



## On the Immune Relationship Between Urea, Human Mononuclears and Colon Cancer Cells

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### Abstract

**Background:** Increased serum urea level is an early herald of chronic renal failure. Inflammatory cytokines produced by Peripheral Blood Mononuclear Cells (PBMC) upon the effect of urea and other toxins initiate and maintain inflammation that persist and converts to a chronic state. Since chronic inflammation is a predisposing factor for carcinogenesis it was the aim of the study to examine the effect of urea on the immune dialogue between PBMC and human colon cancer cells from two lines (HT-29 and RKO).

**Methods:** Cytokine production by non-stimulated human PBMC or cells stimulated with either mitogen or with HT-29 and RKO colon cancer cells were incubated for 24 hrs with various concentrations of urea (40-200 mg/dl) and the secretion of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IFN $\gamma$ , IL-2, IL-10 and IL-1ra, was determined by ELISA.

**Results:** Urea caused increased secretion of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$  and the anti-inflammatory cytokine IL-10 by non-stimulated PBMC and enhanced production of IFN $\gamma$  by PMA/ionomycin stimulated cells. In addition, the secretion of TNF $\alpha$ , IFN $\gamma$  and IL-2 induced by RKO colon cancer cells was elevated following 24 hrs of incubation with urea, whereas the synthesis of IL-1 $\beta$  induced by HT-29 cells was reduced.

**Conclusion:** Although the results indicate that urea may exert a certain concentration- and cell dependent effect on the immune equilibrium between PBMC and colon cancer cells, the impaired immunity observed in patients with chronic renal failure cannot be the principal promoter for increased cancer risk reported in those individuals.

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### Introduction

Although the initial stages of chronic kidney disease could be asymptomatic, the results of appropriate laboratory investigations, such as impairment of glomerular filtration rate, increased serum urea values and proteinuria serve as early omens of its progression [1]. With advancement of the disease the gradually increasing urea levels are associated with monocyte activation, promotion of chronic inflammation with consequent immune alterations and renal fibrosis [2-5]. At the end-stage of renal disease the damage of the immune system involves not only functionally altered monocytes, but also other cellular blood components, such as lymphocytes, macrophages and polymorpho nuclear cells [6,7]. It is well established that uremic patients are at high risk for infections and that at the end-stage renal failure is associated with a premature aging of the immune system affecting the lymphoid cells predominantly [8]. It is of interest that uremia-associated alterations induced on the T-cell system remain constant and are not reversed upon kidney transplantation [3]. Notable, the immune dysfunction at end-stage renal failure is an intricate process involving not only negative effects of urea but also a substantial number of cellular and humoral factors circulating in patients' blood [6]. Furthermore, epidemiological studies have indicated that uremia is associated with a great risk for carcinogenesis [9]. However, reports on the subject reveal that there is no general agreement as for linkage between these two conditions [10,11]. Based on the well-established association between chronic inflammation and carcinogenesis [12] and the feasible effect of uremia on both processes, it was the aim of the present study to elucidate the influence of various urea concentrations on the capability of human Peripheral Blood Mononuclear Cells (PBMC) for cytokine production. In addition, since we have previously shown the existence of an immune dialogue between PBMC and HT-29 and RKO colon cancer cells [13] we decided to examine the possibility that urea may alter this equilibrium.

