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LABORATORY INVESTIGATION

Alternative pathway activation of complement by cultured human proximal tubular epithelial cells

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Alternative pathway activation of complement by cultured human proximal tubular epithelial cells. Human proximal tubular epithelial cells (PTEC) incubated with normal human serum (NHS) were found to fix on their surface C3, properdin, terminal complement components and C5b-9 MAC neoantigen, but not C1q and C4, by immunofluorescence. Complement fixation was abrogated if PTEC were incubated with EDTA-treated NHS or C3-deficient human serum, but not with Mg EGTA-treated NHS or C1q-deficient human serum, showing the prevalent activation of the alternative pathway of complement. This event was followed by marked cytoskeleton alterations with disruption of the actin cortical network, redistribution of actin throughout the cytoplasm and formation of blebs, and by cell cytolysis. In addition, superoxide anion and hydrogen peroxide production and chemiluminescence response were detected in consequence of MAC insertion on PTEC plasma membrane. The dependency on MAC of the observed biological effects of complement fixation on PTEC surface was shown by using sera selectively deficient of terminal components of complement (C6 or C8), and therefore unable to form the C5b-9 MAC, and by restoring the ability to form MAC after addition of purified C6 or C8. The possible pathogenetic relevance of these observations in tubulointerstitial injury occurring in patients with complementuria due to non-selective proteinuria, is discussed.

Tubular interstitial injury, rather than glomerular damage, was shown to correlate with renal functional impairment and progression of a variety of glomerulonephritis [reviewed in 1]. It was suggested that alteration in glomerular functions and/or extension of the immune injury to the interstitium may promote tubulointerstitial damage [1].

One of such alterations is the loss of glomerular permeselectivity leading to proteinuria [2–6]. In several experimental models tubulointerstitial damage correlates with the severity of proteinuria [4–6], suggesting that plasma proteins escaped into urine, such as lipoproteins [7], transferrin [8] and complement fractions [9, 10], and may account for the tubular injury. Histological studies demonstrated that the alternative pathway of complement can be directly activated by the brush border of proximal tubules of normal rat [11] and human kidney [9]. The occurrence of tubular deposits of complement in patients with altered glomerular permeability was shown to correlate with urinary excretion of native and breakdown products of complement [10]. Recently, the urinary excretion of C5b-9 membrane attack complex (MAC) and its deposition in tubular epithelium was detected in proteinuric nephropathies either in the presence or in the absence of MAC glomerular deposition [12, 13]. Thus, the increased urinary excretion of MAC may reflect an immune glomerular activation and/or an activation of complement by the tubular brush border. However, the pathogenetic implications of complement activation by proximal tubules are at present unknown.

The aim of the present study was to investigate whether cultured human proximal tubular cells (PTEC) could activate complement with fixation of MAC on their surface and whether these events may lead to cell stimulation with generation of reactive oxygen species (ROS) and/or to cell injury.

Methods

Materials

Ferricytochrome c (Type IV), superoxide dismutase, phenol red, 5,5-dimethylpyrroline N-oxide (DMPO), horseradish peroxidase, catalase, luminol, human anti-myosin antibodies, FITC-conjugated anti-mouse and anti-goat antibodies were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Anti-human C1q, C4, C3, IgG, IgA and IgM antibody were from Behringwerke AG (Marburg, Germany); anti-human properdin, C3, C5, C7, C8 antibodies were from Miles-Yeda (Kickatweizman, Rehovot, Israel). Anti-human C9 monoclonal antibody was from ICN Flow (Milano, Italy). Monoclonal mouse anti-human C5b-9 reacting with a neoepitope in activated C9 complement exposed in the solid phase and membrane form and in the fluid phase form of the terminal complement complex but not in native C9 [14, 15], was from Dako s.p.a. (Milano, Italy). Human factor VIII antiserum was from Nordic Immunology (Tilburg, The Netherlands); mouse monoclonal anticytokeratin antibody was from Labometrics (Milano, Italy). The specificity of these reagents was established by immunodiffusion and by immunoelectrophoresis. Before use the reagents were appropriately adsorbed in order to avoid nonspecific staining.

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Table 1. Human complement components on PTEC or HUVEC

	C3	Properdin	C1q	C4	C5	C8	C9	C5b-9 MAC neoantigen
Treatment of PTEC								
NHS	+++	+++	0	0	++	+++	+++	+++
HIHS	0	0	0	0	0	0	0	0
NHS + EDTA	0	0	0	0	0	0	0	0
NHS + Mg EGTA	+++	+++	0	0	++	+++	++	+++
C1q-DEF HS	++	++	0	0	++	++	++	++
C3-DEF HS	0	0	0	0	0	0	0	0
C6-DEF HS	+++	++	0	0	++	0	0	0
C6-DEF HS + C6	+++	++	0	0	++	++	++	++
C8-DEF HS	+++	+++	0	0	++	0	0	0
C8-DEF HS + C8	+++	+++	0	0	++	++	++	+++
Treatment of HUVEC with NHS	+/−	0	0	0	0	0	0	0

In vitro fixation of various human complement components on the surface of PTEC or of HUVEC incubated at 37°C for one hour with 25% NHS, HIHS, NHS with 10 mM EDTA, NHS with 5 mM Mg-EGTA, C1q-def HS, C3-def HS, C6-def HS, C6-def HS with 50 μg/ml C6, C8-def HS, C8-def HS with 70 μg/ml C8 was studied by immunofluorescence as described in the **Methods**.

