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Biological Activity of Interleukin-2 Bound to *Candida albicans*

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The lymphokine interleukin-2 (IL-2), which is necessary for the generation of an optimal cell-mediated immune response, has recently been shown to have lectinlike properties, with specificity for high-mannose groups. Therefore, the ability of IL-2 to bind to the mannose-rich fungus *Candida albicans* was examined. Heat-killed fungi preincubated with IL-2 stimulated, in a dose-dependent manner, proliferation of the IL-2-dependent cell line CTLL20. Soluble mannan, which is rich in exposed mannose groups, inhibited binding of IL-2 to *C. albicans* by approximately 60%, suggesting that the lectinlike properties of IL-2 are partially responsible for its fungal binding capacity. Binding of IL-2 to fungi appeared to be reversible, as *C. albicans* preincubated with IL-2 stimulated CTLL20 proliferation even when the fungi and cells were separated by an 0.4-μm-pore-size membrane. The lymphoproliferative response of normal human peripheral blood mononuclear cells to *C. albicans* was augmented when the fungus was preincubated with IL-2. Binding of ^{125}I-IL-2 could not be inhibited by unlabeled IL-2, suggesting the absence of high-affinity receptors on *C. albicans* for IL-2. While the in vivo relevance remains to be determined, these data demonstrate that IL-2 can bind to *C. albicans* in vitro and thereby influence the host response to this medically important fungus.

Mucocutaneous infections due to *Candida albicans* are particularly common in the setting of immunosuppression from a defective cell-mediated immune (CMI) system, such as that which occurs with AIDS, lymphomas, and corticosteroid therapy (4, 9). Despite advances in our understanding of the immunology of candidiasis, many of the mechanisms in the normal host by which the CMI system responds to prevent such infections remain to be determined.

An effective CMI response requires stimulation of T cells to secrete the lymphocytotrophic hormone interleukin-2 (IL-2). IL-2 interacts with T cells expressing IL-2 receptors (IL-2R) to stimulate proliferation. The degree of IL-2 production directly influences the magnitude and duration of the T-cell proliferative response (25, 26). In addition to the effects described above, IL-2 activates non-major histocompatibility complex-restricted cytotoxic cells, resulting in the generation of lymphokine-activated killer cells with the capacity to kill tumor and fungal targets (14, 20, 28).

Recently, IL-2 was also shown to have lectinlike properties (23). Specific binding of IL-2 to the high-mannose glycoproteins uromodulin, ovalbumin, and mannan was demonstrated. The mannose-binding site on IL-2 appears to be distinct from the site that binds to IL-2R. Since the cell wall of *C. albicans* is rich in exposed mannose groups, we sought to determine whether IL-2 can bind to this fungus, thereby potentially affecting the host response to candidiasis.

MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise stated. Human recombinant IL-2, with a specific activity of 18×10^6 IU/mg, was a gift of Cetus (Emeryville, Calif.). RPMI 1640 (GIBCO, Grand Island, N.Y.) was supplemented with 0.4 mM glutamine, 20 U of penicillin per ml, and 20 μg of streptomycin per ml. For experiments with CTLL20 cells, 5 mM 2-mercaptoethanol was also added to the supplemented RPMI 1640. Dulbecco's phosphate-buffered saline (PBS) (GIBCO) containing 0.1% bovine serum albumin (BSA) or 0.1% fetal calf serum (FCS) was used for incubations and washes. These concentrations of BSA and FCS have been shown to reduce nonspecific binding of IL-2 to undetectable levels (11). Sterile polypropylene test tubes (1.5 ml) (Brinkmann Instruments Co., Westbury, N.Y.) and glass test tubes (10 by 75 mm) (Fisher Scientific Co., Pittsburgh, Pa.) were siliconized with dimethyl-dichlorosilane as described previously (16). All incubations took place in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C, unless otherwise stated.

Fungi. Blastoconidia from a well-defined clinical isolate of *C. albicans* were harvested from 2-day-old cultures grown on Sabouraud's dextrose agar at 22°C (15). Where indicated, the fungus was heat killed by immersion in a 60°C water bath for 30 min. Prior to use in experiments, *C. albicans* was washed twice with PBS containing 0.1% BSA.

