-hand side of the oscillographic record and continued in the upper traces. Membrane area, 0.2 mm²; $V_m = 50$ mV. Note the increase in noise level with time.

shown in Fig. 5. The increase in conductance was accompanied by a simultaneous increase in the noise level of current traces. In addition, no defined pores could be detected down to a conductance value of about 5 pS at 1 M NaCl in experiments with an even larger current resolution than in Fig. 5. These results indicated that the increase in conductance of lipid bilayer membranes caused by the burn toxin and its analog from native skin cannot simply be explained by the formation of pores in the membranes. It is likely that the toxin inserted hydrophilic patways for ions into the membranes which are not as defined as a pore. Along these pathways, however, larger ions such as $Tris^+$ or $Hepes^-$ are permeable.

Zero current potential measurements

Zero current potential measurements were performed in order to test whether cations or anions are preferentially permeable through the pathways created by the burn toxin in lipid bilayer membranes. Membranes from oxidized cholesterol or egg phosphatidylcholine were formed in an aqueous solution of 10^{-2} M salt, 200 ng/ml toxin (or its analog from unburned skin) and 40 ng/ml SDS. Under these conditions the specific conduc-

tance of the membranes exhibited values between about 10^{-6} S · cm^{-2} (egg phosphatidylcholine) and 10^{-4} S · cm^{-2} (oxidized cholesterol) 20–30 min after blackening of the membranes. Subsequently, the salt concentration on one side of the membrane was raised by addition of a concentrated salt solution to this side under stirring. The zero-current membrane potential was always found to be positive at the more dilute side of the membrane. The membrane potentials for a 10-fold salt gradient are listed for a variety of different conditions in Table I. We were able to fit the potential versus salt gradient curve with the Goldman-Hodgkin-Katz equation as published elsewhere [21]. The ration of the cation permeability P_c divided by the anion permeability P_a as calculated on the basis of this equation is also given in Table I. The values for P_c/P_a range between 2 and 4, reflecting more or less the mobility sequence of the single cations in the aqueous phase.

Experiments with isolated cells

The alternations in membrane conductance identified by the bilayer experiments were confirmed in studies with isolated hepatocytes of the rat and human red blood cells. In the isolated hepatocytes mitochondrial respiration was measured using a modified Clarke electrode according to Delieu and Walker [26]. Succinate is known to stimulate respiratory oxygen consumption by isolated mitochondria. In cells with an intact membrane structure mitochondrial respiration does not change after addition of 1 mM succinate to the medium due to an effective exclusion of this anion. As can be seen from Table II, liver cells preincubated with the toxin or its analog from unburned skin increase their respiratory oxygen consumption by a factor of 1.4. Even after a preincubation time of 100 min. a highly significant stimulatory effect of the succinate is detectable, indicating severe damage of the cellular membrane associated with loss of compartmentalization of ions.

Incubation of human red blood cells with burn toxin or its analog from unburned skin results in a release of K^+ and hemoglobin from the intracellular space into the supernatant. Treshold concentration of the toxic protein was about 0.01 mg/ml for the release of K^+ and 0.2 mg/ml for release of hemoglobin from red blood cells (Fig. 6). A significant decline of the hematocrit and visible hemolysis was observed at a burn toxin concentration of 0.25 mg/ml.

Morphological examination of the isolated liver cells after incubation with the toxic material isolated from burned skin revealed characteristic alterations of the structure of the cellular membrane. As can be seen in transmission electron micrographs (Fig. 7), the cellular membrane protrudes and the organelles of the hepatocyte concentrate in

TABLE I

ZERO-CURRENT MEMBRANE POTENTIALS, V_m, IN THE PRESENCE OF A 10-FOLD SALT CONCENTRATION GRADIENT

V_m is the electrical potential of the dilute side minus the potential of the concentrated side (0.1 M). The membranes were formed either from oxidized cholesterol (ox. chol.) or egg phosphatidylcholine (egg pc) in *n*-decane. The aqueous solutions were buffered with 5 M Tris-HCl (pH 7.5), $t = 25°C$. P_c/P_a (the ratio of the permeabilities of the cationic species to the permeabilities of the anionic species) were calculated from the Goldman-Hodgkin-Katz equation as described previously [21]. V_m was derived from at least three membranes.

