

Gamma Interferon Gene Expression and Release in Human Lymphocytes Directly Activated by *Cryptococcus neoformans* and *Candida albicans*

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Previous studies in our laboratory and others have demonstrated that T and/or NK cells can directly bind to and inhibit the growth of the medically important fungal pathogens *Cryptococcus neoformans* and *Candida albicans* by apparently non-major histocompatibility complex-restricted mechanisms. Here, we examined whether this direct interaction between lymphocytes and fungi also results in cytokine gene expression and release. Nonadherent lymphocytes (NAL), isolated from human peripheral blood mononuclear cells by depletion of cells adherent to plastic and nylon wool, released gamma interferon (IFN- γ), but not interleukin-4 (IL-4) and IL-10, following stimulation with *C. neoformans* yeast cells and *C. albicans* yeast cells, hyphae, and supernatants. The fungal stimuli also induced IFN- γ mRNA, with peak gene expression seen at or after 18 h. IFN- γ release was still seen even when either NK cells or T lymphocytes were depleted by negative selection, suggesting that both cell types can be stimulated by fungi to produce IFN- γ . Release of IFN- γ from fungus-stimulated NAL occurred in the absence of an intact complement system and was not especially enhanced by culture with IL-2 or IL-12. These data expand the mechanisms by which the direct interaction of NAL with fungal targets can lead to immune activation. Moreover, to our knowledge, this is the first demonstration of direct stimulation of T-cell cytokine release by microbial pathogens.

A considerable body of literature has accumulated demonstrating that T and NK cells can directly bind to and inhibit the growth of a variety of microbial pathogens by apparently non-major histocompatibility complex-restricted mechanisms (reviewed in references 28 and 33). This interaction has been studied perhaps most extensively with the fungal pathogens *Cryptococcus neoformans* and *Candida albicans*. These two fungi are major causes of opportunistic mycoses in persons with impaired lymphocyte function, especially those with AIDS (9, 22, 35). By electron and Nomarski differential interference contrast microscopy studies, an intimate association occurs between human peripheral blood T and NK cells and *C. neoformans* (25, 26, 28, 34). Moreover, lymphocyte binding to *C. neoformans* is a reversible event that appears to be associated with degranulation (25, 26). Following binding of human T and NK cells to *C. neoformans*, growth inhibition and, perhaps, killing of the fungus occur (17, 25, 26, 34). While human NK cells also bind *C. albicans*, growth inhibition has not been demonstrated, even when the lymphocytes are activated with interleukin-2 (IL-2) (1, 41). However, although not defined with human T cells, murine IL-2-activated CD8⁺ T cells bind to and inhibit the growth of *C. albicans* hyphae (3, 5).

In addition to lymphocyte-mediated growth inhibition of *C. neoformans* and *C. albicans*, binding of lymphocytes to their fungal targets may result in other contributions to host defenses, in particular, the release of lymphokines. Indeed, purified populations of human NK cells have been shown to secrete granulocyte-macrophage colony-stimulating factor and

tumor necrosis factor alpha (TNF- α) when stimulated with *C. albicans* yeast phase cells (6, 12). Moreover, TNF- α released by *Candida*-activated NK cells can augment the capacity of human polymorphonuclear neutrophils to kill *C. albicans* (11). In the present study, we examined cytokine gene expression and release in purified human T and NK cells directly stimulated by *C. neoformans* and *C. albicans*.

MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless stated otherwise. All experiments were performed under conditions carefully designed to minimize endotoxin contamination as previously described (30). RPMI 1640 and phosphate-buffered saline were obtained from Biowhitaker, Inc. (Walkersville, Md.), and contained less than 0.005 endotoxin U/ml. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was prepared as previously described (30). Pooled human serum (PHS) was prepared by combining serum from more than 10 healthy donors under conditions designed to minimize endotoxin contamination and preserve complement activity. Heat-inactivated PHS was prepared by heating PHS to 56°C for 30 min. IL-2 and IL-12 were generous gifts of Cetus Corporation (Emeryville, Calif.) and Genetics Institute (Cambridge, Mass.), respectively. Unless otherwise indicated, medium is defined as RPMI 1640 containing 10% PHS, and all incubations were performed in humidified air supplemented with 5% CO₂ at 37°C.

