Title

PMN Inactivation of *Candida albicans*-MTT-Reduction: A Three Hour Model System Suggesting that MTT Reduction Detects "Dead" Cells.

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Introduction:

Considering polymorphonuclear neutrophils (PMNs) play a major role in preventing yeast pathology, too little is known concerning PMN interaction with Candida albicans, (Rogers and Balish, 1980, Murphy 1991). Such knowledge is particularly important today since Candida infections are rising due to increases in individuals immunocompromised by AIDS , chemotherapy , and premature birth. It is important to have convenient assays available for such mechanisms to study the underlying mechanisms of protection against such pathogens (Edwards 1991).

We report here a more rapid modification of the earlier method of Ashman (1986), to study "killing" of yeast by PMNs. This optical density, microplate assay of yeast viability, as reflected by MTT reduction, has the advantage of rapidly, 2-3 hours, measuring PMN anti-yeast activity in several hundred wells, without the laborious process of counting yeast colonies (Beilke et al, 1989) or microscopic evaluation (Lehrer, 1970). Lost MTT reduction capability has often been equated with cell death (Mosmann 1883, Ashman 1986, Twentyman et al 1989, Berg et al 1990). With this method we detected 60-75% suppression of yeast MTT reduction within 60-90 minutes by 1-$5x10^5$ human PMNs, but not by sonicated PMNs or the promyelocytic cell line HL60. Yeast suppression by PMNs was reversed by cytochalasin B, and partially reversed by doxycycline, both findings suggesting the importance of phagocytosis. Anti-yeast PMN activity was not enhanced by PMN stimulants, formyl-methionyl-leucyl-phenylalanine, FMLP, or phorbol myristate acetate, PMA. Nor was suppression reversed by the inhibitors of oxygen radical activity, superoxide dysmutase, catalase or N-monomethyl-arginine, although, oxygen radical-mediated mechanisms are thought to play a major role during PMN killing of Candida (Lehrer and Cline 1969, Ferrante 1989, Gabler 1987).

The above suggested that suppressed yeast MTT reduction may not depend on killing of yeast cells but only depend on phagocytosis. When direct comparisons were made between initial yeast vs surviving yeast "indicators" i.e. yeast particle counts, yeast MTT reduction and yeast colony-forming-units, it was learned that a major source of difference between yeast-only

cultures and yeast+PMN cultures, was the growth of yeast in yeast-only cultures during the initial incubation step designed to permit PMN "killing" of yeast cells. In fact, a slight increase in MTT reduction was observed even in PMN-treated yeast cultures, compared to starting levels of activity, in spite of a concomitant 66% loss in colony-forming units, suggesting that MTT reduction does not accurately measure yeast cell death. Rather these findings can be interpreted as continued MTT-reduction-potential by yeast cells, released from PMNs by TX-100, which are "killed", i.e. can not divide, but are still able to reduce MTT, and that yeast cells sequestered within phagolysosomes in the initial incubation period are metabolically blocked via lost access to substrate etc, markedly inhibiting their growth compared to yeast-only cultures.

Thus this assay provides a very rapid and convenient quantitative assay of phagocytosis. Secondly these findings suggests that growth inhibition of yeast within the PMN phagolysosome could play a major role in delaying both the replication and invasion of Candida albicans during in situ disease processes.

Materials and Methods:

PMN preparation: The method used was a modification of the one-step method of Ferrante and Thong (1982). Blood was drawn via venipuncture from normal consenting adults into a 60-ml syringe and expressed into 5ml of PBS containing 0.15 ml of heparin sodium (10,000 units/ml). The flask was swirled and the blood gently pipetted into six 16x125 mm plastic tubes preloaded with 5 ml of Ficoll-Hypaque solution and held at a 45 angle to minimize mixing. Ficoll-Hypaque consisted of 40 g sodium diatrizoate and 25.4 g Ficoll 400, brought to 267 ml with distilled water, passed through a 0.22 um filter, and stored at 4 C. The tubes were spun at 312-372g for 45 minutes. The neutrophil-rich bands were removed, pooled, washed, and resuspended at 10^7 PMNs/ml in HBSS and held at room temperature for at least 30 minutes prior to use. Such preparations typically contained greater than 98% viable leukocytes, of which greater than 94% were PMNs (2,3,12). Remaining RBC (30-60%) were usually not removed by lysis to minimize damage to PMNs. However in a limited number of experiments RBC-free preparations were found to produce comparable results.

PMN SONICATION: PMNs were sonicated in ice via three 10 second bursts at 4'C. using a Sonifier Cell Disruptor model W185 at 100 watts.

MTT Assay: This was a modification of Ashman's method (1986) using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium Bromide),MTT, 5mg/ml HBSS standard solution, 1.25 mg/ml in the wells(13), to assay neutrophil killing of yeast cells. Candida Blastospores were used at target ratios of 1-16 yeast cells/PMN. Cultures usually contained $5X10^5$ PMNs/well unless stated differently in the results section. This concentration produces a "solid layer" of cells in the bottom of the well. Plates with PMNs only were often "pre-incubated" with PMN inhibitors or activators at 37^0C for one hour to modulate PMN activity. Yeast cells were then added to the appropriate wells and the plates centrifuged, to enhance PMN/yeast contact. Plates were then incubated at 37^0 C or 4^0 C for 0-2 hours to permit PMN yeast cell interaction. PMNs were then lysed by incubation with either TX-100 (0.06%) and succinate (6.2 mM) or with TX-100 and iodoacetamide (50mM) for 10 minutes prior to adding MTT (1.25mg/ml). Lysis was followed by one hour incubation to detect MTT reduction. We have observed this dose of iodoacetamide blocks virtually all MTT reduction. MTT Formazan was dissolved by bringing the well volume to 0.2 with DMSO added directly to the small volume of culture fluid or following removal of the culture fluid. Plates were read at 550nm on an ELISA reader. All MTT values presented are the mean of six replicate wells minus the iodoacatamide-treated wells +/- the standard deviation or standard error as appropriate.

Assay of colony-forming units: 0.025 ml of culture fluid from wells was added to 10 ml of RPMI to dilute the yeast cells. From this dilution tube 0.025 ml was spread onto agar plates of the Trypticase soy agar mixture used for growing yeast (Beilke et al 1989). Plates were incubated and counted 1-2 days later. Colonies were counted within 30-500 colonies/plate.

Candida albicans: Our departmental stock C albicans culture was originally obtained from a student isolate and the recent clinical, C. albicans isolate was kindly provided by Dr. J. W. Rourke. Both strains were recently speciated as C. albicans using germ tube morphology and carbohydrate assay. Yeast cells were prepared by culture on Tryptocase Soy Agar (40g/L) plates with added

glucose (20g/L). Plates were incubated for 24 hours prior to yeast harvest. Yeast cells were suspended in HBSS, washed and counted on a Coulter Counter.

Statistics test: Student's Paired T Test, Wilcoxon Signed Rank Test and The Bonnferroni/Dunn ANOVA tests were employed using StatView Software. A 5% Significance level was used throughout.

