Effect of Friend Leukemia Virus Infection on Susceptibility to
Candida albicans

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Previous studies have demonstrated that Friend leukemia virus (FLV) induces a profound immunosuppression in susceptible mice. The studies described in this report indicate that mice infected with FLV have an increased susceptibility to subsequent infection with the opportunistic pathogen Candida albicans, as measured by increased numbers of C. albicans CFU in the kidneys of FLV-infected mice relative to uninfected controls. Experiments in which the NB-tropic and N-tropic strains of FLV were used suggest that virus replication or the resulting virus burden may be important in the observed increased susceptibility to C. albicans. Since neutrophils are believed to be important in the response of mice to systemic Candida infections, the effect of FLV infection on neutrophil candidacidal activity was investigated. The percentage of neutrophils present in unfractionated Proteose Peptone-elicited peritoneal exudates of mice infected with FLV for 14 days was significantly lower than in uninfected control mice or mice infected with FLV for 6 or 10 days. When neutrophils from FLV-infected and control mice were purified, adjusted to equal concentrations, and tested for in vitro candidacidal activity, neutrophils from mice infected with FLV for 14 days were deficient in their ability to kill C. albicans relative to normal controls and mice infected with FLV for 6 or 10 days. Addition of normal mouse serum increased killing in all groups but did not restore candidacidal activity of neutrophils from mice infected with FLV for 14 days to levels of control neutrophils or neutrophils from mice infected for 6 or 10 days with the virus. These results suggest a defect in neutrophil function, at the later stages of FLV infection, involving in vitro candidacidal activity. In addition, neutrophils from FLV-infected mice may be deficient in in vivo chemotactic activity. These defects in neutrophil function could account, at least in part, for the observed increased susceptibility of FLV-infected mice to C. albicans.

Infection of susceptible strains of mice with the murine retrovirus complex Friend leukemia virus (FLV) (3) results in a rapid development of erythroleukemia accompanied by a profound immunosuppression (16). It has been demonstrated that susceptibility to viral infection and the resulting immunosuppression are under genetic control (26, 31, 37). This FLV-induced immunosuppression has been shown to affect B-cell function (12, 37, 40), T-cell function (14, 33, 34, 38, 39), macrophage function (9, 30), natural killer cell function (20, 35), leukocyte migration (19), and suppressor cell generation (25). A number of studies have shown that FLV infection suppresses cellular (38) and humoral (11, 12, 37, 40) responses to nominal antigens such as purified protein derivative and sheep erythrocytes. The effect of FLV-induced immunosuppression on the immune response to pathogenic organisms, however, has not been extensively investigated. In the present study, the effect of FLV infection on the susceptibility of mice to Candida albicans was examined.

C. albicans is a yeast which is part of the normal gastrointestinal flora of humans and is often observed as an opportunistic infection in immunocompromised hosts (41). Mice are not normal hosts for C. albicans, but they can become infected in an experimental model by intravenous (i.v.) inoculation with live Candida yeasts, resulting in a disseminated disease similar to that seen in humans (41). The mechanism of resistance to the disseminated form of candidiasis is not entirely clear. Clinical evidence as well as several experimental animal studies suggest that resistance is dependent, at least in part, on neutrophil function (4, 28, 29, 43). The role of activated T cells (2, 13, 15, 21, 44) and/or activated macrophages (5, 43) in resistance to the disseminated form of the disease is less clear. In contrast, resistance to the chronic mucocutaneous form of the disease appears to be mediated primarily by T-cell-dependent immunity (24).

Our results indicate that mice infected with FLV have an increased susceptibility to C. albicans, as measured by greater numbers of Candida CFU in the kidneys of mice infected with FLV than in mice infected with C. albicans alone. This observation suggests that FLV-infected mice are unable to generate an effective response against systemic Candida infection. In order to investigate a possible mechanism for the observed increased susceptibility to C. albicans induced by FLV, we studied the effect of FLV infection on neutrophil function. Our studies demonstrate that deficiencies in in vitro neutrophil candidacidal activity as well as in vivo neutrophil chemotaxis in the later stages of FLV-induced disease may contribute to the increased susceptibility of FLV-infected mice to Candida infection. In addition, it appears that virus replication or the resulting increase in virus burden may be an important factor in this increased susceptibility to Candida infection.

