Lack of Enhanced T-Helper Cell Function in Uremic Patients With Circulating Alloreactive Antibodies

Ahmed Shoker, Richard Miller, Robert Uldall, Eva Friedman, Sarita Angra, and Carl J. Cardella

A. Shoker, R. Uldall, E. Friedman, S. Angra, C.J. Cardella, Division of Nephrology, Department of Medicine, University of Toronto, Toronto, Ontario, Canada
R. Miller, Department of Immunology, University of Toronto, Toronto, Ontario, Canada


ABSTRACT
Some uremic patients with a history of blood transfusion, pregnancy, and previous transplantation maintain high levels of alloreactive cytotoxic antibodies in the absence of continuous exogenous allogenic stimuli and are thus considered sensitized to the major histocompatibility proteins. To differentiate into antibody-producing cells, B lymphocytes must interact with T-helper (CD4+) cells. Whether ongoing help from these cells is necessary for the B cells to continue producing cytotoxic alloreactive antibodies in these sensitized uremic patients is unknown. To gain insight into the cellular mechanisms that are associated with sustained alloantibody production, T cell activation markers were measured and specific and nonspecific T-helper cell function was studied in three uremic groups with different levels of panel reactive antibodies: 10 patients whose sera reacted to more than 80% of a panel of normal lymphocytes for at least 6 months before the study were highly sensitized, 20 patients whose sera reacted to less than 80% of the panel were moderately sensitized, and 10 nonsensitized patients whose sera did not react to any cell on the panel. The number of total and activated T-helper cells was similar in the highly sensitized and nonsensitized patients. Peripheral blood lymphocyte proliferation in response to plant lectins, soluble OKT3, or alloantigens was similar in the three uremic groups. The spontaneous proliferation of pure T-helper cells and proliferative responses to immobilized OKT3 or alloantigens were also similar in highly sensitized and nonsensitized patients. Alloreactive interleukin-2-producing cell frequencies with pure CD4+ cells as responding cells were 771 ± 77.9/10^6 cells in highly sensitized, 945 ± 252/10^6 cells in nonsensitized, and 793 ± 114/10^6 cells in controls (P = not significant). Panel reactive antibody levels did not correlate with any of the measures of T helper responses. There was a significant decrease of peripheral blood lymphocyte responses to alloantigens and anti-CD3 antibody in all uremic patients as compared with normals, suggesting a dysfunction in accessory cells that was quantitatively similar in sensitized and nonsensitized patients. In spite of the continuous production of alloantibodies by B cells, there is no evidence of either specific or nonspecific enhancement of T-helper cell function in sensitized patients. The absence of T cell immunity to alloantigens suggests that sustained activation of T-helper cells with subsequent interleukin-2 production is not necessary to maintain alloreactive B cell function.

Key Words: T-helper cell, interleukin-2, uremia, highly sensitized patients, B cell function, CD4+ cells

Although uremia is an immune-deficient state, 15% of uremic patients maintain in their sera high levels of alloreactive cytotoxic antibodies directed against a panel of normal lymphocytes (panel reactive antibody [PRAl]) and are considered sensitized to the major histocompatible complex (MHC) proteins (1). Sensitization has been linked to blood transfusion, pregnancy, and previous transplantation. The incidence of sensitization in the normal population is 1% and is usually due to low-titer immunoglobulin (IgM) antibody (2). In renal transplant recipients, exposure to alloantigens occurs after the implantation of the kidney under immunosuppressive therapy. As the graft fails, a small number of patients develop cytotoxic antibodies to graft alloantigens that persist after the kidney is removed and therapy is stopped. When these sensitized patients return to dialysis, they wait a longer time for a second transplant and have a higher risk of rejecting a subsequent graft as compared with those patients who did not develop cytotoxic antibodies (3,4). It is paradoxical that uremia, which is associated with poor humoral responses, permits a sustained level of alloreactive cytotoxic antibody of the IgG subtype in the absence of continuous exogenous allogenic challenge (5).

There are several possibilities that could explain persistent alloantibody production in the absence of continued exogenous exposure to foreign proteins. Central to the production of alloantibody is an activated CD4+ helper cell, which directs B cells to produce antibody by both cell-to-cell contact and soluble cytokines (6,7). The frequency of specifically activated T and/or B cells may become altered by the uremic state and may fail to respond to normal regulatory
signals. Alternatively, the host may retain foreign MHC peptides that could sustain T cell activation. Finally, the development of counterregulatory mechanisms may be deficient or absent in uremia, enabling either alloreactive T or B cells to persist in an activated state for a prolonged period of time. Thus, the finding of altered T cell function over a prolonged period of time would support one or more of these possibilities. Four months after MHC exposure via blood transfusions, there may be a fall, an increase, or no change in the frequency of donor-specific T cell precursor frequencies (8,9). Interleukin (IL)-2 production from T cells may be decreased after blood transfusion (10). However, T cell function 6 months or more after MHC exposure via organ transplantation in patients with persistent alloantibody production is unknown. Sustained T cell activation has been found in animals with autoimmune diseases, characterized by antibody responses to non-MHC alloantigens, and persistent nonspecific preactivation of T cells has been described in uremia (11–13). Therefore, specific or nonspecific T cell activation over a prolonged period of time could contribute to sustained alloantibody production from specific B cell precursors.