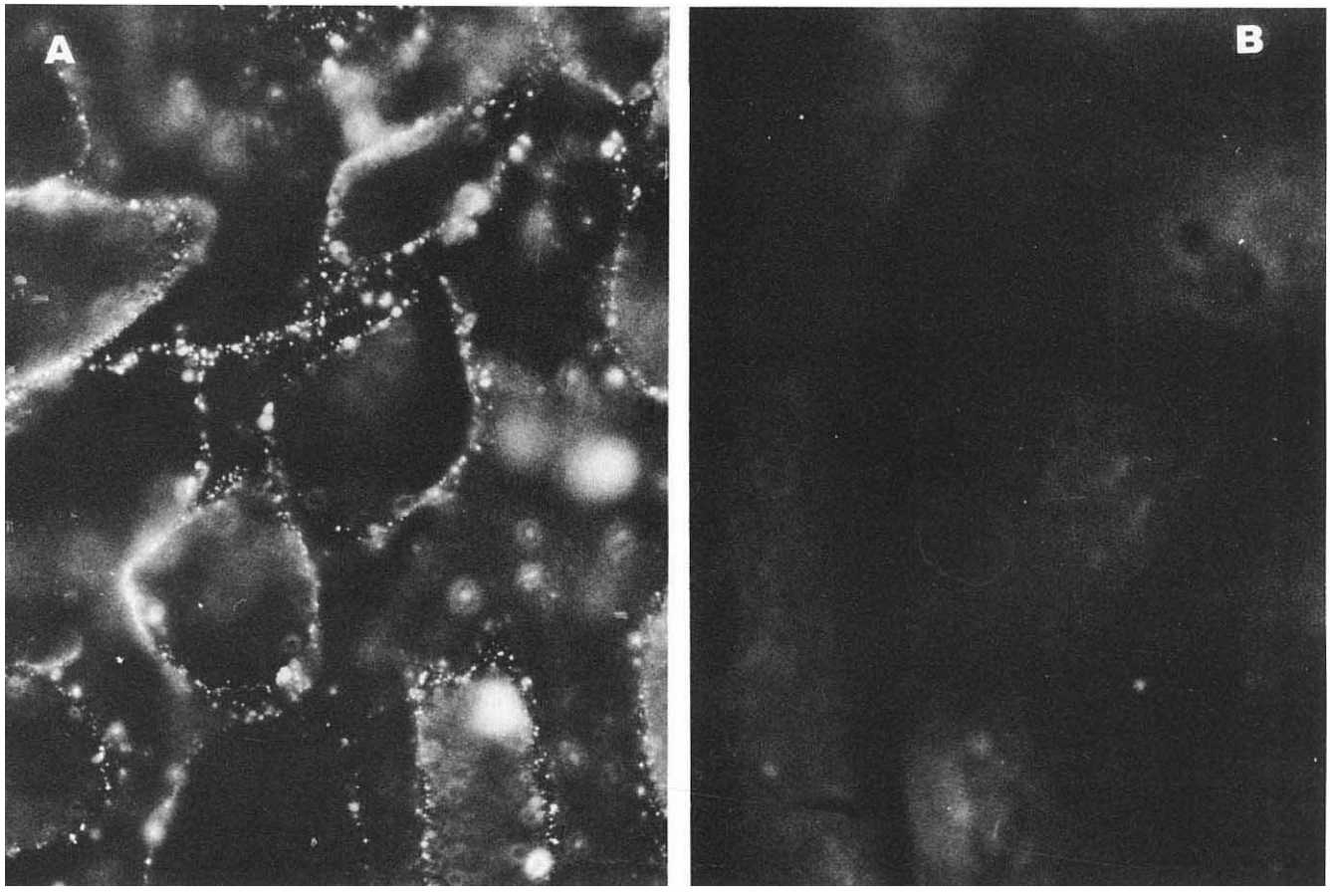

Fig. 1. *Micrographs illustrating the results of immunofluorescence tests performed on cultured PTEC.* **(A)** C3 fixation on the surface of PTEC incubated at 37°C for 1 hour with 25% NHS; **(B)** negative control for C3 fixation after PTEC incubation at 37°C for 1 hour with 25% HIHS (× 400).

PTEC preparation and characterization

Surgical specimens of kidneys were used as source for establishing cultures. The fibrous capsule was removed and portions of tissue were dissected from the outer cortex, minced and forced through a graded series of meshes to remove interstitial fragments and glomeruli [16]. PTEC were pelleted, resuspended and plated in RPMI 1640 supplemented with 17% fetal calf serum (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin; culture flasks were kept in a 95% air-5% CO_2

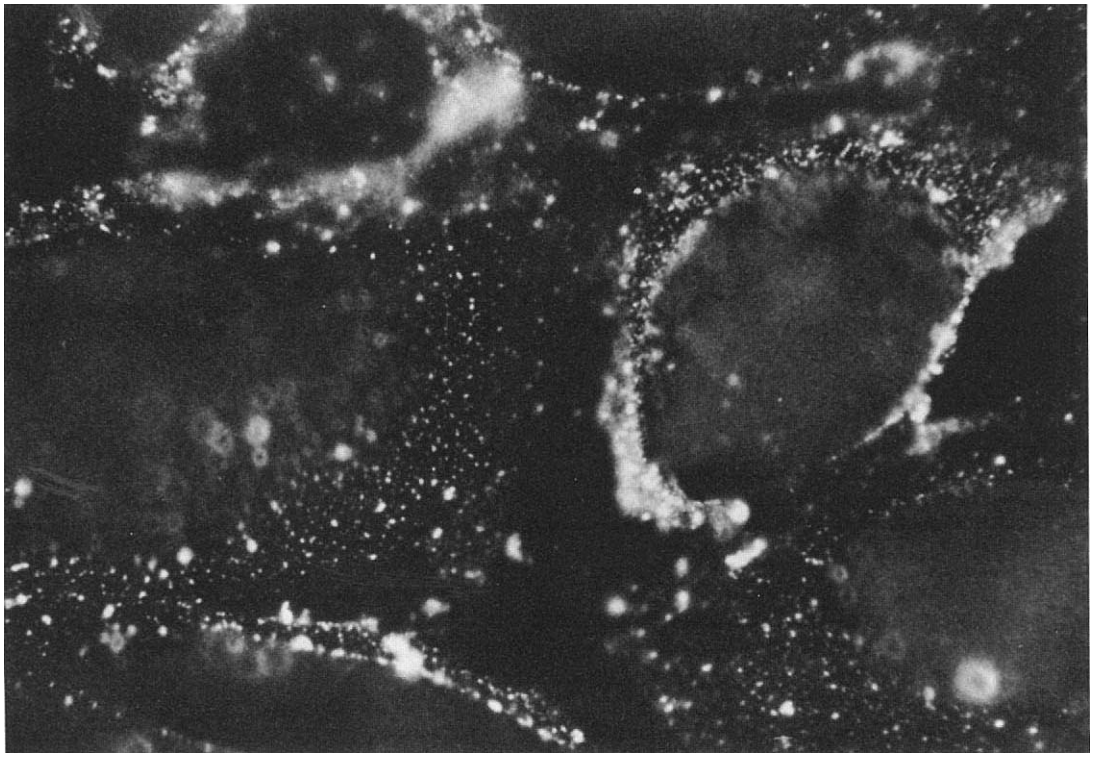

Fig. 2. *The immunofluorescence micrograph shows fine granular deposits of C5b-9 MAC neoantigen on the plasma membrane of PTEC incubated at 37°C for 1 hour with 25% NHS (× 400).*

