Hyperosmolality impairs ammonia-mediated inflammation: implications for the renal medulla

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Clark, Edward C., Karl A. Nath, Thomas H. Hostetter, and Margaret K. Hostetter. Hyperosmolality impairs ammonia-mediated inflammation: implications for the renal medulla. *Am. J. Physiol.* 263 (*Regulatory Integrative Comp. Physiol.* 32): R148–R155, 1992.—Although ammonia modifies the third component of complement (C3) and activates the alternative pathway, inflammation is not seen in the renal medulla where ammonia concentrations are normally elevated. We examined the effect of the unique hyperosmolar milieu of the renal medulla on the interaction of ammonia with C3 and the capacity of ammonia-modified C3 ($NH_3 \cdot C3$) to induce cytolytic injury and stimulate neutrophils (PMN). Incubation of purified human C3 with ammonia in concentrations found in urine results in significant disruption of the C3 thiolester bond compared with ammonia-free controls. Coincubation with urinary osmolytes and hyperosmolar NaCl and urea does not impair thiolester disruption over a range of ammonia concentrations. However, hyperosmolar NaCl and urea virtually abolish cytolytic injury mediated by the alternative pathway. Coincubation with the organic osmolytes betaine, sorbitol, and inositol fails to reverse this inhibitory effect of hyperosmolar NaCl and urea. Hyperosmolar NaCl and urea also suppress lytic injury mediated by ammonia and complement in MDCK, a cell line derived from canine distal tubular epithelium. Both PMN degranulation and respiratory burst responses to $NH_3 \cdot C3$ are significantly blunted in the presence of hyperosmolar NaCl and urea. Hyperosmolality also impairs PMN responses to the formyl peptide *N*-formyl-Met-Leu-Phe and phorbol 12-myristate 13-acetate (PMA). Therefore, in an in vitro setting of hyperosmolar NaCl and urea, amidation of C3 occurs, but subsequent membrane-directed and receptor-mediated functions of $NH_3 \cdot C3$ are markedly impaired. These findings suggest that hyperosmolar NaCl and urea protect the medulla from immune injury triggered by ambient ammonia and C3 and also provides a mechanism underlying the diminished inflammatory response characteristic of the renal medulla.

C3 thiolester; alternative complement pathway

SEVERAL MODELS of renal (20, 31) and extrarenal disease (14) implicate ammonia as contributing to tissue injury. This injury results in part from the ability of free-base ammonia (NH_3) to disrupt the internal thiolester bond of the third component of human complement (C3) endowing it with "C3b-like" properties. C3 modified by ammonia, also called amidated C3 ($NH_3 \cdot C3$), forms the alternative pathway convertase, initiates cleavage of C3 and C5, and generates the cytolytic membrane attack complex (13, 17). Additionally, the binding of $NH_3 \cdot C3$ to membrane complement receptors on phagocytic cells leads to the release of superoxide ($O_2^-\cdot$), myeloperoxidase (MPO), and lactoferrin (10, 14).

Because of its vital role in urinary acidification and water balance, the renal medullary interstitium constitutes a unique osmolar milieu. Extracellular concentrations of NaCl, urea, and ammonia greatly exceed those in other tissue sites (5). Ammonia concentration in particular rises nearly 100-fold along a corticomedullary axis (27). Also accumulating within the mammalian renal medulla are numerous organic solutes or osmolytes. During antidiuresis, concentrations of these solutes increase in the medulla, where their proposed role is maintenance of cell volume and optimal enzyme function during hyperosmolar stress (26, 34).

Studies implicating increased concentrations of ammonia as an instigator of inflammatory injury in models of renal disease have focused on the renal cortical interstitium (20, 31). Under physiological conditions, however, the renal medulla possesses high interstitial concentrations of ammonia; yet evidence of ongoing complement activation and inflammation is absent. We propose that the hyperosmolar milieu of the renal medulla impairs alternative complement activation and neutrophil function. This impairment serves to protect the renal medulla from immune injury triggered by ambient ammonia. Additionally, the suppressive role of hyperosmolality may provide an explanation for the less exuberant inflammatory response seen in the renal medulla in bacterial pyelonephritis (2, 9) and allograft rejection (11). To test this hypothesis, both membrane-directed functions and ligand-receptor interactions of the alternative complement pathway were studied under conditions of hyperosmolar NaCl and urea.

METHODS

Isolation of human C3. Purified human C3 was isolated from fresh plasma by the method of Tack et al. (29) with sequential chromatography on lysine-Sepharose 4B, DEAE Sephacel, and Sepharose CL-6B. Possible trace contaminants immunoglobulin (Ig) G, IgA, and C5 were absorbed by affinity chromatography on cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to which goat antibodies to human IgG, IgA, and C5 had been coupled. C3 utilized in these experiments was therefore free of contamination with IgG, IgA, and C5 as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and double immunodiffusion against the above-named antibodies.