Therefore, we studied T lymphocyte surface markers and function in a cross-section of uremic patients with different levels of cytotoxic alloantibody. When T cells are activated, a number of new cell surface molecules are expressed. Therefore, we measured early markers of T cell activation such as CD 25 (IL-2 receptor) and Class II antigens. In addition, we assessed the proportion of naive and memory T cells in the circulation by using monoclonal antibodies that identify different isoforms of the CD45 (leukocyte common antigen) surface molecule on CD4+ cells. Antigens identified by monoclonal antibodies against CD45 R and 2H4 are highly expressed on naive T cells, whereas antigens identified by the monoclonal antibodies directed against CD45 RO and 4B4 (CDW29) are highly expressed on CD4+ cells with memory function (14). The response of lymphocytes and T helper cells to alloantigens and nonspecific mitogens was assessed by proliferation, by IL-2 and IL-4 production, and by IL-2-producing cell precursor frequencies. These results were correlated to levels of circulating cytotoxic antibody.

**MATERIALS AND METHODS**

**Patients**

Blood samples were taken at regular intervals from 40 nondiabetic uremic patients on hemodialysis awaiting a renal transplant and were analyzed to determine the level of cytotoxic antibody. On the basis of reactivity to a panel of normal lymphocytes, the patients were divided into three groups. The 10 highly sensitized (H.S.) patients demonstrated cytotoxic antibody to at least 80% of the panel on each of several samples taken over the previous 6 months. The 20 patients who reacted to less than 80% of the panel were considered moderately sensitized (M.S.). The 10 nonsensitized (N.S.) patients did not demonstrate cytotoxic antibody on several serum samples taken over the previous 6 months. The panel consisted of lymphocytes from 20 normal individuals of known HLA phenotype.

The ages, ratio of men to women, and initial kidney diseases were similar in the three groups (Table 1). The duration on dialysis was greater in the H.S. patients (8.2 ± 1.2 versus 4.0 ± 0.59 yr; \( P = 0.01 \)), but the adequacy of dialysis as measured by the KT/V was similar in the three groups. K is a constant equal to the urea clearance of the dialyser; \( T \) is the time on dialysis, and \( V \) is the volume of distribution of urea. KT/V was determined by the formula KT/V = 0.4 times the percentage reduction of urea minus 1.2 (15). The KT/V was measured monthly, and the mean over the last 6 months is given in Table 1. All patients received at least 3 U of packed red blood cells in the past but not within the 6 months before the study. The 18 patients who had lost a previous transplant had the graft removed at least 2 yr before the study. All patients with failed grafts were induced with rabbit antithymocyte sera and were maintained on cyclosporine, prednisone, and azathoprine in the posttransplant period. Of the seven graft failures in the H.S. patients, one was lost to acute rejection and the other six to chronic rejection. Of the eight

<table>
<thead>
<tr>
<th><strong>Group</strong></th>
<th><strong>Age (yr)</strong></th>
<th><strong>Sex</strong></th>
<th><strong>Initial Kidney Disease</strong></th>
<th><strong>Duration (yr)</strong></th>
<th><strong>KT/V</strong></th>
<th><strong>Previous Transplantation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H.S.</strong></td>
<td>39.7 ± 3.49</td>
<td>8M, 2F</td>
<td>Glomerulonephritis (4)</td>
<td>8.2 ± 1.24</td>
<td>1.44 ± 0.12</td>
<td>7</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Structural (4)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Systemic lupus (1)</td>
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<td>Pyelonephritis (1)</td>
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<tr>
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<td></td>
<td></td>
<td>Glomerulonephritis (9')</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>Structural (4)</td>
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<tr>
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<td>Uncertain (7)</td>
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<td>Glomerulonephritis (5)</td>
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<tr>
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<td></td>
<td></td>
<td>Structural (1)</td>
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<td>Hypertensive nephrosclerosis (3)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Pyelonephritis (1)</td>
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<tr>
<td><strong>M.S.</strong></td>
<td>39 ± 14</td>
<td>12M, 8F</td>
<td>Glomerulonephritis (9')</td>
<td>5 ± 2.1</td>
<td>1.37 ± 0.17</td>
<td>8</td>
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<tr>
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<td></td>
<td>Uncertain (7)</td>
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<tr>
<td><strong>N.S.</strong></td>
<td>45.9 ± 5.08</td>
<td>8M, 2F</td>
<td>Glomerulonephritis (5)</td>
<td>4 ± 0.59</td>
<td>1.48 ± 0.05</td>
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<tr>
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<td></td>
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<td>Structural (1)</td>
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<td></td>
<td></td>
<td></td>
<td>Pyelonephritis (1)</td>
<td></td>
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</tr>
</tbody>
</table>

\( a \) \( P \) value = N.S as compared with N.S. or M.S. group.

\( b \) Significantly higher than N.S. or M.S. group (\( P = 0.01 \)).
From each N.S. patient, two were lost to acute rejection, five to chronic rejection, and one to recurrent focal segmental glomerulosclerosis. Three patients in the N.S. group rejected their transplant and had a transient increase in cytotoxic antibody that was not detectable in the 6 months before the study. One of the 10 women was multiparous and was in the H.S. group. No patient was taking immunosuppressive drugs at the time of the study. The 10 controls were normal health care workers with a mean age of 39 ± 7 yr. Blood for cellular studies was taken in stable patients before their dialysis.

