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8. A multistep procedure was used to prepare T lymphoblasts and memory cells that were highly enriched (relative to unprimed cells or cells primed in vivo) in carrier-specific helper functions. DC's ($5 \times 10^4$) and Lyt-2$^-$, Ia$^-$ T cells ($5 \times 10^6$) were cultured with defined proteins (KLH, HSA, HGG, and ovalbumin have all been used) for 2 days. Cellular aggregates, which contained most of the DC's and responding T cells, formed in culture and were separated by velocity sedimentation in Percoll (*7*) into cluster ($2 \times 10^5$ to $3 \times 10^5$ cells) and noncluster ($2 \times 10^6$ to $3 \times 10^6$ cells) fractions. The former were cultured with antigen for 3 days, whereupon many of the clustered cells proliferated and released lymphoblasts. The latter were separated from residual clusters (containing most of the DC's) by velocity sedimentation in Percoll. The blasts were used immediately or cultured 2 to 7 days with or without feeder macrophages to provide rested or memory helper cells.
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# Uromodulin: A Unique 85-Kilodalton Immunosuppressive Glycoprotein Isolated from Urine of Pregnant Women

Abstract. *Crude fractions of urine from pregnant women are immunosuppressive in vitro. An 85-kilodalton immunosuppressive glycoprotein purified to homogeneity from such urine inhibited in vitro assays of human T-cell and monocyte activity at concentrations of $10^{-9}$ to $10^{-11}$ molar. This material was nontoxic and blocked early events required for normal T-cell proliferation in vitro. On the basis of its tissue source and its in vitro activity, the name "uromodulin" is proposed for this glycoprotein.*

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Major advances in our understanding of mechanisms involved in cellular regulation of immunity have occurred when previously described supernatants and factors were purified to homogeneity. For example, the purification of interleukin-2 (IL-2) has resulted in extensive characterization of the chemistry and biology of this important factor and has allowed the characterization and cloning of the cell-surface IL-2 receptor (*1, 2*). Similar advances have been associated with the purification of IL-1 and the various interferons. In 1973 two groups of investigators reported that human chorionic gonadotropin (HCG) was immunosuppressive in vitro (*3, 4*). However, the observed immunosuppression proved to be the result of contaminants found in commercial preparations of HCG (*5, 6*).

Crude fractions of urine from pregnant women exhibit nontoxic reversible immunosuppression in vitro. These immunosuppressive compounds might have a role in protecting the placenta from maternal immunosurveillance. Despite the theoretical importance of immunosuppressive factors in human pregnancy urine, there has been little further characterization of these compounds. We isolated from human pregnancy urine a compound with marked immunoregulatory activity in vitro and purified it to homogeneity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This material is an 85-kilodalton glycoprotein that suppresses antigen-specific proliferation in vitro at concentrations as low as 30 p*M*. Using this purified material as an immunogen we developed a sensitive enzyme-linked immunosorbent assay (ELISA) and Western blot assays. We propose the name "uromodulin" for this material because of its source and its ability to modulate immune responses in vitro.

The purification of uromodulin is outlined in Fig. 1. At each fractionation step, bioactivity was assayed in vitro by measuring inhibition of the T-cell proliferation that occurs in response to the antigen tetanus toxoid (*7*). It is not possible to calculate a relative biologic specific activity during the purification procedure since we have found several immunosuppressive factors and have confirmed the presence of mitogenic factors in human pregnancy urine (*8*).

The procedure schematically outlined in Fig. 1 is based on several unique properties of uromodulin that result in a simple three-step purification procedure. Starting with crude pregnancy urine, step 1 takes advantage of uromodulin's being a glycoprotein that binds to immobilized concanavalin A (Con A); this binding results in the removal of approximately 90 percent of the starting protein while retaining 80 to 100 percent of the immunoreactive uromodulin (Fig. 1). Step 2 exploits its relatively large molecular weight and its tendency to form aggregates. Thus, after dialysis against distilled water, uromodulin migrates in the void volume of a Fractogel 55 S column (Merck; exclusion limit 700 kD) despite its much lower apparent molecular weight on SDS-PAGE. This results in resolution of uromodulin from most of