environment at 37°C. Confluent monolayers were passaged after treatment with 0.05% trypsin-0.02% EDTA (ICN Flow). Endotoxin was not detectable in either preparation when tested by the limulus assay. PTEC were characterized on the basis of: (a) minimal staining for desmin and negative staining for factor VIII-vWF [16]; (b) strong staining for cytokeratin and actin [16]; (c) staining of 90 to 96% of cells for alkaline phosphatase as determined by the naphtal AS-MX method [17]; (d) increased cAMP production after stimulation with 100 nM PTH but not 1 μM ADH [17].

Sources of complement

For *in vitro* experiments the following sera were used as a source of complement: (1) normal human sera pooled from 10 healthy donors; in some experiments sera were complement-inactivated by heating at 56°C for 1 hour (HIHS); (2) C1q-deficient human serum (C1q-def HS), C3-deficient human serum (C3-def HS), C6-deficient human serum (C6-def HS), C8-deficient human serum (C8-def HS) were purchased by Sigma Chemical Co. The CH_{50} levels in sera were determined by hemolytic assay, as previously described [18]. CH_{50} level of NHS was 45 ± 4.8 U/ml. C6-def HS and C8-def HS had no detectable hemolytic activity. Restoration of the normal hemolytic activity was obtained after addition of 50 μg/ml human purified C6 (Sigma) to C6-def HS or 70 μg/ml human purified C8 (Sigma) to C8-def HS.

Experimental protocol

10^6 confluent PTEC, plated on 35 mm plastic dishes, were incubated for the indicated periods of time at 37°C with 25% NHS, HIHS, C1q-def HS, C3-def HS, C6-def HS, C8-def HS or with C6- or C8-def HS reconstituted with purified C6 or C8, respectively. Human endothelial cells from umbilical cord vein (HVEC) were used as control.

Immunofluorescence studies

After stimulation, cells were fixed in 3% paraformaldehyde in PBS (pH 7.6) containing 2% sucrose for five minutes at 4°C and then stained for C1q, C4, C3, IgG, IgA, IgM, properdin, C5, C7, C8, C9 or C5b-9 neoantigen. Immunofluorescence tests on PTEC in culture and the relevant control experiments were performed as previously described [19].

Cytoskeleton alterations were studied on fixed PTEC after permeabilization with 0.1% triton X-100 in PBS and stained for F-actin with 2 μg/ml FITC-conjugated phalloidin (F-PHD) [20].

Assay for cytotoxicity

The cytotoxic effect of complement on PTEC in culture was measured as percentage of ^{51}Cr release in the supernatant, as previously described [21]. Confluent cultures of PTEC in 35-mm culture dishes were incubated for two hours at 37°C with 250 μCi of $Na^{51}CrO_4$. The cells were washed five times with

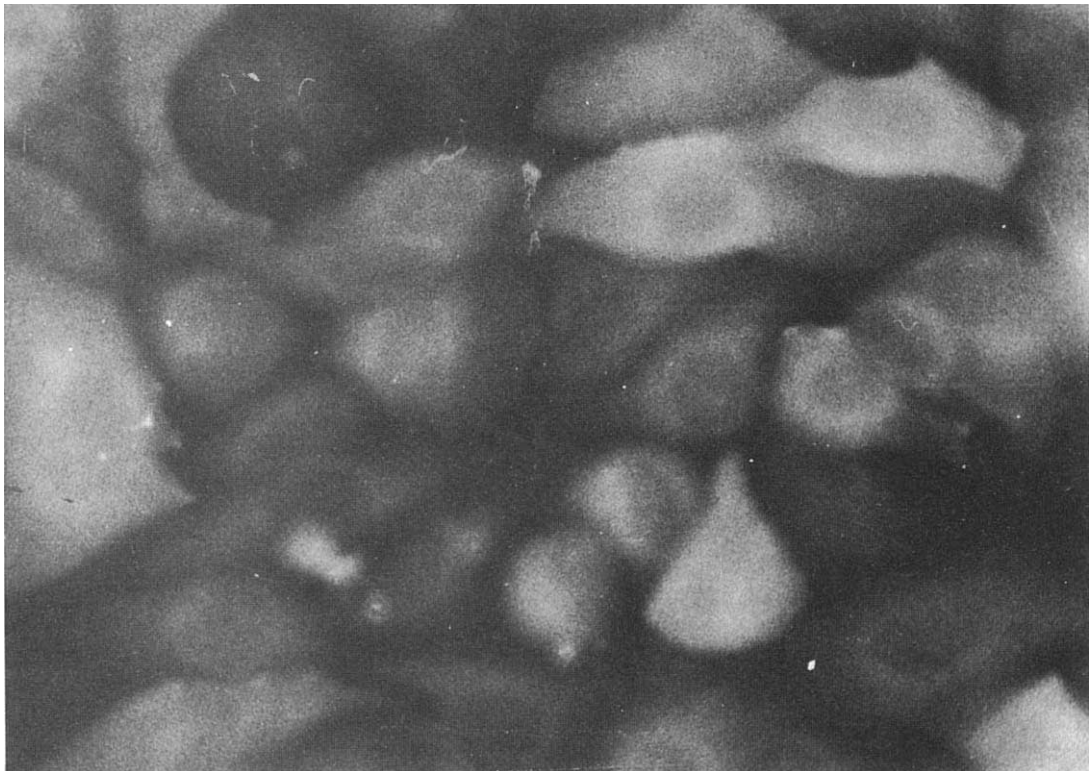

Fig. 3. *The immunofluorescence micrograph demonstrates absence of C5b-9 MAC neoantigen deposition on the surface of PTEC incubated at 37°C for 1 ourwith 25% C6-def HS (× 400).*