RESULTS:

Correlation of Yeast Cell Concentration with MTT Reduction:

To determine the appropriate concentration of yeast cells to use in our cultures and to learn if MTT reduction in our system was related to numbers of yeast cells, we correlated yeast particle counts with MTT reduction. Yeast cells were added to two microtiter plates, one incubated directly and one centrifuged prior to incubation (as done in PMN experiments to increase interaction of yeast with added PMNs). Wells were treated with TX-100/Succinate or TX-100/Iodoacetamide, a negative control (Bullock et al, 1990), incubated 10 minutes, MTT added and plates incubated for an additional hour. As shown in figure 1, a linear response was obtained between 10^5-10^6 cells with R^2 values, for this limited range, of 0.97 and 0.99 respectively. This suggested that, within this range, short term incubation with MTT can be used for in vitro assessment of relative concentrations of yeast in the same growth phase. Similar short term cultures of Listeria monocytogenes also demonstrated rapid MTT reduction related to cell concentration, as shown in figure 2, suggesting the value of this approach to study this important bacterial pathogen in vitro. Additional experiments (not shown) indicated that the reduction activity of both organisms varies with the growth phase.

Role of Serum Concentration in Yeast MTT Reduction:

Since PMNs often require serum for optimum activity, it was important to observe the effects of various concentrations of serum on MTT reduction by yeast cultures. Cells without serum or in various serum concentrations were preincubated 1.5 hour (as a control for subsequent experiments using 1.5 hour pretreatment of yeast with PMNs), treated with TX-100/Succinate or TX-100/Iodoacetamide, incubated 10 minutes, followed by addition of MTT and incubated for an additional hour. As can be seen in table I , in the

presence of HBSS medium, yeast activity was poor in the absence of serum, whereas, the highest 20% concentration depressed the response. Optimal responses were obtained with 0.625-10% serum. This high dose 20% serum suppression was seen, in the absence of neutrophils, with both fetal calf serum and human serum. In figure 3, it can be seen that the use of RPMI medium can supplant the serum requirements for maximum yeast MTT reduction and therefore RPMI was used in appropriate experiments were serum was deleted.

TX-100 Blocks PMN But Not Candida Reduction of MTT

PMNs can reduce MTT and this assay can be used to assay the functional state of PMNs (Oez et al 1990). Distilled water has been used to lyse PMNs while retaining yeast MTT activity (Ashman). Since, we routinely use TX-100 to lyse PMNs (Creamer et al, 1983, Bullock et al, 1990) we set up pilot experiments to assess yeast action in the presence of TX-100. TX-100 not only lysed PMNs but, in 5 sequential experiments, enhanced, 9-43% (mean of 23%) yeast MTT reduction.

MTT Reduction Kinetics and Succinate Enhancement

To determine a) optimum incubation time and b) whether sodium succinate would enhance yeast MTT reduction, as reported for mouse sarcoma cells (Maehara et al 1988), yeast cells were cultured with MTT for 30, 60, or 90 minutes in HBSS or in the presence of succinate (6.25 mM). All wells received TX-100. As shown in figure 4, maximum dye reduction occurred at 60 minutes with no increase at 90 minutes. Therefore subsequent experiments were assayed at one hour incubation with MTT. The strong enhancement seen in this experiment with succinate was larger than usually observed, although, 4 of 5 such experiments comparing with and without succinate resulted in improved MTT reduction in the presence of succinate. Therefore 6.25mM succinate was added as indicated.

Inactivation of Yeast Cells by PMNs:

Having established a culture protocol for the rapid O.D. measurement of yeast cells in microtiter plates and an approach to eliminate PMNs, we asked if we could detect short-term PMN inactivation of yeast cells. Yeast, 0.4 $x10^6$ cells/well, and PMNs $0.5x10^6$/well were incubated alone or together as shown in figure 5. These doses were chosen because this PMN concentration approximates a monolayer of cells at the bottom of each well insuring that each yeast will

contact a neutrophil. In addition, after adding yeast and PMNs, plates were centrifuged to enhance the potential for rapid phagocytosis. Sonicated PMNs were also tested to learn if metabolically-active PMNs were needed for observed effects or merely PMN products. As can be seen, 68% suppression occurred in the presence of whole PMNs. No significant suppression was obtained with the sonicated PMN preparation. Similar results were observed in 10 sequential experiments using our original Candida albicans isolate, as shown in Table II, with a mean of 63% suppression.

MTT Reduction also Detects PMN Inhibition of *Listeria monocytogenes*.

When tested in the same system as used above Listeria were also inhibited by PMNs as shown in figure 6 where 71% inhibition was observed. Again, no suppression was obtained with sonicated PMNs indicating the requirement for whole cells to bring about inhibition.

PMN/Target Dose Response:

To learn if detectable short term inactivation of yeast required large numbers of PMNs, a series of PMN concentrations was tested with a constant number of yeast targets as shown in figure 7. Comparable, marked inactivation of yeast cells occurred with up to 8 yeast cells/PMN. Table III provides the percent inactivation obtained in three such experiments.

Correlation of MTT Reduction With Recovered Yeast Colony Counts:

MTT reduction measures dehydrogenase activity at various cell sites, largely within mitochondria, and loss of MTT reduction has been equated with cell death of yeast cells (Ashman, 1986, Berg et al, 1990), but, such reduction could also be related to specific inactivation of enzymes without concomitant cell death, or, cells unable to divide but capable of short term metabolism. Therefore, we removed a 0.025ml aliquant from wells, after adding TX-100, and just before adding MTT to each well. These samples were then diluted, plated, incubated 24 hours and the yeast colonies counted. The results of three such experiments are displayed in figure 8. In each experiment there was a direct relationship between the number of colony forming units and the MTT reduction. The number of colonies are 25-79% decreased in the presence of PMNs and 113-236% increased in the absence of PMNs compared to the number of yeast

particles added at the start of the experiment, suggesting both killing and growth of yeast cells respectively. However, these data do not rule out the possibility that growth alone accounts for the differences observed in the MTT assay. This is particularly important since it has been reported that PMNs inhibit yeast activity but produce much less detectable killing. In addition, most assays compare yeast alone cultures with yeast+PMN cultures, assayed over several hours, permitting the unobstructed yeast to divide several fold. Therefore, the question to be asked is, what PMN activities suppress MTT reduction?

Effects of Cytochalasin B on PMN Yeast Suppression: Cytochalasin B both inhibits phagocytosis and enhances extracellular release of lysosomal contents. We therefore assessed the effects of cytochalasin B on suppression of yeast MTT reduction by PMNs. As shown in Table IV, when incubated together with PMNS, Cytochalasin B virtually eliminated PMN suppression, i.e. from 69% down to 4%. This cytochalasin B inhibition was dose dependent, with higher concentrations completely blocking the MTT inhibition, bringing it to the control levels, as shown in Figure 9. Exposure of C. albicans to PMN cell contents did not result in suppressed MTT reduction, as shown by incubation of C. albicans with sonicated PMNs.

Failure of PMN Stimulants to Enhance Yeast Inactivation: PMA and FMLP were added to the incubated PMNs and C. albicans to ask if potent PMN stimulants would increase the degree of PMN inhibition of yeast MTT activity. These reagents did not enhance yeast inactivation; nor did these PMN stimulants exert any effect on the C. albicans alone, figure 10. Suprisingly, a small reversal of MTT suppression was observed with PMA. Phorbol myristate acetate is one of the most potent stimulators of PMN function and mimics the long term activation of PMNs by yeast particles (Burnham et al, 1989). Therefore several experiments were carried out to compare the effects of PMA, Table V. In each of 5 experiments the PMA-treated cultures displayed less suppression of yeast MTT reduction.