Proliferation assay with CTLL20. The IL-2-dependent cytotoxic T-lymphocyte line CTLL20 was a gift of S. Burakoff (Harvard Medical School, Boston, Mass.) (10, 19). Cells were propagated in supplemented RPMI 1640 containing 10% FCS and 10 U of IL-2 per ml. A 24-h-old culture was harvested, washed five times with RPMI 1640 containing 10% FCS to remove excess IL-2, resuspended, and plated at 10^4 cells per 0.1 ml of RPMI 1640 containing 10% FCS per well in a 96-well sterile flat-bottom tissue culture plate (Falcon #3072; Becton Dickinson Labware, Lincoln Park, N.J.). Heat-killed fungi were suspended in 1 ml of PBS containing 0.1% BSA in siliconized polypropylene test tubes to which a specific amount of IL-2 was added. For inhibition studies, variable amounts of soluble mannan prepared from *Saccharomyces cerevisiae* by the Cetavlon method (1) were added prior to the addition of IL-2. The mannan was free of detectable impurities, as determined by thin-layer chromatography performed by the manufacturer (Sigma Chemical Co.). The *C. albicans* cells were then rotated at room

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temperature for 30 min, washed five times with 1 ml of PBS containing 0.1% BSA, and resuspended in 0.1 ml of PBS containing 0.1% BSA, and 0.025 ml each was added to wells containing CTLL20 cells. The plates were then incubated for 24 h. Four hours prior to harvesting on a cell harvester (Cambridge Technology, Watertown, Mass.), the wells were pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, Mass.). Proliferation, reflected by the amount of [*methyl*-^{3}H]thymidine incorporated, was then measured by scintillation spectroscopy. For every assay, a standard curve was constructed with known amounts of soluble IL-2, ranging from 0.01 to 10 U per well.

Membrane assay. A proliferation assay consisting of CTLL20 cells in a 24-well sterile flat-bottom tissue culture plate with 0.4-μm-pore-size membranes (Costar, Cambridge, Mass.) was also utilized. CTLL20 cells were prepared as described above and then added at 10^5/0.5 ml of RPMI 1640 containing 10% FCS per well below the membrane. *C. albicans* was incubated with or without 100 U of IL-2 in 1 ml of PBS containing 0.1% BSA for 30 min at room temperature, washed five times, and resuspended, and 10^7 fungi in a volume of 0.1 ml were added per well either above or below the membrane. The plates were incubated for 24 h. Four hours prior to harvesting, the wells were pulsed with 5 μCi of [^{3}H]thymidine.

Proliferation assay with PBMC. Heparinized blood was obtained by venipuncture from healthy human volunteers, and the peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque sedimentation (17). PBMC were washed and resuspended at 10^6/ml in RPMI 1640 containing 10% human AB serum, and 0.1 ml was added per well to a 96-well sterile flat-bottom tissue culture plate. Organisms preincubated with or without IL-2, as described above, were added at 10^6 fungi per well with the PBMC. The plates were then incubated for 3, 5, 8, 11, or 15 days. The wells were pulsed with 0.5 μCi of [^{3}H]thymidine 24 h prior to harvesting.

^{125}I-IL-2 binding assay. ^{125}I-IL-2 with a specific activity of 45.4 μCi/μg was purchased from New England Nuclear. Fungi were washed and aliquoted at 2×10^8/ml of RPMI 1640 containing 1% FCS in siliconized glass tubes. In some experiments, up to 10^4 U of unlabeled IL-2 was added per tube prior to the addition of 13.5 U (0.75 ng) of ^{125}I-IL-2. The samples were incubated at 37°C in a water bath for 30 min and washed five times with RPMI 1640 containing 1% FCS, and percent binding was determined with a gamma counter according to the formula [(experimental cpm − background cpm)/(total cpm of 0.75 ng of ^{125}I-IL-2 − background cpm)] × 100 with background counts per minute (cpm) measured from tubes treated exactly as described above except that fungi were not added. A similar protocol was followed with CTLL20 cells, except that 10^6 CTLL20 cells (rather than fungi) were added per tube in RPMI 1640 containing 10% FCS.

Statistics. Means and standard errors of the mean (SEM) were compared with the two-sample, two-tailed Student's *t* test for independent means (Number Cruncher Statistical System, version 5.03; Kaysville, Utah). Curve fitting and coefficient of determination (r^2) were calculated with a Hewlett Packard 97 calculator.