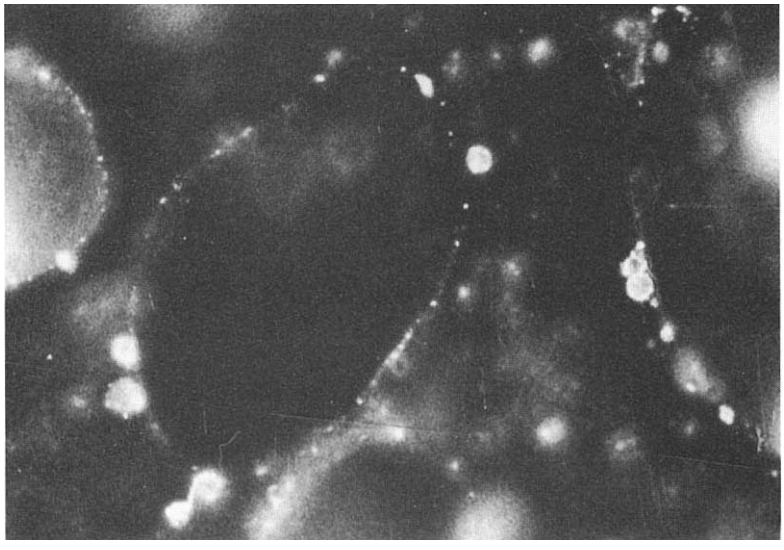

Fig. 4. *The immunofluorescence micrograph shows the formation of C5b-9 MAC neoantigen-containing blebs on the surface of PTEC incubated at 37°C for 1 hour with 25% C6-def HS after reconstitution with 50 μg purified human C6 (× 400).*

Eagle's modified Dulbecco medium (GIBCO, Grand Island, New York, USA) supplemented with 10% complement-inactivated fetal calf serum and covered with 1 ml of the same medium. After appropriate treatment, the cell-free supernatant was collected and the radioactivity was counted in a gamma counter (Packard autogamma scintillation spectrometer, Packard Instrument Co., Clarence, New York, USA). After treatment with 5% Triton X-100, the radioactivity that remained associated with the cells was also measured. The spontaneous release of ^{51}Cr into the supernatant and the amount of radioactivity associated with untreated cells were evaluated in separate wells. The percentage of ^{51}Cr release in each individual well was calculated, assuming as 100% the radioactivity in the supernatant plus the value extract from the cells.

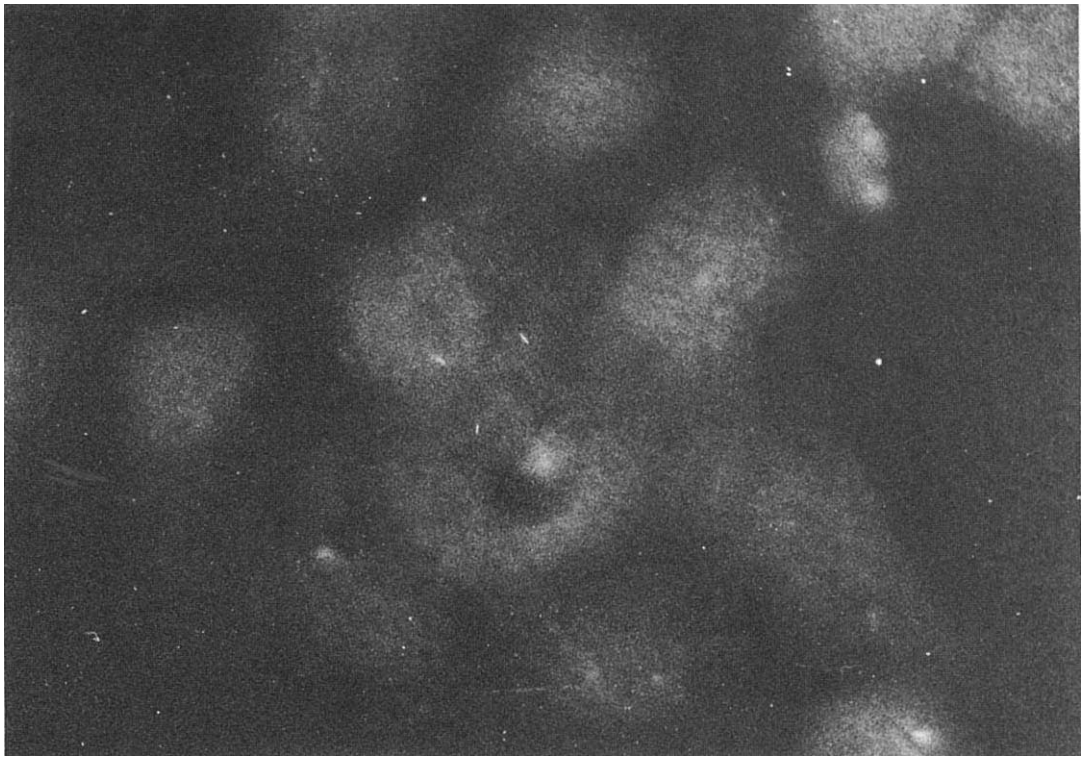

Fig. 5. *The immunofluorescence micrograph demonstrates the absence of IgG deposition on the surface of PTEC incubated at 37°C for 1 hour with 25% NHS; similar results were obtained for IgA and IgM (× 400).*