MATERIALS AND METHODS

Mice. In these experiments, 5- to 10-week-old female BALB/cAn mice obtained from the National Cancer Insti-
tute (Bethesda, Md.) or from the Temple University Central Animal Facility (Philadelphia, Pa.) were used. Five mice were used per experimental group for both in vivo and in vitro assays.

**FLV infection.** Two polychromic strains of FLV were used in these experiments. The NB-tropic strain (NBFLV) is maintained by passage in susceptible (Fv-1<sup>+</sup> homozygous) BALB mice (31). The N-tropic strain (NFLV) is maintained by passage in susceptible (Fv-1<sup>-</sup> homozygous) DBA/2 mice (31). For in vivo experiments to measure growth of *C. albicans* in virus-infected mice, virus stocks were prepared as 10% spleen homogenates from pooled spleens of animals inoculated with FLV 14 days earlier. Homogenates were clarified by centrifugation to remove cellular debris. Virus titers were determined by the spleen focus assay (3) to be 3.3 × 10<sup>5</sup> focus-forming units (FFU) per ml for NBFV and 3.9 × 10<sup>5</sup> FFU/ml for NFLV. The virus was diluted in ice-cold saline to yield the indicated FFU per mouse in 0.2 ml volume. Mice received virus intraperitoneally (i.p.) 5 days before inoculation of *C. albicans*. For in vitro assays to measure candidacidal activity of neutrophils, neutrophils were taken from mice which received i.p. injections of 0.5 ml of virus diluted in ice-cold saline to yield the indicated dose. For these experiments, virus stocks of NBFLV were prepared as supernatants from infected SC-1 cells (22) and had a titer of 8.7 × 10<sup>5</sup> FFU/ml.

*C. albicans*. *C. albicans* B311 (ATCC 32354) was maintained at 23°C by weekly transfer on Sabouraud dextrose agar (SDA) plates. For all experiments, *C. albicans* was subcultured onto a fresh SDA plate 24 h prior to use. For in vivo assays, *C. albicans* was inoculated into 50 ml of medium described by Lee et al. (27) and incubated for 18 to 24 h at 25°C with shaking. Cells were washed and adjusted to 3.75 × 10<sup>6</sup>/ml in sterile saline, and mice were injected i.v. in the tail with 0.2 ml of the suspension (7.5 × 10<sup>6</sup> cells per mouse) on day 0. The 50% lethal dose for *C. albicans* in BALB/c mice is 2.5 × 10<sup>5</sup> cells. For in vitro neutrophil assays, a colony of yeasts was taken aseptically from a 24-h SDA plate, washed once in sterile saline, and adjusted to 5 × 10<sup>5</sup> cells per ml in neutrophil assay medium.

**Enumeration of C. albicans in the kidneys.** Mice were sacrificed by cervical dislocation on days 1, 7, 10, and 14 after i.v. inoculation with 7.5 × 10<sup>6</sup> live *C. albicans* yeast cells. Both kidneys from individual mice were removed aseptically and homogenized with a mechanical tissue homogenizer, in a final volume of 10 ml of sterile phosphate-buffered saline (PBS). The homogenate was serially diluted and inoculated into plates containing warm SDA. The number of viable *C. albicans* CFU recovered from the kidneys of individual mice was determined after incubation of SDA plates for 48 h at 37°C. Results are expressed as the mean CFU of *C. albicans* recovered from the kidneys of five animals ± the standard error of the mean. Statistical significance was determined by the Student t test.