**Table 1:** Effect of urea on pro-inflammatory cytokine production by PBMC.

	Non-stimulated	LPS-stimulated	HT-29	RKO
<b>UREA concentration</b>	<b>TNF<math>\alpha</math>, pg/ml</b>			
<b>0</b>	339 $\pm$ 51	1222 $\pm$ 237	1597 $\pm$ 172	1917 $\pm$ 96
<b>40 mg/dl</b>	329 $\pm$ 45	1334 $\pm$ 206	1686 $\pm$ 121	1709 $\pm$ 86**
<b>100 mg/dl</b>	289 $\pm$ 54	1270 $\pm$ 216	1591 $\pm$ 129	1673 $\pm$ 96
<b>200 mg/dl</b>	615 $\pm$ 136**	1284 $\pm$ 211	1668 $\pm$ 120	1588 $\pm$ 88
<b>Repeated measures</b>	F <sub>3,15</sub> =10.4, P=0.0006	F <sub>3,15</sub> =1.43, P=0.273	F <sub>3,15</sub> =0.85, P=0.48	F <sub>3,15</sub> =8.77, P=0.0013
<b>UREA concentration</b>	<b>IL-1<math>\beta</math>, ng/ml</b>			
<b>0</b>	1.83 $\pm$ 0.25	6.67 $\pm$ 1.23	9.79 $\pm$ 0.54	8.44 $\pm$ 1.01
<b>40 mg/dl</b>	1.77 $\pm$ 0.26	6.75 $\pm$ 1.14	8.99 $\pm$ 0.78**	8.69 $\pm$ 0.82
<b>100 mg/dl</b>	1.81 $\pm$ 0.29	7.09 $\pm$ 1.42	9.28 $\pm$ 0.75†	8.71 $\pm$ 1.14
<b>200 mg/dl</b>	2.01 $\pm$ 0.29***	6.87 $\pm$ 1.13	9.74 $\pm$ 0.72	8.75 $\pm$ 0.87
<b>Repeated measures</b>	F <sub>3,15</sub> =14.51, P=0.0001	F <sub>3,15</sub> =1.12, P=0.373	F <sub>3,15</sub> =6.7, P=0.0043	F <sub>3,15</sub> =0.48, P=0.701
<b>UREA concentration</b>	<b>IL-6, ng/ml</b>			
<b>0</b>	8.82 $\pm$ 1.12	29.87 $\pm$ 0.93	28.02 $\pm$ 0.77	27.62 $\pm$ 1.41
<b>40 mg/dl</b>	7.13 $\pm$ 0.63	29.31 $\pm$ 1.10	26.94 $\pm$ 0.32	27.68 $\pm$ 1.42
<b>100 mg/dl</b>	6.73 $\pm$ 0.79	29.48 $\pm$ 1.35	27.52 $\pm$ 1.11	28.52 $\pm$ 1.99
<b>200 mg/dl</b>	8.06 $\pm$ 0.69	28.36 $\pm$ 1.00	28.85 $\pm$ 0.80	26.29 $\pm$ 1.51
<b>Repeated measures</b>	F <sub>3,15</sub> =2.3, P=0.119	F <sub>3,15</sub> =1.4, P=0.28	F <sub>3,15</sub> =2.88, P=0.70	F <sub>5,15</sub> =1.53, P=0.23

Non-stimulated PBMC or cells stimulated with either LPS or with one of the colon cancer cell lines HT-29 or RKO, were incubated for 24 hrs without (0) or with urea at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean $\pm$ SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without urea (\*\*p<0.01, \*\*\*p<0.001, †p=0.06).

## Materials and Methods

### Cell preparation

**Peripheral Blood Mononuclear Cells (PBMC):** Venous blood was obtained from adult donors who gave written agreement and informed consent that components of the blood might be used for medical research. PBMC were separated from by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The cells were washed twice in Phosphate Buffered Saline (PBS) and suspended in RPMI-1640 medium containing 1% penicillin, streptomycin and nystatin, 10% fetal bovine serum (FBS, Biological Industries, Beith Haemek, Israel) and was designated as Complete Medium (CM).

**Preparation of urea:** Urea was purchased from Sigma-Aldrich, Israel and was freshly dissolved in CM for each experiment. Urea was added at the onset of cultures at a volume of 10 $\mu$ l/ml in final concentrations of 40 mg/dl, 100 mg/dl and 200 mg/dl.

**Colon cancer cell lines:** HT-29 and RKO human colon cancer cell lines were obtained from American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-Coy's 5A medium and Dulbecco Modified Eagle Medium (DMEM) respectively, supplemented with 10% FBS, 2mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin-Biological Industries Co, Beith-Haemek, Israel). The cells were grown in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Effect of urea on cell proliferation:** The effect of urea on PBMC and colon cancer cells proliferation was determined using XTT proliferation assay kit (Biological Industries, Beith Haemek, Israel). Briefly, 0.1 ml aliquots of PBMC suspended in CM or colon cancer cells obtained after typosinization and suspended in appropriate CM (10<sup>5</sup>/ml of CM) were added to each one of 96 well plates and incubated for 24 hrs in the absence or presence of urea at concentrations as

indicated. At the end of the incubation period the cells were stained according to the manufacturer's instructions. The plates were incubated for 2-4 hrs at 37°C in a humidified incubator containing 5% CO<sub>2</sub> and the absorbance was measured at 450 nm using ELISA reader.