Effect of Doxycycline on PMN Yeast Suppression: Doxycycline has been shown to suppress PMN phagocytosis of Candida albicans (Forsgren et al, 1974). We therefore tested the activity of this agent on PMN suppression of yeast MTT

reduction. As shown in table VI and figure 11, Doxycycline reversed MTT reduction by 50% supporting the earlier cytochalasin B findings that phagocytosis is key to PMN suppression.

Fresh Clinical Isolate is Equally Susceptible to PMN MTT Inhibition:

Considering the above data, it seemed that the PMNs were too effective! Their "killing" activity should have been easier to disrupt. Therefor we considered the idea that the long-term laboratory strain of C. albicans, used in the above experiments, may have lost virulence over time and may be more susceptible to PMN inactivation. We therefore tested a fresh clinical isolate of Candida albicans in the same system. As shown in figure 13, This recent isolate was equally susceptible to inhibition by PMNs, with 63% suppression of yeast MTT reduction by PMN activity. PMN suppression of yeast MTT reduction by this fresh clinical isolate was equally unresponsive to reversal by oxygen radical inhibitors.

Yeast Phagocytosis in the Absence of Opsonins:

There appears to be no requirement for opsonins under these conditions, since as shown in figure 13, removal of serum in the presence of RPMI did not prevent PMN suppression of yeast MTT reduction, unlike the findings of Gabler and Hunter, 1987. The enhanced uptake of yeast may be due to the centrifuge step or the use of RPMI medium, see figure 3, RPMI vs HBSS. We have also found heat inactivated serum to work equally well in bringing about PMN inactivation of yeast, not shown.

Cells May Reduce MTT While Being Unable to Divide i.e. Dead:

The above findings suggest that decreased MTT activity in the presence of PMNs, compared to yeast alone, was the result of phagocytosis but not due to damage by oxygen radicals, did not require opsonization, and occurred with a presumably virulent fresh clinical isolate. Since these findings correlate poorly with several earlier studies of yeast destruction by PMNs, we decided to compare several actions of PMNs on yeast cells. We reasoned that since the incubation time involved for "killing" does not take into account yeast cell growth in the PMN-free cultures, phagocytized yeast, cut off from nutrients may not grow, while yeast cultured without PMNs likely continue to grow. Thus

the observed lesser MTT activity in PMN -treated yeast cultures may be due to yeast growth not cell death. Alternatively, phagocytized yeast may be killed by non-oxidative mechanisms resulting in the observed decrease in MTT reduction even in the presence of inhibitors, or both mechanisms could occur. Therefore particle counts, colony-forming-unit counts and MTT-reduction-over-time were compared in parallel cultures. As shown in figures 14a-c, neither the yeast particle count nor the number of colony-forming-units reflects the large, early increase in MTT reduction observed in PMN-free yeast cultures (greater than 10x at 60 minutes), suggesting increased cell growth but not increased colony-forming units explains the difference in MTT reduction. In Fact the MTT activity within PMN-containing cultures does not decrease over this same period, but stays constant as does the activity in parallel 4'C cultures. In contrast to no decreased MTT activity, PMNs killed 63% of the colony-forming units; suggesting mitotically-blocked cells still reduce MTT. Thus although cell killing as defined by colony counts does occur, the major effect was blocked reproduction, possibly by nutrient deprivation of yeast within the phagolysosomes.

Discussion:

The work presented here and previously reported indicate that, within an appropriate range, short term incubation with MTT can be used for in vitro assessment of relative concentrations of yeast in the same growth phase. Similar short term cultures of Listeria monocytogenes also demonstrated rapid MTT reduction related to cell concentration, suggesting the value of this approach to study this important bacterial pathogen in vitro. We report here a modification of the earlier method of Ashman (1986) to study inactivation of yeast by PMNs. An optical density, microplate assay of MTT reduction by active yeast was employed for this study, which has the advantage of rapidly, 2-3 hours, measuring PMN anti-yeast activity in several hundred wells, without the laborious process of counting yeast colonies. With this method we detected 60-75% suppression of 10^6 Candida albicans within 60-90 minutes by 1-$5x10^5$ human PMNs. The short term culture 1-2 hours can reproducibly detect PMN suppression of MTT reduction by Candida and Listeria. No suppression was obtained with sonicated PMNs indicating the requirement for whole PMNs.

The suppression of yeast MTT reduction in RPMI serum-free medium argues against the need for serum-derived opsonins such as complement or

antibody, suggesting that yeast cells present ligands reactive with PMN surface receptors, enhancing uptake. This finding is in contrast to the serum requirements for PMN uptake of yeast reported by Gabler and Hunter, 1987, possibly the difference is due to the centrifuge step used here, the monolayer of PMNs or the use of RPMI medium, see figure 3, RPMI vs HBSS. We also found heat-inactivated serum supported PMN yeast suppression (not shown).

When incubated together with PMNs, Cytochalasin B, an inhibitor of phagocytosis, reversed PMN suppression of yeast MTT reduction suggesting that phagocytosis by PMNs is required.. This effect was dose dependent, with higher concentration of Cytochalasin B completely blocking the MTT inhibition, bringing it to control levels. Doxycycline, also reported to block PMN Phagocytosis of yeast (Forsgren et al, 1974), also inhibited the PMN suppression 50%. Anti-yeast PMN activity was not enhanced by PMN stimulants FMLP or PMA. Nor did the anti-oxygen-radical agents, superoxide dysmutase, catalase or N-monomethyl arginine reverse the suppression of yeast MTT reduction. Although, oxygen radical mechanisms are thought to play a major role on PMN damage to yeast cells (Sasada et al 1987, Pruzanski et al1988, Wysong et al 1989, Ferrante,1989, Djeu et al 1990). These findings suggested that the suppression of MTT reduction may not depend on killing mechanisms. When direct comparisons were run of particle counts, MTT reduction and colony-forming-units, it was learned that a major source of difference between yeast only cultures and yeast + PMN cultures, was the growth of yeast in yeast-only cultures during the initial PMN "killing" incubation. In the initial hour there was no actual decrease in the MTT reduction activity in the presence of PMNs but instead a small 2.5-fold increase, whereas, the yeast only cultures displayed a large 11.2-fold increase, suggesting overall suppressed growth in the presence of PMNs, rather than yeast cell death, as an important mechanism producing the large difference in MTT reduction rates. Increased MTT reactions in cultures were not observed at 4'C where growth does not occur.