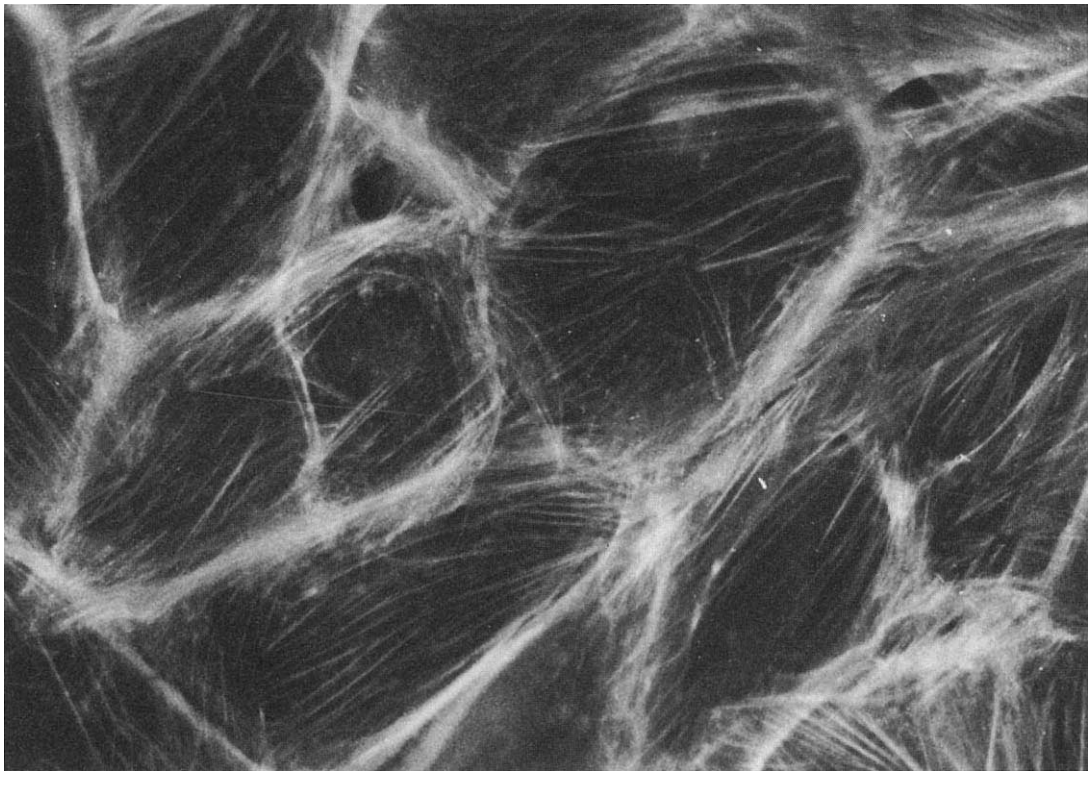

Fig. 6. *F-actin distribution in fixed and permeabilized PTEC showing the normal distribution of actin-containing stress fibers at the periphery of the cells in a ring-like pattern.* The same pattern was observed after PTEC incubation at 37°C for 1 hour with 25% HIHS or C6-def HS (× 400).

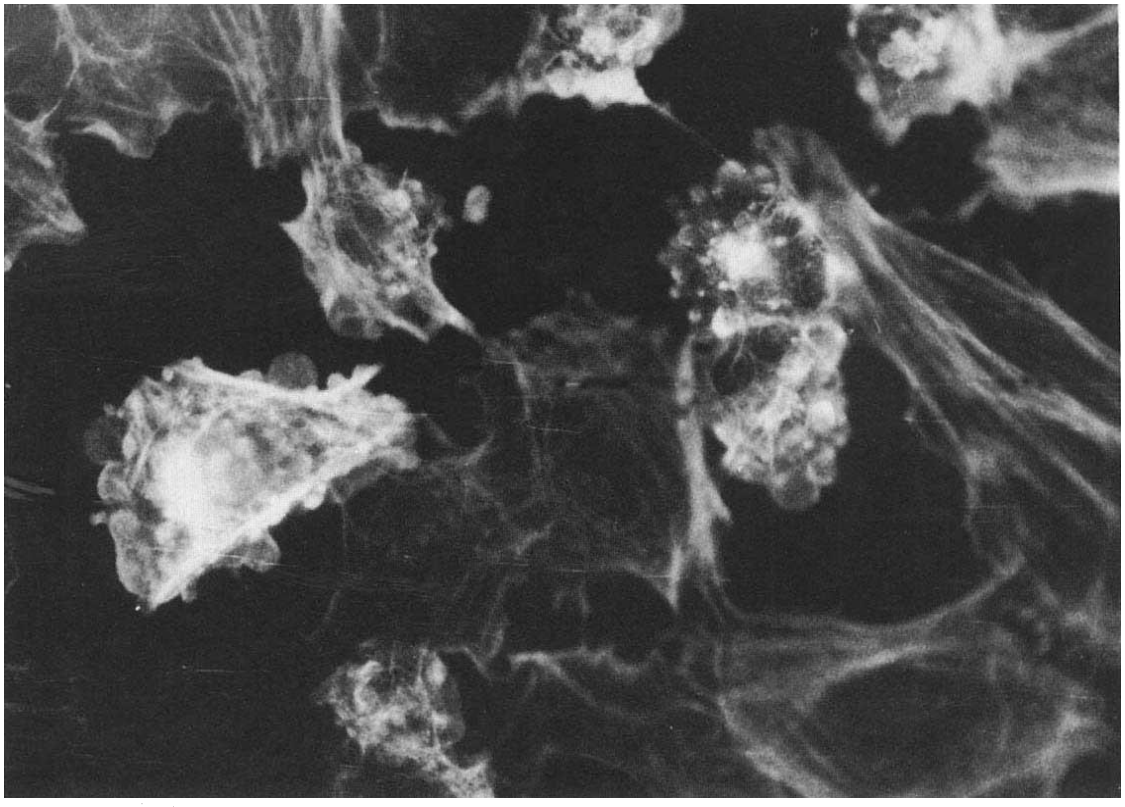

Fig. 7. *Altered distribution of F-actin after exposure of PTEC to 5% NHS for 1 hour at 37°C.* The cortical network of actin-containing stress fibers tends to disappear, cells separate from each other, become round and develop blebs (× 400).

