Humoral Inhibitors of the Immune Response in Uremia

V. Induction of Suppressor Cells in Vitro *by Uremic Serum*

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The mechanism of inhibition of mixed lymphocyte reaction (MLR) by serum of chronically uremic rats has been studied. The inhibitory activity of the serum has been associated with a discrete subset of very low density lipoproteins (VLDL) of S_f 100–400. The degree of the inhibitory activity of uremic serum correlates with the severity of uremia. Spleen cells from normal rats incubated for 20 hours with uremic serum or its VLDL fraction suppress the response of control syngeneic cells in the MLR. Induction of such suppressor activity does not require cell proliferation because it is not inhibited by mitomycin C. Although the exact identity of the induced suppressor cells has not been established, they may be macrophages. The suppressor activity of induced spleen cells can be markedly reduced by filtration of spleen cells on glass wool or on nylon wool columns. Reconstruction experiments show that the adherent cell fraction of spleen cells exposed to uremic serum suppresses the response of the nonadherent fraction of control spleen cells. These results indicate that the immunosuppressive effects of rat uremic serum *in vitro* involve the induction of suppressor cells. (Am J Pathol 1983, 111:149–155)

CHRONIC RENAL FAILURE is associated with significant changes of immune potential; cell-mediated immunity is more uniformly affected than antibody production.[1-3] The mechanism of this pathologic suppression of the immune response has not been elucidated. Most studies of the immunity in clinical and experimental uremia have concentrated on two areas: the response of uremic lymphocytes to various mitogenic stimuli *in vitro* and the effects of uremic serum on functions of normal lymphoid cell populations. Although the early data on the immune potential of uremic lymphocytes show considerable variability, more recent studies indicate that the response of uremic lymphocytes to various *in vitro* stimuli is usually suppressed.[4-7] Uremic serum often contains humoral factors that affect various lymphocyte functions *in vitro*. Particular attention has been given to the effects of uremic serums on the mixed lymphocyte reaction (MLR).[3,8-10] Such studies in clinical uremia are complicated by the heterogeneity of the patient populations, by diversity of the causes of the uremic state, and by different therapeutic regimens. We have developed an experimental model of slowly developing uremia in the inbred rat that allows us to study the changes of the immune response under much better defined conditions.[11] Rejection of skin allotransplants is significantly delayed, and antibody formation to T-cell-dependent antigen is reduced in such animals.[12,13] Reactivity of uremic lymphocytes in the graft-versus-host reaction is suppressed,[11] as is their reactivity to various mitogenic stimuli *in vitro*.[14] Serum of uremic rats inhibits the response of syngeneic control lymphocytes in the MLR and after lectin stimulation.[14] Uremic serum has been extensively fractionated,[15] and its immunosuppressive effects have been associated with the very low density lipoprotein (VLDL) fraction.[16]

In this report we demonstrate that the inhibitory effects of uremic serum are associated with a discrete subset of VLDL. We also demonstrate that inhibition of the MLR by uremic serum or by its VLDL fraction involves an indirect mechanism: the induction of suppressor cell activity.

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Material and Methods

Animals

Inbred adult male Lewis (LEW) and Brown-Norway (BN) rats were obtained from Microbiological Associates, Walkersville, Maryland.

Chronic Renal Insufficiency

Chronic renal insufficiency was induced in LEW rats by a two-stage 5/6 nephrectomy as previously described.[11] Control rats were subjected to a sham operation by stripping of both kidney capsules. Blood was obtained from the tail vein, and the blood urea nitrogen (BUN) level was determined by use of a Beckman BUN analyzer. Prior to use in the MLR, serum was heat-inactivated and sterilized by filtration through a membrane filter.

Mixed Lymphocyte Reaction

The one-way reaction of LEW spleen cells as responders against mitomycin-C–treated BN spleen cells as stimulators was performed as described previously.[17] The effects of uremic serum were tested at final concentrations of 1% or 10% as indicated for individual experiments. The isolated VLDL fractions were added to cultures in complete medium. On the 6th day, the cultured lymphocytes were labeled with ^{3}H-thymidine (1 μCi) for 24 hours. Incorporation of ^{3}H-thymidine into control responder cells incubated without stimulator cells was substracted from all experimental MLR data. The variability of incorporation observed in control MLR reflects differences in specific radioactivity of the ^{3}H-thymidine used in different experiments.