The one hour incubation of yeasts produced no change in particle count (with or without PMNs) but caused a 33% loss in colony-forming units (With PMNs), suggesting growth but not separation of yeast particles in yeast-only cultures and killing but not destruction of yeast cells. These effects are even more apparent at two hours, where, the particle count was unchanged and, in the presence of PMNs, the yeast MTT reaction was still increased 2-fold in spite of a 63% loss in colony-forming units. Lehrer and Cline 1969 also

Anti-yeast PMN activity was not enhanced by either FMLP, or PMA. Nor did the anti-oxygen-radical agents, superoxide dysmutase, catalase or N-monomethyl arginine reverse the suppression of yeast MTT reduction. These findings suggest that the suppression of MTT reduction may not depend on killing but rather the growth of yeast in yeast-only cultures during the initial PMN "killing" incubation as compared to PMN-containing cultures where phagocytosis blocked this process. In the initial hour there was no actual decrease in the MTT reduction activity in the presence of PMNs, but instead, a small 2.5-fold increase, whereas, the yeast only cultures displayed a large 11.2-fold increase. The one hour incubation of yeasts produced no change in particle count (with or without PMNs) but caused a loss in colony-forming units The increased MTT reduction in PMN-treated cultures, with the concomitant loss of colony-forming units, suggest that cells which are "killed", i.e. can not divide, are still able to metabolize MTT. Alternatively, the increased MTT reaction in the presence of PMNs could be due to 4-fold growth of some cells (without separation), and actual killing, preventing both growth and MTT reduction, of other yeast cells. However, the finding that SOD, catalase and NMMA did not reverse suppressed MTT reduction suggests that killing is not the mechanism but rather that sequestration of yeast cells within phagolysosomes blocks access to substrate or oxygen, thus blocking their growth. Such a mechanism could play a major role in delaying the replication and invasion of infective cells in situ.

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TABLE I

	PERCENT FETAL CALF SERUM ADDED						
EXPERIMENT	HBSS	0.625	1.25	2.5	5	10	20
7/29/92	0.48	0.61	0.63	0.65	0.65	0.57	0.35
8/1/91	0.34	nd	nd	0.57	0.74	0.67	nd
7/24/92	0.18	0.43	0.52	0.53	0.66	0.66	0.11
7/23/92	0.28	0.51	0.58	0.53	0.71	0.79	nd
7/21/92	0.18	nd	0.40	0.41	0.46	0.48	nd
MEAN	**0.29**	**0.52**	**0.53**	**0.54***	**0.64***	**0.63***	**0.23**
SEM	**0.06**	**0.05**	**0.05**	**0.04**	**0.05**	**0.05**	**0.12**

* these groups are significantly higher than HBSS only group $p<0.002$

TABLE II

SUMMARY OF YEAST SUPPRESSION BY PMNS:
TEN SEQUENTIAL EXPERIMENTS (5/25/92)

EXP	DATE	YEAST*	YEAST+PMNS*	% SUPP
1	10/11/90	0.84	0.21	75
2	7/25/91	0.47	0.13	72
3	8/13/91	0.53	0.22	58
4	8/23/91	0.38	0.10	74
5	8/29/91	0.59	0.15	75
6	3/14/92	0.31	0.08	74
7	3/25/92	0.45	0.14	69
8	4/6/92	0.33	0.20	39
9	5/14/92	0.65	0.43	35
10	5/20/92	0.56	0.21	62
	MEAN RESPONSE	**0.51**	**0.19#**	**63**
	SEM	0.05	0.03	

MTT reduction significantly suppressed $p<0.0001$

* indicates the reduction of MTT by viable yeast cells as OD at 550nm

TABLE III.

	EXPERIMENTS			MEAN
TARGET-CELL RATIO	8/23/91	8/29/91	3/14/92	
YEAST ONLY	100.0%	100.0%	100.0%	**100.0%**
16 YEAST/PMN	64.5%	79.7%	ND	**72.1%**
8 YEAST/PMN	74.0%	37.3%	57.9%	**56.4%**
4 YEAST/PMN	51.6%	35.6%	36.8%	**41.3%**
2 YEAST/PMN	0.258	0.254	0.263	**25.8%**

TABLE IV

CYTOCHALASIN-B REVERSES PMN-BLOCKED YEAST MTT REDUCTION

DATE	YEAST ONLY	YEAST+PMNS	(% SUPP)
8/13/91	0.53	0.22	58
8/23/91	0.38	0.10	74
8/29/91	0.59	0.15	75
3/14/92	0.31	0.08	74
5/20/92	0.56	0.21	62
MEAN	**0.47**	**0.15***	**69**
SEM	0.06	0.03	

12.5 mcg/ml CYTOCHALASIN-B ADDED

DATE	YEAST ONLY	(% SUPP)	YEAST+PMNS	(% SUPP)
8/13/91	0.45	15	0.54	-20
8/23/91	0.39	-3	0.43	-10
8/29/91	0.26	56	0.22	15
3/14/92	0.29	6	0.24	17
5/20/92	0.61	-10	0.50	18
MEAN	**0.40**	**13**	**0.39**	**4**
SEM	0.06	26	0.07	

* indicates significant suppression of MTT reduction by yeast $p<0.01$

TABLE V.

EFFECT OF PMA ON PMN YEAST SUPPRESSION:

DATE	YEAST ONLY*	YEAST+PMNS*	(% SUPP)
8/13/91	0.53	0.22	58
8/23/91	0.38	0.10	74
8/29/91	0.59	0.15	75
3/14/92	0.31	0.08	74
4/6/92	0.33	0.20	39
MEAN	**0.43**	**0.15**	**64**
SEM	0.06	0.03	

.025-0.25 mcg/ml PMA ADDED

DATE	YEAST ONLY*	(% SUPP)	YEAST+PMNS*	(% SUPP)
8/13/91	0.58	-9	0.25	57
8/23/91	0.43	-13	0.18	58
8/29/1991+	0.51	14	0.24	53
3/14/1992+	0.30	3	0.20	33
5/20/92	0.35	-6	0.34	3
MEAN	**0.43**	**-2**	**0.24!**	**41**
SEM	0.05		0.03	

! significantly less suppression than without PMA $p<0.01$

* indicates the reduction of MTT by viable yeast cells as OD at 550nm

+ indicates the use of 0.25mcg/ml PMA

TABLE VI.

EFFECT OF DOXYCYCLINE ON PMN YEAST SUPPRESSION:

DATE	YEAST ONLY*	YEAST+PMNS*	(% SUPP)
3/25/92	0.45	0.14	69
4/6/92	0.33	0.20	39
5/14/92	0.65	0.43	35
5/20/92	0.56	0.21	62
MEAN	**0.50**	**0.24**	**51**
SEM	0.07	0.06	

80 mcg/ml DOXYCYCLINE ADDED

DATE	YEAST ONLY*	(% SUPP)	YEAST+PMNS*	(% SUPP)
3/25/92	0.43	4	0.25	42
4/6/92	0.35	-6	0.34	3
5/14/92	0.76	-17	0.59	22
5/20/92	0.52	6	0.35	33
MEAN	**0.52**	**-3**	**0.38!**	**25**
SEM	0.09		0.07	

* indicates the reduction of MTT by viable yeast cells as OD at 550nm

! significantly less suppression with doxycycline added $p<0.001$

FIGURE LEGENDS:

Figure 1. Direct Correlation Between Yeast Cell Concentration and MTT Reduction: Freshly cultured, washed Candida albicans Blastospores 0.12-1.3 $x10^6$/well were added to two microtiter plates 0.05ml/well as indicated. Each dilutions was counted separately on a Coulter Counter. Cells were incubated 60 minutes at 37'C with 5% fetal calf serum in HBSS, MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM). One plate was centrifuged prior to incubation (as in later PMN experiments to increase interaction of yeast with added PMNs). After incubation formazan was dissolved with 0.15 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. R^2 values for the linear increase range are 0.97 and 0.99 respectively.