Reactive oxygen species assays

Superoxide anion (O_2^-) assay. Production of O_2^- was measured as the superoxide dismutase inhibitable reduction of ferricytochrome C [22]. Cell monolayers were incubated at 37°C with Tyrode's buffer containing 80 μM cytochrome C and appropriately stimulated. Basal O_2^- production was assessed in the absence of any of the stimulating factors. Supernatants were removed and centrifuged and the absorbance was measured in a spectrophotometer at 550 nm. The extinction coefficient of ferricytochrome C at 550 nm was taken as 2.1×10^4 M^{-1} cm^{-1}. Protein content of the monolayer was measured by the Lowry technique. O_2^- production was expressed as nM of cytochrome C reduced/mg protein/time [22].

Hydrogen peroxide (H_2O_2) assay. Production of H_2O_2 was measured by the method of Pick and Keisari [23]. The assay is based on HRPO-mediated oxidation of phenol red by H_2O_2 resulting in a production with maximal adsorbance at 610 nm. Adsorbance is compared to that of a standard curve of hydrogen peroxide. PTEC were incubated with 0.28 mM phenol red in PBS, containing 50 μg/ml of HRPO. At the specified time, supernatants were removed from the cell monolayers, centrifuged, alkalinized with 10 μl of 1 N NaOH and adsorbance determined at 610 nm. Results were expressed as nM H_2O_2 produced/mg protein/time [22].

Chemiluminescence

Luminol (50 μM) enhanced chemiluminescence was measured on 5×10^6 EDTA-detached and suspended PTEC incubated as described in the experimental protocol in Hepes-buffered Krebs medium containing 0.05% BSA in a LKB 1250 luminometer. Data are expressed in mVolt [24].

Statistical analysis

All data within groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett's or Newman-Keul's multiple-comparison tests where appropriate. Values are given as mean ± SD. Values of $P < 0.05$ were considered statistically significant.

Results

The results of immunofluorescence studies are summarized in Table 1. PTEC incubated with NHS but not with HIHS fixed C3 (Fig. 1) and properdin, but not C1q and C4, on their surface. The complement activation occurred via the alternative pathway since it was not prevented by 5 mM Mg-EGTA or by incubation with C1q-def HS (Table 1). In contrast, 10 mM EDTA and C3-def HS completely prevented complement fixation. As shown by the staining for C5, C8, C9 (Table 1) and C5b-9 MAC neoantigen (Fig. 2), the terminal components of complement inserted in PTEC plasmamembrane. As shown in Figure 2, numerous plasma membrane blebs containing C9 neoantigen were observed. C5b-9 MAC neoantigen was not detectable when PTEC were incubated with C6- or C8-def HS (Fig. 3, Table 1). The immunofluorescence staining for C5b-9 MAC neoantigen was restored after addition of purified human C6 (Fig. 4) or C8 (Table 1), respectively, to C6- or C8-def HS.

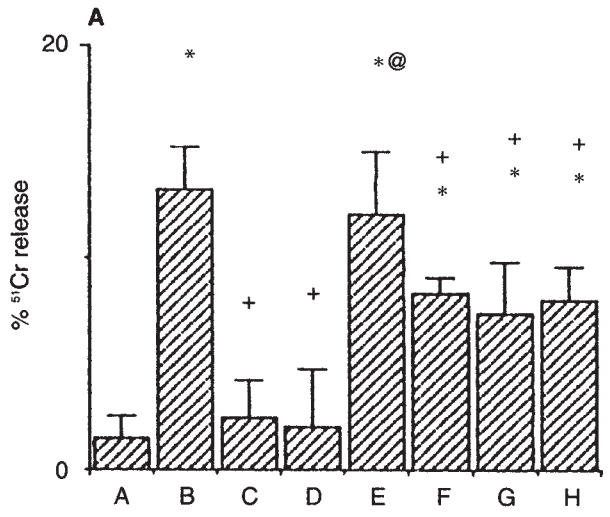

Fig. 8. (A) *Percentage of ^{51}Cr release from PTEC after incubation for 1 hour at 37°C without any addition (A) or with addition of 25% NHS (B), HIHS (C), C6-def HS (D), C6-def HS plus 50 μg/ml C6 (E), NHS plus 100 μg/ml SOD (F), NHS plus 100 μg/ml catalase (G), NHS plus 10 mM DMPO (H).* ANOVA with Newman-Keul's multiple-comparison test was performed within groups: A versus B, C, D, E, F, G or H (*$P < 0.05$); B versus C, D, E, F, G or H (+$P < 0.05$) D versus E (@$P < 0.05$). **Panel (B)** Percentage of ^{51}Cr release from PTEC after incubation for 1 hour at 37°C with various dilutions of NHS (hatched bars) or C1q-def HS (open bars), and from HUVEC (solid bars) after incubation for 1 hour at 37°C with various dilutions of NHS. ANOVA was performed between PTEC incubated with NHS and PTEC incubated with C1q-def HS (not significant), and between PTEC and HUVEC (*$P < 0.05$).

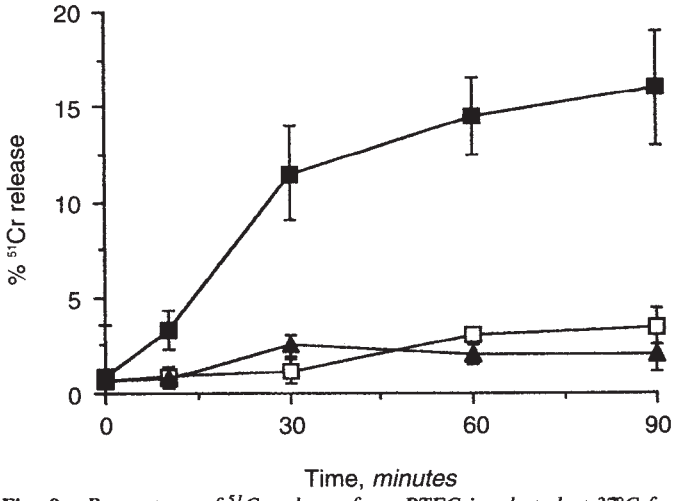

Fig. 9. *Percentage of ^{51}Cr release from PTEC incubated at 37°C for various periods of time without any addition (▲) or with addition of 25% NHS (■) or HIHS (□).*

In all the experimental conditions, the staining of PTEC for IgG, IgA or IgM was consistently negative (Fig. 5). Moreover, control immunofluorescence stainings performed with irrelevant monoclonal mouse IgG were negative. Complement fixation was not observed after incubation of HUVEC with a source of complement following the same protocol used for PTEC (Table 1).