Titration of thiolester disruption. Disruption of the C3 thiolester by ammonia is a concentration-dependent reaction and has been previously reported (20). We studied the effect of osmolar conditions representative of the renal medulla on this reaction in the following manner. C3 thiolester disruption was quantitated by radioalkylation of the exposed sulfhydryl with [^{3}H]iodoacetic acid (28). Human C3 (500 μg) was added to both control and experimental solutions and incubated with 1.5 μl [^{3}H]iodoacetic acid (sp act 12.37 mCi/mmol, Amersham, Arlington Heights, IL) for 80 min in a 37°C water bath. Control buffer was 100 mM tris(hydroxymethyl)aminomethane (Tris)--10 mM EDTA (pH 8.0). Experimental solutions included

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pooled human urine collected from normal volunteers (pH 6.22, osmolality 585 mosmol/kg, total ammonia 17.5 mM), Tris-EDTA buffer with 17.5 mM NH_4Cl, and Tris-EDTA buffer with 17.5 mM NH_4Cl, 500 mosmol/kg NaCl, and 500 mosmol/kg urea. Urine served as an osmolar analogue of medullary interstitial fluid. All experimental solutions were titrated to pH 6.22. Given that the negative logarithm of the acid dissociation constant of NH_3 at 37°C is 9.27, a total ammonia concentration of 17.5 mM at pH 6.22 translates to a free base NH_3 concentration of 15.6 μM. To ensure that concentrations of ammonia lower or higher than that measured in urine did not affect thiolester disruption in the presence of hyperosmolar buffers, a dose-response curve was also performed to quantitate thiolester disruption in the presence of 5, 10, and 50 mM NH_4Cl in buffer containing 500 mosmol/kg NaCl and 500 mosmol/kg urea.

Total ammonia (NH_3 + NH_4^+) was measured by the glutamate dehydrogenase assay system (Sigma Chemical, St. Louis, MO). Osmolality of all solutions was verified by a vapor pressure osmometer (Wescor, Logan, UT). After incubation C3 was separated from unbound [^{3}H]iodoacetic acid by gel-filtration chromatography on Sepharose G-25 (Pharmacia). Total C3 concentration of eluted fractions was determined by absorbance at 280 nm. Radioalkylation of exposed sulfhydryl sites was quantitated by scintillation counting (Beckman Instruments, Palo Alto, CA). Results are expressed as a percentage of thiolester disruption calculated as (mol [^{3}H]iodoacetic acid/mol total C3) × 100.

Hemolytic studies. Studies examining complement-mediated membrane injury under hyperosmolar conditions utilized standard hemolytic assays. For alternative pathway studies, a rabbit erythrocyte (RBC) hemolytic assay was employed (21). Rabbit whole blood was defibrinated on glass beads and RBC suspended at a concentration of 5.0×10^7 RBC/ml in 142 mM NaCl, 0.1% gelatin, 11.0 mM glucose, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2.5 mM $MgCl_2$, and 10 mM barbital sodium (Merck, Rahway, NJ) (Mg-EGTA). Rabbit RBC (0.5 ml) were incubated for 45 min at 37°C in the presence of serial dilutions of Mg-EGTA-chelated normal human serum. Incubations were performed in the presence of isosmolar control buffer and with graded increments in NaCl and urea. This was achieved by supplementing Mg-EGTA buffer with NaCl and urea to achieve the desired concentration of each in a final incubation volume of 1.0 ml. The reaction was halted by the addition of 4 ml 150 mM NaCl, RBC were pelleted, and hemolysis was quantitated by the absorbance at 414 nm of free hemoglobin in the supernatant. Results are expressed as CH_{50} units (21). After the initial reading, supernatants were decanted and RBC pellets were resuspended in isotonic saline. These were again centrifuged and additional hemolysis assayed spectrophotometrically.

Additional rabbit RBC studies were performed to examine the effect of organic osmolytes on alternative pathway function in a hyperosmolar milieu. Osmolytes studied included betaine, sorbitol, and inositol, each at a concentration of 50 mM as approximated from the studies of Yancey and Burg (34). Glycerophosphorylcholine was also studied; however, it consistently induced hemolysis of rabbit RBC in serum-free buffer, thereby preventing its use in this assay system. Hemolytic studies were performed with each osmolyte and graded increments of NaCl and urea identical to the above studies. Mannitol (50 mM) and 10 mM NH_4Cl were also studied as additional controls.

Membrane-directed injury mediated by the classical complement pathway under hyperosmolar conditions was studied using a standard hemolytic assay (12). Briefly, citrated sheep RBC (Kroy Laboratories, Stillwater, MN) were washed in 10 mM EDTA, 142 mM NaCl, 3 mM $NaHCO_3$, 5 mM barbital sodium (EDTA-GVB) and adjusted to 10^9 RBC/ml. Washed RBC were then incubated with a 1:300 dilution of goat anti-sheep RBC IgG (Cappel Laboratories, West Chester, PA) in EDTA-GVB for 30 min at 37°C. After sensitization sheep RBC were washed once in EDTA-GVB and then twice in 0.1% gelatin, 5 mM barbital sodium, 142 mM NaCl, 3 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$ and suspended at a concentration of 5×10^8 RBC/ml. Under osmolar conditions identical to those used in the alternative pathway studies, sensitized sheep RBC were incubated with serial dilutions of normal human serum for 60 min at 37°C in a final incubation volume of 7.5 ml. Suspensions were centrifuged, and hemolysis was determined by absorbance at 541 nm of supernatant. Results are expressed as CH_{50} units.