Cytotoxic Antibody and HLA Determination

To determine cytotoxic antibody, sera from patients were tested against a panel of lymphocytes from 20 normal donors every 1 to 3 months by the Histocompatibility Laboratory at The Toronto Hospital. HLA determination was performed with known antisera and patient lymphocytes. A standard microcytotoxicity test was used to determine cell death for both assays (16). PRA was expressed as a percent kill of lymphocytes from 20 normals by the standard microcytotoxicity technique.

To confirm that the sera of sensitized patients contained cytotoxic antibody against specific stimulator cells used in the mixed lymphocytes reaction (MLR), a 51Cr release assay was also performed (17). The peripheral blood lymphocytes (PBL) that were used as stimulators in the MLR were activated by the addition of 10 µg/mL of phytohemagglutinin (PHA) to 106 cells/mL, incubated for 5 days, and then labeled by the incubation of 200 µCi of 51Cr for 1.5 h. Of these 51Cr-labeled PHA blasts, 105 were then incubated for 2 h at 37°C in RPMI containing 20% sensitized sera and 10 µg of rabbit complement. After a low-speed spin for 10 min, the number of counts per minute (cpm) from 0.1 mL of the supernatant was measured with a gamma counter. The percent specific lysis was calculated as follows:

\[
\text{Percent Specific Lysis} = \left(1 - \frac{\text{Experimental Release}}{\text{Maximum Release} - \text{Spontaneous Release}}\right) \times 100
\]

All results were done in triplicate. The sera from each N.S. patients were confirmed to have cytotoxic antibody against the stimulator cells if the specific lysis was greater than 50%. Sera from N.S. patients had less than 10% 51Cr release.

Proliferative Response to Mitogens

PBL were obtained from defibrinated blood by density gradient centrifugation over ficoll-hypaque at 400 g for 30 min (18). Cells were washed three times and resuspended in RPMI-1640 culture medium (GIBCO, Grand Island, NY) containing 100 U/mL of penicillin and 100 µg/mL of streptomycin. Cells (105/well) were cultured in 96-well microtiter plates for 72 h with or without the following mitogens: PHA, 2 µL/well (GIBCO); concanavalin A (Con A), 2 mg/well (Pharmacia, Piscataway, NJ) or soluble OKT3 (Ortho Pharmaceutical, Raritan, NJ), 1 µg/well. To immobilize anti-CD3 to the surface of microtiter plates, 100 µL of a solution containing 50 µL of OKT3 (50 µg/mL) and 50 µL of culture medium containing 20% human pooled serum (HPS) was added to the plates and incubated for 12 h at 4°C and then washed thoroughly before cells were added to each well. For each mitogen, triplicate cultures were pulsed with 1 µCi of [3H]thyidine (specific activity, 2.5 µCi/10 mL; Amer sham, N.E.N., Boston, MA). Results were expressed as mean cpm of triplicate cultures ± SE.

Mixed Lymphocyte Response

PBL (105/well) or pure CD4+ (105/well) responder cells were isolated and added to mitomycin-treated (70 µg of mitomycin per 106 cells in 1 mL of medium for 40 min at 37°C and washed three times) stimulator cells (105/well) in a one-way MLR containing RPMI-1640, 25 mM N-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 17% vol/vol pooled human serum that had been heat inactivated at 56°C for 60 min. After 96 h in culture, plates were pulsed with [3H]thymidine (as above) and harvested 18 h later. Results are expressed as mean cpm ± SE of triplicates after 5 days in culture. The kinetics of the proliferative response during Days 1 to 4 were similar in uremic patients and controls.

CD4+ cells were isolated by negative selection. Briefly, PBL were incubated in plastic dishes at 37°C and 5% CO2 atmosphere for 12 h. Nondetectable target cells were washed through nylon wool columns (19), and the effluent cells were then incubated with iron filings to allow the removal of the remaining monocytes with a magnet. The remaining B and CD8 cells were then removed with magnetizable polystyrene beads coated with the monoclonal antibodies (Dyna, Lake Success, NY) anti-CD19 (10 µL/105 cells) and then anti-CD8 (30 µL/106 cells) in sequential steps. Before use, each preparation was negative for monocytes and contained less than 1% B cells, less than 3% CD68 cells, and more than 90% CD4+ positive cells.