Lipoprotein Isolation

Lipoprotein fractions of control and uremic serum were isolated by precipitation with dextran sulfate (Pharmacia, Piscataway, NJ) and divalent cations followed by ultracentrifugation as originally described for human serum[18] and adapted for isolation of rat lipoproteins.[16] The quantities of lipoprotein are expressed as micrograms of protein as determined after lipid extraction by diethyl ether by the method of Lowry et al.[19]

Ultracentrifugal Fractionation of VLDL

The fractions of VLDL of rat control and uremic serum were isolated by sequential flotation in a buffer of density of 1.0055 g/ml. Centrifugations were performed at 4 C as described by Gustafson et al.[20] Three distinct fractions of VLDL were used: $VLDL_1$ (S_f 100–400), $VLDL_2$ (S_f 50–100) and $VLDL_3$ (S_f 20–50).

Generation of Suppressor-Cell Activity by Uremic Serum or by VLDL

The spleen-cell suspensions were incubated with medium containing 10% uremic serum at 37 C in a CO_2 incubator. In some experiments the cells were incubated in complete control medium supplemented with 100 μg/ml of isolated uremic VLDL. After 20 hours the cells were collected by centrifugation, washed three times in complete medium, and tested for suppressor-cell activity. In some experiments the LEW spleen cells were treated before or after uremic serum treatment with mitomycin C (25 μg/ml) for 30 minutes.

Assay for Suppressor-Cell Activity

Various numbers of induced LEW cells were added to standard MLR cultures containing 1×10^6 LEW responder and 1×10^6 BN stimulator cells. To control cultures identical numbers of cells induced with control serum were added. The degree of suppression was calculated according to the following formula:

$$\%\ \text{Suppression} = \left(1 - \frac{\text{cpm of cultures containing cells induced with uremic serum}}{\text{cpm of cultures containing cells induced with control serum}}\right) \times 100$$

Fractionation of Spleen Cells

Both uremic-serum–treated and control-serum–treated spleen-cell populations were fractionated by various procedures. The fraction of spleen cells nonadherent to glass wool was prepared as described by Raskova and Morrison[11] with the use of a column containing 1 g of packed glass wool (Owens-Corning Fiberglass Corp., Corning, NY). The cell suspension in medium supplemented with 10% control serum was incubated on the column at 37 C for 30 minutes and then flushed with 30 ml of medium. The fraction of cells nonadherent to nylon wool was prepared on nylon wool columns (Leuko-Pak, Fenwall, Deerfield, Il) under conditions described previously.[21] For preparation of fractions of cells nonadherent and adherent to plastic, the spleen cell suspensions were allowed to adhere to 100-mm plastic culture dishes for 2 hours at 37 C in assay medium. Nonadherent cells were removed by vigorous washing, and adherent cells were recovered by use of a rubber spatula.

The individual cell fractions were added to complete MLR cultures for assays of suppressor activity.

In some experiments the responder cell population was reconstructed from adherent and nonadherent fractions and used in the MLR.

Results

Development of Uremia in Partially Nephrectomized Rats

Subtotal (~5/6) nephrectomy induces a slowly developing renal failure (Figure 1). This experimental model thus offers an opportunity to study the changes in immune potential under conditions free of acute surgical stress encountered in models associated with rapid development of uremia. The uremic animals survive for long periods of time. In all experiments, control animals of the same age, and at the same time period after operation, were used as controls.

Suppression of Mixed Lymphocyte Reaction by Serum of Uremic Rats

Serum of uremic rats is strongly inhibitory to the MLR. In cultures supplemented with 10% uremic serum, greater than 90% inhibition of ^{3}H-thymidine incorporation was seen with serum of animals with BUN levels of 60 mg/dl or greater (Figure 2A). In cultures supplemented with uremic serum diluted 1:

