roform d). The coupling constant J (in Hz) for each proton was $J_{a,b}$ (7.7), $J_{b,c}$ (15.7), $J_{b,d}$ (1.2), $J_{c,d}$ (4.7), $J_{e,f}$ (7.4). The value of $J_{b,c}$ (15.7 Hz) identifies a *trans* configuration between H^b and H^c; R. M. Silverstein, G. C. Bassler, T. C. Morrill, in *Spectrometric Identification of Organic Compounds* (Wiley, New York, 1974), p. 226. Spectra were recorded at ambient temperatures (23° to 25°C) with a Nicolet magnetic NMC500 spectrometer. Spectral resolution was <0.002 ppm. Resolution enhancement was achieved by double exponential apodization [A. G. Ferrige and J. C. Lindon, *J. Magn. Reson.* **31**, 337 (1978)]. Spectral ^{1}H assignments were achieved by nuclear spin decoupling, resonance integration, and comparison with previously published spectral assignments of PA's, dihydropyrrolizine alkaloids, and their metabolites [C. C. J. Culvenor, J. A. Edgar, L. W. Smith, H. J. Tweeddale, *Aust. J. Chem.* **23**, 1853 (1970); H. J. Segall and J. L. Dallas, *Phytochemistry* **22**, 1271 (1983)].
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Protein-Specific Helper T-Lymphocyte Formation Initiated by Dendritic Cells

Abstract. *Antibody responses to hapten-polypeptide conjugates require peptide-specific helper T cells. The latter can be primed in tissue culture by providing small numbers of dendritic cells. Primed, irradiated helper T cells then induce B-cell growth and differentiation in the apparent absence of dendritic cells. Both stages of the antibody response—the induction of helper T lymphoblasts by dendritic cells and the delivery of help from T to B cell—occur in discrete cell aggregates that can be isolated by velocity sedimentation. If helper T blasts revert to smaller "memory" lymphocytes, dendritic cells again are needed to initiate the antibody response.*

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Helper T lymphocytes enhance antibody responses to many antigens, such as foreign red blood cells and defined haptens coupled to polypeptide carriers (*1*). Carrier-specific helper cells usually are obtained from animals that have been primed by exposure to antigen in vivo. This approach, however, makes it difficult to control the determinants that are to be recognized and to analyze the cellular requirements for the induction of helper cells. These topics are assuming increasing importance because of the interest in understanding the initiation of immune responses and the use of polypeptide vaccines. We report here the successful priming of polypeptide-specific helper T-lymphocytes in vitro.

The experimental approach required the use of dendritic cells (DC's) as accessory cells (*2*). DC's are irregularly shaped leukocytes first identified in the adherent population of mouse spleen cells (*3*). At that time it was known that cells adherent to plastic or glass enhanced antibody formation by nonadherent B and T lymphocytes (*4*). Once DC's were separated from other adherent cells, particularly macrophages (*5*), it became apparent that DC's were specialized stimulators of many immune responses, including antibody formation to T cell–dependent antigens (*2, 6*). We will show here that a special role of DC's is to generate active helper cells from unprimed and memory T lymphocytes.

Spleen cell suspensions (from CXD2 F1 mice unless otherwise indicated) were passed over Sephadex G10 columns to provide mixtures of B and T lymphocytes. The latter mixtures were effectively depleted of DC's, as evidenced by their inability to stimulate mixed-leukocyte reactions (MLR's) and to form antibodies (*2*). B cells were G10-nonadherent cells that were treated with antibodies to Thy-1 and Lyt-1 and complement to eliminate T cells. Helper T cells were nylon wool–nonadherent spleen and lymph node cells that were treated with antibodies to I region–associated antigen (Ia) and to Lyt 2.2 and with complement to eliminate residual B cells, DC's, and cytotoxic and suppressor cells. Macrophages were firmly adherent peritoneal or spleen cells. DC's were plastic-adherent spleen cells that had been depleted of macrophages after overnight culture. Although DC's and macrophages are both derived from an initial adherent population, DC's are nonphagocytic, become nonadherent after overnight culture, express the 33D1 DC-specific marker, and lack Fc receptors (*5*). The efficacy of these enrichment procedures was verified by sensitive assays. The B cells responded to the B-cell mitogen LPS (33,000 count/min per 10^5 cells) but not to the T-cell mitogen concanavalin A (Con A) (300 count/min per 10^5 cells) nor to the fetal calf serum that was used in our cultures. The T cells did not respond to LPS (300 count/min per 10^5 cells) but did respond to Con A if small numbers of DC's were present (282,000 count/min per 10^5 T cells and 10^4 DC's). The DC's did not respond to either mitogen.