RESULTS

Initial experiments confirmed the dependency of CTLL20 cells on IL-2 for proliferation (Fig. 1). Minimal activity was measured with 0.03 U of IL-2 per well. Half maximal activity was obtained at between 0.10 and 0.30 U per well, and maximal activity was obtained with 3.0 U or more per well. Between 0.03 and 3.0 U of IL-2 per well, logarithmic increases in IL-2 concentrations resulted in linear increases in proliferation ($r^2 = 0.98$).

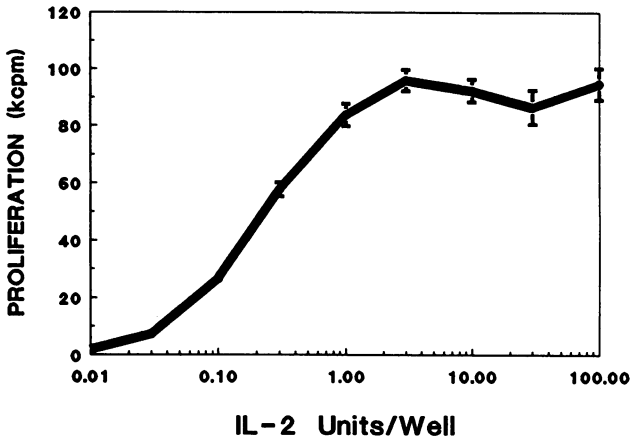

FIG. 1. Proliferation of CTLL20 in response to soluble IL-2. CTLL20 cells (10^4 per well) were stimulated for 24 h with known amounts of IL-2. The total volume per well was 0.15 ml; therefore, to obtain units per milliliter, units per well must be multiplied by 6.67. The data represent the means ± SEM (bar) of four separate experiments, each performed in triplicate.

We next sought to determine the optimal amount of IL-2 that would bind to a specific amount of *C. albicans* by measuring CTLL20 proliferation following stimulation with 10^7 heat-killed *C. albicans* cells that had been preincubated with various amounts of IL-2 (Fig. 2). Minimal proliferation was seen with up to 2.5 U of IL-2 per ml, approximately half maximal proliferation was obtained with 25 U of IL-2 per ml, and maximal stimulation was achieved with 250 U of IL-2 per ml. In the experiments described above, 0.1% BSA was present when IL-2 was incubated with *C. albicans*. Increasing the amount of BSA to 4% or substituting up to 10% FCS

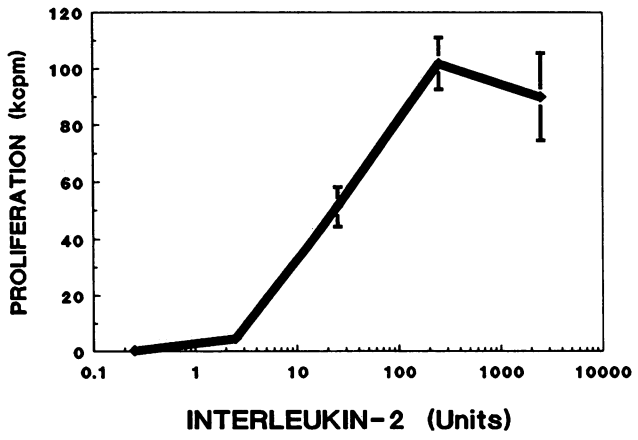

FIG. 2. CTLL20 proliferation stimulated by *C. albicans* preincubated with various amounts of IL-2. Heat-killed *C. albicans* cells (4×10^7) were incubated with various amounts of IL-2, ranging from 0.25 to 2,500 U in 1 ml of PBS containing 0.1% BSA, washed, and then added at 10^7 fungi per 10^4 CTLL20 cells per well. The data represent the means ± SEM (bar) of three separate experiments, each performed in triplicate.