Figure 2. Direct Correlation Between Listeria Concentration and MTT Reduction: Freshly cultured, washed Listeria monocytgenes (0.5-32 $x10^7$/ml HBSS) were added to two microtiter plates 0.05ml/well as indicated. Cells were incubated 60 minutes at 37'C with 5% fetal calf serum in HBSS, MTT (1.25mg/ml), succinate (6.2 mM) and either no additional agents or the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM). After incubation formazan was dissolved with 0.15 ml DMSO. Plates were read at 550nm on an ELISA reader. Values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. R^2 values for the linear increase range of 0.97 and 0.99 respectively.

Figure 3. The Importance of Fetal Calf Serum in MTT Reduction by Yeast Cells: Freshly cultured, washed Candida albicans Blastospores 0.5 $x10^6$/well were added as indicated. Cells were incubated 60 minutes at 37'C with 0-20% fetal calf serum in HBSS, RPMI , or RPMI with glucose 17.5gm/L. MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) were then added and plates incubated 60 minutes at 37'C.. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the

standard deviation. RPMI values for 0-5% serum were all significantly higher than HBSS values and unaffected by low serum level $p<0.05$. Serum of 20% significantly suppressed all cultures even over no serum $p<0.05$.

Figure 4. Succinate Substrate Enhances Yeast MTT Reduction: Freshly cultured, washed Candida albicans Blastospores 1.0 $x10^6$/well were added to microtiter plates as indicated. Cells were incubated with 5 % fetal calf serum in HBSS with TX-100 (0.06%), and with or without succinate (6.2 mM) and with or without the antimetabolite iodoacetamide (50mM). Plates were incubated 30, 60 or 90 minutes at 37'C in MTT (1.25mg/ml). After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells +/- the standard deviation. All succinate added wells were significantly higher at all time periods, $p<0.0001$.

Figure 5. Whole, but Not Sonicated, PMNs Suppress Yeast MTT reduction: Freshly cultured, washed Candida albicans Blastospores 0.5 $x10^6$/well and freshly harvested whole or sonicated (greater than 95% lysis) human neutrophils 0.5 x 106/well were cultured separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS. Wells then received MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) and plates were reincubated 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. The PMN only Tx-100 lysate MTT reduction is uniformly insignificant and not presented in the remaining figures. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. PMNs significantly suppresses yeast $p<0.0001$, whereas, sonicated PMNs did not $p>0.05$.

Figure 6. Whole, but Not Sonicated, PMNs Suppress Listeria MTT reduction: Freshly cultured, washed Listeria monocytogenes strain #10403 (kindly provided by Dr. David Heinrich) 0.5 $x10^6$/well and freshly harvested whole or sonicated (greater than 95% lysis) human neutrophils 0.5 x 106/well were cultures separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS. Wells then received MTT (1.25mg/ml),

succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) and plates were reincubated 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. PMNs significantly suppresses L. monocytogenes $p<0.0001$, whereas sonicated PMNs did not $p>0.05$.

Figure 7. PMN Dose Response Curve for Suppression of Yeast MTT Reduction: Freshly cultured, washed Candida albicans Blastospores 1.0 $x10^6$/well and freshly harvested human neutrophils 0.06-0.5 x 10^6/well were cultures separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS. Wells then received MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) and plates were reincubated 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. All PMN-treated target yeast MTT reduction cultures were significantly suppressed $p<0.005$.

Figure 8. Positive Correlation Between PMN-Suppressed Yeast MTT Reduction and Numbers of Recovered Viable Yeast Cells: Freshly cultured, washed Candida albicans Blastospores, 0.5 $x10^6$/well, and freshly harvested human neutrophils, 0.5 x 10^6/well, were cultured separately or in combination, as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS. The PMN lytic agent TX-100 (0.06%) was added, cells were resuspended (PMNs are lysed by this procedure) and 0.025ml samples removed for yeast colony assay. Then each culture received MTT (1.25mg/ml), succinate (6.2 mM) and, to parallel negative control cultures, the antimetabolite iodoacetamide (50mM). Plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. Cultures samples for plating were diluted with HBSS and aliquants spread onto plates containing the glucose-supplemented tryptocase soy medium used for

yeast culture. Plates were incubated 24-30 hours at 37' C.and yeast colonies counted. Three separate experiments are shown. All values are the mean of six replicates: either wells minus the parallel iodoacetamide-treated control wells or colony counts, +/- the standard deviation.

Figure 9. Reversal of PMN-Mediated Suppression of Yeast MTT Reduction by Cytochalasin b: Freshly cultured, washed Candida albicans Blastospores, 1.0 $x10^6$/well, and freshly harvested whole or sonicated (greater than 95% lysis) human neutrophils, 0.5 x 10^6/well, were cultured separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS alone or with 1.25-125 mcg/ml of cytochalasin b. Plates were removed and MTT 1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) added and plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation.

Figure 10. Failure of PMN Stimulants to Enhance PMN Inhibition of Yeast-Cell-Induced MTT Reduction: Freshly cultured, washed Candida albicans Blastospores, 1.0 $x10^6$/well, and freshly harvested human neutrophils, 0.125 or 0.5 x 10^6/well, were cultured separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS alone or with 12.5 mcg/ml of cytochalasin b, 0.025 mcg/ml PMA or 2.5 x 10^{-7}M FMLP. Plates were removed and MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) added and plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. *=significant suppression.

Figure 11. Failure of Catalase to Reverse PMN Inhibition of Yeast-Cell-Induced MTT Reduction: Freshly cultured, washed Candida albicans Blastospores, 1.0 $x10^6$/well, and freshly harvested human neutrophils, 0.5 x 10^6/well, were cultured separately or in combination as indicated. Cells were incubated 90

minutes at 37'C in 5% fetal calf serum in HBSS alone or with catalase 250 U/ml or Doxycycline, 80mcg/ml. Plates were removed and MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) added and plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. *=significant suppression.

Figure 12. Failure of Superoxide Dismutase to Reverse PMN Inhibition of Yeast-Cell-Induced MTT Reduction: Freshly cultured, washed Candida albicans Blastospores, 1.0 $x10^6$/well, and freshly harvested human neutrophils, 0.5 x 10^6/well, were cultured separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS alone or with superoxide dismutase 10-40 U/ml. Plates were removed and MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) added and plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. *=significant suppression.

Figure 13. Failure of Superoxide Dismutase and N-Mono-Methyl Arginine to Reverse the PMN action on MTT Reduction by a Fresh Clinical Isolate of Candida albicans: Freshly cultured, washed Candida albicans Blastospores, 1.0 $x10^6$/well, grown from a recent clinical isolate, and freshly harvested human neutrophils, 0.5 x 10^6/well, were cultured separately or in combination as indicated. Cells were incubated 90 minutes at 37'C in 5% fetal calf serum in HBSS alone or with superoxide dismutase 40 U/ml or with 500 mcg/ml N-monomethyl arginine. Plates were removed and MTT (1.25mg/ml), succinate (6.2 mM) and either the PMN lytic agent TX-100 (0.06%) or the antimetabolite iodoacetamide (50mM) added and plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader. All values are the mean of six replicate wells minus the parallel iodoacetamide-treated control wells +/- the standard deviation. *=significant suppression.