Fungi. Serotype A strain 145 (26, 30, 32) of *C. neoformans* was grown in RPMI 1640 (without bicarbonate), pH 6.0, at 37°C for 4 days. Under such conditions, capsule thickness averaged 1.2 μ m as measured with a light microscope equipped with a calibrated ocular micrometer following negative staining with India ink (30). A well-described isolate of *C. albicans* (27, 38) was grown in the yeast phase on Sabouraud dextrose agar at 25°C for 4 days. *C. albicans* was grown in the hyphal phase by incubation of 10⁷ yeast phase organisms per ml of RPMI 1640 at 37°C for 4 h (27). *C. albicans* hyphal supernatants were obtained by centrifuging *C. albicans* hyphae and passing the supernatants through a 0.4- μ m-pore-size filter. Prior to use, fungi were heat killed at 50°C for 30 min, washed at least five times in phosphate-buffered saline, and stored at 4°C. Overgrowth of cultures during the incubations precluded the use of live fungi. Fungi were free of significant amounts of endotoxin as determined by the inability of the LPS antagonist *Rhodobacter sphaeroides* lipid A (a gift of Nilo Qureshi, Middleton VA Hospital, Madison, Wis.) to inhibit TNF- α release from fungus-stimulated peripheral blood mononuclear cells (30).

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TABLE 1. IFN- γ release from NAL stimulated by *C. neoformans* and *C. albicans*^a

Donor	Unstimulated	PHA-PMA	Cn 3:1	Cn 10:1	Ca Yst	Ca Hyp	Ca Sup	LPS
1	< 10	8,774	63	81	82	21	< 10	16
2	41	10,000	751	618	292	780	216	ND
3	< 10	5,998	12	< 10	11	26	< 10	< 10

^a NAL were incubated for 18 h with no stimulus; 50 ng of PHA per ml plus 1 μ g of PMA per ml (PHA-PMA); *C. neoformans* at 3:1 and 10:1 fungus-to-lymphocyte ratios (Cn 3:1 and Cn 10:1 respectively); *C. albicans* yeast cells at a 10:1 ratio (Ca Yst); *C. albicans* hyphae at a 1:1 ratio (Ca Hyp); *C. albicans* supernatants (Ca Sup), or LPS. Supernatants were collected and assayed for IFN- γ by ELISA. Data are expressed as picograms of IFN- γ milliliter of supernatant. ND, not determined.

Nonadherent lymphocytes (NAL). Human peripheral blood was obtained by venipuncture from normal volunteers. For each set of experiments, the same blood donor was not used more than once. Blood was treated with the anticoagulant heparin, and the peripheral blood mononuclear cells were purified by centrifugation on a Ficoll-Hypaque density gradient. Monocytes and B cells were then depleted by adherence to polystyrene tissue culture petri dishes for 1 h, and then the nonadherent cells were passed over a nylon wool column (25, 26, 29). The resulting cells, referred to here as NAL, were over 95% T or NK cells and had undetectable numbers of monocytes and less than 2% B cells by flow cytometric analysis.

NK and T-cell depletion. NAL were further purified by negative selection with magnetic beads in accordance with the manufacturer's (DynaL, Inc., Lake Success, N.Y.) recommendations. Briefly, to deplete NK cells, NAL were incubated for 30 min at 4°C with saturating concentrations of mouse monoclonal antibodies (MAb) (Biosource International, Camarillo, Calif.) directed against CD14 (clone B-A8), CD16 (clone B-E16), and CD56 (clone B-A19). Antibodies against the monocyte marker CD14 were included to ensure complete elimination of any contaminating monocytes. Control NAL were incubated with MAb to CD14 alone. NAL were washed three times and incubated with magnetic beads coated with sheep anti-mouse immunoglobulin G, and the cells with attached beads were eliminated by adherence to a magnet. A second cycle whereby the lymphocytes were again sequentially incubated with MAb and magnetic beads was then performed. To deplete T cells, the above-described procedure was performed, except that antibodies to CD3 (clone B-B11) and CD5 (clone B-B8) were substituted for antibodies to CD16 and CD56. The surface phenotypes of the depleted cells were determined by two-color flow cytometry as in previous studies by using MAb directed against CD3, CD4, CD8, CD14, CD16, CD20, and CD56 (25, 26).

