Effect of Uremia on Lymphocyte Transformation and Chemiluminescence by Spleen Cells of Normal and Cryptococcus neoformans-Infected Mice

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The effect of uremia on immune competence was studied. BALB/c mice were infected with a minimally virulent strain of Cryptococcus neoformans 6 weeks before immune assay. Uremia was induced by intramuscular injection of 0.15 ml of glycerol. Pooled spleen cells from four experimental groups (normal, uremic, infected, and infected and uremic) were assayed by lymphocyte transformation (LT) and luminol-dependent chemiluminescence (CL) 24 h after induction of uremia. A greater response to phytohemagglutinin and concanavalin A stimulation in tests of LT and CL was exhibited by uremic cells than by nonuremic cells; however, the presence of BALB/c uremic serum resulted in lower responses by both LT and CL. Infected mice showed a greater response to mitogens than did noninfected mice, but no significant stimulation in response to heat-killed whole cells of C. neoformans. Spleen cell populations of uremic mice had a lower viability and a different composition of spleen cell subpopulations than did cell preparations from nonuremic mice.

Uremia literally means urine in the blood. Specifically, it is a broad range of manifestations of acute or chronic renal dysfunction, which may lead to death by generalized toxemia or infection (12). The pathogenic yeastlike fungus Cryptococcus neoformans is a frequent fungal invader of patients with kidney disease (6). This opportunistic fungus prefers low-molecular-weight nitrogen compounds as nutritive sources; thus, uremic states, particularly if the patient's immune competence is impaired, are of interest in view of the pathogenesis of cryptococcosis.

Modifying the Beijerinck auxanographic method, Staib (20) developed a technique suitable for the detection of nonprotein nitrogen compounds in clinical samples. This technique has recently been applied by Staib et al. (23) and Fromling et al. (9) to murine models of cryptococcosis and experimentally induced uremia kinetics, respectively. In animals infected with a minimally virulent strain of C. neoformans, induction of uremia resulted in more severe symptoms of cryptococcosis and increased mortality (23).

Cell-mediated immunity plays an important role in host defense against fungal infection. Cryptococcosis has been reported to be rare in healthy individuals (25). More than half of patients with cryptococcosis have been observed to be suffering from an abnormality in cell-mediated immunity (11, 18). Thus, a thorough understanding of cell-cell interactions and functions in immunity during a uremic state would be valuable in preventing opportunistic infections in patients with renal insufficiency.

The luminol-dependent chemiluminescence (CL) assay is a measure of early events of lymphocyte activation and cell-cell interactions (26). Lymphocyte transformation (LT) reflects later events in immune reactivity. The early events of lymphocyte activation as measured by CL recently have been correlated with later reactions seen in the LT test (17). Mitogen-induced LT and CL are valuable in the study of alterations in immune competence by pathological conditions.

The purpose of this investigation was to determine the effect of uremia on immunity in a murine model as measured by LT and CL in response to mitogens. Additionally, immunity to C. neoformans was studied in infected and noninfected animals.

MATERIALS AND METHODS

Organism, C. neoformans A94 (ATCC 34543), isolated from bird excreta, was maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) at 4°C with routine subculturing. This strain is heavily encapsulated and has a low virulence for mice (22). Live inoculum was grown on Sabouraud dextrose agar
at 25°C for 48 h. Cells were harvested by repeated washings with sterile saline and centrifugation at 10,000 × g for 10 min. Packed cells were resuspended in saline, and the concentration was adjusted to 2 × 10⁸ cells/ml as determined by hemacytometer counts. Whole-cell antigen for LT and CL assays was grown as above; washed cells were killed by heating at 60°C for 2 h. A portion of the heated cell suspension was plated on Sabouraud dextrose agar to verify killing.