**Effect of urea on cytokine production:** For TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, and IL-1ra production 1 ml of PBMC (2x10<sup>6</sup>/ml of CM) was incubated without (non-stimulated) or with LPS (50ng/ml), and for IL-2 and IFN $\gamma$  secretion the cells were incubated with PMA 1 $\mu$ g/ml and ionomycin 0.5 $\mu$ g/ml (Sigma, Israel). In another set of experiments, 0.5 ml aliquots of PBMC (4x10<sup>6</sup>/ml of CM) were incubated with 0.5 ml of either HT-29 or RKO colon cancer cells (4x10<sup>5</sup>/ml) suspended in appropriate CM. Urea was added at the onset of cultures at concentrations as indicated. The cultures were maintained for 24 hrs at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. At the end of the incubation period the cells were removed by centrifugation at 250g for 10 min., supernatants were collected and kept at -70°C until assayed for cytokines content.

**Cytokine content in the supernatants:** The concentration of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IFN $\gamma$ , IL-2, IL-10 and IL-1ra in the supernatants was tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA) as detailed in the guide-line provided by the manufacturer. The detection level of these kits was 30 pg/ml.

**Statistics:** A linear mixed model with repeated measures and assumption of Compound Symmetry (CS) was used to assess the effect of different concentrations of urea on cytokine secretion by non-stimulated or stimulated PBMC. SAS vs 9.4 were applied for this analysis. Paired t-test was used to compare between the level of cytokines produced following incubation with various concentrations of urea and that found in control cultures. Probability values of p<0.05 were considered as significant. The results are expressed as

**Table 2:** Effect of urea on IL-2 and IFN $\gamma$  production by PBMC.

UREA concentration	IFN $\gamma$ , ng/ml			
	Non-stimulated	PMA-stimulated	HT-29	RKO
0	2.32 $\pm$ 0.30	18.61 $\pm$ 1.21	5.82 $\pm$ 0.54	7.53 $\pm$ 0.63
40 mg/dl	2.24 $\pm$ 0.39	20.31 $\pm$ 1.16†	5.82 $\pm$ 1.13	8.10 $\pm$ 0.84
100 mg/dl	2.97 $\pm$ 0.68	25.55 $\pm$ 3.57**	5.83 $\pm$ 1.04	10.20 $\pm$ 1.97*
200 mg/dl	3.36 $\pm$ 0.74*	19.61 $\pm$ 1.61	6.57 $\pm$ 0.96	6.93 $\pm$ 0.67
Repeated measures	F <sub>3,15</sub> =3.62, P=0.038	F <sub>3,15</sub> =6.46, P=0.005	F <sub>3,15</sub> =0.9, P=0.46	F <sub>3,15</sub> =4.08, P=0.026
IL-2, ng/ml				
0	-	31.96 $\pm$ 2.87	1.43 $\pm$ 0.07	1.54 $\pm$ 0.16
40 mg/dl	-	29.74 $\pm$ 4.76	1.66 $\pm$ 0.21	1.61 $\pm$ 0.08
100 mg/dl	-	33.47 $\pm$ 3.01	1.47 $\pm$ 0.08	2.58 $\pm$ 0.35**
200 mg/dl	-	33.17 $\pm$ 3.02	1.47 $\pm$ 0.13	1.53 $\pm$ 0.12
Repeated measures	-	F <sub>3,15</sub> =0.82, P=0.516	F <sub>3,15</sub> =0.69, P=0.57	F <sub>3,15</sub> =8.96, P=0.0012

Non-stimulated PBMC or cells stimulated with either PMA and ionomycin, or with one of the colon cancer cell lines HT-29 or RKO, were incubated for 24 hrs without (0) or with urea at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean $\pm$ SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without urea (\*p<0.05, \*\*p<0.01, †p=0.07).

mean  $\pm$  SEM.

## Results

**Effect of urea on cell viability:** Urea at concentrations between 40 and 200 mg/dl added to PBMC or HT-29 and RKO colon cancer cells for 24 hrs did not exert any effect on cell proliferation as tested by XTT assay (data not shown).