Culture of NAL with IL-2 and IL-12. NAL were cultured with IL-2 as in previous studies (25, 26). Briefly, 10⁶ NAL per ml were incubated for 7 days in medium containing 100 U of IL-2 per ml. Lymphocytes were washed, counted, and resuspended in medium without IL-2 prior to stimulation with fungi. Culture with IL-12 was performed exactly as described above, except that IL-12 (100 U/ml) was substituted for IL-2.

Lymphocyte stimulation. Incubations were performed in 24-well plates (Costar; Cambridge, Mass.) containing 2.5 \times 10⁶ lymphocytes and the stimulus indicated in Results at a final volume of 1 ml per well. For some experiments, 48-well plates containing 1.25 \times 10⁶ lymphocytes and 0.5 ml of medium were substituted with similar results. After 18 h, supernatants were collected and frozen at -70°C and/or cell pellets were collected for RNA extraction.

Cytokine ELISA. Cytokine concentrations in cell supernatants were quantitated by enzyme-linked immunosorbent assay (ELISA) with antibody pairs purchased from Genzyme Corporation (Cambridge, Mass.) for gamma interferon (IFN- γ) and from PharMingen (San Diego, Calif.) for IL-4 and IL-10 in accordance with the manufacturer's directions. All ELISAs were sensitive to \leq 10 pg/ml.

Reverse transcription-PCR. Total cellular RNA was isolated by using TriReagent (Leedo Medical Laboratories, Houston, Tex.), phenol-chloroform extraction, and ethanol precipitation. Quality of the RNA was assessed on an ethidium bromide-stained 1.2% agarose gel. mRNA from 1 μ g of total cellular RNA was reverse transcribed by using a commercial kit (Promega, Madison, Wis.) in accordance with the manufacturer's instructions. Aliquots of cDNA were amplified with oligonucleotide primers specific for IFN- γ and β -actin cDNA (Clontech Laboratories Inc., Palo Alto, Calif.). β -Actin cDNA was amplified as a control to ensure that approximately equal amounts of cDNA were obtained from all samples. cDNA (2 μ l) was added to a 25- μ l reaction mixture containing PCR buffer, MgCl₂ (2 mM), deoxynucleoside triphosphates (0.2 mM), primers (0.4 μ M), and 1 U of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.). A thermocycler (MJ Research Inc., Watertown, Mass.) ran 30 cycles for IFN- γ cDNA and 22 cycles for β -actin cDNA as follows: 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of extension at 72°C. Each experiment included negative controls without cDNA and positive controls with IFN- γ or β -actin sequences (Clontech Laboratories Inc.). The reaction product was visualized by electrophoresis on a 1.8% agarose gel stained with ethidium bromide. Preliminary experiments demonstrated that under these conditions, a semiquantitative relationship, sensitive to twofold differences, existed between the amount of the starting cDNA and that of the amplified product.

Statistics. Means and standard errors were compared by the two-tailed, two-

sample *t* test with a statistical software program (SigmaStat for Windows; Jandel Scientific Software, San Rafael, Calif.). For comparisons when tests for normality or equal variances failed, the Mann-Whitney rank sum test was utilized.

RESULTS

Cytokine release. Initial experiments examined IFN- γ release from NAL following 18 h of stimulation (Table 1). *C. neoformans* yeast cells and *C. albicans* yeast cells, hyphae, and supernatants stimulated modest release of IFN- γ . As is apparent from Table 1 and later experiments, there was some heterogeneity of response with some donors' NAL failing to release appreciable levels of IFN- γ following fungal stimulation. As expected, the positive control, which consisted of a combination of phytohemagglutinin (PHA) and phorbol myristate acetate (PMA), stimulated massive release of IFN- γ from NAL. LPS failed to stimulate appreciable quantities of IFN- γ .