**Animals.** Male BALB/c mice (Zentralamt für Versuchstiere, Hannover, West Germany), 6 to 8 weeks old, were used. Animals were caged individually and given food and water ad libitum. Mice were divided into four groups: normal (group 1), uremic (group 2), infected (group 3), and infected and uremic (group 4). Groups 3 and 4 were inoculated intraperitoneally with 2 × 10⁷ C. neoformans cells. Six weeks later, uremia was induced in groups 2 and 4 by intramuscular injection of 0.15 ml of glycerol (87%) (24). Serum nitrogen levels were monitored by the auxanographic method (9, 20). Maximum serum levels of low-molecular-weight nitrogen compounds are reached by 24 h after glycerol injection (9). Twenty-four hours after glycerol injection in groups 2 and 4, animals from all four groups were sacrificed by cervical dislocation for LT and CL assays.

**Mitogens.** Phytohemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, England) and concanavalin A (ConA; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were diluted in phosphate-buffered saline and frozen at −20°C until used.

**LT and CL assays.** Spleens were aseptically removed from sacrificed animals and pooled by group in 5 ml of Hank's balanced salt solution. Splenic capsules were cut, and cells were suspended by gently massaging spleens between two ground-glass slides. Cells were passed through a 50-μm wire mesh and washed three times in Hank's balanced salt solution. For LT assays, spleen cells were suspended in RPMI 1640 supplemented with 6% heat-inactivated normal BALB/c serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) to a concentration of 5 × 10⁶ cells/ml. For CL assays, cells were suspended in Dulbecco minimal essential medium buffered with HEPES (N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.2; 50 mM) and supplemented with 10% fetal calf serum to a concentration of 10⁶ cells/ml. Cell concentrations were based on viable cells determined by trypan blue exclusion.

LT methods used were modifications of those reported by Fromting et al. (8). Into flat-bottomed wells of microtiter plates (Falcon Plastics, Oxnard, Calif.) were pipetted 100 μl of spleen cell suspension and 100 μl of appropriate mitogen, fungal antigen, or control medium. All combinations were prepared in quadruplicate. Cells were incubated for 48 h at 37°C in a 5% CO₂ humidified atmosphere. At 6 h before harvest, tritiated thymidine (0.5 μCi; specific activity, 2 Ci/mmol) was added to each well. The cultures were harvested on glass fiber filters, dried, and placed in scintillation vials with 5 ml of toluene cocktail (Packard Instrument Co., Inc., Rockville, Md.). Samples were counted in a Berthold LB 5004 (Wildbad, West Germany) liquid scintillation counter.

Five hundred microliters of cell suspensions prepared for CL was divided among plastic tubes, and the tubes were maintained at 37°C until used. Cell suspensions were preincubated with 10 μl of luminol (6-amino-2,3-dihydro-1,4-phenthahalazinedione; Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml in phosphate-buffered saline) for 10 min at 37°C. CL was evoked by adding mitogen or fungal antigen and measured in a Biolumat LB 9500 (Berthold). Magnitude was expressed as counts per minute, using a calculator (Hewlett-Packard 97 SI/0) connected to the Biolumat.

**RESULTS**

The results of preliminary experiments showed that the minimally virulent strain of C. neoformans (A94) could be isolated only from the brains of intraperitoneally inoculated mice 4 weeks post-inoculation (22). At the time of splenocyte harvest (6 weeks post-inoculation) for LT and CL assays, no viable yeast cells could be isolated from the spleens.

Preliminary LT experiments were performed to determine the optimal incubation time and concentration of mitogens. Optimal luminol and mitogen concentrations were determined in preliminary CL experiments.

Spleen cell preparations of the four groups exhibited a marked difference in cell viability and composition. Cells from groups 2, 3, and 4 showed lower viability and greater percentage of accessory cells versus lymphocytes than the normal control group 1 (Table 1).