The relative efficacy of DC's and mac-

rophages as accessory cells for T cell–dependent antibody responses was examined with two approaches and two types of antigen: heterologous sheep red cells (SRC's) and a hapten-carrier conjugate (trinitrophenyl–keyhole limpet hemocyanin) (TNP-KLH) (Table 1). By positive selection, enriched populations of DC's were active accessory cells whereas macrophages purified from the spleen and peritoneal cavity were not. Lymphocytes could not act as accessory cells, since G10-nonadherent spleen cells (mixtures of B and T cells) never generated responses to antigen in the absence of DC's. For negative selection DC's were eliminated with 33D1-specific antibody and complement. This ablated function as was described previously in other systems (*2, 6*).

Two approaches were taken to generate Lyt-2$^-$, carrier-specific, helper lymphocytes that could interact with B cells to induce antibody responses. First we cultured Lyt-2$^-$ T cells for 5 days with protein antigens and adherent cells. DC's but not macrophages induced helper activity, but it was not carrier-specific. The second approach took advantage of the fact that DC's cluster with responding T cells during several lymphocyte responses in tissue culture (*2, 7, 8*). These clusters generated populations of helper T blasts that had only a few DC's by direct examination and by functional criteria and that doubled in cell number in less than 20 hours after addition of interleukin-2 (IL-2). Clusters that had developed in the presence of specific proteins [KLH and human α-globulin (HGG)] generated lymphoblasts with radioresistant carrier-specific helper activity (Table 2). The nonclustered fraction lacked helper function. When clusters developed in the absence of carrier (the syngeneic MLR), comparable numbers of T blasts were released but helper function was polyclonal, that is, α-TNP and α-SRC responses developed in the absence of antigen (Table 2). The polyclonal activity of cluster-derived T blasts decreased progressively as the dose of KLH, which was used to prime specific helper cells, was raised from 0.2 to 2.0 to 20 μg/ml. Therefore, the presence of carrier protein during priming preempted the response to self or to self plus fetal calf serum.

The specificity and antigen-presenting cell requirements of T blasts primed in vitro were studied in proliferative assays (Fig. 1). Syngeneic MLR blasts responded vigorously to low doses of syngeneic

Table 1. Accessory cell requirements for primary antibody responses in tissue culture. Positive and negative selection techniques were used to prepare DC's and macrophages (*2, 6*). Accessory cells were cultured for 4 days with 4 × 10^6 B and T cells with or without SRC's or TNP-KLH. Results are PFC responses by spleen B and T cells to no antigen, TNP-KLH, or SRC's. Abbreviations: Ab, antibody; C, complement.

Cell type	No antigen		TNP-KLH		SRC's	
	α-TNP	α-SRC's	α-TNP	α-SRC's	α-TNP	α-SRC's
Accessory cells prepared by positive selection						
Spleen dendritic						
1 × 10^5	116	24	736	56	60	1040
3 × 10^4	20	8	616	40	40	956
1 × 10^4	8	4	472	16	44	656
Spleen macrophages						
1 × 10^5	4	0	16	4	4	4
3 × 10^4	4	0	8	4	8	0
1 × 10^4	4	0	8	4	4	4
Peritoneal macrophages						
1 × 10^5	12	4	44	8	12	4
3 × 10^4	16	8	28	8	8	4
1 × 10^4	8	4	12	8	12	0
None	8	0	12	4	4	0
Accessory cells prepared by negative selection						
Spleen adherents (5 × 10^4)						
No Ab, no C	8	0	572	28	48	852
No Ab, with C	12	0	548	24	40	812
33D1, no C	8	0	572	32	40	872
33D1, with C	4	0	64	12	8	44