Supernatants were also assayed for IL-4 and IL-10. However, in three separate experiments, levels of both of these cytokines were below the limit of detection (<10 ng/ml) following stimulation with *C. neoformans*, *C. albicans* yeast cells, *C. albicans* hyphae, and *C. albicans* supernatants. In contrast, the combination of PMA and PHA stimulated NAL to release both IL-4 and IL-10 (77 \pm 15 and 197 \pm 101 pg/ml, respectively, mean \pm standard error of three experiments).

IFN- γ gene expression in fungus-stimulated NAL. The next set of experiments examined whether fungal stimulation of NAL results in IFN- γ gene expression. RNA was extracted from NAL following incubation with *C. neoformans* yeast cells and *C. albicans* yeast cells, hyphae, and hyphal supernatants. Reverse transcription-PCR was then performed with primers specific for IFN- γ and β -actin. All of the fungal stimuli induced IFN- γ mRNA (Fig. 1), with peak gene expression at or after 18 h. Unstimulated NAL did not have detectable IFN- γ mRNA. NAL stimulated with PMA and PHA gave the strongest signal. Approximately equal amounts of reverse transcription-PCR products were seen in all samples when the cDNA was amplified with β -actin primers (data not shown).

NK and T-cell depletions. By flow cytometric analysis, NAL consisted almost entirely of a mixed population of NK and T cells, with no detectable monocytes and \leq 1% B cells (data not shown). As both NK and T cells are known to be capable of IFN- γ production, we next sought to determine whether either cell type (or both) was primarily responsible for fungus-stimulated IFN- γ release. NK or T cells were depleted by two rounds of negative selection by using magnetic beads. In the first set of experiments, NAL were treated with MAb to CD14, CD16, and CD56 to deplete NK cells and any residual monocytes while control NAL were treated with MAb to CD14 alone. Five experiments were performed. The mean percentages of T and NK cells, as determined by flow cytometry, were 95.0 and <0.1%, respectively, in the NK cell-depleted group and 83.4 and 11.5%, respectively, in the control group. Overall, NK cell depletion had no significant effect on IFN- γ release (Fig. 2A). However, consistent with previous experiments,

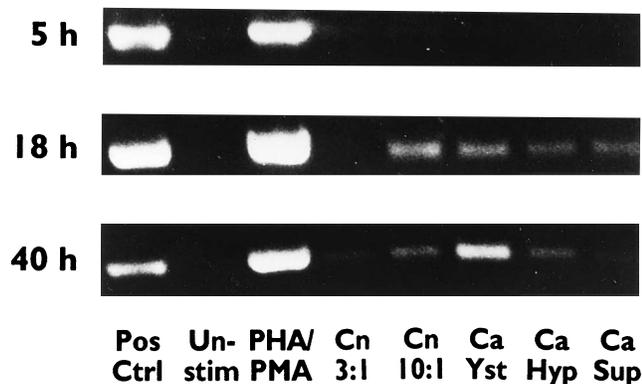


FIG. 1. IFN- γ gene expression in fungus-stimulated NAL. NAL were incubated with the indicated stimuli for 5, 18, or 40 h. Total RNA was extracted, and RT-PCR was performed as described in Materials and Methods. Three other experiments yielded similar results, except that in those experiments a band was seen with Cn 3:1 at the 18-h time point. Abbreviations for the stimuli are the same as in Table 1. Pos Ctrl, positive control; Un-stim, unstimulated.

there was heterogeneity of response: NAL from two of the five donors failed to make detectable IFN- γ in response to any of the fungal stimuli. Of the three donors whose NAL made IFN- γ when stimulated with fungi, NK cell depletion had no effect on two donors and nearly completely abrogated detectable IFN- γ release in the third donor. This last donor's NAL made unusually large amounts of IFN- γ in response to cryptococcal stimulation (3,807 and 1,655 pg/ml at effector-target cell ratios of 3:1 and 10:1, respectively).