Cytolytic injury of MDCK cells. Madin-Darby canine kidney cells (MDCK) were obtained from American Type Culture Collection (Bethesda, MD) at passage 60. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) in 95% air-5% CO_2 at 37°C. DMEM was supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Studies were performed on cultures over a range of no more than six passages. After reaching confluence, cells were loaded with ^{51}Cr for quantitation of cytolytic injury as previously described (3). After aspiration of DMEM, each well was incubated with 2.0 μCi sodium [^{51}Cr]chromate (Amersham) in 0.2 ml Hanks' balanced salt solution (HBSS; GIBCO) for 3 h at 37°C. Cells were then gently washed twice with HBSS to remove excess label. To explore the effect of hyperosmolar NaCl and urea on cytolytic injury triggered by ammonia, MDCK were incubated in either isotonic human serum or a serum supplemented with 400 mosmol/kg NaCl and 400 mosmol/kg urea. Both isotonic and hypertonic sera were supplemented with 500 μM NH_4Cl and cells were incubated for 12 h at 37°C. To enhance the susceptibility of MDCK to complement-mediated lysis, these studies were also performed after the treatment of MDCK with neuraminidase for 30 min at a concentration (4.0 U/ml), which is not itself cytolytic (32). After incubation, serum was aspirated and centrifuged to pellet detached cells, and the supernatant removed. ^{51}Cr in the supernatant, which corresponds to cytolytic ^{51}Cr release, was quantitated by gamma counting (Beckman Instruments, Richmond, CA). Percentage lytic release was calculated as the lytic ^{51}Cr release divided by the total ^{51}Cr in each well.

Neutrophil studies. Neutrophils were separated from heparinized whole blood drawn from healthy human volunteers by dextran sedimentation and density centrifugation with lymphocyte separation medium (Litton Bionetics, Kensington, MD). After lysis of RBC with isotonic NH_4Cl, neutrophils were washed twice in calcium- and magnesium-free HBSS with 0.1% gelatin (GHBSS). Neutrophil purity and viability (assessed by trypan blue exclusion before and after incubation studies) were >97%. Neutrophils were suspended in GHBSS with 1.3 mM $CaCl_2$ and 0.9 mM $MgCl_2$ and studied immediately after separation.

Studies of neutrophil responses utilized both soluble and particulate stimuli. Soluble stimuli included *N*-formyl-Met-Leu-Phe (fMLP; Sigma) and phorbol 12-myristate 13-acetate (PMA; Sigma), and microspheres coated with either gelatin or $NH_3 \cdot C3$ served as particulate stimuli. $NH_3 \cdot C3$ was prepared according to published methods (14) by incubating purified human C3 in 100 mM NH_4Cl, 100 mM Tris-10 mM EDTA (pH 8.0) for 80 min in a 37°C water bath. Excess NH_4Cl was removed by exhaustive dialysis of $NH_3 \cdot C3$ in phosphate-buffered saline (PBS). Thiolester disruption was quantitated as described above with all preparations demonstrating >85% disruption at this concentration of ammonia. Coupling of $NH_3 \cdot C3$ to 0.9-μm microspheres (Covaspheres, Duke Scientific, Palo Alto, CA) was achieved as previously described (14) by first dialyzing $NH_3 \cdot C3$ (0.4 mg/ml) overnight at 4°C in 100 mM $NaHCO_3$-500 mM NaCl (pH 8.5) and then incubating with 10^{10}

microspheres for 1 h at room temperature. This method deposits ~15,000 molecules of $NH_3 \cdot C3$/microsphere (10). $NH_3 \cdot C3$-coated microspheres were washed three times in PBS with 0.1% gelatin and suspended in GHBSS to yield a final incubation ratio of 100 microspheres/neutrophil for degranulation studies and 1,000/neutrophil for $O_2^-\cdot$ generation studies. For degranulation studies, microspheres coated with gelatin were prepared in a manner identical to that for $NH_3 \cdot C3$-coated microspheres. After pretreatment of neutrophils with cytochalasin B (5 μg/ml, Sigma) for 5 min at room temperature, degranulation studies were performed by incubating neutrophils at a concentration of 5×10^6/ml with gelatin-coated microspheres, $NH_3 \cdot C3$-coated microspheres, PMA (10 ng/ml) and soluble fMLP (10^{-7} M) for 30 min at 37°C in a shaking incubator. Incubation media consisted of isotonic GHBSS either alone or adjusted to 400 mosmol/kg NaCl and 400 mosmol/kg urea in a final volume of 0.4 ml. After incubation, neutrophil suspensions were centrifuged and MPO released into the supernatant was measured by the microtiter method of Webster and Henson (33). This assay utilizes the HOCl induced oxidation of *o*-dianisidine quantitated at 450 nm. Total MPO content was determined for each experiment by lysis of 2×10^6 neutrophils (PMN) with Triton X-100 (Sigma). MPO release in experimental samples was expressed as a percentage of total cellular MPO. In separate studies, 2×10^6 PMN at each osmolar increment were sonicated and assayed for MPO to assess any direct effect of hyperosmolality on MPO enzymatic function.