**Preparation of neutrophils.** Mice were injected i.p. with 1 ml of 10% Proteose Peptone in sterile saline. Four hours later the peritoneal cavities were lavaged with 10 ml of ice-cold PBS (Dulbecco PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>) plus 5 U of heparin per ml. Peritoneal-exudate cells were centrifuged for 10 min at 300 × g, and when necessary, erythrocytes were removed by hypotonic lysis. Cells were resuspended in PBS plus 5 mM EDTA at 10<sup>7</sup>/ml and layered onto gradients of Lymphoprep (Ficoll-Hypaque, 1 g/ml; Accurate Chemicals, Westbury, N.Y.) in 15-ml polypropylene conical tubes. Gradients were centrifuged at 400 × g for 30 min at 23°C. Neutrophil-rich pellets were washed three times with PBS, and the cells were adjusted to desired concentrations in neutrophil assay medium (RPMI 1640 supplemented with 15% fetal calf serum, glutamine, penicillin, and streptomycin). Cell samples taken before and after fractionation were cytocentrifuged and stained with Diff-Quik (Baxter, McGaw Park, Ill.). Pellets were found to consist of 70 to 95% neutrophils.

**Neutrophil killing of C. albicans.** The assay for neutrophil candidacidal activity was modified from the protocol reported by Djeu et al. (18). Fifty microliters of neutrophils adjusted to 1.5 × 10<sup>6</sup>, 8 × 10<sup>5</sup>, or 1 × 10<sup>5</sup> was added to triplicate wells of a 96-well microtiter plate. After incubation for 30 min at 37°C in 5% CO<sub>2</sub>, contaminating nonadherent cells were removed by gentle washing with warm assay medium, and 50 ml of fresh medium with or without 5% normal mouse serum was then added to the wells. Fifty microliters of *C. albicans* yeast cells adjusted to 5 × 10<sup>5</sup>/ml in neutrophil assay medium with or without 5% normal mouse serum was added to the wells with neutrophils. These concentrations of neutrophils and *C. albicans* yielded effector/target ratios of 300:1, 160:1, and 20:1, respectively. Control wells contained either neutrophils only or *C. albicans* only. Plates were incubated for 18 h at 37°C in 5% CO<sub>2</sub>, after which the wells were washed twice with sterile distilled H<sub>2</sub>O. Twenty-five microliters of [3,5,6-<sup>3</sup>H]glucose (NET-807, 60 to 90 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.), adjusted to 10 μCi/ml in sterile distilled H<sub>2</sub>O, was added to the wells, and the plates were incubated for 20 min at 37°C. Fifty microliters of bleach was added to each well to remove adherent hyphae, the wells were harvested onto glass fiber filters by using a Ph.D. cell harvester (Cambridge Technology, Inc., Watertown, Mass.), and label was counted on a beta counter. This protocol was found to yield results in which the uptake of <sup>3</sup>H-glucose was proportional to the amount of *Candida* growth in the wells (data not shown). Results are expressed as percent inhibition of *C. albicans* growth by neutrophils, which was calculated as follows: percent inhibition = [1 − (cpm of wells containing *C. albicans* plus neutrophils/cpm of wells containing *C. albicans* only)] × 100.

**RESULTS**

**Effect of FLV infection on clearance of C. albicans from the kidneys.** Experiments were conducted to determine the effect of FLV infection on susceptibility of mice to *C. albicans*. Animals were inoculated with NBFLV 5 days before i.v. challenge with *C. albicans* to eliminate the possible effect of transient (1- to 3-day) interferon-mediated immunosuppression on *C. albicans* growth (6, 10). We chose to measure *C. albicans* growth in the kidneys, since previous studies have shown that the kidneys are the primary site of replication of *C. albicans* in infected mice (42). For these studies, mice were divided into two groups: group 1 received NBFLV 5 days prior to challenge with *C. albicans*, and group 2 received *C. albicans* alone. Mice were sacrificed 1, 7, 10, and 14 days after challenge with *C. albicans*. Results of a representative experiment are shown in Table 1. On day 1 after inoculation with *C. albicans*, there was no difference in the numbers of *C. albicans* CFU recovered from the kidneys of mice in groups 1 and 2, indicating that both groups received the same dose of yeast cells. At days 7, 10, and 14 after inoculation with *C. albicans*, the number of CFU in the kidneys of mice which had received FLV continued to rise. In contrast, mice which received *C. albicans* alone had significantly fewer CFU in their kidneys.
TABLE 1. Enumeration of C. albicans in the kidneys of FLV-infected and control mice