T-cell depletion was performed as in the above-described NK cell depletion experiments, except that antibodies to CD3 and CD5 were substituted for antibodies to CD16 and CD56. Lymphocytes from four donors were tested. The mean percentages of T and NK cells, as determined by flow cytometry, were 2.6 and 33.0%, respectively, in the T-cell-depleted group and 83.3 and 7.9%, respectively, in the control group. NAL from three of four donors responded to all of the fungal stimuli. T-cell depletion (and consequent NK cell enrichment) did not significantly affect IFN- γ release (Fig. 2B). It should be noted that for *C. albicans* hyphae, nearly 10-fold less IFN- γ release was seen following T-cell depletion. Although this was not statistically significant ($P = 0.1$), the possibility that a truly significant result was obscured by the relatively small sample size (type II error) must be considered. In contrast, T-cell depletion profoundly and significantly reduced IFN- γ release in response to the combination of PMA and PHA ($6,669 \pm 1,887$ and 167 ± 138 pg/ml [means \pm standard errors; $P = 0.005$ by the Mann-Whitney rank sum test]) in the T-cell-depleted and control groups, respectively.

Requirement for complement. In the above-described experiments, PHS was included in all incubations. As NK cells and some T cells have receptors which recognize breakdown products of the third component of complement (C3), we next assessed the contribution of complement opsonization to fungus-stimulated IFN- γ release by comparing responses in the presence of PHS versus heat-inactivated PHS. Fungi stimulated NAL to release IFN- γ in four of seven donors. The substitution of heat-inactivated PHS for PHS resulted in a modest, albeit not significant, decrement in fungus-stimulated IFN- γ release (Fig. 3).

Effect of IL-2 and IL-12 and fungus-stimulated IFN- γ release. IL-2 and IL-12 have been shown to be inducers of IFN- γ in both T and NK cells (40). Moreover, IL-2 is known to

activate lymphocytes for enhanced growth inhibition of *C. neoformans* and *C. albicans* (4, 5, 17a, 25, 26). Therefore, it was of interest to determine whether fungi can stimulate IFN- γ production in NAL cultured for 7 days with IL-2 and/or IL-12. As expected, IL-2 and IL-12 each induced baseline IFN- γ secretion (Tables 2 and 3). Release of IFN- γ was moderately augmented when the lymphocytes were stimulated with *C. neoformans* and *C. albicans*.

DISCUSSION

The data presented herein expand the mechanisms by which the direct interaction of T and NK cells with fungal targets can lead to immune activation. *C. neoformans* and *C. albicans* directly stimulated human peripheral blood lymphocyte IFN- γ gene expression and release. While mitogens and cross-linking antibodies are well-established stimulators of cytokine release in T cells (15, 16), to our knowledge this is the first demon-