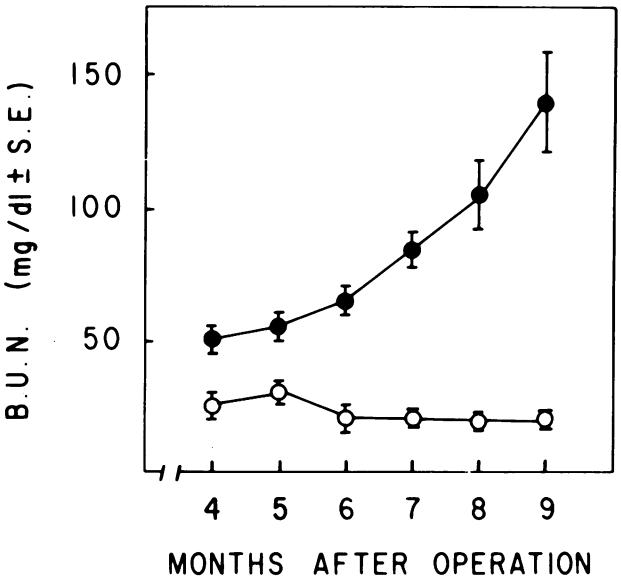

Figure 1 – Development of uremia in LEW rats. Fifty adult male rats were subjected to a two-stage 5/6 nephrectomy (●). Control animals (○) underwent sham operations (stripping of both kidney capsules). The BUN levels were determined with a Beckman BUN analyzer.

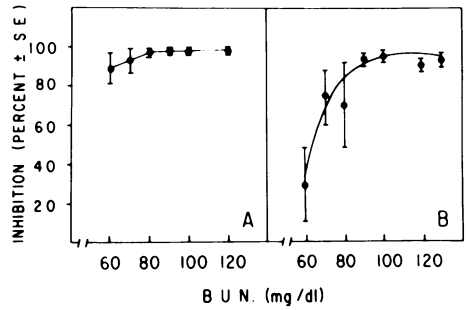

Figure 2 – Correlation of severity of uremia with the degree inhibition of one-way mixed lymphocyte reaction by uremic serum. Reaction of LEW cells as responders against mitomycin-C-treated BN cells as stimulators was inhibited by serum with different BUN levels. **A** – The MLR in modified Eagle's medium (MEM) supplemented with 10% of different uremic serums. **B** – The MLR in MEM supplemented with 9% of control LEW serum and 1% of various uremic serums. The results show the means of results with a minimum of five serums for each BUN level (± SE).

10 with control syngeneic serum, strong inhibitory effects were seen with serum of animals with BUN levels of 70 mg/dl and greater (Figure 2B). It is apparent that serums of rats with BUN greater than 90 mg/dl completely inhibit the MLR at a final concentration of 1%. This result demonstrates that uremic serum contains an inhibitor of the MLR rather than showing the lack of a factor required for support of the mitogenic response. In the experiments described below we used serums of animals not less than 6 months after surgical intervention, with BUN levels between 90 and 110 mg/dl.

Inhibitory Effects of Uremic VLDL Are Associated With a Discrete $VLDL_1$ Fraction

We have previously established that the inhibitory effects of uremic rat serum are associated with its VLDL fraction.[16] At analogous concentrations, the VLDL fraction of control serum was not inhibitory.[16]

The VLDL fraction of rat serum is a heterogeneous mixture of particles that contain a heterogeneous family of apoproteins. To establish whether the inhibitory activity of the uremic VLDL can be associated with a VLDL subset of characteristic density, the VLDL was fractionated according to flotation rate into 3 fractions.[20] The effects of the total VLDL fraction and its three subfractions were tested in the MLR (Figure 3). It is apparent that both total VLDL and its discrete subset of $VLDL_1$ (S_f 100–400) exert strong inhibitory effects. The other two VLDL fractions, $VLDL_2$ (S_f 50–100) and $VLDL_3$ (S_f 20–50) were not inhibitory at the concentrations used. The inhibitory activity of uremic VLDL is thus associated with a discrete subset of VLDL.