Neutrophil production of $O_2^-\cdot$ was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm according to the microassay of Pick and Mizel (22). Neutrophils were pretreated with 5 μg/ml cytochalasin B (except for PMA studies), adjusted to 10^6/ml, and incubated with $NH_3 \cdot C3$-coated microspheres (1,000/neutrophil), fMLP (10^{-6} M), and PMA (100 ng/ml) for 15 min at 37°C under osmolar conditions identical to those in degranulation studies. Total incubation volume was 0.2 ml. Results were calculated using a millimolar extinction coefficient at 550 nm of 21.1 cm^{-1} with a light path of 0.6 cm (22) and expressed as nanomoles of $O_2^-\cdot$ per 2×10^5 PMN.

Statistical analysis. Data are presented as means ± SE. Comparisons between two groups employ the unpaired Student's *t* test. For comparisons involving three or more groups, a one-way analysis of various (ANOVA) with subsequent analysis by an appropriate multiple comparisons test (Dunnett, Newman-Keuls, Bonferroni) was used. Differences were considered significant for $P < 0.05$.

RESULTS

In vitro complement studies. Figure 1 depicts the results of thiolester titration studies. Incubation of C3 in Tris-EDTA buffer without ammonia (controls) revealed 4.8 ± 0.1% thiolester disruption. This finding is in agreement with previous measurements of thiolester disruption in fresh C3 preparations from human plasma (14, 15, 18). However, all three ammonia-containing media produced significant and equivalent degrees of thiolester disruption during incubation. Percentage thiolester disruption in urine (19.2 ± 3.7%), Tris-EDTA plus NH_4Cl (21.0 ± 0.2%), and Tris-EDTA plus NH_4Cl and hyperosmolar NaCl and urea (19.2 ± 3.8%) were all significantly greater than that in Tris-EDTA alone ($P < 0.05$). In a single dose-response experiment, the measurements of C3 thiolester disruption in 5, 10, and 50 mM NH_4Cl and hyperosmolar NaCl and urea were 7.3, 18.3, and 34.5%, respectively, after subtraction of the background thiolester disruption in the presence of hyperosmolar NaCl and urea without NH_4Cl. These results demonstrate that if pH and ammonia concentration are held constant, concentrations of hyperosmolar Na, urea, and urinary osmolytes analogous to those within the medullary interstitium do not impede disruption of the C3 thiolester by ambient ammonia. In addition, as shown by the dose-response experiments, hyperosmolar NaCl and urea do not impair thiolester disruption by concentrations of ammonia higher or lower than those observed in urine.

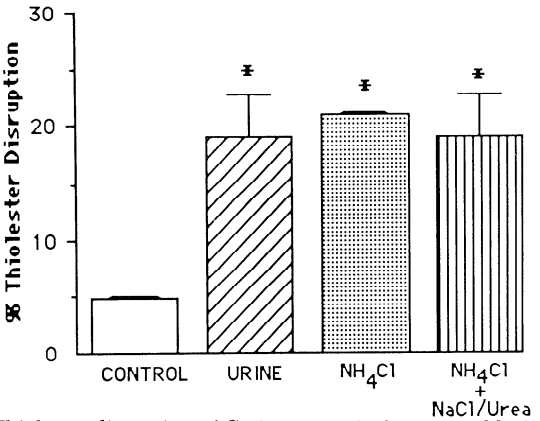

Fig. 1. Thiolester disruption of C3 in ammonia-free control buffer, human urine, buffer and NH_4Cl, and buffer with NH_4Cl and hyperosmolal NaCl (500 mosmol/kg) and urea (500 mosmol/kg). Values represent results of 4 experiments. * $P < 0.05$ vs. control by Dunnett's test. Total ammonia concentration in ammonia-containing solutions is 17.5 mM, which results in 15.6 μM NH_3 at pH 6.22.

The effect of hyperosmolar conditions on the function of the alternative complement pathway as a whole was assessed by quantitating membrane-directed cytolytic injury. A rabbit RBC hemolytic assay served as our model system. As shown in Fig. 2, increments in NaCl and urea concentration resulted in a dramatic and stepwise suppression of membrane injury mediated by the alternative complement pathway. CH_{50} values at each osmolar increment were significantly different from those at all other increments ($P < 0.05$) and ranged from 20.14 ± 0.09 under isotonic conditions to 0.04 ± 0.02 in a buffer containing 400 mosmol/kg NaCl and 400 mosmol/kg of urea. The mammalian renal medulla easily attains NaCl and urea concentrations of this magnitude (5). Studies of membrane-directed injury mediated by the classical complement pathway revealed similar stepwise inhibition of

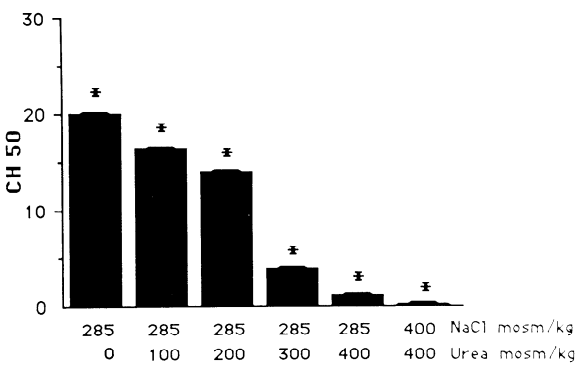

Fig. 2. Hemolytic activity of alternative complement pathway under varying osmolar conditions. Values represent 4 separate CH_{50} determinations at each osmolar increment. * $P < 0.05$ vs. all groups by Newman-Keuls test.