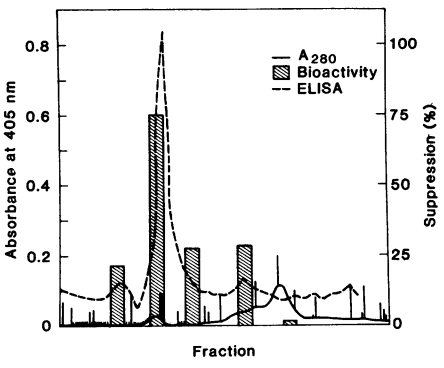


Fig. 1. Elution pattern, bioactivity, and immunoreactive material of a representative experiment. First void morning urine samples from individual donors between the weeks of 20 and 40 of gestation were collected and stored at −20°C. Unfractionated urine (6 liters) from one donor was run over Con A–Sepharose columns with a total bed volume of 200 ml. The Con A–Sepharose columns were then washed with four bed volumes of phosphate-buffered saline before elution with two bed volumes of 250 m*M* α-methyl mannose in phosphate-buffered saline. This material was dialyzed against three changes of 50 volumes of deionized water for 48 hours at 4°C. After dialysis the sample was lyophylized and resuspended in 15 ml of phosphate-buffered saline. This was placed on a 2.5- by 90-cm Fractogel 55 S column and eluted with phosphate-buffered saline. Similar elution patterns have been seen from five donors. At this stage the material is substantially homogeneous (lane B of Fig. 2). The first peak from this column was pooled and dialyzed against distilled water and resuspended in 0.01*M* phosphate buffer at *p*H 7.0. This material was loaded onto preabsorbent wicks and focused on precast isoelectric focusing (IEF) gels (*p*H 4.5 to 9.0, LKB, Broma, Sweden). The area directly under the wick was collected and eluted with distilled water. This sample was concentrated in Centricon filters (Amicon) with a cut-off of 30 kD. A molecular sieving HPLC column (TCK 3000, Bio-Rad) was also used when necessary to remove all of the focusing buffers. An ELISA assay based on the rabbit heteroantiserum shown in Fig. 3 was used to calculate the approximate yield. The material eluted from the isoelectric focusing gel was defined as pure, and the yield of uromodulin is based on this assumption. The ELISA assay consisted of a sandwich assay (*19*).

Table 1. Purification of uromodulin.

| Source | Volume (ml) | Total protein (μg) | Uromodulin (μg) | Yield (%) |
|---|---|---|---|---|
| Crude pregnancy urine | 6000 | 720,000 | 7080 | 100 |
| Eluate Con A column | 42 | 92,400 | 5950 | 84 |
| Peak 1 Fractogel | 6 | 2,800 | 1810 | 26 |
| Eluted IEF gel | 1.2 | 300 | 300 | 4 |

the smaller weight materials found in the Con A fractionation step. At this stage of purification, the only consistent contaminant on SDS-PAGE is a 30-kD band that copurifies with the 85-kD material under a variety of conditions. The final purification step is based on uromodulin's failure to migrate in isoelectric focusing gels. Uromodulin migrates freely through the application paper, but on entering the isoelectric focusing gel it apparently aggregates in the presence of the buffers, since it fails to migrate regardless of where it is placed. Contaminants, however, move freely, resulting in final purification. Occasionally even under these conditions the 85-kD uromodulin is contaminated with trace amounts of the 30-kD material. The gel is then extracted with phosphate-buffered saline, and the focusing buffers are removed on a TCK 3000 high-performance liquid chromatography (HPLC) molecular sieving column. This procedure yields a broad single 85-kD band on unreduced 12.5 percent SDS-PAGE (Fig. 2). Under reducing conditions with dithiothreitol SDS-PAGE of uromodulin yields a single band at 95 kD, suggesting that uromodulin is a single peptide with intrachain disulfide linkages.