IL-2 and IL-4 Measurements

IL-2 and IL-4 activity was measured in the supernatant of bulk MLR cultures with pure CD4 responder cells. Stimulator cells (105) were used to activate 105 responder cells for the IL-2 assay and 2 × 106 responder and stimulator cells were used to measure IL-4. After 4 days of incubation, the CTLL bioassay (ATCC, Rockville, MD) was used to measure IL-2 (20). Supernant (150 µL) was added to 96-well microtiter plates (Flow Laboratories, McLean, VA) in triplicate, and to each well, 5,000 CTLL cells were added in 50 µL of 10% vol/vol human pooled sera and RPMI. After 24 h, 2 µCi of [3H]thydrine (TDR) incorporation. Each plate contained a set of background wells that received supernatants from stimulator cells only. The mean uptake of [3H]thydine for each experimental datum point was background subtracted. Each experiment was accompanied by a standard IL-2 titration curve that was derived so that IL-2 activity in MLR cultures could be expressed as units per milliliter. One unit was defined as the concentration giving a 50% maximum response. To determine IL-4, the samples of supernatants were brought to room temperature. A total of 200 µL of each sample in triplicate or the known IL-4 serial dilution standard was added to 50 µL of the assay diluent RDI and incubated for 2 h in a 96-well plate. After aspiration, each well was washed three times, and then, 200 µL of the IL-4 conjugate was added. The plate was covered with an adhesive strip and incubated for 2 h. Each well was then aspirated and washed three times. Two hundred microliters of the substrate solution was then added to each well and incubated for 20 min, at which time, 50 µL of the stop solution was added to each well. The optical density of each well was determined within 30 min with a spectrophotometer set to 550 nm. The IL-4 concentration...
was interpolated from a standard IL-4 titration curve after the background activity was subtracted. The standard IL-4 titration curve is exponential between 10 to 1,000 pg/mL. All samples were done in duplicate, and all incubations were done at room temperature. The IL-4 levels in our sample were measured at Day 4 (maximum level) as predetermined from a previous time kinetic study.

Measurement of IL-2-Producing Cell Frequency by Limiting Dilution

In a limiting dilution analysis (LDA), 0.1 x 10^6 mitomycin-treated stimulator PBL were used to activate increasing numbers of responder cells, as previously described (20–22). In brief, LDA cultures containing a limiting number of responder cells were established in U-bottomed microtiter plates at a final volume of 200 μL/well in RPMI supplemented with 17% FBS. The responding cell concentration varied from 0 to 10,000 cells/well for each sample; 20 replicate wells were seeded for each dilution. After 4 days in culture, 150 μL of supernatant from each well was harvested and stored at −20°C until IL-2 production was measured. Cultures were scored as positive when the [3H]thymidine incorporation as cpm from 5 x 10^3 CTLL cells per well was higher than the mean plus 3 SD of background cultures with stimulator cells only. Frequency estimates were obtained by computer with χ² minimization by the method of Porter and Berry (23). The CTLL line used in our assay is strictly reactive to IL-2; thus, the alloreactive precursors measured by this assay are only the IL-2 producer cells.

Determination of Cell Surface Markers

The number of total and activated CD4 cells was assessed by flow cytometry (Coulter Electronics, Hialeah, FL) with fluorescein-labeled mouse monoclonal antibodies directed against the surface markers CD3, CD25, CD4, CD4 (HLA-DR), CD4 (CD45RO), CD4 (2H4), CD4 (4B4) and B1 (B cells), MO2 (monocytes), and CD8 cells. Fluorescein isothiocyanate-conjugated Fab′2 and normal mouse IgG and IgM (for monocytes) were used as negative controls. Briefly, 100-μL aliquots of the PBL or T cell suspensions (concentration of 0.5 x 10^6/mL) were stained by incubation for 15 min at 20°C with various combinations of fluorescein isothiocyanate, phycoerythrin, and energy-coupled dye-conjugated monoclonal antibodies (Coulter; Becton Dickinson, Mississauga, and Cedarlane Hornby, Ontario, Canada). After incubation, the cell suspensions were washed once with phosphate-buffered saline (pH 7.2, with 0.02% NaN₃; Coulter) and resuspended in phosphate-buffered saline before analysis. The percentage of positive cells was determined relative to the nonspecific binding of the appropriate isotypic mouse antibody control, which was nonreactive with human lymphocytes.

Statistical Analysis

Wilcoxon’s unranked t test was used to compare groups, and linear regression analysis was used to study correlations. Results are given as means ± SE. Analysis of variance was used to compare the responses of the three M.S. patients to allogenic stimuli by the two different stimulators.

RESULTS

Activation Surface Markers on PBL and T Cells

By immunofluorescence, the percentage of PBL that were positive for CD3 was 78 ± 12% in controls; 69 ± 15% in N.S. patients, and 76 ± 14% in H.S. patients (P = not significant [NS]). The percentage of PBL cells positive for the CD4 marker was 38 ± 7% in controls, 31 ± 12% in N.S. patients, and 32 ± 9% in H.S. patients (P = NS). The activation marker CD25 (IL-2 receptor) was found on 9 ± 6% of PBL from controls, 10 ± 3% of PBL from N.S. patients, and 11 ± 4% of PBL from H.S. patients (P = NS). The percentage of CD4 cells positive for the activation marker HLA DR was 13 ± 6% in controls, 16 ± 9% in N.S. patients, and 14 ± 8% in H.S. patients (P = NS).

Among peripheral blood T lymphocytes, the percentage of T4+ cells with the phenotype CD45RO, which promotes immunoglobulin production and reflects the number of memory cells, was 39 ± 10% in controls, 41 ± 12% in N.S. patients, and 36 ± 9% in H.S. patients (P = NS). The percentage of CD4+ cells that were positive for the activation marker 4B4, which reflects memory T cells that provide help for B cells, was 24 ± 7% in controls, 25 ± 8% in N.S. patients, and 22 ± 10% in H.S. patients (P = NS). The percentage of CD4+ cells with the phenotype marker 2H4, which reflects a subpopulation of naive T cells that are necessary for response to alloantigens, was 46 ± 11% in controls, 49 ± 15% in N.S. patients, and 42 ± 12% in H.S. patients (P = NS).