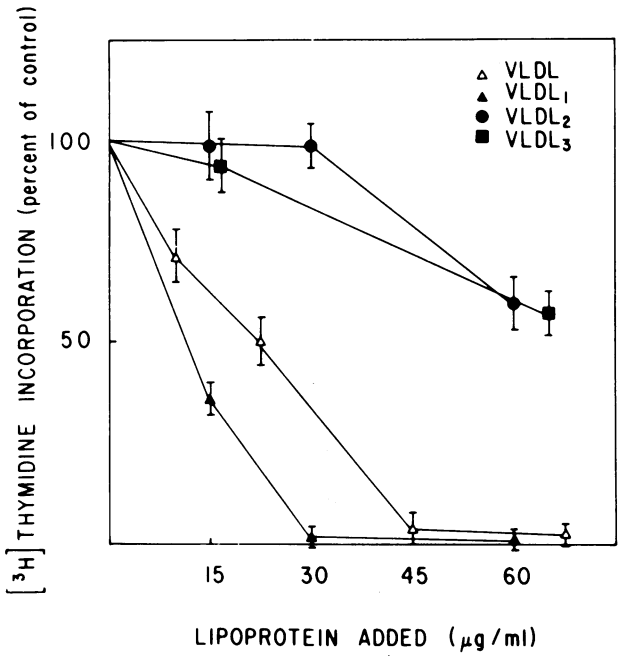

Figure 3 — Inhibition of the MLR by uremic VLDL. The complete MLR cultures were supplemented with increasing concentrations of the total VLDL fraction of uremic serum (△) and its three subfractions prepared by sequential ultracentrifugation-flotation: ▲, $VLDL_1$ (S_f 100-400); ●, $VLDL_2$ (S_f 50-100); ■, $VLDL_3$ (S_f 20-50). The data show the mean ± SE of a minimum of three determinations. The mean incorporation in control cultures was 4430 ± 345 cpm.

In control experiments, none of the 3 VLDL fractions of the control serum exerted significant inhibitory effects at concentrations up to 55 μg/ml: 24% inhibition was seen with control $VLDL_1$, no inhibition with $VLDL_2$ (96% of control), and 18% inhibition with $VLDL_3$.

Suppression of MLR by Cells Exposed to Uremic Serum or VLDL

The MLR is inhibited when LEW cells are preincubated for 18 hours in uremic serum or VLDL, but the late stages of incubation are resistant to inhibition.[14] Experiments were performed to determine whether uremic serum inhibits the MLR directly or via an indirect suppressor-cell mechanism. Control LEW cells were incubated for 20 hours in medium supplemented with 10% uremic serum, washed, and added in varying ratios to standard MLR cultures. The results shown in Figure 4A indicate that cells exposed to uremic serum suppress the response of normal untreated syngeneic cells in the one-way MLR: the inhibitory effects directly correlate with the number of cells induced with uremic serum in the reaction. The addition of untreated cells or cells pretreated with serum of rats that underwent sham operations did not result in suppression of the response. Similar suppressor activity was observed with spleen cells exposed to isolated uremic VLDL (Figure 4B).

Short-term (2-hour) exposure to uremic serum failed to induce the suppressor effect (Table 1).

Effects of Mitomycin-C Treatment on Induction of Suppressor-Cell Activity

Different requirements for cell proliferation have been reported for *in vitro* induction of suppressor-cell activity.[23-25] In order to establish whether cell proliferation is a prerequisite for induction of the observed suppressor effect, control spleen cells were treated with mitomycin C before or after induction with uremic serum. Such treatment was without effect on the suppressor activity of spleen cells incubated with uremic serum (not shown).

Suppressor Activity of Fractionated Spleen-Cell Populations

Spleens of chronically uremic rats contain increased numbers of suppressor cells, which belong to the adherent cell class[11,22] and are readily removed by adherence to glass wool[11] or rayon wool.[22] In order to determine whether the suppressor-cell activity induced *in vitro* had a similar property, the inhibitory spleen-cell populations were fractionated on glass wool and nylon wool columns, and the suppressor activity of nonadherent fractions was tested (Table 2). It is apparent that filtration through both nylon wool and glass wool reduces the suppressor activity of induced spleen cells. When similarly treated cells induced with control serum were added to the MLR, the observed mitogenic response was also significantly higher than that observed in cultures supplemented with unfractionated cells.

The MLR With Responder Cells Reconstructed From Adherent and Nonadherent Spleen Cells

An excess of adherent cells strongly inhibits the mitogenic response of rat spleen cells.[26,27] Addition of both control and uremic serum-exposed adherent cells to the complete MLR resulted in strong suppression (not shown). In order to test directly the effects of adherent cells treated with uremic serum, a reconstruction experiment was performed. Spleen cells induced with uremic serum and control spleen cells were fractionated by adherence to plastic dishes. The responder cell population in MLR cultures was reconstructed from the adherent and nonadherent cell fractions (Table 3). Strong inhibition of the mitogenic response was seen only in cultures containing