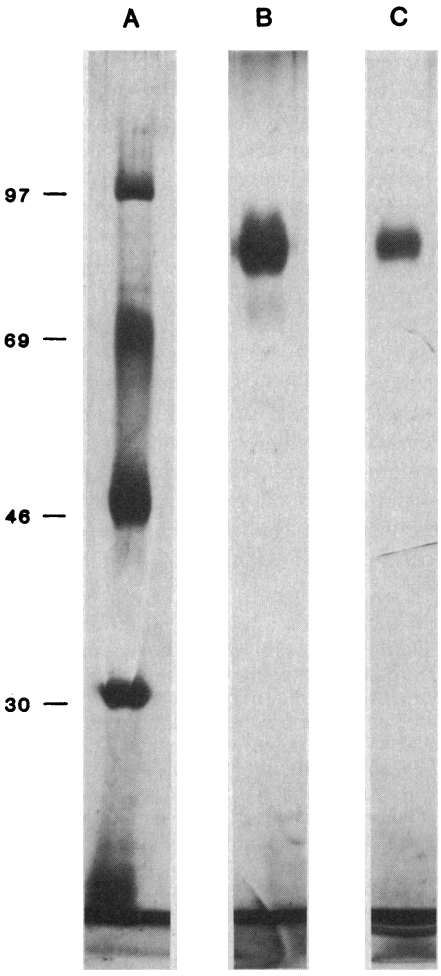


Fig. 2. SDS-PAGE of fractions of uromodulin obtained at various fractionation steps. (Lane A) Molecular weight markers. (Lane B) Uromodulin after concentration of peak 1 from Fig. 1. This material was almost homogeneous: this step resulted in material that was routinely contaminated with a 30-kD contaminant. Multiple attempts at size fractionation under nondenaturing conditions failed to resolve the 85-kD band from the 30-kD one, which suggests that the 85-kD glycoprotein was tightly associated with the 30-kD material. (Lane C) Final purification after elution from an isoelectric focusing gel.

A hetero antibody to this material was raised in a rabbit. This antiserum was used as a probe in Western blot analysis of crude pregnancy urine and exhibited a single major band at 85 kD with two minor bands seen only on overloaded gels (Fig. 3). As further evidence that this antiserum specifically recognized an immunosuppressive molecule, it was conjugated to cyanogen bromide–activated Sepharose (Pharmacia, Uppsala, Sweden) and used as a solid-phase immunosorbent. Crude pregnancy urine was adsorbed to this column, washed extensively, and eluted with 0.1*M* glycine buffer (*p*H 2.8). The eluted material had a molecular weight of 85 kD on SDS-PAGE and was immunosuppressive in vitro (data not shown). A purified immunoglobulin G (IgG) fraction of this antiserum was conjugated to alkaline phosphatase, and a sensitive direct quantitative ELISA was developed. Total yield is shown in column 3 of Table 1.

We used this purified material to examine the in vitro bioactivity of uromodulin. Using our screening assay, which measures the inhibition of antigen-specific T-cell proliferation, we found that uromodulin exhibited a broad dose-response curve with activity demonstrable from $10^{-9}$ to $10^{-11}$*M* (Fig. 4A). Uromodulin exhibited no apparent antigen specificity, since inhibition of proliferation was seen with the antigens tetanus toxoid, streptokinase-streptodornase, and *Candida* (data not shown). Furthermore uromodulin had no effect on cell viability even after 7 days of culture in vitro (Fig. 4C). In an effort to further characterize the cellular specificity and mode of action of uromodulin, it was added to in vitro assays of B-cell and monocyte function.