Table 2. Priming of carrier-specific helper T cells in vitro. Helper T lymphoblasts were generated from DC-T cell clusters (*8*) that had been cultured in the presence of HSA or KLH (experiment 1) or in the presence of no carrier (syngeneic MLR) or KLH (experiment 2). The T blasts were used with or without exposure to ionizing irradiation and were added in the indicated doses to 2 × 10^6 B cells in the absence of antigen or in the presence of TNP-modified proteins or SRC's. After 4 days α-TNP and α-SRC PFC's were measured on TNP-treated SRC's or SRC indicator cells. B lymphocytes did not form PFC's in the absence of helper cells.

Helper T cells		No antigen		TNP-KLH		TNP-HSA or TNP-HGC		SRC's	
Specificity	Number	α-TNP	α-SRC's	α-TNP	α-SRC's	α-TNP	α-SRC's	α-TNP	α-SRC's
				Experiment 1					
HSA (3000 rads)	1 ×10^5	156	42	84	54	416*	60*	Not tested	
	3 ×10^4	12	0	44	0	428*	72*		
	1 ×10^4	0	0	48	30	280*	60*		
KLH (3000 rads)	2 ×10^5	4	0	28	12	0*	0*	Not tested	
	1 ×10^5	0	0	164	30	0*	0*		
	3 ×10^4	0	0	504	54	0*	0*		
	1 ×10^4	4	0	548	42	0*	0*		
	3 ×10^3	0	0	500	66	0*	0*		
				Experiment 2					
Syngeneic MLR (3000 rads)	1 ×10^5	352	96	480	174	432†	138†	328	884
	3 ×10^4	332	108	516	180	470†	168†	312	924
Syngeneic MLR (no ^{137}Cs)	1 ×10^5	172	72	332	144	236†	90†	188	732
	3 ×10^4	268	122	628	264	468†	216†	276	798
KLH (3000 rads)	1 ×10^5	8	0	392	66	40†	18†	12	12
	3 ×10^4	4	6	484	48	36†	12†	8	16
KLH (no ^{137}Cs)	1 ×10^5	12	6	300	9	24†	6†	20	32
	3 ×10^4	4	0	372	24	6†	20†	20	28

*TNP-HSA. †TNP-HGC.

Table 3. Helping of MHC-compatible B cells by T cells primed in vitro when hapten and carrier are linked. Three groups of helper T blasts (syngeneic MLR, KLH, and HSA) were generated from dendritic T-cell clusters and cultured with B cells for 4 days with various antigens at 10 μg/ml. Values represent helper activity (PFC's) for B cells cultured as shown.

Helper T		No antigen		SRC's		TNP-KLH		TNP-HSA		TNP-KLH + HSA		TNP-HSA + KLH	
Number	Carrier	TNP	SRC's	TNP	SRC's	TNP	SRC's	TNP	SRC's	TNP	SRC's	TNP	SRC's
						B6							
		25	0	15	0	30	15	35	8	35	8	35	23
10^5		145	79	150	360	270	75	165	68	280	128	205	113
10^5	KLH	20	0	40	40	580*	23	35	0	660*	38	40	23
10^5	HSA	35	8	45	55	35	30	500*	15	50	30	605*	38
						B6.H-2k							
		20	8	33	5	35	15	40	8	30	15	33	23
10^5		40	23	53	5	45	38	25	0	80	30	55	23
10^5	KLH	5	0	30	10	15	8	5	0	20	8	25	8
10^5	HSA	30	15	30	20	45	0	5	8	35	23	25	8
						B6.H-2k + B6 DC's							
		40	8	60	8	50	23	45	8	55	23	70	8
10^5		55	8	38	640	50	23	50	8	40	15	40	8
10^5	KLH	20	0	45	35	40	8	40	0	40	0	5	0
10^5	HSA	0	0	38	35	30	8	25	0	15	8	35	8

*Hapten and carrier were physically linked.