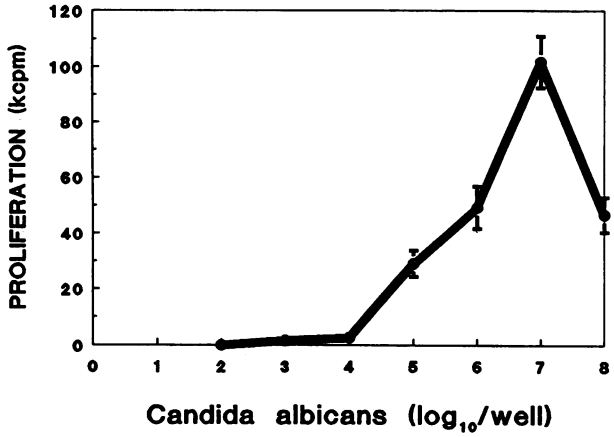

FIG. 3. CTLL20 proliferation stimulated by various numbers of *C. albicans* cells preincubated with IL-2. Heat-killed *C. albicans* cells (4×10^2 to 4×10^8) were incubated with IL-2 in 1 ml of PBS containing 0.1% BSA, washed, and then added at 10^2 to 10^8 fungi per 10^4 CTLL20 cells per well. The data represent the means ± SEM (bar) of three separate experiments, each performed in triplicate.

or pooled human serum for the BSA did not affect results (data not shown).

Subsequent experiments were performed to determine CTLL20 proliferation following stimulation by various amounts of heat-killed *C. albicans* preincubated with a saturating concentration of 250 U of IL-2 per ml (Fig. 3). Minimal proliferation was observed with up to 10^4 *C. albicans* cells per well. Thereafter, proliferation increased as the number of *C. albicans* cells was increased, up to 10^7 per well. However, when the number of *C. albicans* cells was increased to 10^8 per well, proliferation decreased below peak levels.

Since IL-2 has been shown to have mannose-binding properties (23), we next explored whether IL-2 was binding to *C. albicans* via its surface mannose groups. To do this, proliferation of CTLL20 stimulated by *C. albicans* preincubated with IL-2 in the presence or absence of mannan derived from *S. cerevisiae* was determined. When mannan was added to the incubation of *C. albicans* with IL-2, CTLL20 proliferation was inhibited in a dose-dependent fashion, with 32% inhibition of proliferation seen at the highest concentration of mannan tested (4%) ($P < 0.01$) (Fig. 4A). However, since logarithmic changes in IL-2 concentrations result in only linear changes in CTLL20 proliferation (as demonstrated in Fig. 1), simply calculating inhibition of proliferation on a linear scale underestimates the ability of mannan to inhibit the binding of IL-2 to *C. albicans*. Therefore, CTLL20 proliferation in response to *C. albicans* preincubated in IL-2 was converted into the equivalent units of soluble IL-2 that would be required to give the same amount of proliferation. By using this conversion, CTLL20 proliferation in response to *C. albicans* incubated with 25 U of IL-2 per ml was equivalent to the proliferation that would be obtained with 0.33 U of soluble IL-2. However, when 4% mannan was added to the incubation with *C. albicans*, CTLL20 proliferation was equivalent to that obtainable with only 0.14 U of IL-2, which is a 58% decrement (Fig. 4B). Inhibition mediated by mannans did not appear to be the result of the nonspecific effects of having carbohydrate or protein in the incubation mixture, since in parallel experiments, inhibition of IL-2 binding was not seen when up to