Proliferative Responses to Mitogens and Alloantigens

Table 2 outlines the proliferative responses of uremic PBL and CD4+ cells to nonspecific mitogens and alloantigens. The spontaneous proliferation of uremic cells from sensitized and N.S. patients did not differ from controls. The proliferation of PBL to the nonspecific T cell mitogens Con A and PHA was normal in the three uremic groups. After activation with soluble OKT3 or alloantigens, the proliferative responses of the PBL from the three uremic groups was statistically inferior to that of controls, but the pure CD4+ responses to immobilized OKT3 and alloantigens were normal. Compared with controls, the mean percent reduction of proliferative response of PBL to soluble OKT3 ranged from 48% in N.S. patients (P = 0.01) to 37% in H.S. patients (P = 0.01) and 38% in M.S. patients (P = 0.01). There was no statistical difference between the response of sensitized and M.S. PBL to soluble OKT3. Similarly, the proliferative response of PBL to alloantigens was reduced in uremic patients as compared with controls. The mean reduction was 18% for N.S. patients (P = 0.04), 23% for M.S. patients (P = 0.03) and 30% in H.S. patients (P = 0.01). There was no difference in the response of PBL from sensitized and N.S. uremic patients to alloantigens.

Alloreactive CD4+ Cell Frequencies

Table 3 summarizes the frequency of alloreactive cells in H.S. and N.S. patients. The frequency of alloreactive cells in H.S. patients was 771 ± 78 cells per 10⁶ CD4+ cells, and this was similar to the fre-
TABLE 2. Proliferative responses of uremic PBL and CD4+ cells

<table>
<thead>
<tr>
<th>Cell Groups</th>
<th>Stimulus Preparation</th>
<th>N.S.</th>
<th>M.S.</th>
<th>H.S.</th>
<th>Control</th>
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<tbody>
<tr>
<td></td>
<td>Spontaneous (72 h)</td>
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<tr>
<td></td>
<td>PBL</td>
<td>927 ± 61</td>
<td>886 ± 47</td>
<td>896 ± 38</td>
<td>944 ± 30^a</td>
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<tr>
<td></td>
<td>T4</td>
<td>443 ± 33</td>
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<td>420 ± 24</td>
<td>476 ± 53^a</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td>58,800</td>
<td>104,201</td>
<td>107,440</td>
<td>133,432</td>
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<tr>
<td></td>
<td>± 15,802</td>
<td>± 17,930</td>
<td>± 25,180</td>
<td>± 25,180</td>
<td>± 5,156^b</td>
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<tr>
<td></td>
<td>Con A</td>
<td>124,745</td>
<td>123,036</td>
<td>102,520</td>
<td>137,260</td>
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<tr>
<td></td>
<td>± 5,243</td>
<td>± 14,908</td>
<td>± 6,937</td>
<td>± 6,937</td>
<td>± 6,586^c</td>
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<td>Soluble OKT3</td>
<td>20,808</td>
<td>25,406</td>
<td>31,968</td>
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<td>37,967</td>
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<td>35,499</td>
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<td>± 3,085</td>
<td>± 1,636</td>
<td>± 5,595</td>
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<td>Immobilized Alloantigens</td>
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<td>29,467</td>
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<td>Alloantigens^a</td>
<td>± 3,381</td>
<td>—</td>
<td>± 1,671</td>
<td>± 2,544^d</td>
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</table>

a Proliferation (cpm) to all stimuli was not significantly different between N.S., M.S., and H.S. groups.
b P = NS between all groups.
c P = 0.01 versus any uremic groups.
d P = 0.01 versus H.S. group; 0.03 versus M.S. group; 0.04 versus N.S. group.

* H.S. patients demonstrated alloreactive cytotoxic antibodies in their sera against the respective stimulators.

TABLE 3. IL-2-producing cell frequencies^a and cross-match (percent killing of T and B cells) against the respective stimulators