DC's. Addition of several carrier proteins had no effect. In contrast, carrier-primed T lymphoblasts exhibited proliferation dependent on KLH or TNP-KLH and were markedly depleted of antigen-independent or "self" reactivity (Fig. 1). Three hundred DC's raised the level of [^{3}H]thymidine uptake by 3×10^4 T cells at 32 to 38 hours from 400 to 25,000 count/min. The sensitivity of the blasts to exogenous DC's indicated that the T-cell preparations contained much less than 1 percent DC's. Irradiated (900 rads) DC's and B cells both presented antigens to the blasts. Helper T cells recognize soluble proteins in association with Ia (*9*), so the observed superiority of DC's could be attributed to the fact that DC's express about ten times more Ia than B lymphocytes (*10*). In additional experiments unirradiated B cells proliferated actively (more than 20 percent were blasts at day 2 to 3) when cultured with antigen and irradicated T cells.

The function of carrier-specific helper cells required that hapten and carrier be physically linked (values with asterisks in Table 3) and that the responding B cells be histocompatible (compare B6 and B6.H-2k B cells). Allogeneic B cells did not generate antibody responses even if the cultures were supplemented with excess DC's, indicating that the interaction between T and B cells could not be bypassed by soluble, DC-induced helper factors. The latter did mediate an α-SRC response [640 plaque-forming cells (PFC's)], as anticipated (*2*). Helper cells also mediated secondary immunoglobulin G responses when the dose of hapten was suitably low (*11*) and when hapten and carrier were linked.

Cell aggregates also developed when primed T blasts were cocultured with B cells for 2 days. Clustering required the addition of carrier and did not occur with nonspecific antigen [SRC's or human serum albumin (HSA)] or with unprimed T cells. The KLH-dependent clusters were isolated by velocity sedimentation. By indirect immunofluorescence (*2*), there were roughly equal numbers of B cells (Ia$^+$, Ig$^+$, TIB 146$^+$—a B-cell monoclonal antibody from the American Type Culture Collection) and T cells (Thy-1$^+$). In functional assays the entire α-TNP response developed in clusters (Table 4).

To simulate memory cells, KLH-primed helper blasts were cultured an additional 2 to 7 days in the absence of carrier or DC's. Within 40 hours most of the blasts reverted to smaller cells. Viable cell recoveries were greater than 90 percent if the blasts were "rested" on small numbers of syngeneic peritoneal macrophages (1×10^4 macrophages per 3×10^5 blasts). These memory cells were IL-2–unresponsive, indicating that they had converted from an activated to a resting state. Graded doses of helper T blasts and memory cells were then added to B cells plus TNP-KLH or SRC's. The freshly sensitized blasts were active helper cells even at low doses of T cells