Figure 1 shows LT in response to ConA, PHA, and heat-killed cells of C. neoformans. Spleen cells altered by uremia, infection with C. neoformans, or both were more responsive to mitogen stimulation than normal cells. Neither the noninfected or infected groups responded to the heat-killed whole-cell C. neoformans antigen. Three concentrations (5 × 10⁵, 5 × 10⁴, and 5 × 10³ per ml) of yeast antigen were tested. No significant response was elicited at any concentration. CL in response to ConA stimulation was similar to the LT response (Fig. 2). Groups 2, 3, and 4 exhibited a greater response than did group 1. The initial peak elicited in response to ConA better reflected LT response than did the second phase of the CL response, that occurring after 3 min. CL responses to PHA (Fig. 3) also reflected the greater reactivity of spleen cells from groups 2, 3, and 4. No detectable CL was elicited in response to the addition of 10⁵ heat-killed cells of C. neoformans by any of the four groups.
Figures 4 through 6 show results of additional experiments performed to determine the effect of uremic serum on LT and CL response to mitogens. Lymphocyte transformation by ConA and PHA significantly decreased in the presence of uremic serum (Fig. 4). The presence of uremic serum in the CL assay also decreased the spleen cell response to ConA (Fig. 5) and PHA (Fig. 6).

**DISCUSSION**

We have shown that experimental acute uremia alters spleen cell populations, resulting in a significant increase in cell reactivity to the mitogens ConA and PHA as measured by LT and CL. We also have shown that the spleen cells of mice surviving infection with a minimally virulent strain of *C. neoformans* exhibit a similar increase in mitogen reactivity, but no antigen specific reactivity measurable by LT or CL. Additionally, the presence of uremic serum has been shown to diminish LT and CL response.

The literature contains mixed reports on the change in immune cell reactivity during uremia as measured by LT. Authors have reported decreased (6), normal (14), and increased (2, 3) LT in uremic cells. Our data show a clear increase in LT reactivity to ConA and PHA or uremic spleen cells compared with normal cells (Fig. 1 and 4). These results were repeated several times with different groups of animals.

Further verification of cell-cell interaction and general immune reactivity as measured by LT can be obtained by CL assays of the same suspension of pooled cells. CL is a rapid quantitative procedure which reflects early cell reactivity to stimulating agents by the generation of high-energy photons (27). The luminol-dependent CL assay detects reactive products of oxygen (O$_2^-$, H$_2$O$_2$, OH$^-$) and of singlet O$_2$ ($\Delta^1$O$_2$) which are released during a "respiratory burst," a coordinated series of metabolic events that take place upon immune cell stimulation (1). CL results have been shown to be correlated with those obtained by LT of identical spleen cell prepara-

**TABLE 1. Viability and composition of pooled spleen cell preparations**

<table>
<thead>
<tr>
<th>Group</th>
<th>% Viability</th>
<th>% Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>96.9</td>
<td>81.8</td>
</tr>
<tr>
<td>Uremic</td>
<td>89.0</td>
<td>61.5</td>
</tr>
<tr>
<td>Infected</td>
<td>91.2</td>
<td>67.3</td>
</tr>
<tr>
<td>Infected and uremic</td>
<td>83.4</td>
<td>59.0</td>
</tr>
</tbody>
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**FIG. 1**. LT in response to ConA (2 μg/ml), PHA (2 μg/ml), and heat-killed cells of *C. neoformans* (Cn; 5 x 10$^6$/ml) by spleen cells of normal, uremic, infected, and infected and uremic BALB/c mice.

**FIG. 2.** Luminol-dependent CL in response to addition of ConA (2 mg/ml) in spleen cells of normal (○), uremic (●), infected (□), and infected and uremic (■) BALB/c mice.
Our CL results show a clearly increased activity of uremic spleen cells after stimulation with ConA (Fig. 2 and 5) and PHA (Fig. 3 and 6) compared with normal cells. These results paralleled those obtained with LT studies using the identical cell suspensions and were repeated with several groups of mice.