The incubation of PTEC with NHS induced changes in the cell cytoskeleton. Actin-containing stress fibers which in unstimulated PTEC were concentrated at the periphery of the cells in a ring-like pattern (Fig. 6), were disrupted in 60 ± 11% of the cells after incubation for one hour with 5% NHS (Fig. 7), a concentration that was found to induce only minimal cytotoxic effects, as detected by Trypan blue staining (10 ± 3% of the cells) and ^{51}Cr release (Fig. 8B). Stress fibers were decreased and randomly distributed in the cytoplasm with disappearance of the cortical network. Cells tended to lose the reciprocal contact, reducing their surface to became round and developed blebs (Fig. 7). These alterations were absent when cells were incubated with HIHS or with C6-def HS and were restored by addition of purified C6 to the C6-def HS. For concentration of 25% NHS the deposition of MAC on the plasma membrane of PTEC as well as changes in the cytoskeleton distribution were associated with cytotoxic effects, as detected by Trypan blue staining of lethally injured cells (49 ± 6%). Cell cytotoxicity was quantitated measuring the release of ^{51}Cr. As shown in Figure 8A, incubation of PTEC with NHS, but not with HIHS, induces release of ^{51}Cr. The release of ^{51}Cr became significant after 20 minutes incubation and reached the plateau after 60 minutes (Fig. 9). That cytolysis is mediated by the MAC was shown by the absence of cell lysis when C6-def HS was used and by restoration of cytolytic activity with human purified C6 (Fig. 8A). Similar results were obtained with C8-def HS and with C8-def HS reconstituted with human purified C8 (data not shown). Cytotoxicity studies performed in parallel with C1q-def HS and NHS at various dilutions confirmed that the complement activation was independent from the classical pathway activation (Fig. 8B). Figure 8B also shows the absence of cell lysis when the HUVEC were incubated with various dilutions of NHS, suggesting that the alternative pathway activation and the consequent MAC-dependent cytolysis was unique to the PTEC.

Experiments performed in the presence of superoxide dismutase, catalase or DMPO suggested that complement induced cytolysis may at least in part depend on ROS generation (Fig. 8A). Production of O_2^- and H_2O_2 from PTEC was detectable 10

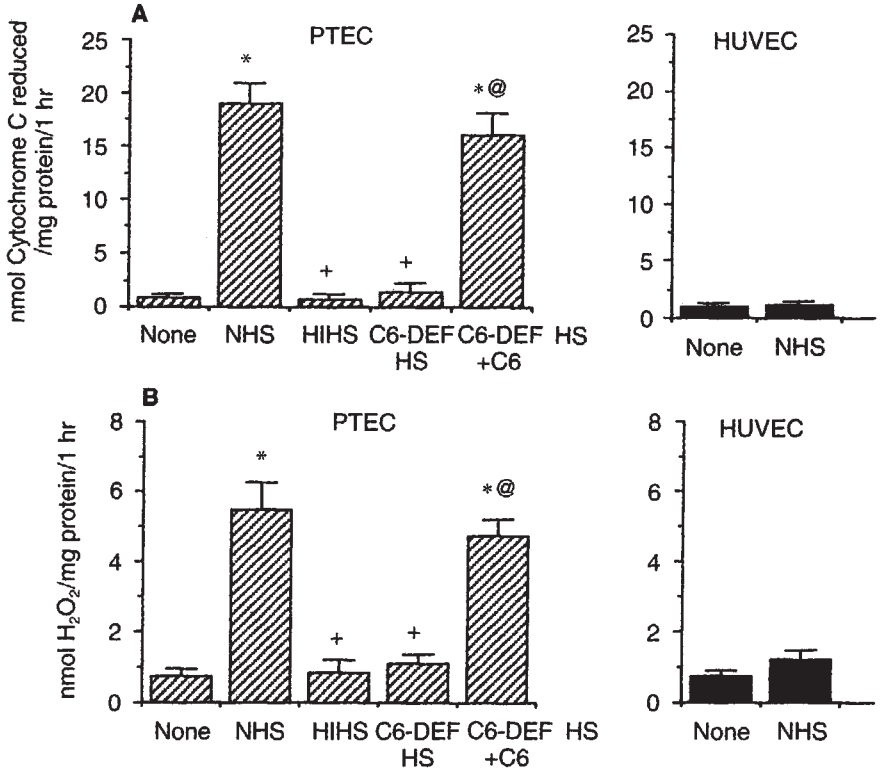

Fig. 10. *Production of O_2^- (A) and H_2O_2 (B) from PTEC after incubation for 1 hour at 37°C without any addition (None) or with addition of 25% NHS, HIHS, C6-def HS or C6-def HS plus 50 μg/ml C6 and from HUVEC after incubation for 1 hour at 37°C without any addition (None) or with addition of 25% NHS. ANOVA with Newman-Keul's multiple-comparison test was performed within treated groups versus None (*$P < 0.05$); NHS versus HIHS, C6-def HS or C6-def HS plus C6 (+$P < 0.05$); C6-def HS versus C6-def HS plus C6 (@$P < 0.05$).*