Figure 14. Modulation of Yeast MTT Reduction, Yeast Particles and Cultivatable Yeast cells Over Time in The Presence and Absence of PMNs: Freshly cultured, washed Candida albicans Blastospores, 0.5 x10^6/well, and freshly harvested human neutrophils, 0.5 x 10^6/well, were cultured in 5% fetal calf serum in HBSS, separately or in combination as indicated. Separate plates were incubated 90 minutes at 4'C or 37'C. The PMN lytic agent TX-100 (0.06%) was added, cells were resuspended (PMNs are lysed by this procedure) and 0.025ml samples removed for yeast colony assay (Figure 14a) and particle counts with the Coulter Counter (Figure 14b). Then each culture received MTT (1.25mg/ml), succinate (6.2 mM) and, to parallel negative control cultures, the antimetabolite iodoacetamide (50mM). Plates were reincubated for 60 minutes at 37'C. After incubation formazan was dissolved with 0.2 ml DMSO. Plates were read at 550nm on an ELISA reader (Figure 14c). Separate aliquants of the 0.025 ml Culture samples were counted on the Coulter Counter and spread onto agar plates containing the glucose-supplemented tryptocase soy medium used for yeast culture. Agar plates were incubated 24-30 hours at 37'C and yeast colonies counted. All values are the mean of six replicates +/- the standard deviation.

FIGURE 1.

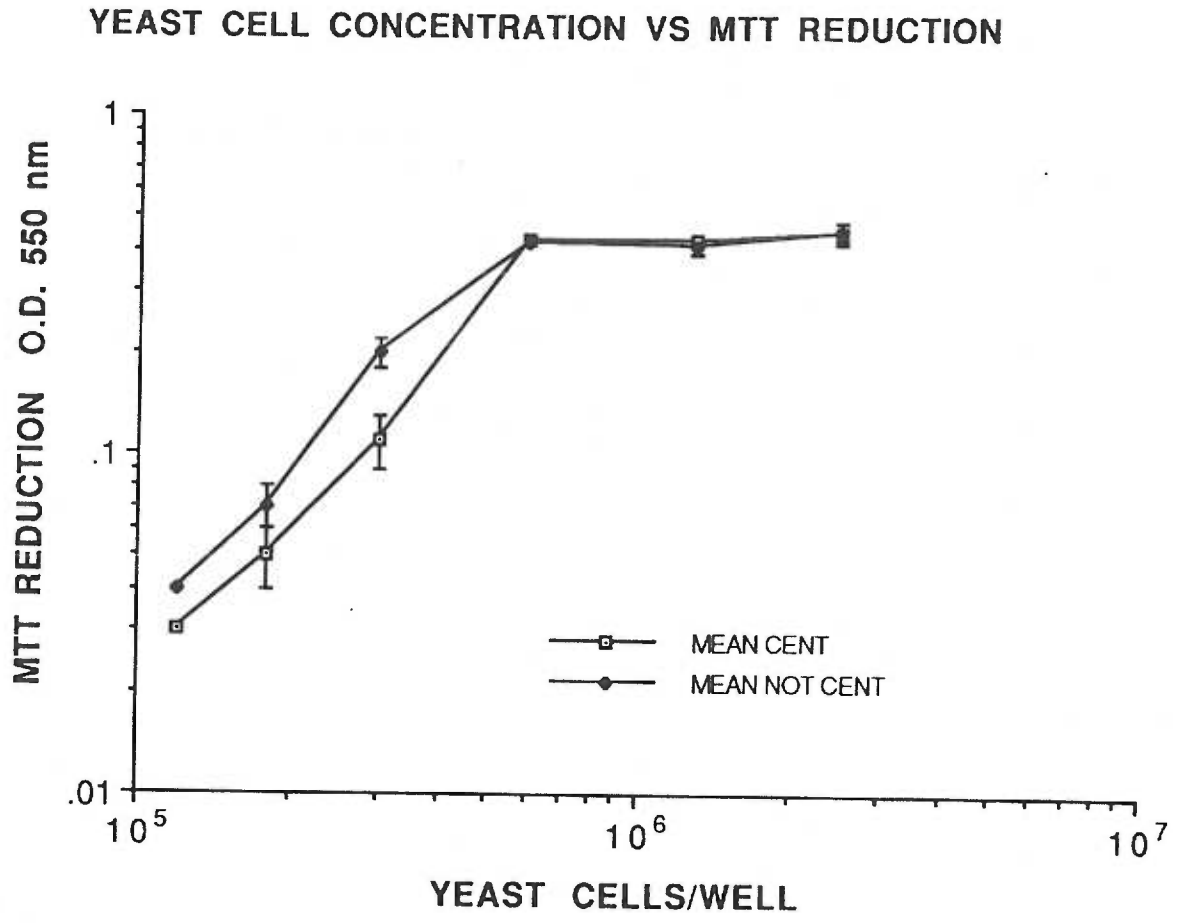

YEAST CELL CONCENTRATION VS MTT REDUCTION
MTT REDUCTION O.D. 550 nm
1
.1
.01
10^5
10^6
10^7
YEAST CELLS/WELL
MEAN CENT
MEAN NOT CENT

FIGURE 2.