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>H.S. group</th>
<th>F Value^b</th>
<th>95th Percentile Limit</th>
<th>Target T/B^c</th>
<th>Patient No.</th>
<th>F Value</th>
<th>95th Percentile Limit</th>
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<td>1</td>
<td>870</td>
<td>1,226–617</td>
<td>80/70</td>
<td>1</td>
<td>1,270</td>
<td>1,818–888</td>
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<td>2</td>
<td>938</td>
<td>1,277–690</td>
<td>90/80</td>
<td>2</td>
<td>547</td>
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<td>3</td>
<td>445</td>
<td>548–326</td>
<td>90/80</td>
<td>3</td>
<td>372</td>
<td>505–275</td>
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<tr>
<td>4</td>
<td>321</td>
<td>467–220</td>
<td>70/60</td>
<td>4</td>
<td>377</td>
<td>552–257</td>
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<tr>
<td>5</td>
<td>698</td>
<td>995–490</td>
<td>70/50</td>
<td>5</td>
<td>334</td>
<td>509–219</td>
<td></td>
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<tr>
<td>6</td>
<td>949</td>
<td>1,305–690</td>
<td>80/40</td>
<td>6</td>
<td>2,222</td>
<td>3,137–1,577</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>719</td>
<td>1,090–475</td>
<td>90/40</td>
<td>7</td>
<td>365</td>
<td>954–229</td>
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<tr>
<td>8</td>
<td>1,117</td>
<td>1,513–824</td>
<td>70/40</td>
<td>8</td>
<td>478</td>
<td>678–337</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>696</td>
<td>992–488</td>
<td>70/40</td>
<td>9</td>
<td>1,044</td>
<td>1,490–731</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>959</td>
<td>1,406–654</td>
<td>90/90</td>
<td>10</td>
<td>2,449</td>
<td>3,508–1,700</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>771</td>
<td>± 52 ± 78</td>
<td>78*</td>
<td></td>
<td>945</td>
<td>± 252</td>
<td></td>
</tr>
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</table>

a All curves gave a significant fit to the limiting dilution model according to χ² analysis.
b F value = frequency of alloreactive IL-2-producing T4/10^6 cells.
c Target T/B = frequency of alloreactive IL-2-producing T4/10^6 cells.
d Control LDA = 973 ± 114 was not statistically different from either group (P = 0.1).

* p = 0.1 as compared with the N.S. group.

quency in N.S. patients of 945 ± 252 cells per 10^6 CD4+ cells and to that in controls, which was 973 ± 114 cells per 10^6 CD4+ cells (P = NS).

At the time the limiting dilution assay was performed, the sera of each H.S. patient contained cytotoxic antibody directed against the specific stimulator cells that were used to determine the frequency of alloreactive cells. The range of killing by the sera of each patient to the respective stimulator cell was 70 to 90% for T cells and 40 to 90% for B cells, as measured by reactivity to a panel of normal lymphocytes by the standard microcytotoxicity test.

Alloreactivity to Sensitized and N.S. HLA Targets

Three M.S. patients with cytotoxic antibody directed against 44 to 66% of a normal panel were studied with a target to which the patient had developed specific cytotoxic antibody and to a target to which the patient had not. When the cellular responses to alloantigens were measured, the sera of the responder contained specific cytotoxic antibody, measured by 51Cr release lysis and by the standard microcytotoxicity assay, directed toward the sensitized target. The test subjects' sera did not contain specific cytotoxic antibody.
toward the N.S. target, as indicated by a negative microcytotoxicity test and by the low Cr release of less than 10%. The four measures of alloreactivity—proliferation in MLR, IL-2 producing cell frequencies, and IL-2 and IL-4 released into the supernatant of MLR cultures—were similar when either a sensitized or an N.S. HLA stimulator was used (Table 4).

**Correlation of Alloreactivity to Degree of Sensitization and Duration of Uremia**

In Figure 1, the relationship between alloreactivity in MLR, expressed as cpm per 10^6 cells, was correlated to the PRA of each responder in the MLR reaction. In each case, the HLA-A,B and HLA DR antigens of the stimulator cell were different from those of the responding cell. The cpm ranged from 26,000 to 55,000 in N.S. patients and from 32,000 to 48,000 in sensitized patients (P = NS). There was no correlation between the MLR and PRA reactivity (r = 0.01; P = NS).

The relationship between the degree of sensitization, duration on dialysis, and IL-2 precursor frequencies is given in Figure 2. There was no correlation between duration on dialysis and IL-2 precursor frequency (r < 0.02; P = NS) in either the sensitized or N.S. patients.

**DISCUSSION**

The mechanism whereby alloreactive cytotoxic antibody production persists in some uremic patients is unexplained. Initially, a small subset of CD4^+ positive T cells is activated by recognizing the Class II MHC on cells expressing the foreign MHC antigens. These CD4^+ cells not only activate CD8 cells but also activate B cells by cell-to-cell contact and lymphokine secretion and cause them to proliferate, produce specific IgM alloreactive antibody against the foreign MHC, and then “class switch” to IgG alloantibody production (24-26). When antigen is eliminated, both B and T cells would normally be down-regulated and become memory cells and alloantibody production would stop (27-29).

**TABLE 4. Comparison between pure T4 alloreponses directed toward a sensitized and N.S. target**

<table>
<thead>
<tr>
<th>Patient HLA</th>
<th>Target HLA</th>
<th>Proliferation In MLR (cpm)</th>
<th>IL-2 Producing Cell Frequency (95th Percentile Limit)</th>
<th>IL-2 Level In MLR (IU/mL)</th>
<th>IL-4 Level In MLR (pg/mL)</th>
<th>PRA</th>
<th>Lysis of Target Cells % kill % ^51Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>A24,30,835,W62,DRW2</td>
<td>A2,3,827,40,DR4,3,4</td>
<td>21,780</td>
<td>1,118.8-962.7</td>
<td>2.9</td>
<td>150</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>A1,28,87,8,DR3,W15</td>
<td>20,079</td>
<td>664.3-878.3</td>
<td>2.7</td>
<td>140</td>
<td>44</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A2,30,813,W62,DR2,7</td>
<td>23,658</td>
<td>1,248.2-681.9</td>
<td>3.0</td>
<td>180</td>
<td>65</td>
<td>70</td>
<td>50</td>
</tr>
<tr>
<td>A24,32,827,-,DR4,5</td>
<td>24,900</td>
<td>1,021.2-559.1</td>
<td>3.2</td>
<td>200</td>
<td>65</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A23,26,844,38,DR7,W14</td>
<td>19,187</td>
<td>1,118.8-612.7</td>
<td>2.2</td>
<td>220</td>
<td>60</td>
<td>80</td>
<td>72</td>
</tr>
<tr>
<td>A24,3,827,40,DR3,4</td>
<td>20,658</td>
<td>1,021.2-559.1</td>
<td>2.6</td>
<td>240</td>
<td>60</td>
<td>0</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