Salt	Toxin	V_m (mV)	P_c/P_a	Membrane
NaCl	burned	18 ± 3	2.4 ± 0.4	ox. chol/
KCl	burned	26 ± 3	3.6 ± 0.7	*n*-decane
KCl	native	27 ± 2	3.7 ± 0.4	
NaCl	burned	12 ± 2	1.8 ± 0.2	egg pc/
KCl	burned	24 ± 3	3.3 ± 0.4	*n*-decane
KCl	native	23 ± 2	3.2 ± 0.3	

TABLE II

STIMULATION OF RESPIRATORY OXYGEN CONSUMPTION AFTER ADDITION OF SUCCINATE TO ISOLATED HEPATOCYTES OF THE RAT

(a) Cells preincubated with burn toxin; (b) cells incubated with the analog from unburned skin; (c) control. The values give the ratio: respiration after/respiration before addition of succinate

	40 min	120 min
(a) Burned	1.4 ± 0.1	1.2 ± 0.1
(b) Unburned	1.4 ± 0.1	1.2 ± 0.1
(c) Control	1.0	1.0

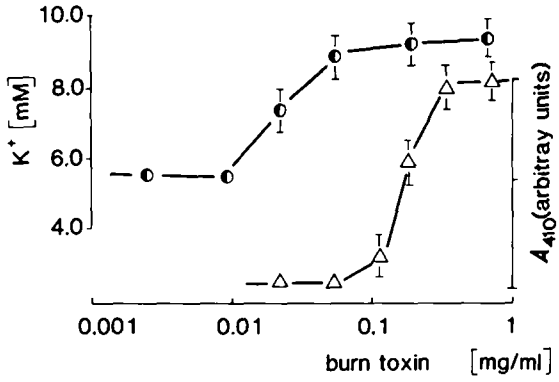

Fig. 6. Release of K^+ (○) and hemoglobin (△) from red blood cells 30 min after incubation with burn toxin. Points and triangles represent mean values from five experiments.

the center of the cell. This result is indicative of secondary alterations of the cytoskeleton caused by the change in the ionic milieu of the cell consequent to the membrane damage. Scanning electron microscopy (Fig. 8) exhibits a picture very similar to that observed after phalloidin intoxication of the cells.

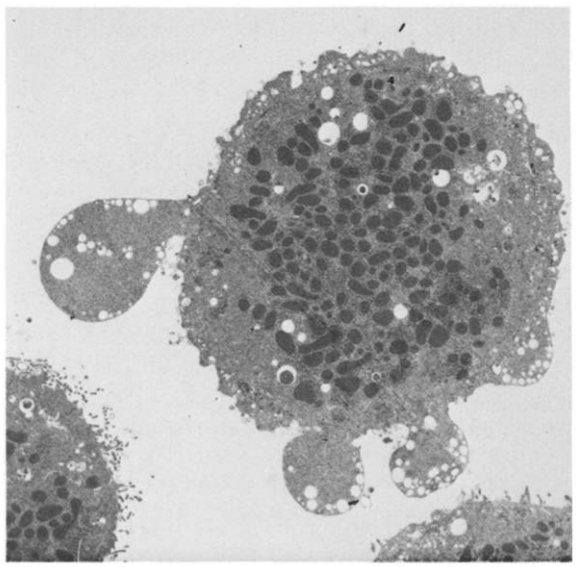

Fig. 7. Transmission electron micrograph of hepatocytes after incubation with burn toxin. Organelles such as mitochondria concentrate in the center of the cells and the membranes show protrusions.