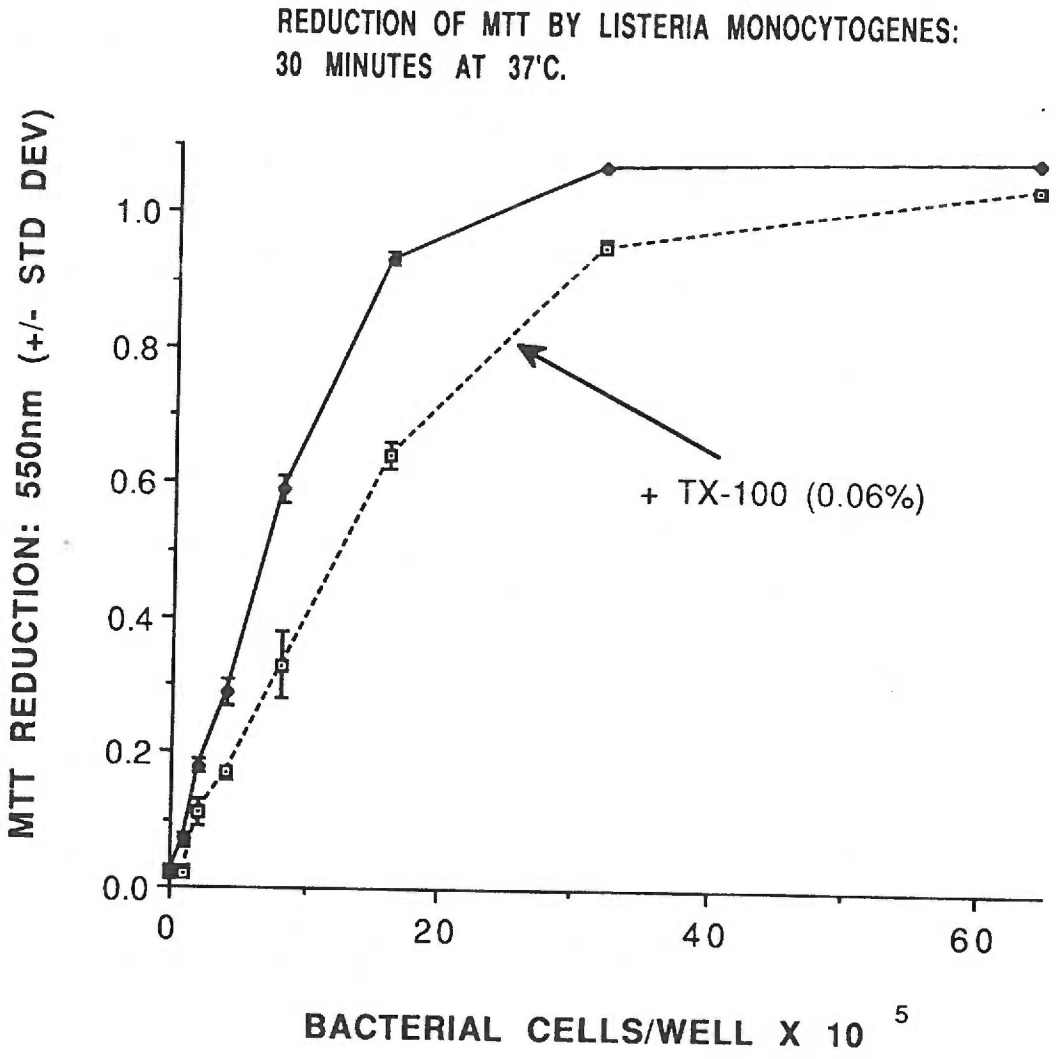
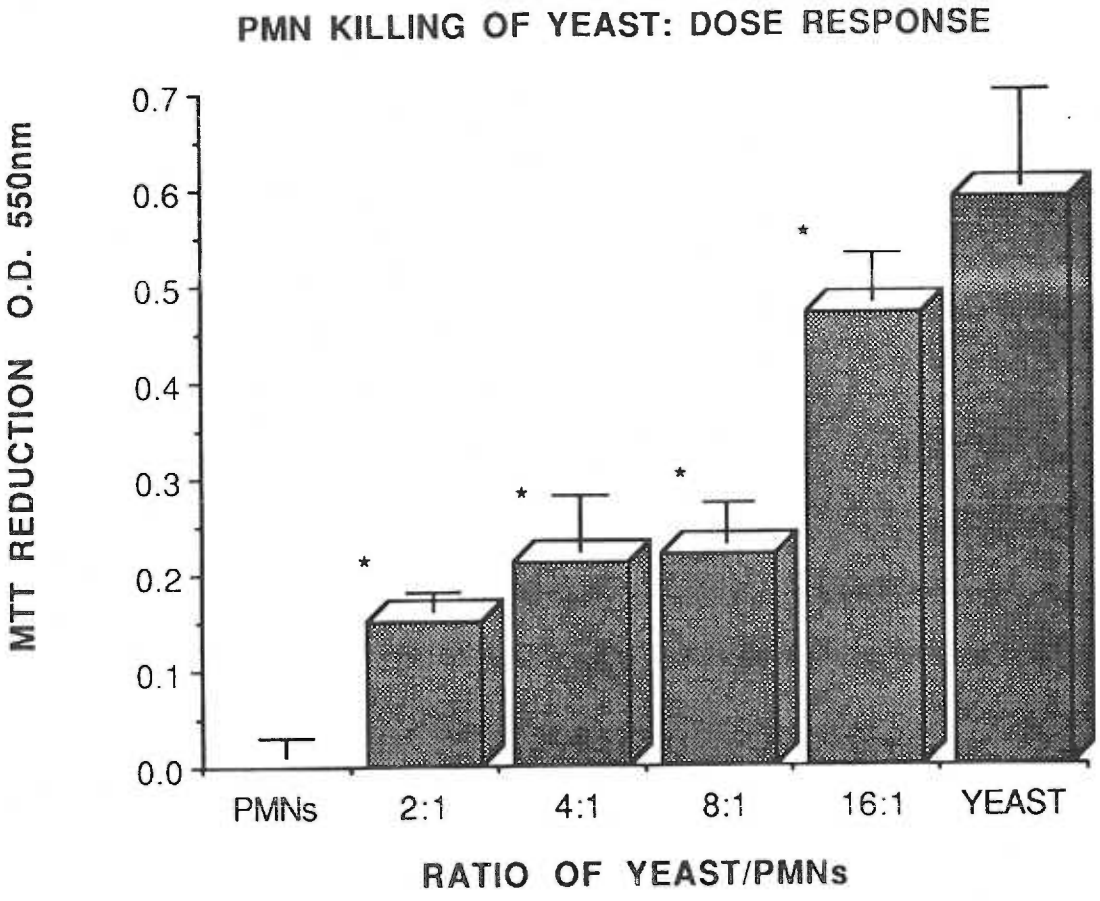
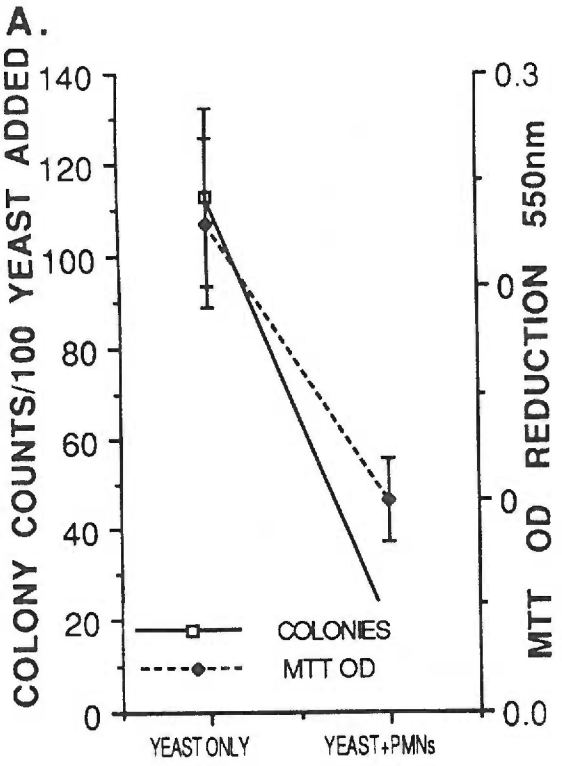
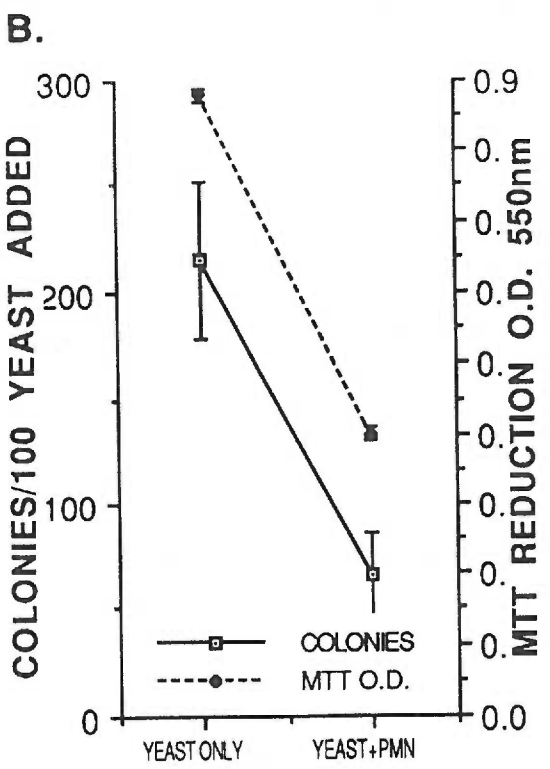