^a IL-2 levels measured in bulk MLR with 10^6 cells/mL at Day 4.
^b IL-4 levels measured in bulk MLR with 2 x 10^6 cells/mL at Day 4.
^c Microcytotoxicity indicated as % kill, % ^51Cr release from blast cells as targets. Antibody-mediated cytotoxicity measured in patients’ sera by both the microcytotoxicity assay and % ^51Cr release with 10,000 target cells in 0.2 mL of 20% serum.
The percentage of CD4 cells and the number of these cells was normal, but the response of PBL to PHA and alloantigens differed in all patients, in spite of differing levels of circulating uremic cells to respond normally. A qualitative defect in accessory cell function has been described (41). The normal responses of pure CD4⁺ cells to immobilized OKT3 or to alloantigens supports the possibility of an accessory cell defect, because neither of these stimuli requires accessory cell function. However, the depression of PBL responses was similar in both sensitized and N.S. patients, and thus, a quantitative accessory cell dysfunction in uremia may not explain the sustained alloantibody production. Because Con A and PHA responses also require accessory cell function, the normal response of uremic patients to these mitogens may be the result of different stimulation kinetics or mode of stimulation by soluble anti-CD3 antibody (42) and may suggest that the accessory cell dysfunction is not generalized but may be qualitatively abnormal and dependent on the type of stimuli used.

Because PBL responses to alloantigens were slightly impaired, we examined the possibility that a variable defect might exist that could be detected by correlating responses to PRA. However, we could not correlate proliferative responses or IL-2-producing cell frequencies to either PRA alone or the duration of dialysis and PRA. Finally, a subtle but significant abnormality in alloreactive responses may be evident if different targets were used. There were, however, no demonstrable differences in proliferative responses to either a sensitized or N.S. target.

Our results are different from those of Grimm and Ettenger (43), who suggested that the proliferation of PBL to alloantigens was enhanced in H.S. patients. They studied the response of PBL from H.S. children to alloantigens expressed on a pool of cells with a wide variety of HLA phenotypes. Our patients differed from theirs in that we studied adult sensitized patients whose PBL were challenged with allogenic cells expressing disparate HLA antigens from a single stimulator to whom the sensitized patient had produced circulating alloreactive antibody. Furthermore, in unpublished experiments in our laboratory, the cellular responses of sensitized and N.S. patients to pooled stimulator cells were not different (data not shown). The reasons for the differences between our results and theirs are unknown.

Observations in other models have shown a similar discordance between T cell function and B cell responses. In a smaller and more limited study, with homozgygous B lymphoblastoid cell lines as stimulators and an LDA, Deacock and Lechler could not demonstrate a relationship between either total or primed circulating alloreactive T-helper cell frequencies and PRA (44). That study supports the use of peripheral blood as a source of specifically activated T cells in renal failure patients because those investigators were able to measure an increased frequency of alloreactive T cells directed toward DR antigens expressed on grafts that had been rapidly rejected. Roelen et al. have also failed to demonstrate an increase in cytotoxic T cell precursor frequencies in uremia.

Our results show that the number and function of CD4⁺ cells in this population of well-dialyzed patients is normal, regardless of their state of sensitization. The percentage of CD4⁺ cells and the number of activated and memory CD4⁺ cells in peripheral blood is normal in all uremic patients, regardless of their level of PRA. Furthermore, the function of pure CD4⁺ cells, as measured by spontaneous proliferation and proliferative responses to Con A, PHA, immobilized OKT3, and alloantigens, is normal. Other assessments of CD4⁺ function, such as IL-2 and IL-4 production after alloantigen stimulation, were also normal. Finally, the number of precursors that can recognize alloantigens as measured by IL-2-producing cell frequencies and immunofluorescence is the same in all patients, in spite of differing levels of circulating alloantibody. These results agree with and extend those of Meur et al., who also found that CD4⁺ responses are normal in uremia (40). We have not found any evidence to support an impaired (38) or activated (12,13) population of CD4⁺ cells in uremia.