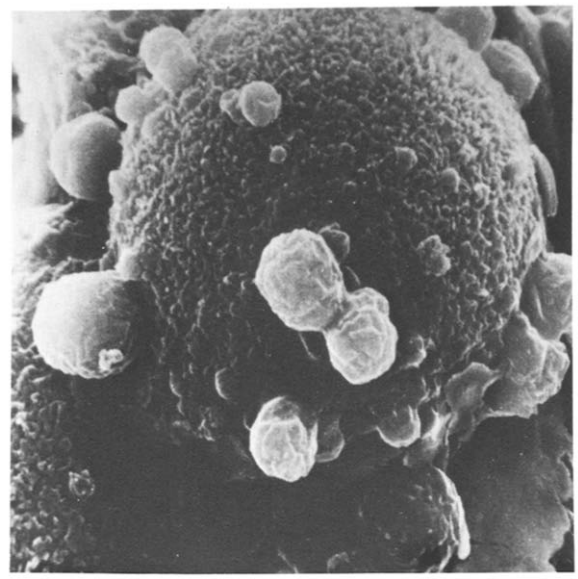

Fig. 8. Scanning electron microscopy of isolated hepatocytes after incubation with burn toxin. The cell membrane shows protrusions which cannot be demonstrated in control cells.

Discussion

Among the consequences of a severe thermal injury, alterations in the permeability of cellular membranes are a characteristic finding. In the early clinical course after burning, damage of membrane structure and function is an important etiological factor of secondary alterations such as uncontrolled exchange of water and electrolytes. The normally strictly separated intra- and extracellular spaces are open and the significantly different electrolyte concentrations equilibrate. In particular, the sodium ions which dominate the extracellular compartment move into the cells, thus causing a severe hyponatriemia. The potassium ions released into the blood stream are rapidly excreted by the kidneys. Simultanously with the shift of sodium into the interstitial and intracellular compartment, a transfer of water takes place which causes the tissue edema observed after a thermal trauma. This effect is accompanied by a water loss from the blood followed by an increase in blood viscosity and serious hemodynamic alterations.

The mechanisms of the membrane damage are not known in detail. For a long time, however, it

has been assumed that constituents of the burned skin play an etiologic role. It was Fox [26] who described an anti-sodium effect of extracts of burned skin.

Schoenenberger [27] succeeded in isolating a toxic factor from burned murine and human skin which was in part chemically characterized and exhibited a lethal dose in mice of about 300 mg/kg body weight. Schoelmerich et al. [28] were able to show that this purified toxic factor produced similar metabolic changes in the perfused rat liver, in isolated hepatocytes, and in whole animals as an in vivo burning. The primary target and the mechanisms of toxic acitivity, however, remained obscure, although speculations on an interaction of the toxic material with cellular membranes were frequent and contradictory. The experimental work of Dubois [29] and Moati [30] provides additional evidence on the presence of neurotoxic and cardiotoxic compounds in burned individuals. The biological activity of the purified cardiotoxic and neurotoxic fractions is described to involve membrane permeability changes. The data measured in this work demonstrate interactions between extracts from burned or unburned skin with membranes in model systems and in cells as well. The result of the interaction is a marked increase in electrical conductance and ionic permeability. In particular, the permeability of Na^+ and K^+, which create the membrane potential, is altered. The breakdown of the membrane potential due to increasing electrical conductance of the membrane secondarily influences the distribution of anions in the compartments. It is generally accepted that mediators of the inflammatory respons such as histamine, oxygen radicals, prostaglandins or activated complement are involved in the etiology of the local edema after burning [31]. From the results reported here it is obvious that direct thermal activation or release of mediators is possible without participation of inflammatory reactions. This mechanism, in fact, is operative in the case of complement where the alternative activation pathway can be started by heating or shear forces. The membrane attack complex of complement (MAC) is capable of interacting with lipid membranes, increasing the electrical conductance of artificial lipid bilayers similarly to the effects described in this paper for burn toxin [32].

It is likely that the increase in membrane permeability after in vivo burning of the skin is not a one-step reaction but a cascade of events which ultimately result in the production and/or release of the active complex. It is interesting to note that the analog from unburned skin is also active, indicating that the activation or release of the mediators can be induced by mechanical or thermal tissue damage. From these data it is obvious that autointoxication after major tissue damage is not a burn-specific process. Changes in membrane permeability, however, could be of etiological significance in this condition.

Participation of exogenous mediators, for example of bacterial origin, cannot be fully excluded. From experiments with germ-free animals, however, it is clear that exclusion of bacterial contamination does not affect toxic activity.

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