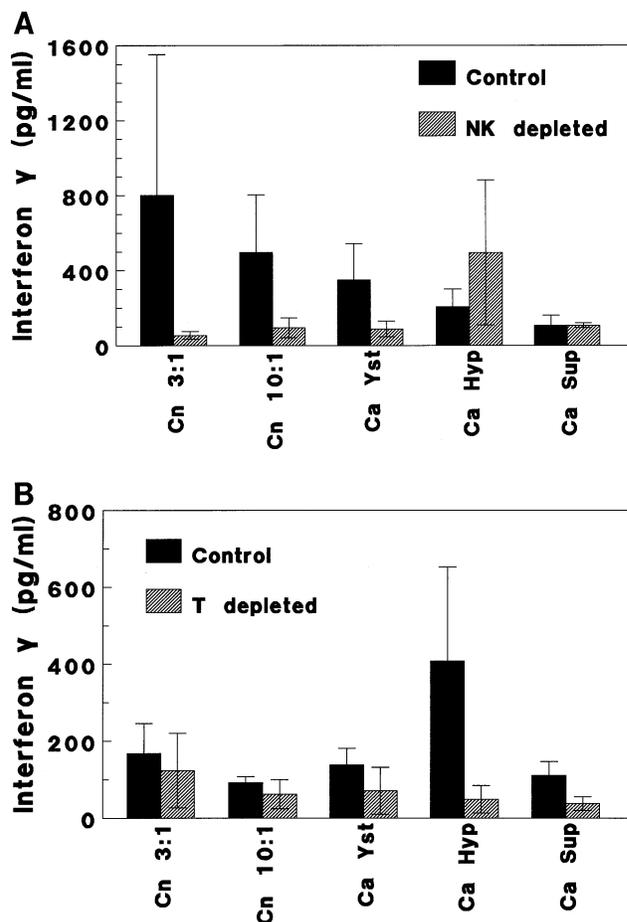


FIG. 2. Effect of NK and T-cell depletion on IFN- γ release by fungus-stimulated NAL. (A) NAL were subjected to negative selection following treatment with MAb to either CD14 alone (Control) or CD14, CD16, and CD56 (NK depleted) and then incubated with the indicated stimuli for 18 h. Supernatants were assayed for IFN- γ by ELISA. (B) Same as A, except that the experimental group was treated with MAb to CD14, CD3, and CD5 (T depleted). Median IFN- γ release in unstimulated cells was below the limit of detection (10 pg/ml) of the assay. Data represent means \pm the standard errors of the means of five (A) and four (B) experiments, each of which was performed singly or in duplicate. There are no significant differences between any of the control and experimental groups. Abbreviations for the stimuli are the same as in Table 1.

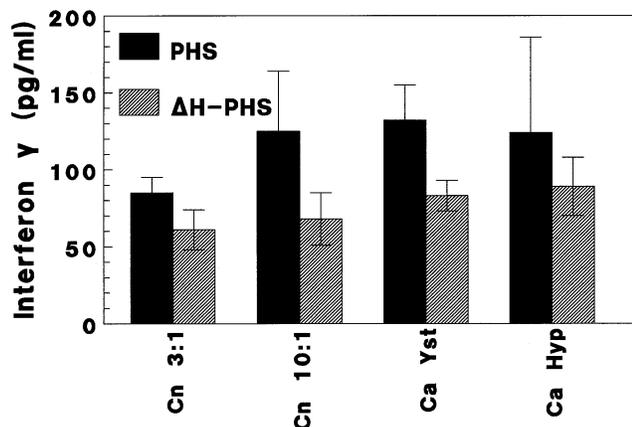


FIG. 3. Effect of complement inactivation on IFN- γ release. NAL were stimulated for 18 h as described in the footnote to Table 1, in the presence of either 10% PHS or 10% heat-inactivated PHS. Median IFN- γ release in unstimulated cells was below the limit of detection (10 pg/ml) of the assay. The data represent means \pm the standard errors of the means of four experiments, each of which was performed singly or in duplicate.

stration of direct stimulation of T-cell cytokine release by microbial pathogens.

Both NK and T cells appear to be capable of being directly stimulated by *C. neoformans* and *C. albicans* to release IFN- γ . Depletion of either NK or T cells failed to significantly lessen IFN- γ release in response to the two fungi, although there was a strong trend towards diminished release when T-cell-depleted NAL were stimulated with *C. albicans* hyphae. The possibility that a cell type other than T or NK cells was responsible for IFN- γ release cannot be entirely excluded but is unlikely for several reasons. First, T and NK cells are the only known sources of human IFN- γ (18). Second, culture of NAL with IL-2 and IL-12, which preferentially expands T and NK cells, did not result in loss of the ability to secrete IFN- γ in response to *C. neoformans* and *C. albicans*. Third, in one donor, IFN- γ release was completely abrogated when NK cells were depleted. Unfortunately, positive selection techniques could not be used in our studies as cross-linking of receptors by the antibodies used for positive selection induce or act as costimulators for IFN- γ production in NK and T cells (7, 8, 37, 39). Studies with T-cell clones are planned to further define the lymphocytic phenotypes competent for IFN- γ release.