B-cell function was assayed with a sensitive reverse-hemolytic plaque assay that measures the total number of antibody-secreting cells after polyclonal stimulation with pokeweed mitogen. Plaque-forming cells were enumerated on day 7 (*9*). Uromodulin did not affect this assay of B-cell function (Fig. 4B). Next, uromodulin was used in an assay measuring the in vitro development of spontaneous monocyte-mediated cytotoxicity in humans. This assay is regulated by suppressor cells and is sensitive to a number of exogenous agents capable of

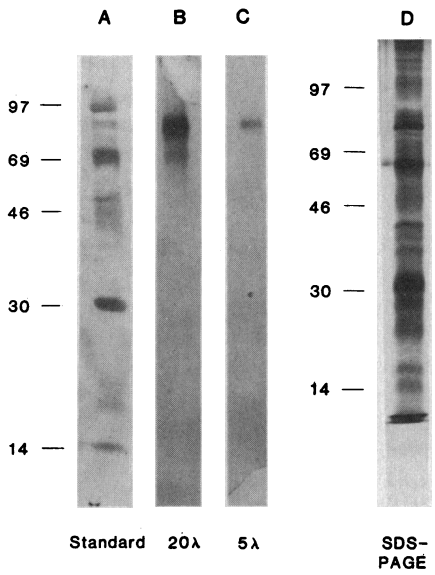


Fig. 3. Western blot analysis of Con A–fractionated pregnancy urine. (Lane A) Molecular size standards stained with Amido black. (Lane D) Protein silver stain of 1 μl of the crude urine fraction. (Lanes B and C) Reactivity of our rabbit serum used as a probe against this crude fraction of pregnancy urine. The crude fractions of pregnancy urine were separated on 12.5 percent SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell protocols, S and S, Keene, New Hampshire). Bound antibody on the nitrocellulose was detected with a biotinylated goat antiserum to rabbit IgG followed by an avidin-biotin-horseradish–peroxidase complex reagent (Vector, Burlingame, California). This antibody failed to bind significantly to any proteins found in normal human serum with a molecular size of less than 150 kD; it did bind weakly in an area consistent with human IgG, which could not be removed by absorption of our antisera with immobilized human serum, suggesting that human antibody was actually recognizing the rabbit IgG.

modulating monocyte function (*10, 11*). According to this measure, uromodulin is a potent inhibitor of spontaneous monocyte cytotoxicity when added at the beginning of culture, acting at concentrations as low as $10^{-11}M$.

These data represent the purification of a potent immunoregulatory compound capable of inhibiting T-cell and monocyte reactivity in vitro. Purification of this compound should allow extensive characterization of its mode of action and cell source. Uromodulin joins but a handful of immunoregulatory compounds available in such purity. Our data suggest that the primary site of action of this molecule is likely at the monocyte or T-cell level. Inhibition by uromodulin was seen only when it was added at the initiation of culture. Addition only 24 hours later resulted in failure of inhibition. Besides suggesting that uromodulin may be interfering with an early cellular collaboration step, these data also suggest that uromodulin is not acting by nonspecifically blocking cellular division, nor is it likely that it blocks IL-2 function, since such mechanisms should inhibit cellular proliferation at later stages as well.

We suspect that uromodulin may be responsible for previously reported observations associated with the immunoregulatory effects of crude fractions of human pregnancy urine that have been variously attributed to human chorionic gonadotropin (hCG) and human placental lactogen. It is also possible that uromodulin could be responsible for immunomodulatory activity previously attributed to α-fetoprotein (70 kD). Although α-fetoprotein has been reported to be immunosuppressive in vitro, the observation has remained controversial (*12, 13*). We have screened homogeneous α-fetoprotein obtained from both amniotic fluid and hepatomas and were unable to show any significant effect of these homogeneous preparations on several in vitro assays. We have screened our uromodulin preparations for the presence of α-fetoprotein through the use of high titer equine antisera and failed to detect any by double radial immunodiffusion. Furthermore, purified α-fetoprotein has a different mobility on SDS-PAGE. A more likely candidate to share properties with uromodulin is pregnancy-specific β-1 glycoprotein, also known as SP1. SP1 purified from pregnancy serum or placental tissue shares many physical and chemical properties with uromodulin including (i) a molecular size on SDS-PAGE of 90 kD, (ii) the presence of carbohydrate (approximately 30 percent), and (iii) a tendency to aggregate in solution (*14*). However, data concerning the potential immunosuppressive properties of SP1 in vitro are in conflict (*15*). In studies showing immunosuppression by SP1 in vitro, suppressive effects on T-cell proliferation were seen only at concentrations of 100 μg/ml. This represents a concentration almost four orders of magnitude greater than that required for immunosuppression by uromodulin in vitro (*16*). Furthermore, SP1 has been purified from pregnancy urine, which is only partially similar to SP1 derived from placenta (*17*). Finally, analysis of SP1 from urine on SDS-PAGE demonstrates that its apparent molecular size is 65 kD and that it comigrates with albumin, which clearly distinguishes urinary SP1 from uromodulin (*17*).