**Effect of urea on TNF $\alpha$ , IL-1 $\beta$  and IL-6 production:** (Table 1) The secretion of the proinflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  by non-stimulated PBMC was promoted upon incubation with urea at the above mentioned concentrations (F<sub>3,15</sub>=10.4, p=0.006 and F<sub>3,15</sub>=14.5, p<0.001, respectively). At 200 mg/dl of urea the secretion of TNF $\alpha$  was 1.8 times higher (p=0.002) and that of IL-1 $\beta$  was enhanced by 10% (p=0.001). At lower urea concentrations the production of both cytokines by non-stimulated PBMC was not modified. There was no effect of urea on the production of TNF $\alpha$  by LPS stimulated PBMC or by PBMC stimulated with HT-29 colon cancer cells (F<sub>3,15</sub>=1.43, p=0.273 and F<sub>3,15</sub>=0.85, p=0.48, respectively). However, TNF $\alpha$  synthesis induced by RKO cells was increased following incubation with urea (F<sub>3,15</sub>=8.77, p=0.0013), and was higher by 5.6% following incubation with 40 mg/dl (p=0.008), whereas at higher concentrations TNF $\alpha$  secretion was not affected. The production of IL-1 $\beta$  by PBMC stimulated with either LPS or RKO cells did not change by urea added at the above mentioned concentrations (F<sub>3,15</sub>=1.12, p=0.37 and F<sub>3,15</sub>=0.48, p=0.70, respectively). On the other hand, IL-1 $\beta$  secretion by HT-29 stimulated PBMC was reduced following incubation with urea (F<sub>3,15</sub>=6.7, p=0.0043) and was lower by 8% at urea concentration of 40 mg/dl (p=0.0043). At higher urea concentrations IL-1 $\beta$  production by RKO stimulated PBMC was reduced but did not reach statistical significance. IL-6 production by non-stimulated PBMC or by cells stimulated with either LPS or by colon cancer cells was not affected following incubation with urea at the three concentrations used (F<sub>3,15</sub>=2.3, p=0.119 and F<sub>3,15</sub>=1.4, p=2.8, F<sub>3,15</sub>=2.88, p=0.07 and F<sub>3,15</sub>=1.53, p=238, respectively).

**Effect of urea on IL-2 and IFN $\gamma$  secretion:** (Table 2) Non-stimulated PBMC incubated without or with urea at the concentrations tested did not secrete IL-2. The production of IL-2 by PMA/ionomycin stimulated PBMC or by cells stimulated with HT-29 cells was not affected by incubation with urea (F<sub>3,15</sub>=0.82, p=0.5 and F<sub>3,15</sub>=0.69,

p=0.57, respectively). However, IL-2 secretion by RKO stimulated PBMC was significantly increased (F<sub>3,15</sub>=8.96, p=0.0012) and was higher by 68% at urea concentration of 100 mg/dl (p=0.0017), but not at the other concentrations applied. IFN $\gamma$  production was increased by non-stimulated PBMC and by cells prompted by PMA/ionomycin and RKO subsequent to 24 hrs incubation with urea (F<sub>3,15</sub>=3.62, p=0.038, F<sub>3,15</sub>=6.46, p<0.005, and F<sub>3,15</sub>=4.08, p=0.026, respectively), but not by HT-29 stimulated PBMC (F<sub>3,15</sub>=0.9, p=0.46). At 100 mg/dl IFN $\gamma$  production by PMA/ionomycin or RKO stimulated PBMC was increased by 35%, whereas that by non-stimulated PBMC was higher by 45% at 200 mg/dl of urea (p=0.04).

**Effect of urea on IL-10 and IL-1ra secretion:** (Table 3) Incubation with urea at the applied concentrations increased IL-10 secretion by non-stimulated PBMC (F<sub>3,15</sub>=3.95, p=0.029) and was higher by 50% at 200mg/dl of urea. IL-10 production by PBMC stimulated with either LPS or with cancer cells of the two cell lines was not affected by 24 hrs of incubation with urea at the three concentrations used in the study. IL-1ra production by non-stimulated, as well as stimulated PBMC by one of the methods used was not influenced by incubation with urea.

## Discussion

A survey of the present results indicates that higher urea concentrations (200 mg/dl) promoted non-stimulated PBMC to increase significantly the production of all inflammatory cytokines examined except IL-1ra. On the other hand, when urea was added to PBMC stimulated with either LPS or PMA this effect was not observed. Considering the fact that both LPS and PMA caused an optimal expression of cytokine production reaching a level more than three times higher in comparison to non-stimulated cells, it is plausible that the PBMC in that setting were not able to respond to further stimulation. According to Girndt et al. [14], while 15%-20% of peripheral blood monocytes may be activated for cytokine production, this percentage is much higher in patients with end-stage renal disease and may reach 50%. The great quantity of inflammatory cytokines secreted by these cells induces chronic inflammation and predisposition to infections. It should be underlined that although all inflammatory cytokines produced in uremic patients play important role in progression of chronic inflammation, the activity of the pro-inflammatory IL-6 and TNF $\alpha$  and that of the anti-inflammatory IL-