hemolysis under hyperosmolar conditions (Fig. 3). CH_{50} in isotonic media (33.76 ± 1.07) differed significantly ($P < 0.05$) from all other media tested. Again, greatest inhibition of cytolysis was found at 400 mosmol/kg NaCl and 400 mosmol/kg of urea, where CH_{50} was 5.97 ± 1.68. Figure 4 compares the inhibition of the alternative pathway by hyperosmolality with that of the classical pathway. Expressed at each osmolar increment as a percentage of isosmolar CH_{50}, the alternative pathway demonstrated greater sensitivity to the inhibitory effect of hyperosmolality at the three highest osmolalities tested ($P < 0.05$).

After initial determinations of hemolysis, rabbit RBC were resuspended in isotonic saline. Additional hemolysis remained <1.5% of total for all osmolar increments after resuspension. This failure of an isotonic milieu to support further hemolysis argues against the possibility that a functional membrane attack complex exists but is masked by hypertonicity, which impairs transmembrane water flux and prevents hemolysis. Additional rabbit hemolytic studies are depicted in Table 1. Although several solutes studied further inhibited hemolysis, none counteracted the perturbing effect of hyperosmolar NaCl and urea on alternative pathway cytolytic function. Table 2 illustrates the results of cytolytic studies of MDCK cells. In cells not treated with neuraminidase, release of ^{51}Cr in isotonic serum supplemented with 500 μM NH_4Cl was 62.9 ± 1.1%; in hyperosmolar serum supplemented with 500 μM NH_4Cl, ^{51}Cr release was reduced by nearly 40%, to 38.0 ± 0.9% ($P < 0.05$). The same relationship obtained for MDCK cells after treatment with neuraminidase. Again, hyperosmolar serum reduced cytolytic injury by 37.6% (75.1 ± 0.5% in isotonic serum to 46.8 ± 1.4% in hyperosmolar serum, $P < 0.05$). Hyperosmolar NaCl and urea significantly inhibited cytolytic injury induced by NH_3 and complement in cells treated and untreated with neuraminidase.

In vitro neutrophil studies. $NH_3 \cdot C3$ stimulates phagocytic cells by interacting with membrane complement receptors and, as demonstrated above, can be formed in a hyperosmolar milieu from ambient ammonia. We therefore studied the ability of $NH_3 \cdot C3$ to stimulate neutrophil degranulation and oxidative responses under hyperosmolar conditions. Initial studies of MPO enzymatic function under hyperosmolar conditions (Fig. 5) demonstrated significant inhibition of MPO-induced oxidation of *o*-dianisidine ($P < 0.01$). Subsequent degranulation results were therefore corrected for this 30% inhibition of MPO activity by hyperosmolar NaCl and urea.

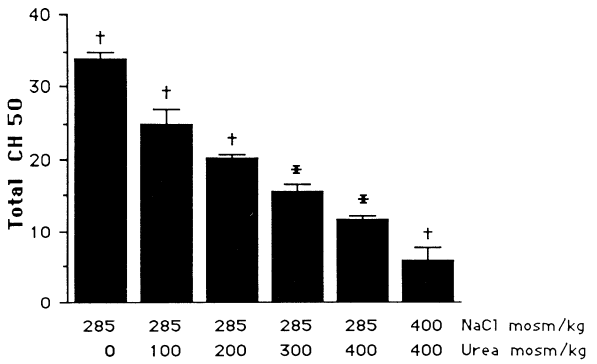

Fig. 3. Classical pathway hemolytic activity under varying osmolar conditions. Values represent 3 total CH_{50} determinations at each osmolar increment. * $P < 0.05$ vs. control by Dunnett's test. $P < 0.05$ vs. all groups by Neuman-Keuls test.

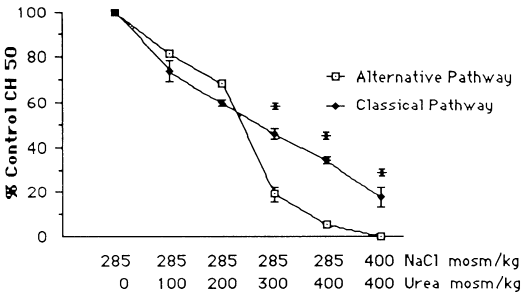

Fig. 4. Hemolytic activity of both complement pathways expressed at each osmolar increment as a % of isotonic CH_{50}. * $P < 0.05$ vs. alternative pathway by unpaired Student's *t* test.

Control studies of MPO release under isosmolar conditions from neutrophils incubated with gelatin-coated microspheres (100/PMN) revealed 1.64 ± 0.24% release. This is significantly less than release produced by $NH_3 \cdot C3$-coated microspheres under identical conditions (6.53 ± 0.46%, $P < 0.01$); these results confirm previous reports that $NH_3 \cdot C3$ serves as a specific neutrophil stimulus (10, 14). Basal production of O_2^-• by unstimulated neutrophils was 0.96 ± 0.55 nmol/2 × 10^5 PMN in an isotonic buffer and 0.27 ± 0.19 nmol/2 × 10^5 PMN in hyperosmolar NaCl and urea.