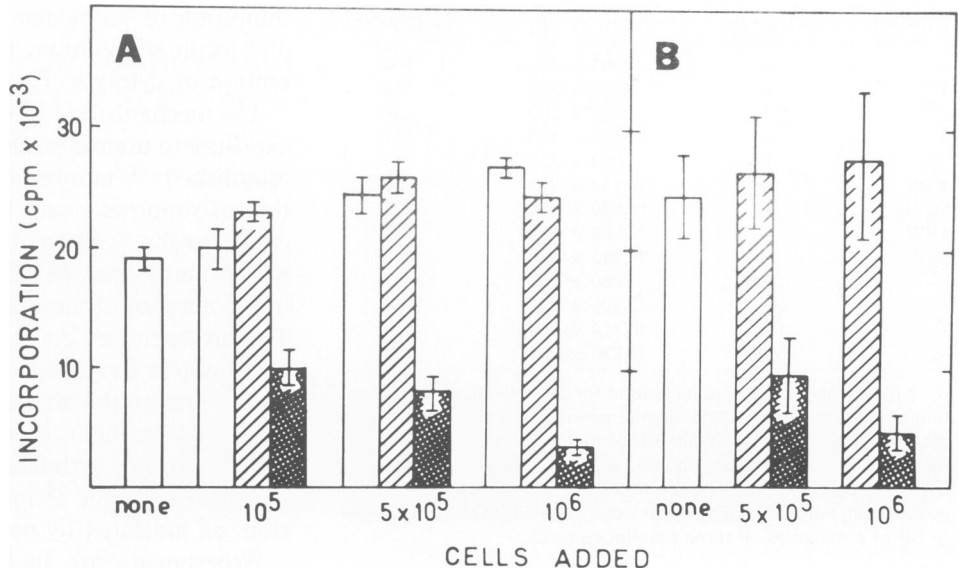

Figure 4—Suppression of MLR by LEW cells exposed to uremic serum and its isolated VLDL fraction. **A**—LEW cells were incubated for 20 hours in MEM medium supplemented with 10% control or uremic serum. The cells were then thoroughly washed in complete medium, and different numbers of viable cells were added to standard MLR cultures containing 10^6 LEW responder cells and 10^6 mitomycin-C-treated BN stimulator cells. There was no significant difference in the viability of cells preincubated in control and uremic serum, as determined by trypan blue dye exclusion. *Empty columns* are untreated cells; *hatched columns* are cells pretreated with control serum; *cross-hatched columns* are cells pretreated with uremic serum. The results show the mean ± SE of a minimum of four determinations. **B**—LEW cells were incubated for 20 hours in medium supplemented with 100 μg/ml control or uremic VLDL and processed and used as described in **A**. *Empty columns* are untreated cells; *hatched columns* are cells induced with control VLDL; *cross-hatched columns* are cells induced with uremic VLDL. The data show the means ± SE of a minimum of three determinations.

adherent cells induced with uremic serum. A MLR containing responder cells constructed from nonadherent cells exposed to uremic serum and control adherent cells was not significantly reduced.

Discussion

The results presented here demonstrate that the concentration of the inhibitor of the MLR in serum of uremic rats correlates with the severity of uremia. It has been previously demonstrated that such inhibitory activity of uremic serum is recovered in the VLDL fraction.[16] We now present experimental evidence that the inhibitory effects of uremic VLDL are associated with a discrete subset of $VLDL_1$ (S_f 100–400). Immunoregulatory properties of serum lipoproteins have been demonstrated in other systems. The isolated VLDL fraction of normal human serum has immunosuppressive properties.[28] In addition, normal human serum contains a discrete species of immunoregulatory lipoprotein of intermediate density, LDL-In.[29] The mechanism of immunoregulatory effects of human serum lipoproteins has not been established. Inhibitory human LDL-In has been reported to act by direct suppression of lymphocyte activation with no apparent role of adherent cells or indirect suppressor mechanisms.[30]

Chronic uremia in the rat, however, is associated with markedly increased suppressor cell activity.[11,22] Results reported here indicate that exposure of rat spleen cells *in vitro* to uremic serum or its VLDL fraction also results in induction of suppressor cell activity. Fractionation experiments indicate that such suppressor activity is present in the adherent cell fraction of the splenic cell population. Suppressor cells detected in spleens of uremic rats are also of the adherent type.[11,22] Observation that the short-term ex-

Table 1—Short-term Preincubation of Lew Cells with Uremic Serum Fails to Induce Suppressor Activity

Time of induction (hours)	^{3}H-thymidine incorporation (cpm ± SE)	% Suppression
None	4076 ± 320	–
2	4250 ± 280	−4
20	745 ± 206	82

Five × 10^5 Control LEW cells or LEW cells incubated for indicated time periods in medium supplemented with 10% uremic serum were added to complete MLR cultures containing 10^6 LEW cells as responders and 10^6 mitomycin-C-treated BN cells as stimulators. Suppression was calculated as described in Materials and Methods. Results show the mean ± SE of quadruplicate cultures.