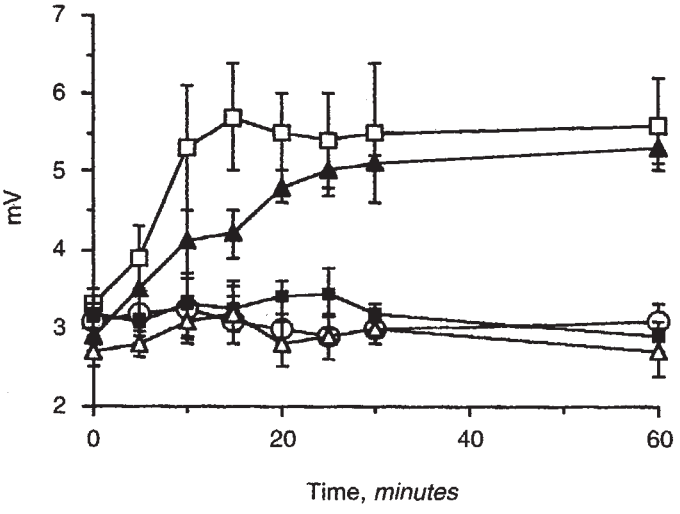

Fig. 11. *Chemiluminescence of PTEC incubated at 37°C for various periods of time with 25% NHS (□), HIHS (○), C6-def HS (■), C6-def HS reconstituted with 50 μg/ml C6 (▲) or without any addition (△).*

minutes after incubation with NHS (data not shown) and reached a plateau after one hour. Figure 10 shows the production of significant amounts of O_2^- and H_2O_2 from PTEC after one hour incubation whit NHS. In contrast, incubation with HIHS did not stimulate significant ROS generation. The amount of O_2^- (Fig. 10A) and H_2O_2 (Fig. 10B) produced by PTEC was significantly reduced using C6-def HS. The addition of purified C6 completely restored the generation of ROS. HUVEC after incubation with NHS did not produce significant amounts of O_2^- and H_2O_2. NHS, but not HIHS or C6-def HS, stimulated chemiluminescence of PTEC in the presence of luminol. Reconstitution of C6-def HS with human purified C6 restored generation of chemiluminescence by PTEC (Fig. 11).

Discussion

Incubation of sections of frozen normal rat [11] and human kidney tissue [9] with fresh serum is associated with the activation of the alternative pathway of complement by the brush border of proximal tubular cells. Studies on patients with nonselective proteinuria demonstrated a positive correlation between the occurrence of tubular C3 deposits and the urinary complement excretion, as well as with the presence of C3 breakdown products in urine, suggesting that complement components once filtered through the glomerular barrier might be activated by the brush border of the proximal tubules [10]. Baker et al demonstrated the formation and stabilization of cell-bound C3 convertase activity on heat-killed human kidney cells, suggesting that cell injury may locally intensify C3-activation [25–27]. The results of the present study extend these observations by showing that cultured human proximal tubular epithelial cells activate complement via the alternative pathway leading to binding of the C5b-9 MAC to the cell surface. These events are followed by marked cytoskeleton alterations with disruption of the cortical network of actin-containing stress fibers, redistribution of actin within the cytoplasm, formation of blebs and cytolysis. That these alterations are produced by MAC is shown by the absence of cytolysis after cell incubation with sera deficient of the C6 or C8 components of complement, and thus they are incapable of forming the C5b-9 MAC. Furthermore, the present study demonstrates that the susceptibility to cell injury is restored when sera were reconstituted with the purified C6 or C8 complement fractions. MAC could

produce injury either by stimulating the release of other mediators or by producing direct plasma membrane damage.

Insertion of C5b-9 MAC into the lipid bilayer of glomerular epithelial cells was shown to induce structural changes comparable to membrane defects produced in erythrocytes incubated with specific antibodies and complement [21]. Alternatively, MAC may produce cell injury by stimulating the release of cytokines or by generation of autacoids and reactive oxygen radicals [28–32]. The present experiments, performed with C6 deficient and C6 reconstituted sera, indicated that MAC insertion in the plasma membrane of PTEC stimulates synthesis of reactive oxygen radicals. However, experiments performed with superoxide dismutase and catalase [33] suggest that the cytolytic effect of MAC is only partially dependent on reactive oxygen radical generation and that the direct mechanism of cell injury [34] is prevalent. Nevertheless, if oxygen radical generation would occur *in vivo* after complement fixation by proximal tubular cells in patients with nonselective proteinuria, it may favor development of inflammatory lesions in the interstitium. Indeed, it was proposed that hydroxyl ions and other oxidants may cause tubulointerstitial injury in proteinuric states [reviewed in 35]. This may be catalyzed by iron released from transferrin reabsorbed by PTEC [8, 36] or as proposed herein by MAC insertion in the PTEC plasma membrane. Evidence obtained *in vivo* supports the contention that MAC is activated in membranous glomerulonephritis and other proteinuric glomerulopathies [12, 13]. In patients with membranous nephropathy, increased urinary excretion of C5b-9 is associated with an increased deposition of MAC in glomerular and tubular epithelium and correlates with the severity of the disease [13]. In this disease C5b-9 urinary excretion may reflect the immune complex-mediated intraglomerular activation of terminal components of complement, similar to that observed in Heymann nephritis [37–39]. However, in other proteinuric states, such as diabetic and focal glomerulosclerosis, an increased urinary excretion of C5b-9 occurs in the absence of glomerular deposition of MAC [13], suggesting that it may result from the activation of complement by tubular brush border. By either mechanism, terminal complement activation may contribute to tubular interstitial injury. In the present study we have not explored the production of cytokines or autacoids by tubular epithelial cells. However, it is well-established on other cell types that MAC may stimulate production of cytokines and autacoids that can act as chemoattractants for leukocytes and monocytes [28–32]. Furthermore, by inducing alterations in the cytoskeleton of epithelial cells, MAC plays a critical role in maintaining cell polarity [40] and may affect tubular cell functions. This possibility is supported by results of micropuncture studies demonstrating functional as well as morphological lesions induced by intraluminal perfusion of proximal tubules with fresh serum [41].

In conclusion, the results of the present study provide an experimental support for the hypothesis that in patients with urinary excretion of complement components because of a nonselective proteinuria, tubulointerstitial injury may be, at least in part, sustained from direct activation of the complement system by the plasma membrane of proximal tubular cells. The biological effects of such complement activation are related to insertion of MAC in the cell plasma membrane as shown by dependency of redistribution of cell cytoskeleton, cytolysis and generation of ROS on the activation of MAC.

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Reprint requests to Dr. Giovanni Camussi, Dipartimento di Biochimica e Biofisica, Seconda Universita di Napoli, Via Costantinopoli 16, 80100 Napoli, Italy.

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