Neutrophils incubated with microspheres coated with $NH_3 \cdot C3$ displayed marked inhibition of degranulation and O_2^-• production by a hyperosmolar NaCl and urea medium (Fig. 6). MPO release and O_2^-• production fell from 6.53 ± 0.46% and 2.19 ± 0.39 nmol/2 × 10^5 PMN, respectively, under isotonic conditions to 2.61 ± 0.26% and 0.07 ± 0.05 nmol/2 × 10^5 PMN ($P < 0.01$) in the presence of 400 mosmol/kg of both NaCl and urea. In studies of soluble ligands, both fMLP and PMA were used. Stimulation with the prototypic neutrophil ligand fMLP yielded results similar to those with $NH_3 \cdot C3$ (Fig. 7). O_2^-• production decreased from isotonic values of 3.83 ± 0.60 to 1.49 ± 0.46 nmol/2 × 10^5 PMN ($P < 0.01$) in hyperosmolar conditions. MPO release in response to fMLP trended downward from 73.99 ± 3.80 to 61.30 ± 5.48% but did not achieve statistical significance ($P = 0.085$). Neutrophil stimulation with PMA, a specific activator of protein kinase C, was studied under hyperosmolar conditions to better localize the site in neutrophil signal transduction inhibited by hyperosmolality (Fig. 8). Significant reductions in both MPO release (59.77 ± 1.90 to 13.26 ± 0.61%, $P < 0.01$) and O_2^-• production (4.00 ± 0.28 to 0.58 ± 0.17 nmol/2 × 10^5 PMN, $P < 0.01$) demonstrated potent inhibition of PMA-induced neutrophil activation by hyperosmolar NaCl and urea.

DISCUSSION

The presence of an internal thiolester bond within the α-chain of the C3 molecule and its susceptibility to modification by nucleophiles such as ammonia have been extensively studied (17, 18). This thiolester bond, formed between cysteinyl and glutamyl residues, occupies a central role in the structure-function relationships of the C3

Table 1. *Effect of osmolytes on alternative complement hemolytic function*

NaCl, mosmol/kg:	285	285	285	285	400
Urea, mosmol/kg:	100	200	300	400	400
Added solute					
Control	18.09±0.42	15.95±0.35	11.02±0.83	9.64±0.70	0.60±.043
Betaine (50 mosmol/kg)	18.84±0.29	15.76±0.12	11.21±0.31	11.05±0.33	0.09±0.02
Sorbitol (50 mosmol/kg)	16.04±0.10*	14.14±0.21*	5.33±0.57*	7.94±2.23	0.17±0.10
Inositol (50 mosmol/kg)	16.88±0.14	14.95±0.16	6.84±0.75*	10.64±0.60	0.07±0.03
Mannitol (50 mosmol/kg)	17.22±0.35	15.44±0.10	10.65±0.66	8.92±0.71	1.37±0.40
NH_4Cl (10 mM)	13.38±0.14*	6.13±0.94*	1.86±0.54*	1.85±0.87*	0.46±0.15

Values are means ± SE expressed as CH_{50}. Each value represents a minimum of 5 separate CH_{50} determinations. * $P < 0.05$ vs. control.

Table 2. *Effect of isotonic and hyperosmolar buffers on release of ^{51}Cr from MDCK cells*

Neuraminidase	^{51}Cr Release, %	
	Isotonic serum	Hypertonic serum
−	62.9±1.1	38.0±0.9*
+	75.1±0.5	46.8±1.4*

Values are means ± SE from MDCK cells after incubation with serum and 500 μM NH_4Cl. Experiments were performed in absence and presence (4 U/ml) of neuraminidase. Values represent results from 5 replicate wells in each group. * $P < 0.05$ vs. isotonic serum.

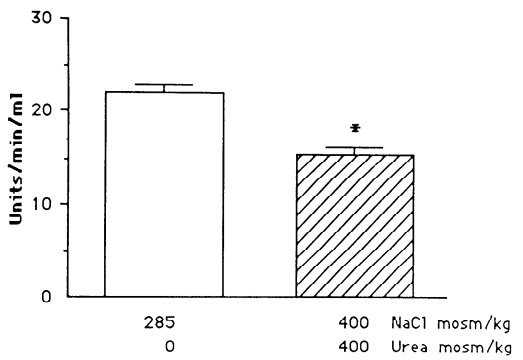

Fig. 5. Myeloperoxidase (MPO) enzymatic function in a hyperosmolar milieu. Values represent results of 5 experiments. * $P < 0.01$ by unpaired Student's t test.