Table 2—Effects of Removal of Subpopulations of Spleen Cells Adherent to Glass or Nylon Wool on the Suppression of MLR

Cells added (× 10^5)*		^{3}H-thymidine incorporation	
Control	Uremic	(cpm ± SE)	% Control
–	–	8300 ± 230	100
1	–	9354 ± 385	112
–	1	2425 ± 157	29
5	–	8396 ± 450	101
–	5	933 ± 265	11
1GW	–	15,150 ± 245	182
5GW	–	18,920 ± 390	227
1NW	–	16,262 ± 180	195
5NW	–	24,880 ± 310	299
–	1GW	8950 ± 35	107
–	5GW	12,525 ± 680	150
–	1NW	10,714 ± 286	129
–	5NW	14,230 ± 450	171

* LEW spleen cells were incubated for 20 hours in medium containing 10% control or 10% uremic serum, washed and added to standard MLR cultures. GW identifies results with the nonadherent fraction of spleen cells fractionated after incubation with serum on the glass wool column. NW identifies results with the fraction of spleen cells nonadherent to nylon wool. The results show the mean ± SE of a minimum of three parallel cultures.

posure (2 hours) of control spleen cells fails to induce suppressor effect is in agreement with the earlier observation that longer exposure to uremic serum or VLDL is required for inhibition of mitogenic response.[14]

Normal rat spleen contains an adherent suppressor cell that has been shown to inhibit the MLR,[31] response to lectins,[32] antibody production *in vitro*,[33] and the generation of cytolytic T cells *in vitro*.[34] It has been concluded that although such a cell probably belongs to a macrophage class,[33,34] its suppressor activity *in vitro* is dependent on a T lymphocyte, which also appears to be adherent to glass wool.[27]

In spleens of uremic rats, a considerably more potent suppressor-cell activity has been detected.[11,22]

Table 3—The MLR with Responder Cell Population Reconstructed from Adherent and Nonadherent Cells

Cells added (× 10^6)		^{3}H-thymidine incorporated	
Adherent	Nonadherent	(cpm ± SE)	% Control
0.5 Control	0.5 Control	2541 ± 680	100
0.5 Control	0.5 Uremic	2084 ± 320	83
0.5 Uremic	0.5 Control	486 ± 212	19
0.5 Uremic	0.5 Uremic	103 ± 62	4
–	0.5 Control	3898 ± 430	153
–	0.5 Uremic	3209 ± 605	126

LEW cells were incubated for 20 hours in medium supplemented with 10% uremic serum, washed, and fractionated by adherence to plastic tissue culture dishes. Adherent and nonadherent cell populations were then mixed with the indicated fraction of control spleen cells and used as responders against 10^6 mitomycin-C-treated BN cells as stimulators. The results show the mean ± SE of a minimum of three cultures.

The experiment presented here indicates that such suppressor activity in the adherent cell population of rat spleen cells can be induced by exposure to uremic serum *in vitro*. Such exposure results, not only in inhibition of mitogenic responses to allogeneic cells and lectin stimulation, but in inhibition of the generation of cytolytic T cells as well.[14]

The mechanism of induction of suppressor cells by exposure to uremic serum in *in vitro* culture has to be established. A number of different modes of induction of suppressor cells *in vitro* have been observed: concanavalin A stimulation,[35] allogeneic cell stimulation,[36] autologous MLR,[37] and even *in vitro* culture in the absence of an identifiable inducing stimulus.[38] The properties of the induced suppressor cells differ from system to system, depending on the species involved and on the method of induction. The requirements for cellular proliferation in the induction process are also variable.[23-25] Induction of suppressor activity by uremic serum does not require cell division, as indicated by its resistance to mitomycin C.

Experiments are in progress to characterize in detail the adherent suppressor cell found in uremia and to elucidate the mechanism of the suppressor activity. It has to be established whether these cells act by direct cell-to-cell interaction or by synthesis and release of a soluble suppressive factor. A number of antigen-specific and nonspecific soluble suppressor substances released by cultured lymphoid cells have been described.[39-41] That such a factor may be involved is an attractive possibility, because it has been shown that cultured uremic spleen cells release a macromolecular inhibitor of the MLR.[17]

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