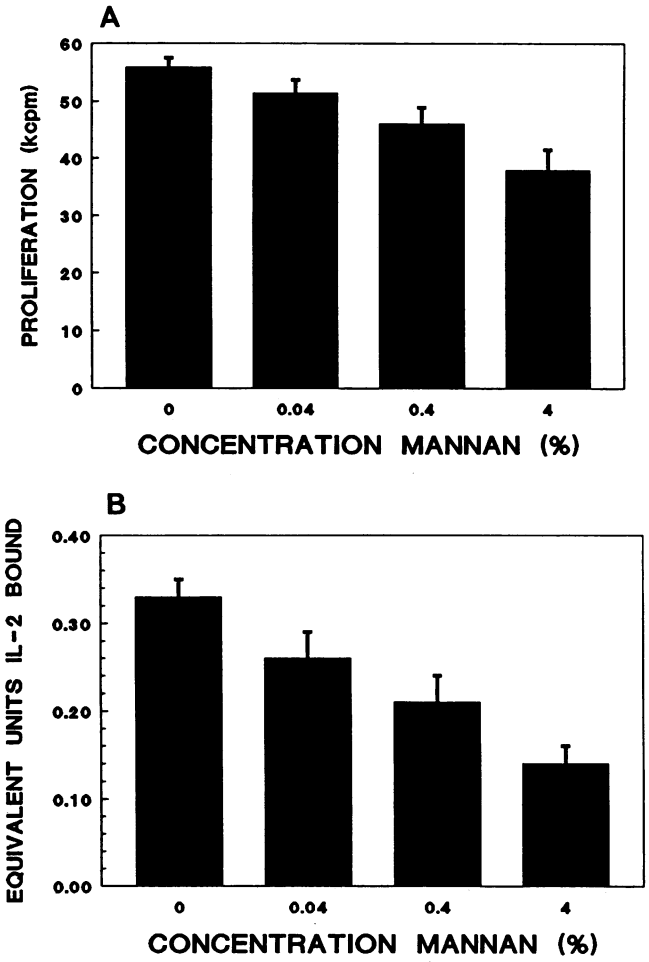

FIG. 4. Inhibition of IL-2 binding to *C. albicans* by mannan. Heat-killed *C. albicans* cells (4×10^7) were incubated with 25 U of IL-2 in 1 ml of PBS containing 0.1% BSA and various concentrations of mannan (wt/vol), washed, and then added at 10^7 fungi per 10^4 CTLL20 cells per well. (A) CTLL20 proliferation. (B) By using a concomitantly generated standard curve of proliferation in response to soluble IL-2, a curve-fitting formula based upon $y = a + b(\ln x)$ was calculated, where y is proliferation (counts per minute) and x is units of IL-2. By this formula, proliferation depicted in panel A was converted into the units of soluble IL-2 that would be needed to give an equivalent amount of proliferation. The data represent the mean ± SEM (bar) of a representative experiment, performed in triplicate. Four other experiments yielded similar results. P was <0.05 and <0.01 when results with no mannan were compared with results with 0.4 and 4% mannan, respectively.

4% (wt/vol) dextran (a high-molecular-weight carbohydrate) or albumin was substituted for mannan (data not shown).

We next sought to determine whether binding of IL-2 to *C. albicans* was reversible. To do this, wells separated into two compartments by a 0.4-μm-pore-size membrane were used. This pore size permits passage of soluble IL-2 but excludes CTLL20 cells and *C. albicans*. CTLL20 cells were added to the lower compartments of the wells, and *C. albicans* preincubated with IL-2 was added to either the upper or lower compartments of the wells. The presence of the membrane separating *C. albicans* from CTLL20 did not significantly affect proliferation (Fig. 5). Approximately equal proliferation was also seen when soluble IL-2 was added above rather than below the membrane.

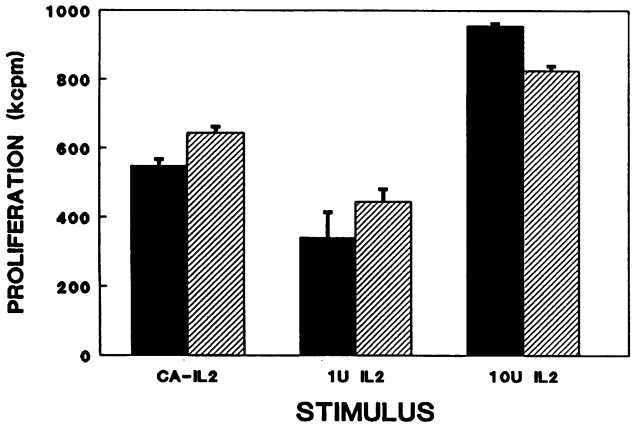

FIG. 5. CTLL20 proliferation by IL-2-treated *C. albicans* across a micropore membrane. CTLL20 cells (10^5 per well) were added to the lower compartments of wells, divided by a 0.4-μm-pore-size membrane. Heat-killed *C. albicans* cells (10^8) were incubated with 100 U of IL-2 (CA-IL2) in 1 ml of PBS containing 0.1% BSA, washed, and then added at 10^7 fungi per well above (■) or below (▨) the membrane. Soluble IL-2 at 1 and 10 U per well was studied in a similar manner for comparison. The data represent the means ± SEM (bar) of two experiments, each performed in triplicate.