**Table 3:** Effect of urea on anti-inflammatory cytokine production by PBMC.

UREA concentration	IL-10, ng/ml			
	Non-stimulated	LPS-stimulated	HT-29	RKO
0	0.48±0.11	1.50±0.17	1.50±0.15	1.42±0.15
40 mg/dl	0.36±0.05	1.45±0.18	1.41±0.12	1.30±0.15
100 mg/dl	0.42±0.08	1.39±0.520	1.41±0.13	1.33±0.13
200 mg/dl	0.72±0.2	1.53±0.19	1.51±0.19	1.34±0.13
Repeated measures	F <sub>3,6</sub> =3.95, P=0.029	F <sub>3,15</sub> =2.24, P=0.125	F <sub>3,15</sub> =1.35, P=0.29	F <sub>3,15</sub> =1.44, P=0.264
UREA concentration	IL-1ra, ng/ml			
	Non-stimulated	LPS-stimulated	HT-29	RKO
0	2.00±0.09	2.44±0.23	2.68±0.22	2.58±0.15
40 mg/dl	2.13±0.12	2.21±0.06	2.53±0.23	2.56±0.24
100 mg/dl	2.06±0.15	2.61±0.17	2.59±0.24	2.67±0.3
200 mg/dl	2.00±0.12	2.34±0.18	2.61±0.24	2.54±0.20
Repeated measures	F <sub>3,15</sub> =0.41, P=0.75	F <sub>3,15</sub> =2.75, P=0.079	F <sub>3,15</sub> =1.24, P=0.330	F <sub>3,15</sub> =0.25, P=0.86

Non-stimulated PBMC or cells stimulated with either LPS or with one of the colon cancer cell lines HT-29 or RKO, were incubated for 24 hrs without (0) or with urea at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean±SEM of 6 experiments.

10 is the principal inducer of immune imbalance in these individuals [15]. It has been reported that increased cytokine production is observed not only in cases with chronic uremia, but also in uremia following acute kidney damage. Uremia in mice subjected to acute kidney injury caused suppressed production of the proinflammatory cytokines TNF $\alpha$  and TGF- $\beta$ 1, while the gene expression of the anti-inflammatory IL-10 was increased. However, the authors acknowledge that the origin of cytokines produced in that system may reflect functional alterations in the proximal tubules [16]. Chronic, persistent inflammation that evokes production of cytokines such as TNF $\alpha$ , TGF- $\beta$  and IL-10 presents a predisposing milieu for carcinogenesis [17]. Since similar conditions exist in uremic patients, the question if malignant tumors develop more frequently in those individuals has intrigued researchers since long. The general concept is that there is a high incidence of cancer in patients with uremia and the subject has been thoroughly reviewed by Digenis et al. [10]. There are a few mechanisms by which uremia may enhance cancer development, including immune alterations [18]. The results of the present study consisting of increased inflammatory IL-2 and IFN $\gamma$  production following addition of urea to PBMC co-cultured with colon cancer cells insinuate the possibility of an additional mechanism, i.e. the impact of urea on the immune dialogue between these cells. The existence of such a cross-talk expressed by altered cytokine production by PBMC upon the influence of HT-29 or RKO colon cancer cells has been demonstrated in a previous study from our laboratory [13]. Factors capable to affect the immune dialogue between these cells have been debated [19]. The results of this work clearly indicate that incubation of a mixture of PBMC and HT-29 cells with concentrations of urea applied in the study did not affect the secretion of any of the cytokines examined. On the other hand, at the same laboratory settings, RKO cells induced a further increase in IL-2 and IFN $\gamma$  secretion by PBMC under the effect of 100 ng/dl urea only. It is conceivable that in our study the influence of urea has been cell- and dose- dependent. Although addition of urea disturbed to a certain degree the immune equilibrium between PBMC and RKO cancer cells, it is ambiguous to assume that the higher urea level in patients with chronic renal failure is the sole promoter of carcinogenesis. It is reasonable that the increased risk for malignancy in uremic patients is not due to the urea itself but to a bulk of toxins and substances existing in the circulation of these patients. Bush et

al. [11] have not found increased percentage of malignant diseases in uremic patients and conclude that the uremic state in these individuals does not predispose to cancer. Working with two groups of sub-totally nephrectomized mice –one kept at normal diet and the second on a low-protein diet and all of them injected thereafter with Erlich's ascites tumor cells, Roll et al. [20] have found that the tumor size in the uremic animals kept on regular diet was significantly smaller than that of mice with urea levels in the normal range.

In short, the results of the study demonstrate that although urea may express a modulatory effect on the immune dialogue between peripheral blood mononuclear cells co-incubated with certain types of cancer cells, this activity cannot be the principal promoter of carcinogenesis in patients with chronic renal failure.

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