The response of PBL from uremic patients to different stimuli was variable. The proliferative response of PBL to the potent nonspecific T cell mitogens Con A and PHA was normal, but the response of PBL to soluble OKT3 and alloantigens in MLR was not. Responses to soluble OKT3 and alloantigens require normal accessory cell function, and the failure of uremic cells to respond normally may be the result of a defect in either the quantitative or the qualitative function of these cells. A qualitative defect in accessory cell function has been described by Girndt et al., who demonstrated that uremic T cells can respond normally when the costimulating pathway B7/CD28 is supplemented (41). The normal responses of pure CD4⁺ cells to immobilized OKT3 or to alloantigens supports the possibility of an accessory cell defect, because neither of these stimuli requires accessory cell function. However, the depression of PBL responses was similar in both sensitized and N.S. patients, and thus, a quantitative accessory cell dysfunction in uremia may not explain the sustained alloantibody production. Because Con A and PHA responses also require accessory cell function, the normal response of uremic patients to these mitogens may be the result of different stimulation kinetics or mode of stimulation by soluble anti-CD3 antibody (42) and may suggest that the accessory cell dysfunction is not generalized but may be qualitatively abnormal and dependent on the type of stimuli used.
sensitized patients (45). However, they did show that CD8 cells in H.S. patients were resistant to anti-CD8 treatment and to cyclosporine, suggesting that a qualitative rather than a quantitative change was present. Our studies agree with those of Roelen et al. and suggest that there is also no increase in CD4+ precursor frequencies in sensitized patients. However, we were unable to demonstrate a qualitative change in CD4+ cells in these patients as assessed by MLR using sensitized and N.S. targets.

The lack of quantitative and qualitative differences in circulating CD4+ cells suggests that other mechanisms are responsible for maintaining T cell activation to alloantigens. Paradoxically, B cell frequencies have been reported to be increased with CD4+ depletion (46). To implicate this mechanism, sustained functional CD4+ inactivation would need to be documented and, if found, would likely be due to extrinsic factors, because intrinsic CD4+ function is normal. B cell frequencies may be increased and sustained independent of CD4+ cells when there is coactivation of the B cell by activated T cells and a B cell mitogen such as Staphylococcus aureus Cowan fraction (47). There is, however, no evidence to support such an activation process in response to alloantigens.

The findings of this study suggest that several new hypotheses will have to be tested to further understand our understanding of allostimulation in uremic patients. A difference in the qualitative accessory cell dysfunction in sensitized and N.S. patients could explain the sustained production of alloreactive cytotoxic antibodies in the absence of exogenous allostimulation. The down-regulation of activated B cells requires T cell activation and the production of anti-idiotypic antibodies. If the B7/CD28-costimulating pathway is impaired in sensitized patients, T cell activation may not be sufficient to generate anti-idiotypic responses. Our data do not address this issue directly, but Tan et al. have found impaired T cell responses to alloantigens when CD28 is blocked by its natural ligand, B7/BB1 (48). Furthermore, monocytes as antigen-presenting cells could be defective in uremia. The Class II molecules on monocytes present MHC antigens to T cells by either a direct or an indirect pathway (49). If this function of uremic monocytes is substantially more defective in sensitized patients, inadequate T cell activation would occur with failure to down-regulate B cells.

MHC antigens could be retained in the sensitized patient by either the sequestration of MHC peptides in host dendritic cells or by the preservation of intact donor cells (microchimerism). The injection of allogenic cells in the neonatal period produces microchimerism in animals and induces both B cell and CD4 activation against endogenous antigens (50). Class II-bearing cells such as dendritic cells are capable of retaining processed proteins for prolonged periods of time and providing a site whereby virgin T cells can convert to memory cells and then recirculate (51,52). The relationship between function, surface phenotype markers, and the migration pathways of memory cells is complex and could be altered by the uremic environment (53). If normal circulation pathways are not followed, memory cells, regardless of their mode of stimulation, may not be detectable in peripheral blood.

Finally, uremic sera or other cell types may contribute to sustained B cell activation. CD4+ cells in sensitized patients may be inactivated or down-regulated by factors in uremic sera, leading to a failure of these cells to generate anti-idiotypic antibody and down-regulate alloreactive B cells (54). The CD8+ cell rather than the CD4+ cell may be responsible for maintaining the sensitized state because it is known that CD8+ cells can stimulate B cells in the absence of CD4+ cells (55).

In conclusion, we report that CD4+ cell function is intrinsically normal in all uremic patients on hemodialysis. Specifically, there is no increase in the spontaneous proliferation of CD4+ cells or in the enhancement of their responses to mitogenic or allogenic stimulation in the H.S. population. There is a subtle but significant defect in the help that accessory cells provide in the response of CD4+ cells to alloantigens and CD3 stimulation, but the degree of quantitative dysfunction of the accessory cells is similar in sensitized and N.S. patients. A qualitative difference in accessory cell dysfunction could explain the sensitized state and needs to be further investigated. These data suggest that mechanisms other than enhanced alloreactive CD4+ cell responses are responsible for maintaining the H.S. state of uremia and that increased IL-2 production may not be necessary to maintain alloreactive memory B cell function in humans.

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REFERENCES

7. Sanders ME, Makgoba MW, Shaw S: Human naive and memory T cells: Reinterpretation of helper-inducer sub-


45. Roelen D, Datema G, Van Bree S, Zhang L, Van Rood J, Claas F: Evidence that antibody formation against a certain HLA alloantigen is associated not with a quantitative but with a qualitative change in the cytotoxic T cells recognizing the same antigen. Transplantation 1992;53:899-903.