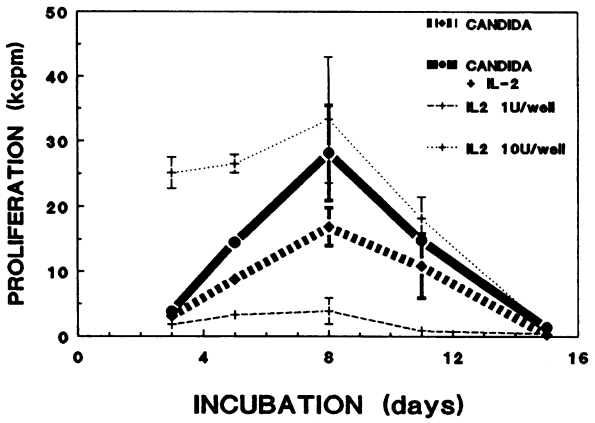

FIG. 6. PBMC proliferation stimulated by *C. albicans* preincubated with IL-2. Heat-killed *C. albicans* cells (2×10^7) were incubated with (CANDIDA + IL-2) or without (CANDIDA) IL-2 in 1 ml of PBS containing 0.1% BSA, washed, and then added at 10^6 fungi per 10^5 PBMC per well. Proliferation was measured on days 3, 5, 8, 11, and 15. Soluble IL-2 at 1 and 10 U per well was studied in a similar manner for comparison. The data are the mean ± SEM (bar) of one representative experiment of four experiments, performed in triplicate. P was <0.001 when proliferation on day 5 in response to *C. albicans* incubated with IL-2 was compared with proliferation without IL-2.

Having established that fungus-bound IL-2 could stimulate a proliferative response in an IL-2-dependent cell line, we next sought to determine whether the presence of fungus-bound IL-2 would influence the lymphoproliferative response of human PBMC to *C. albicans*. Therefore, PBMC were stimulated with heat-killed fungi preincubated with or without IL-2. In the absence of IL-2, *C. albicans* stimulated a lymphoproliferative response, with peak stimulation seen on day 8. Preincubation of the organisms with IL-2 resulted in augmentation of the magnitude of the lymphoproliferative response (Fig. 6).

C. albicans has been shown to have receptors capable of specifically binding a number of mammalian proteins, such as complement proteins (3). Therefore, we examined whether functional receptors capable of high-affinity binding of IL-2 were present on *C. albicans*. If high-affinity receptors for IL-2 were present, then binding of radiolabeled IL-2 to *C. albicans* should be inhibited by excess unlabeled IL-2. However, while a fraction of the radiolabeled IL-2 did bind to *C. albicans*, binding was not affected significantly by the prior addition of up to a 740-fold excess of unlabeled IL-2 (Fig. 7). In contrast, binding of ^{125}I-IL-2 to CTLL20 cells was inhibited by 51% when a 74-fold excess of unlabeled IL-2 was added (7,600 and 3,700 cpm in the absence and presence of unlabeled IL-2, respectively).

DISCUSSION

The data presented establish that IL-2 can bind to *C. albicans*. Binding of IL-2 to *C. albicans* was found to increase in direct proportion to the concentrations of both IL-2 and fungi (Fig. 2 and 3). However, at the highest concentration of *C. albicans* (10^8 per well) tested, there was a marked decrease in proliferation of CTLL20 cells. The reason for this decrement is unclear. With a vast excess of fungi present, IL-2 released by *C. albicans* may have a greater chance of binding to another fungal cell than to an IL-2R on a CTLL20 cell. Alternatively, it is possible that the high number of *C. albicans* cells per well had a direct toxic effect on the CTLL20 cells.

Binding of IL-2 to *C. albicans* was inhibited in a dose-dependent fashion by mannan (Fig. 4). The candidal cell wall is rich in exposed mannose groups (8), and direct binding of IL-2 to compounds, including mannan, that contain high-mannose groups has been demonstrated (23). Thus, our data suggest (but do not prove) the specificity of IL-2 for the mannose groups of the fungal cell wall. Nevertheless, approximately half of the binding of IL-2 to *C. albicans* could not be inhibited by mannan. Future studies will be needed to determine whether this binding is specific for another li-