<table>
<thead>
<tr>
<th>Day postinfection with FLV</th>
<th>C. albicans recovery (10^7 ± SEM) from mice infected with:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>C. albicans</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
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<td>14</td>
<td>19</td>
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a BALB/c mice were inoculated with 6.5 x 10^4 FFU of NBFLV on day -5 and then with 7.5 x 10^9 C. albicans yeast cells on day 0. Mice were sacrificed on the days indicated, and CFU of viable C. albicans in the kidneys were determined as described in Materials and Methods.

b Mean from one of three experiments with kidneys of five mice.

c Not significant compared with value for infection with C. albicans alone.

d Significant at P < 0.05 compared with value for C. albicans alone.

at days 7 and 10 than did FLV-infected mice, and they had virtually cleared the infection by day 14 postchallenge. It is apparent from these experiments that mice inoculated with NBFLV prior to C. albicans infection are less able to eliminate C. albicans or to prevent its replication in the kidney than are uninfected control mice. These results suggest that the previously reported NBFLV-induced immunosuppression which was found to affect cellular and humoral responses to nominal antigens (11, 12, 37, 38, 40) also affects immunity against a pathogenic organism.

Comparison of the effects of NBFLV andNFLV on clearance of C. albicans from the kidneys. Since previous studies have shown that virus replication may be an important factor in the induction of immunosuppression (10, 31), experiments to determine whether virus replication was required for increased susceptibility of mice to C. albicans were performed. For these studies, the growth of C. albicans in mice inoculated with NBFLV was compared with growth in mice inoculated with NFLV. NFLV replicates 100- to 1,000-fold less efficiently in BALB/c cells than does NBFLV, by virtue of the expression of the Fv-1^h allele by this mouse strain (31). The effect of virus replication on susceptibility to C. albicans was examined by infecting mice with 1.3 x 10^3 FFU of NBFLV orNFLV and challenging them 5 days later with C. albicans. Mice were sacrificed 10 days after challenge with C. albicans, and the number of CFU in the kidneys was determined. The mean number of CFU (± standard error of the mean) recovered from the kidneys of NBFLV-infected mice (2,351 ± 1,109) x 10^2; significant at P < 0.05 compared with value for infection with C. albicans alone) was significantly greater than those recovered from control mice which received C. albicans alone (70 ± 19 x 10^2) and from mice which received NFLV and C. albicans ([154 ± 43] x 10^2; not significant compared with value for infection with C. albicans alone). These results suggest that virus replication or the resulting increase in virus burden is an important factor in the observed increased susceptibility of FLV-infected BALB/c mice to C. albicans.

Effect of FLV infection on in vitro neutrophil candidacidal activity. Since polymorphonuclear leukocytes (PMN, or neutrophils) are believed to be important cells in the elimination of a systemic Candida infection in both mice (4, 43) and humans (28, 29), it seemed possible that FLV infection caused an increase in susceptibility to Candida infection by affecting PMN function. To test this possibility, neutrophils purified from Proteose Peptone-elicited peritoneal exudates of FLV-infected and control mice were tested for their ability to inhibit the growth of C. albicans in an in vitro microassay. Neutrophils were elicited into the peritoneal cavities of FLV-infected and control mice by inoculation with Proteose Peptone as described in Materials and Methods. To test in vitro candidacidal activity, equal numbers of purified neutrophils from FLV-infected and control mice were cultured with live C. albicans yeast cells in the presence or absence of normal mouse serum. The inhibition of C. albicans hyphal growth after culture with neutrophils for 18 h was measured by uptake of [3H]glucose by surviving Candida hyphae. Neutrophils from normal mice inhibited the growth of C. albicans in a dose-dependent fashion (Fig. 1). Neutrophils from mice infected with FLV for 6 or 10 days inhibited the growth of C. albicans in a manner indistinguishable from that of normal controls (Fig. 1A and B). Neutrophils from mice infected with FLV for 14 days, on the other hand, were unable to inhibit the growth of C. albicans in this assay, even at a ratio of 300 neutrophils to 1 Candida yeast cell (Fig. 1C).