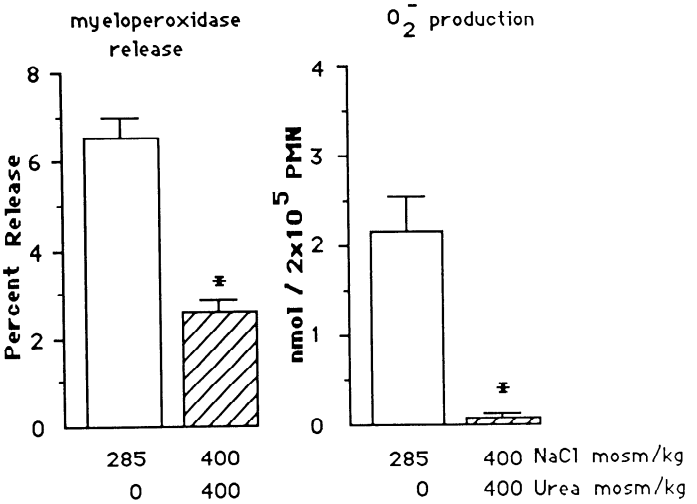

Fig. 6. Neutrophil responses to $NH_3 \cdot C3$-coated microspheres under hyperosmolar conditions. $n = 6$ for MPO release; $n = 7$ for superoxide anion ($O_2^-\cdot$) production. * $P < 0.01$ vs. isotonic controls by unpaired Student's t test. PMN, polymorphonuclear leukocytes.

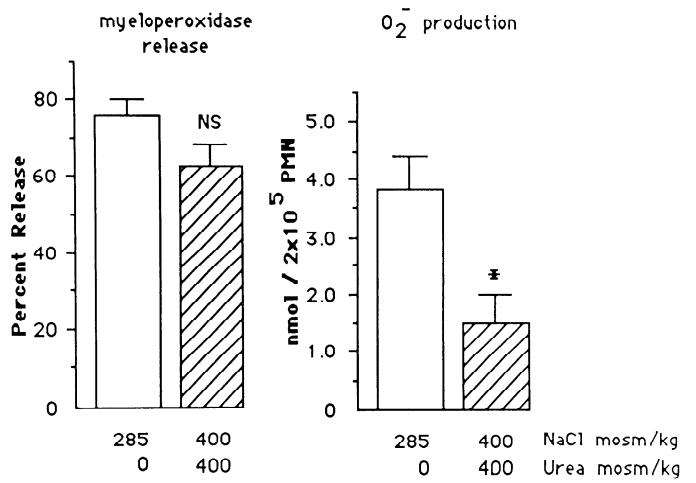

Fig. 7. Neutrophil response to N-formyl-Met-Leu-Phe. $n = 6$ for MPO studies; $n = 8$ for $O_2^-\cdot$ studies. * $P < 0.01$ vs. isotonic control; NS, not significant.

molecule (16). Disruption of the thiolester by ammonia yields an amide linkage at the glutamyl carbonyl site and induces a conformational change in the C3 molecule (13, 17). This $NH_3 \cdot C3$ possesses C3b-like properties and in the presence of complement factors B, D, properdin, and magnesium ions forms the alternative pathway convertase (13). Furthermore, the binding of $NH_3 \cdot C3$ to membrane complement receptors on phagocytic cells stimulates degranulation and triggers the production of reactive oxygen species (10, 14).

Tissue injury directly attributable to modification of C3 by ammonia has been demonstrated in a variety of models characterized by increased local generation of ammonia. In an in vitro model of ischemic tissue injury, Hostetter and Johnson (14) proved that production of ammonia from the action of RBC adenosine deaminase on adenosine was sufficient to form $NH_3 \cdot C3$. In subtotal renal ablation and hypokalemic nephropathy, two models of chronic renal disease in the rat characterized by increased partial pressures of ammonia within the renal cortex, amidation of C3 contributed to cortical tubulointerstitial injury as demonstrated by functional and immunohistochemical studies. Furthermore, amelioration of renal injury occurred in these rat models when ammoniagenesis was suppressed by exogenous alkali (20, 31).

Under physiological conditions, the concentration of ammonia in the renal cortex closely parallels that in renal venous plasma (8). In the renal medulla, however, physiological ammonia concentrations exceed those in the

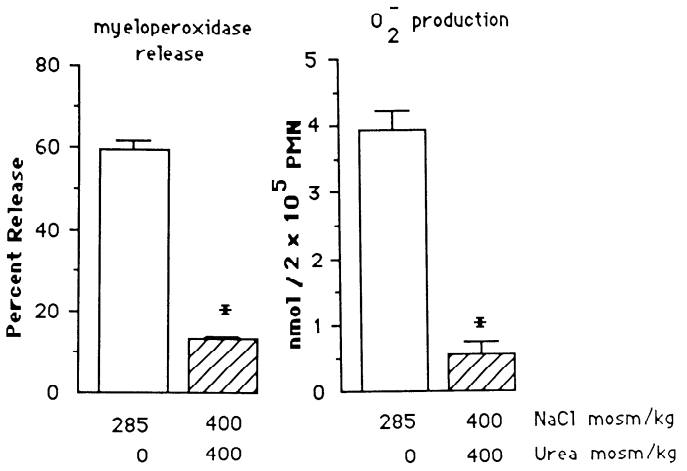

Fig. 8. Neutrophil response to phorbol 12-myristate 13-acetate. $n = 4$ for MPO and O_2^-• values. * $P < 0.01$ vs. control.