The specific ligand(s) on the fungi that stimulates NAL to manufacture IFN- γ remains speculative. The finding that *C. albicans* hyphal supernatants stimulate IFN- γ gene expression and release demonstrates that a product(s) released from growing fungi can directly stimulate lymphocytes. *C. albicans* is known to release a variety of substances with immunomodulatory properties, including cell wall fractions which directly

TABLE 2. IFN- γ release from IL-2-activated NAL^a

Donor	Unstimulated	PHA-PMA	Cn 3:1	Cn 10:1	Ca Yst	Ca Hyp	Ca Sup
1	26	4,331	37	25	186	358	35
2	1,088	10,058	3,059	4,505	7,064	ND	3,159
3	52	255	122	105	141	ND	77

^a NAL were incubated for 7 days with 100 U of IL-2 per ml and then stimulated for 18 h with the indicated stimuli. Supernatants were then collected and assayed for IFN- γ by ELISA. Data are expressed as picograms of IFN- γ per milliliter of supernatant. Abbreviations for the stimuli are the same as in Table 1. ND, not determined.

TABLE 3. IFN- γ release from IL-12-activated NAL^a

Donor	Unstimulated	PHA-PMA	Cn 3:1	Cn 10:1	Ca Yst	Ca Hyp	Ca Sup
1	260	402	434	308	825	351	524
2	405	15,359	4,995	6,227	4,168	ND	416
3	<10	306	<10	<10	62	ND	20

^a Same as Table 2, except that 100 U of IL-12 per ml was substituted for IL-2. ND, not determined.

stimulate macrophages to release the proinflammatory cytokine TNF- α (2, 21). Very recently, Forsyth and Mathews presented data suggesting that human large granular lymphocytes use complement receptor type 3 (CR3 or CD11b/CD18) to bind unopsonized *C. albicans* hyphae (14). Moreover, greater than 95% inhibition of adhesion was obtained by using peptides containing alanine-glycine-aspartic acid (RGD) sequences (14). Further studies are needed to define the component(s) in hyphal supernatants responsible for inducing IFN- γ release from NAL and whether CR3 plays a role in hyphal recognition.

Consistent with our previous studies examining NAL-mediated growth inhibition of *C. neoformans* (26), an intact complement system was not necessary for fungus-stimulated IFN- γ release from NAL. This is in contradistinction to the situation with most phagocyte populations, in which complement in PHS serves to opsonize *C. neoformans* with C3 degradation products for immune recognition by complement receptors (reviewed in reference 23). We cannot dismiss the possibility that in the present studies, immunoglobulins or other heat-stable components in serum opsonized *C. neoformans* for recognition and subsequent IFN- γ production by NAL. However, in previous studies, NAL-mediated growth inhibition of *C. neoformans* occurred even in the complete absence of serum (26). Although the receptor(s) on NAL responsible for immune recognition of *C. neoformans* remains unknown, evidence of a receptor-mediated event can be found in studies demonstrating inhibition of lymphocyte-mediated fungistasis when NAL receptors were cleaved with either trypsin or bromelain (26).

The clinical significance of our findings remains speculative. Direct stimulation of IFN- γ release by lymphocytes could activate bystander phagocytes early in the immune response before a specific cell-mediated immune response has had time to develop. IFN- γ has been shown to have a multitude of potentially salutary effects on phagocytic responses to *C. albicans* and *C. neoformans*, including increases in antimicrobial activity, phagocytosis, TNF- α release, and oxidant generation (10, 13, 20, 24, 30, 31, 36). Moreover, IFN- γ reportedly protects endothelial cells from damage by *C. albicans* (19). The lack of IL-4 and IL-10 release from NAL following direct fungal stimulation suggests a predominantly T_H1-type pattern. Issues of clinical significance notwithstanding, the present studies expand the spectrum by which microbial targets can directly stimulate T cells to include cytokine gene expression and release.

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