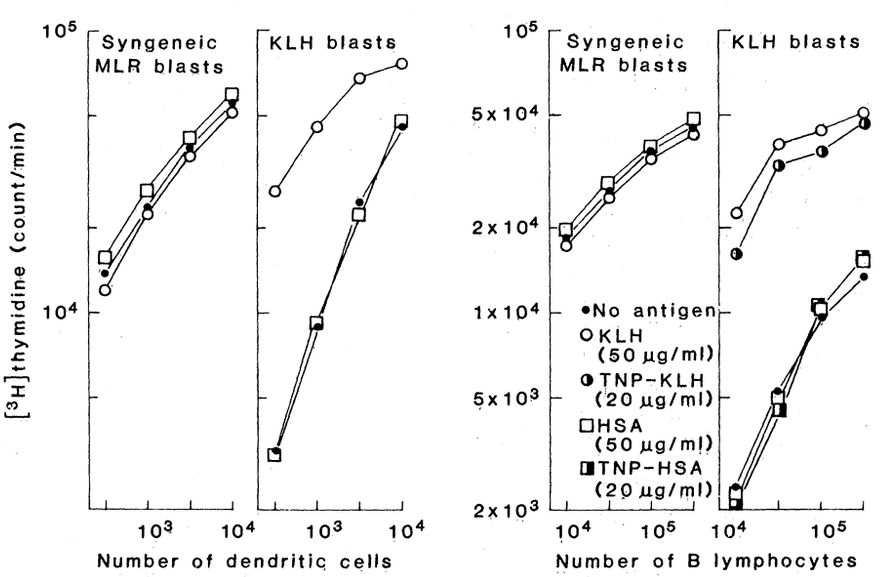

Fig. 1. Proliferative responses of Lyt-2$^-$ T blasts primed in vitro. Blasts (3×10^4) were cultured in microtest wells with graded doses of syngeneic irradiated (900 rads) DC's or B cells with different antigens or with no antigen. Uptake of [^{3}H]thymidine was measured at 32 to 38 hours with a pulse (4 μCi/ml) of label. Background radiation counts were 417 and 294 count/min for the syngeneic MLR and anti-KLH blasts alone, respectively, and less than 200 count/min for irradiated presenting cells alone.

(10^3) and TNP-KLH (0.5 μg/ml) (Table 5). In contrast, 3×10^4 memory T cells did not help B cells unless DC's were added. Higher doses of helper cells were needed with lower doses of carrier. Macrophages provided little or no accessory function. The memory T cells also proliferated in response to carrier presented by DC's, but not by B cells.

In summary, clusters of DC's and Lyt-2^- T cells generated carrier-specific lymphoblasts that behaved like those induced in situ (*11*). The helper cells acted on B cells that secrete immunoglobulins M and G, that are major histocompatibility complex (MHC)–compatible, and when hapten and carrier are physically linked. Function was radioresistant and was delivered in the apparent absence of DC's at T-cell to B-cell ratios of less than 1 to 1000, which seems appropriate considering that less than 0.1 percent of the B cells responded. The need for direct contact between TNP-specific B cells and carrier-specific T cells may represent a major barrier to the efficacy of T cell–dependent antibody responses. Such cell-cell interactions have not been visualized in situ, but comparable events may occur. B cells are readily identified in tissue sections of spleen periarteriolar sheaths, where both DC's and T cells are found and where antibody responses begin (*11*). Optimal interaction between B and T cells would seem to require that the antigen be soluble so that it can associate with two sites on the B cell and still be accessible to the helper T cell. Membrane proteins, like minor histocompatibility and tumor-associated antigens, would not be good thymus-dependent stimuli unless they could be released from the cell surface.

The biology of the helper T cell clarifies the role of different types of antigen-presenting cells, particularly DC's, in T-cell stimulation. The term "antigen-presenting" cell reflects the long-standing view that the main function of accessory cells is to somehow interact with and "present" antigen in association with MHC products, particularly the class II or Ia glycoproteins. However, helper T cell function requires that antigen be presented by B cells as well as by specialized DC's since the same clone that is induced in response to antigen plus accessory cells must also recognize antigen presented by B cells to execute help. Evidence that B cells present antigens to helper T cells in an MHC-restricted fashion has long been available (*13*). However, B cells exert little or no priming or initiating function, for which DC's are critical. The need for DC's applies to unprimed and memory helper cells. Similar conclusions have emerged from studies of alloantigen presentation during the primary MLR (*6, 7*) and allograft rejection (*14*), in that DC's are much more efficient stimulators than other Ia^+ leukocytes. Once the alloreactive helper cell is sensitized, it can interact effectively with alloantigens presented by B cells, macrophages, and presumably other Ia^+ cell types (*7*).

Table 4. Generation of PFC's from clusters of carrier-primed helper cells and hapten-specific B cells. KLH-primed, irradiated T blasts (10^5) were mixed with B cells (6×10^6) and TNP-KLH (0.5 μg/ml) for 40 hours. Cluster and noncluster fractions were isolated and cultured for three more days with or without TNP-KLH (0.5 μg/ml). At 80 to 86 hours a constant volume (75 μl) of each 1-ml culture was exposed to [^{3}H]thymidine (5 μCi/ml), and at 110 hours PFC's were measured on indicator TNP-SRC's and SRC's. Cultures of T and B cells were also set up with no TNP-KLH or with SRC's as antigen. Few clusters or PFC's formed because the T blasts were primarily KLH-specific at the doses used.