cortex by nearly 100-fold (27). Despite these high medullary levels of ammonia under normal conditions, evidence of ammonia-triggered complement activation and inflammation at this tissue site is absent. Additionally, several models of inflammatory disease demonstrate a substantially attenuated inflammatory response within the renal medulla. Freedman and Beeson (9) established bacterial infection in the rat renal medulla by intrarenal injection of as few as 10 organisms, whereas sustained cortical infection required injection of $\sim 10^5$ organisms. Employing another rat model of pyelonephritis, Andreoli and Epstein (2) demonstrated that the medullary inflammatory response and capacity to clear microorganisms improved markedly with osmotic diuresis. The inflammatory response to thermal injury in rats also normalized with osmotic diuresis (1). Studies of acute renal allograft rejection in humans also identified a diminished or delayed inflammatory response in the renal medulla compared with the cortex (11). These findings implicate the hyperosmolar milieu of the renal medulla in suppression of inflammatory responses. An inhibitory role of hyperosmolality has also been demonstrated for several neutrophil functions and hemolysis mediated by the classical complement pathway (6, 7, 19). However, no studies have yet examined the effects of physiological medullary concentrations of urea, NaCl, and ammonia on the C3 molecule specifically and on alternative complement pathway function as a whole.

The current studies demonstrate an unrestrained ability of ammonia to disrupt the C3 thiolester under osmolar conditions representative of the renal medullary interstitium. This finding suggests that the absence of ammonia-triggered injury in the normal renal medulla does not result from impaired amidation of C3 but instead involves inhibition of more distal events in ammonia-mediated inflammation. These include membrane-directed cytolytic injury by complement proteins and ligand-receptor interactions of $NH_3 \cdot C3$ with inflammatory cells. Our findings identify potent suppression by hyperosmolar NaCl and urea of cytolysis mediated by both complement pathways. In relative terms, the alternative pathway is more sensitive to hyperosmolar inhibition than is the classical pathway at higher osmolalities (Fig. 4). Because the terminal membrane attack complex is common to both pathways, this differential sensitivity seems most likely to be due to impaired formation of the alternative pathway convertase. Direct relevance of these studies for renal tissue is suggested by the results of studies with MDCK. Incubation of these cells, derived from canine distal tubular epithelium, with serum and 500 μM NH_4Cl (the latter concentration easily attained in the medulla) results in significant cell lysis in isotonic conditions, even without neuraminidase treatment. However, in the presence of hyperosmolar NaCl and urea, cytolysis is reduced by nearly 40%, both in the absence and the presence of neuraminidase.

The precise mechanism of inhibition of complement-mediated lysis remains unclear. The complement pathways consist of a complex series of interactions among complement proteins, substrates, and ionic cofactors. The capacity of hyperosmolar NaCl and urea to perturb macromolecular structure and function is well documented in both mammalian and nonmammalian enzyme systems (26, 36). Conversely, a number of intracellular osmolytes, primarily trimethylamine compounds and polyhydric alcohols, have been identified in renal medullary cells whose theorized function is to maintain cell volume and preserve macromolecular function in the setting of hyperosmolality (4). The current studies, however, demonstrate an inability of betaine, sorbitol, and inositol to reverse the inhibitory effect of significant hyperosmolality on alternative pathway hemolytic function.

Similar to those in previous studies (10, 14), our findings have demonstrated stimulation of neutrophil degranulation and O_2^-• production by $NH_3 \cdot C3$ in an isotonic medium. These responses are significantly inhibited by hyperosmolar NaCl and urea. Parallel inhibition of responses to fMLP suggests a more generalized impairment of neutrophil responses to surface ligands by hyperosmolality instead of a specific inhibitory effect on $NH_3 \cdot C3$ alone. Potent suppression of neutrophil responses to PMA, a protein kinase C activator, further suggests that this inhibitory effect of hyperosmolar NaCl and urea resides largely at or distal to the site of action of protein kinase C in the signal transduction pathway of the neutrophil. However, concurrent impairment of more proximal events such as ligand-receptor binding, G-protein coupling or phospholipase C function cannot be excluded by these studies. Possible mechanisms underlying this impairment of neutrophil function by hyperosmolar NaCl and urea include acute changes in cell volume (36), shedding of complement receptors (30), and perturbation of the structure and function of macromolecules necessary for signal transduction. Extracellular hypertonicity has also been shown to suppress degranulation in adrenal chromaffin cells by preventing osmotic swelling and lysis of granules (24). A significant role for this mechanism in these studies seems unlikely given the concurrent impairment of O_2^-• generation.

Considered together, these findings confirm that ambient ammonia in concentrations found within the renal medulla modifies the third component of human complement. However, medullary hyperosmolality impairs

subsequent membrane directed and ligand receptor mediated injury triggered by $NH_3 \cdot C3$. In this setting hyperosmolality serves to protect the renal medulla from the phlogistic capacity of ambient ammonia. This generalized suppression of alternative complement and neutrophil function also provides a mechanism that may underlie the less exuberant inflammatory response seen in the renal medulla in a variety of disease states. Additionally, diminished medullary hyperosmolality and impaired urinary concentrating ability as seen in chronic renal insufficiency may contribute to progressive tubulointerstitial injury. In this setting, attenuation of hyperosmolality's suppressive action on complement and neutrophil function may foster injury triggered by ambient ammonia.

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