DISCUSSION

Although the fact that FLV induces immunosuppression has been known for quite some time, the effect of FLV infection (or infection by any other retrovirus) on susceptibility to pathogenic organisms has not been extensively investigated. Certain studies have demonstrated a decrease in the antibody response to Ascaris antigen (48) and Escherichia coli (23). Another retrovirus, LP-DM5, has been demonstrated to cause an abrogation of resistance to severe mousepox in C57BL/6 mice (8). Although a recent study has shown that infection of mice with lymphocytic choriomeningitis virus...
TABLE 2. Percent PMN in Proteose Peptone-elicited peritoneal exudates from normal and NBFLV-infected mice

<table>
<thead>
<tr>
<th>No. of expts</th>
<th>Mouse group</th>
<th>$10^6$ cells recovered/mouse (mean ± SD)*</th>
<th>% PMN (mean ± SD)**</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>Normal FLV (day 10)</td>
<td>8.3 ± 1.5</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>4</td>
<td>Normal FLV (day 14)</td>
<td>4.2 ± 2.1</td>
<td>51 ± 7</td>
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* Peritoneal exudate cells obtained by peritoneal lavage of Proteose Peptone-treated normal and NBFLV-infected (1.3 x 10^6 FFU) mice were counted in a hemacytometer.

** Cytocentrifuged samples of PMN purified from Proteose Peptone-elicited peritoneal exudates as described in Materials and Methods were stained with Diff-Quik, and the percent PMN was determined by nuclear morphology. A total of 100 to 200 cells were scored per sample.

with C. albicans demonstrate increased susceptibility to infection with C. albicans, as measured by a 2- to 4-log increase in the number of C. albicans colonies recovered from the kidneys of NBFLV-infected mice compared with mice infected with C. albicans alone. These results suggest that FLV modulates the activity of a cell(s) involved in eliminating a systemic Candida infection.

The cells involved in mediating resistance to systemic C. albicans infection have not been clearly defined. The role of T-cell-mediated immunity in the response to systemic C. albicans infection is particularly controversial. In this regard, evidence both for (2, 13, 21) and against (15, 44) a role for T cells in systemic Candida infection has been reported. In addition, the extent of involvement of activated macrophages in resistance is unclear (5, 43). Numerous reports, however, have provided evidence that PMN are responsible, at least in part, for eliminating a systemic C. albicans infection in both humans (28, 29) and mice (4, 43). For this reason, in order to investigate the mechanism by which FLV causes increased susceptibility to infection with C. albicans, we decided initially to examine the effect of FLV on PMN candidacidal activity.

Results of these experiments suggest a defect in neutrophil function at day 14 after infection with FLV. Specifically, we found that mice infected with FLV for 14 days, but not for 6 to 10 days, were deficient in their ability to inhibit the growth of C. albicans in vitro. The presence of normal mouse serum in the cultures, which presumably provides a source of opsonin, only partially restored the ability of neutrophils from FLV-infected mice to inhibit Candida growth relative to normal controls. These results indicate that neutrophils from mice infected for 14 days with FLV have a deficiency in vitro candidacidal activity. The fact that decreased candidacidal activity is more dramatic in the absence than in the presence of normal mouse serum suggests that this deficiency may involve effects on non-complement-receptor-mediated uptake and killing of C. albicans.