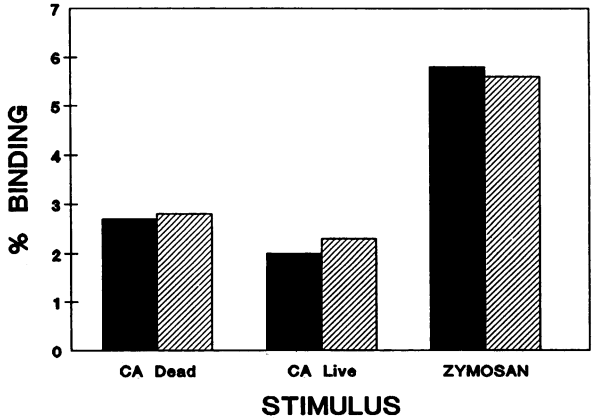

FIG. 7. Binding of ^{125}I-IL-2 to fungi in the presence or absence of unlabeled IL-2. Heat-killed *C. albicans* (CA Dead [2×10^8]), live *C. albicans* (CA Live [2×10^8]), and zymosan (6.5×10^8) were incubated with 13.5 U of ^{125}I-IL-2, in the presence (▨) or absence (■) of 10,000 U of unlabeled IL-2. The data are expressed as percentages of the radiolabeled IL-2 that remained bound to the fungi after washing and are representative of experiments repeated three times.

gand(s) on the candidal cell surface. While it remains possible that some of the binding was nonspecific, the conditions used in the assay, including the presence of 0.1% BSA, have been shown to result in no detectable nonspecific binding with assays similar to ours (11). Moreover, increasing the concentration of BSA or using up to 10% serum did not affect binding of IL-2 to *C. albicans*.

The membrane experiments demonstrate that the binding of IL-2 to *C. albicans* is reversible, for proliferation was equivalent regardless of whether the stimulus was added above or below the semipermeable membrane. Thus, direct contact of organism-bound IL-2 with the cell is not necessary for immune stimulation. Although IL-2 may not bind to the fungi with high affinity, binding does nonetheless occur. Such binding has the potential to affect the CMI response of the host, as demonstrated by the experiments with PBMC (Fig. 6). The ability of IL-2 to bind to fungi could have either beneficial or adverse effects on the host. Beneficial effects could accrue if lymphocytes bearing IL-2R are directly stimulated by fungus-bound IL-2 to kill the fungus itself or to recruit and activate other cell types capable of effector function. Alternatively, IL-2 bound and then released from the fungal surface could invoke a nonspecific immune response leading to inflammation which damages host tissue without effectively destroying the fungus. Moreover, fungi could act as a sink, binding soluble IL-2 before it reaches the intended target of the CMI system.

C. albicans has been shown to have receptors for several mammalian proteins important for host defenses, including complement and basement membrane proteins (3). Such molecular mimicry has been postulated to enhance the virulence of the fungus. Having established that IL-2 could bind to *C. albicans*, it was therefore of interest to determine whether specific IL-2R were present on the organism. However, binding of ^{125}I-IL-2 to *C. albicans* could not be inhibited by a large excess of unlabeled IL-2 (Fig. 7), suggesting that high-affinity, functional IL-2R are not present on *C. albicans*.

In addition to IL-2, tumor necrosis factor and IL-1 have mannose-binding properties (18, 22). While future studies are needed to determine whether these cytokines also bind to *C. albicans*, on the basis of the data presented in this article for IL-2, it can be predicted that they would. Moreover, the binding of IL-2 to microorganisms via this lectin-like interaction likely will not be limited to *C. albicans*, as other fungi, bacteria, parasites, and viruses (including human immunodeficiency virus type 1) have exposed mannose on their surfaces (6, 7, 13).

Clinical trials are under way to evaluate pharmacologic doses of recombinant IL-2 in patients with neoplasms. Initial results have been moderately favorable, with partial or complete responses seen in a minority of patients with particular cancers (20, 27, 28). Therapy has been limited by the frequency of severe toxic reactions which have been postulated to result, at least in part, from transiently high IL-2 levels in serum following bolus administration of IL-2. Indeed, constant infusion of IL-2, although inconvenient for patients, appears to decrease the toxicity of IL-2 (27). Novel systems for delivering IL-2, such as linking IL-2 to biodegradable microspheres or polystyrene beads, are being tested (5, 12). The data presented here, although preliminary, provide a rationale for studying immunotherapy by using IL-2 linked to fungal cell walls. Such an approach could result in decreased toxicity due to the avoidance of the transiently high peak levels of IL-2 seen with bolus injections. Moreover, β-glucans and mannans, components of fungal cell walls, have immunoenhancing effects in and of themselves (2, 21, 24). Finally, IL-2-coated fungal particles administered intravenously would likely concentrate in specific organs such as the lung, liver, and kidneys, ideally resulting in specific targeting of IL-2 to tumors or infections in these organs.

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