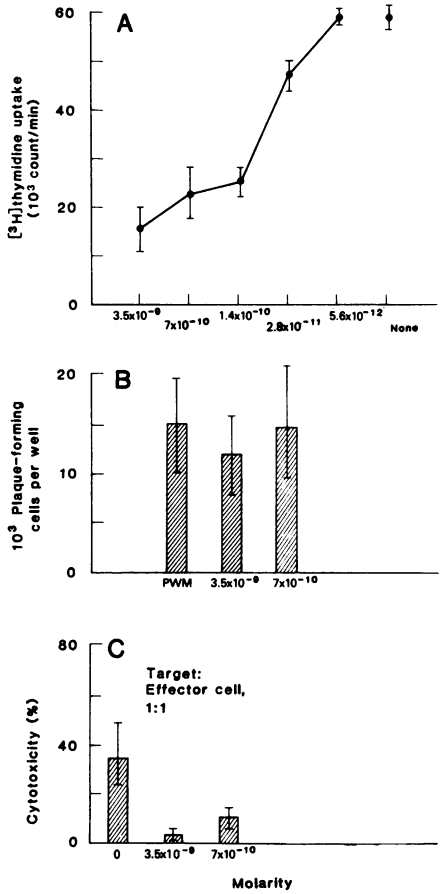


Fig. 4. (A) Dose-response function (mean ± SEM) of uromodulin from a single donor added at the initiation of culture to human peripheral blood mononuclear cells stimulated with tetanus toxoid and harvested as described (*10*). Results are expressed as tritiated thymidine uptake added on day 6 of cultures run in triplicate. Similar results were obtained from 12 different experiments representing five separate batches of uromodulin from different donors. The mean counts per minute of 12 control cultures was 37,736 ± 7,029. Inhibition ranged from 42 percent to 91 percent [$t(11) = 5.89$, $P = 0.0002$]. (B) Dose-response of uromodulin added at the initiation of culture to human peripheral blood mononuclear cells stimulated with pokeweed mitogen. Total reverse hemolytic plaques of cultures run in triplicate were enumerated after 7 days of culture (*12*). The starting culture was 200,000 mononuclear cells, and three individual experiments were performed. (C) Effect of uromodulin on the generation of spontaneous monocyte-mediated cytotoxicity. Uromodulin at the indicated concentrations was added at the initiation of culture. After 6 days the cells were resuspended and counted for viability using trypan blue exclusion. (Viability ranged from 85 to 95 percent with no difference between treated and untreated cultures.) Viable cells ($n$ = 200,000) were added to microtiter dishes and cytotoxicity was assayed in triplicate with $^{51}Cr$-labeled chicken red blood cell targets (*13*).

Other pregnancy-associated compounds with putative immunosuppressive activity such as pregnancy-associated plasma protein A (PAPP-A) or α-2 pregnancy-associated glycoprotein (α-2-PAG) are much larger than uromodulin. A new group of immunosuppressive glycoproteins termed trophoblast antigens-1 (TA-1) has been recently described (*18*). Although TA-1 antigens have not been purified to homogeneity, they share functional similarities with uromodulin (*18*). Finally, we are unaware of other immunosuppressive factors isolated from nonpregnant human sources that share immunologic and physio-chemical properties with uromodulin. Thus we believe that uromodulin represents a unique highly active immunosuppressive glycoprotein. Because of its purity on SDS-PAGE, uromodulin should be amenable to extensive molecular and biologic characterization both in vitro and in vivo.

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