In addition, a deficiency in the in vitro chemotactic function of neutrophils was suggested by the observation that fewer PMN were present in Proteose Peptone-elicited peritoneal exudates of mice infected with FLV for 14 days than in exudates of normal controls. We cannot, however, rule out the possibility that neutrophils are indeed elicited into the peritoneal cavities of mice infected with FLV for 14 days as efficiently as in normal mice but for reasons related to the pathology of the disease are not easily obtained by lavage. In support of our observations, however, are reports of deficiencies in in vitro chemotactic activity of neutrophils iso-

causes increased susceptibility to the opportunistic fungal pathogen Histoplasma capsulatum (47), to our knowledge the present study is the first in which infection with a retrovirus has been demonstrated to increase susceptibility to a fungal infection in a mouse model. It is clear from this study that mice receiving FLV 5 days prior to inoculation

FIG. 1. Inhibition of in vitro C. albicans growth by neutrophils from normal and FLV-infected mice. Mice were injected i.p. with 1.3 x 10^6 FFU of NBFLV or supernatants from uninfected SC-1 cells. PMN were obtained 6 (A), 10 (B), and 14 (C) days later and tested for their ability to inhibit in vitro growth of C. albicans as described in Materials and Methods. Symbols: ■, normal PMN; □, FV PMN; ○, normal PMN plus normal mouse serum; ●, FLV PMN plus normal mouse serum. Results are from three representative experiments (days 6, 10, and 14) and are expressed as the mean percent inhibition of Candida growth from triplicate wells ± the standard deviation. The effector/target ratio indicates the number of PMN relative to the number of C. albicans cells added to the wells.
lated from individuals infected with the human immunodeficiency virus (1).

That an effect of virus infection on PMN activity was not observed until 14 days after infection may reflect the fact that the virus burden did not reach a critical level until this time. The disease progressed markedly from days 10 to 14, as manifested by a twofold increase in the size of the spleen over this 4-day period (data not shown). It is therefore not surprising that defects in neutrophil function were observed at 14 days but not at 10 days after virus infection.

The importance of virus replication or virus burden in the increased susceptibility of FLV-infected mice to C. albicans is suggested by experiments which measure C. albicans growth in NFLV- and NBFLV-infected mice. Unlike NBFLV-infected mice, mice infected with NFLV were able to restrict the growth of C. albicans in the kidneys. This result is consistent with previous studies which have shown that increased viral burden due to viral replication correlates with FLV-induced immunosuppression (10, 31).

The deficiencies in neutrophil function at day 14 after infection with virus may provide an explanation for our in vivo studies. A deficiency in the ability of neutrophils to migrate by chemotaxis to the site of infection, combined with an inability of neutrophils to kill C. albicans efficiently once they reach the site of infection, could explain the increased numbers of C. albicans CFU in the kidneys of mice infected with FLV for 15 and 19 days (days 10 and 14 after inoculation with C. albicans respectively). Although neutrophils are generally thought of as cells involved very early in the response against pathogenic organisms, in the case of a chronic infection it is possible that organisms which escape initial attack by neutrophils reach the kidney and elicit neutrophil-containing inflammatory infiltrates. In the case of the mice infected with FLV for 14 days, neutrophils might not be recruited efficiently and those which are recruited might be unable to efficiently inhibit the growth of C. albicans in the kidney.

These experiments do not address the mechanism by which FLV causes decreased PMN function. One possible mechanism is that FLV has a direct effect on PMN function as a result of infection of the PMN by the virus. Preliminary results from immunofluorescence studies, however, indicate that PMN from virus-infected mice do not stain with a monoclonal antibody directed against the viral gp70, suggesting that PMN do not express viral antigens and thus are not apparently productively infected (data not shown). Another possibility is that the virus affects cells which influence PMN function by producing PMN-activating lymphokines. Numerous studies have provided evidence that neutrophils are activated by lymphokines produced by T cells, including gamma interferon (17, 36, 45), granulocyte-macrophage colony-stimulating factor (32, 46), and interleukin-6 (7). Since FLV has been shown to affect T-cell function (14, 33, 34, 36, 38, 39), the increased susceptibility to fungal infection may be due not to a direct effect on PMN but rather to an effect of virus on T cells which may be needed to activate PMN to kill C. albicans in vivo.

Infection with C. albicans is often seen in individuals infected with human immunodeficiency virus (41). Since a number of recent studies have suggested defects in PMN function in these individuals (1), investigation of the mechanism by which FLV infection modulates PMN function may provide insight into the association between human immunodeficiency virus infection and infection with C. albicans.

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LITERATURE CITED