Cells used to generate PFC's	PFC's, no TNP-KLH		PFC's with TNP-KLH		[^{3}H]thymdine (count/min)	
	α-TNP	α-SRC's	α-TNP	α-SRC's	Without TNP-KLH	With TNP-KLH
Clustered B and T cells (3×10^5)	280	16	463	27	6,591	11,613
Nonclustered B and T cells (3×10^6)	3	3	16	6	881	4,467
Mix of the above	260	16	517	37	8,259	15,002

Table 5. Helper function of carrier-primed T lymphoblasts and memory T cells. T lymphoblasts that had been primed with KLH were cultured without antigen for 3 days to provide memory T cells (*8*). The blast and memory T cell populations were irradiated (3000 rads) and mixed with 2×10^6 B cells for 5 days. In experiment 1 values are PFC responses (α-TNP/α-SRC PFC's) for graded doses of TNP-KLH. In experiment 2 values are α-TNP responses with/without TNP-KLH (1 μg/ml) and different accessory cells.

	Experiment 1				
Cells added to 2×10^6 B lymphocytes	No TNP-KLH	0.05 μg/ml	0.5 μg/ml	5.0 μg/ml	
None	0/0	0/0	0/0	0/0	
KLH-primed T blasts					
1×10^5	104/24	396/24	748/24	400/66	
3×10^4	124/12	920/48	1280/90	648/26	
1×10^4	44/12	620/6	788/30	500/102	
3×10^3	28/10	404/30	584/36	548/78	
1×10^3	4/0	44/6	444/0	508/72	
KLH-primed memory T					
1×10^5	0/0	0/0	48/0	68/18	
3×10^4	0/0	0/0	12/0	60/18	
1×10^4	0/0	0/0	8/0	12/0	
3×10^3	0/0	0/0	4/0	4/0	
10^4 DC's and KLH-primed memory T					
1×10^5	4/6	316/6	212/18	168/24	
3×10^4	0/0	228/0	360/30	268/42	
1×10^4	0/0	16/0	240/6	336/6	
3×10^3	0/0	20/0	248/18	312/0	
	Experiment 2				
Cells added to 2×10^6 B lymphocytes	No accessory	10^4 DC's	3×10^4 DC's	3×10^4 MO's*	10^5 MO's*
KLH-primed memory T					
1×10^5	16/0	420/8	376/0	84/0	176/0
3×10^4	12/8	672/0	404/4	72/4	64/0
1×10^4	4/0	592/4	372/0	16/4	12/0
3×10^3	8/0	504/0	328/8	12/0	0/0
	4/0	4/4	0/4	16/0	12/0

*Peritoneal macrophages.

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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 (5×10^4) and Lyt-2$^-$, Ia$^-$ T cells (5×10^6) 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 (2×10^5 to 3×10^5 cells) and noncluster (2×10^6 to 3×10^6 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

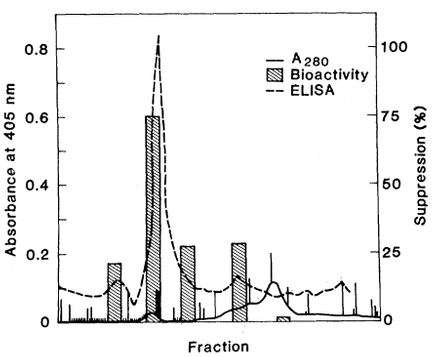

Fig. 1. Elution pattern, bioactivity, and immunoreactive material of a representative experiment. First void morning urine samples from individual donors between the weeks of 20 and 40 of gestation were collected and stored at −20°C. Unfractionated urine (6 liters) from one donor was run over Con A–Sepharose 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*).