Spleen cells of animals infected by one inoculation of C. neoformans strain A94 and sacrificed 6 weeks later repeatedly demonstrated increased LT (Fig. 1) and CL (Fig. 2 and 3) when stimulated with ConA and PHA as compared with normal cells; however, no significant stimulation was observed in response to heat-killed whole cells of C. neoformans strain A94 (Fig. 1). Strain A94 could not be isolated from any organ except the brain 6 weeks after inoculation. This yeast therefore localizes in the brain, but remains nonlethal and does not elicit any symptoms of generalized or cerebral cryptococcosis. Fromtling et al. (7) have shown that multiple inoculations of the live avirulent strain of C. neoformans NU-2-P, elicit detectable LT responses to killed whole-cell antigens of C. neoformans, but only after 6 to 8 weekly inoculations. In addition, their NU-2-P strain could not be isolated from any organs. In light of this observation, the single-inoculation procedure used in the present study may be the reason why...
no LT or CL response to killed yeastlike antigen could be detected. New experimental parameters using the minimally virulent strain A94 are being studied, with particular interest in the persistent but nonlethal presence of the organism in the brains of infected mice. The long-term presence of live cells of \textit{C. neoformans} only in the brain of infected animals may provide a profitable model for the study of immune stimulation and competence in chronic cryptococcosis.

The viability and cell composition of pooled spleen cell preparations from the four groups studied showed marked differences (Table 1). The lower viability of cells in groups 2, 3, and 4 indicates the pathological effects of uremia or infection with \textit{C. neoformans}, or both. Since only viable cells were counted for LT and CL preparations, decreased viability was not responsible for the changes in spleen cell reactivity observed; however, the obvious change in composition of spleen cell populations with a shift toward increased percentage of accessory cells may be responsible for the increased reactivity observed in cells from groups 2, 3, and 4. The role of the accessory cells in immune reactivity and cell-cell interactions is unknown at present, but studies using purified subpopulations of spleen cell preparations are in progress. Preliminary data suggest that macrophages elicit the first CL peak and that the macrophage and granulocyte interactions with activated lymphocytes are largely responsible for the second CL peak observed during cell stimulation (S. Müller et al., manuscript in preparation).

Although increased reactivity to mitogens was observed in uremic cell preparations (Fig. 1–6), the presence of uremic serum as a medium supplement resulted in a significant suppression of spleen cell reactivity (Fig. 4–6). The exact cause of this suppression in immune reactivity in the presence of uremic serum is unknown. However, we suggest that the uremic serum, which contains above-normal levels of urea, uric acid, creatinine, and phenols, may act as a metabolic toxin that decreases cell viability and interferes with cell-cell interaction, thus significantly decreasing the respiratory burst and subsequent generation of active oxygen compounds. Although spleen cells of uremic animals show an increased reactivity, the inhibitory effect of uremic serum may result in a net decrease in immune reactivity in vivo in a uremic state. This hypothesis is supported by the findings of Staib et al. (23) indicating that uremia in infected mice allows the exacerbation of an otherwise latent infection of the brain with \textit{C. neoformans}. Thus, the immunosuppressive effect of uremic serum may be largely responsible for the impaired immune competence that has been observed in uremic human patients (4, 13, 15, 21) and in animal models of uremia (10, 16, 19, 23, 24). The relationship between the immunosuppressive effect of uremic serum and the observed loss of fungistatic effect of the serum of uremic patients...
against *C. neofor mans* (20) should be the subject of further investigation. Staib (20) has shown that the fungistatic effect of serum from such patients is restored by renal dialysis, which eliminates low-molecular-weight nitrogen substances. Our data suggest that these substances contribute to the impairment of immune competence.

We have developed a murine model of uremia which can be used for studies of immune competence in the uremic state. CL and LT assays, procedures for measuring early and late events in immune cell activation, respectively, showed significantly increased spleen cell reactivity to mitogens from uremic animals in comparison with normal animals. However, the presence of uremic serum in an assay significantly suppressed reactivity. This model is being applied to the study of spleen cell subpopulations and specific cell-cell interactions that may occur during an immune response.

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LITERATURE CITED