REDUCTION OF MTT BY LISTERIA MONOCYTOGENES:
30 MINUTES AT 37'C.
MTT REDUCTION: 550nm (+/- STD DEV)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
+ TX-100 (0.06%)
BACTERIAL CELLS/WELL X 10 5

FIGURE 7.


PMN KILLING OF YEAST: DOSE RESPONSE
MTT REDUCTION O.D. 550nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
*
PMNs
2:1
4:1
8:1
16:1
YEAST
RATIO OF YEAST/PMNs


A.
COLONY COUNTS/100 YEAST ADDED
140
120
100
80
60
40
20
0
0.3
0
0
0.0
MTT OD REDUCTION 550nm
COLONIES
MTT OD
YEAST ONLY
YEAST+PMNs


B.
COLONIES/100 YEAST ADDED
300
200
100
0
0.9
0.
0.
0.
0.
0.
0.0
MTT REDUCTION O.D. 550nm
COLONIES
MTT O.D.
YEAST ONLY
YEAST+PMN

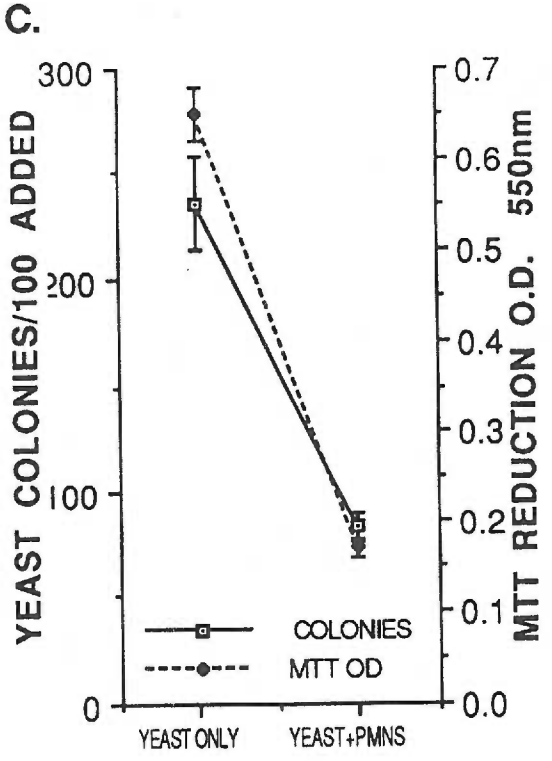

C.
YEAST COLONIES/100 ADDED
300
200
100
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MTT REDUCTION O.D. 550nm
COLONIES
MTT OD
YEAST ONLY
YEAST+PMNS

FIGURE 9.

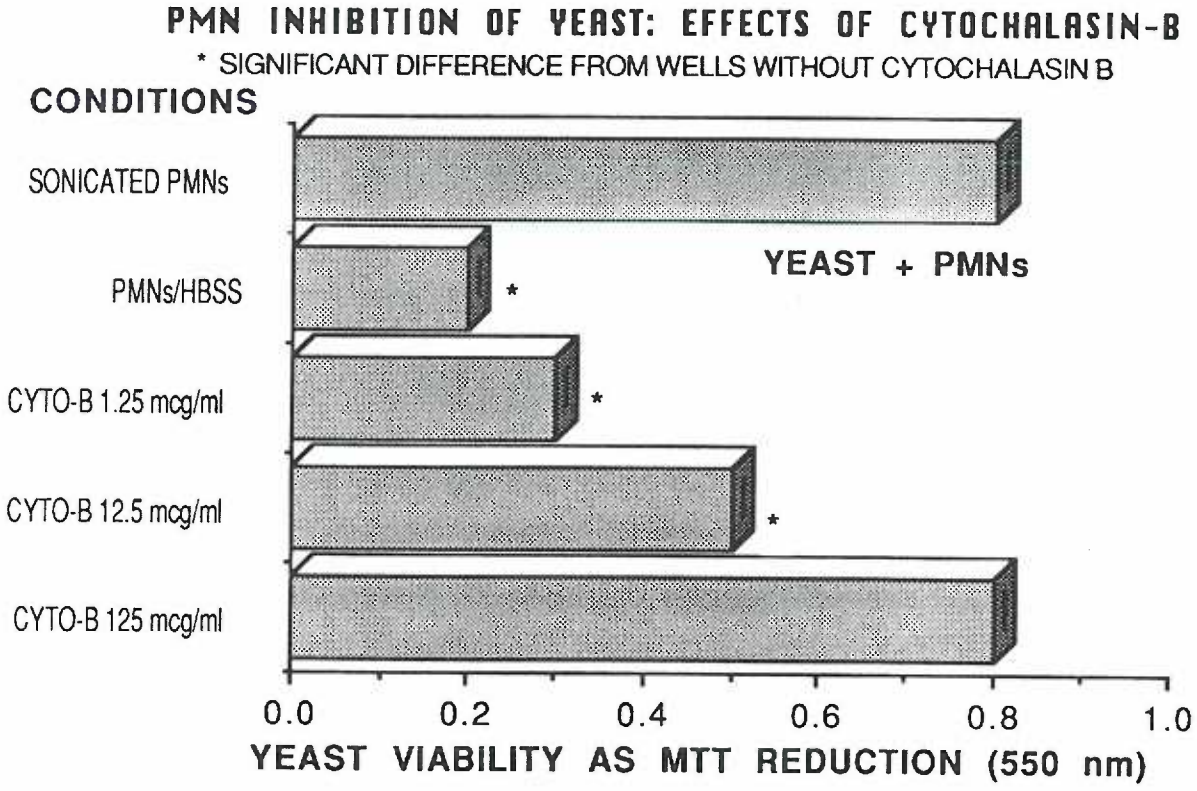

PMN INHIBITION OF YEAST: EFFECTS OF CYTOCHALASIN-B
* SIGNIFICANT DIFFERENCE FROM WELLS WITHOUT CYTOCHALASIN B
CONDITIONS
SONICATED PMNs
PMNs/HBSS
CYTO-B 1.25 mcg/ml
CYTO-B 12.5 mcg/ml
CYTO-B 125 mcg/ml
YEAST + PMNs
*
*
*
0.0
0.2
0.4
0.6
0.8
1.0
YEAST VIABILITY AS MTT REDUCTION (550 nm)

FIGURE 10.

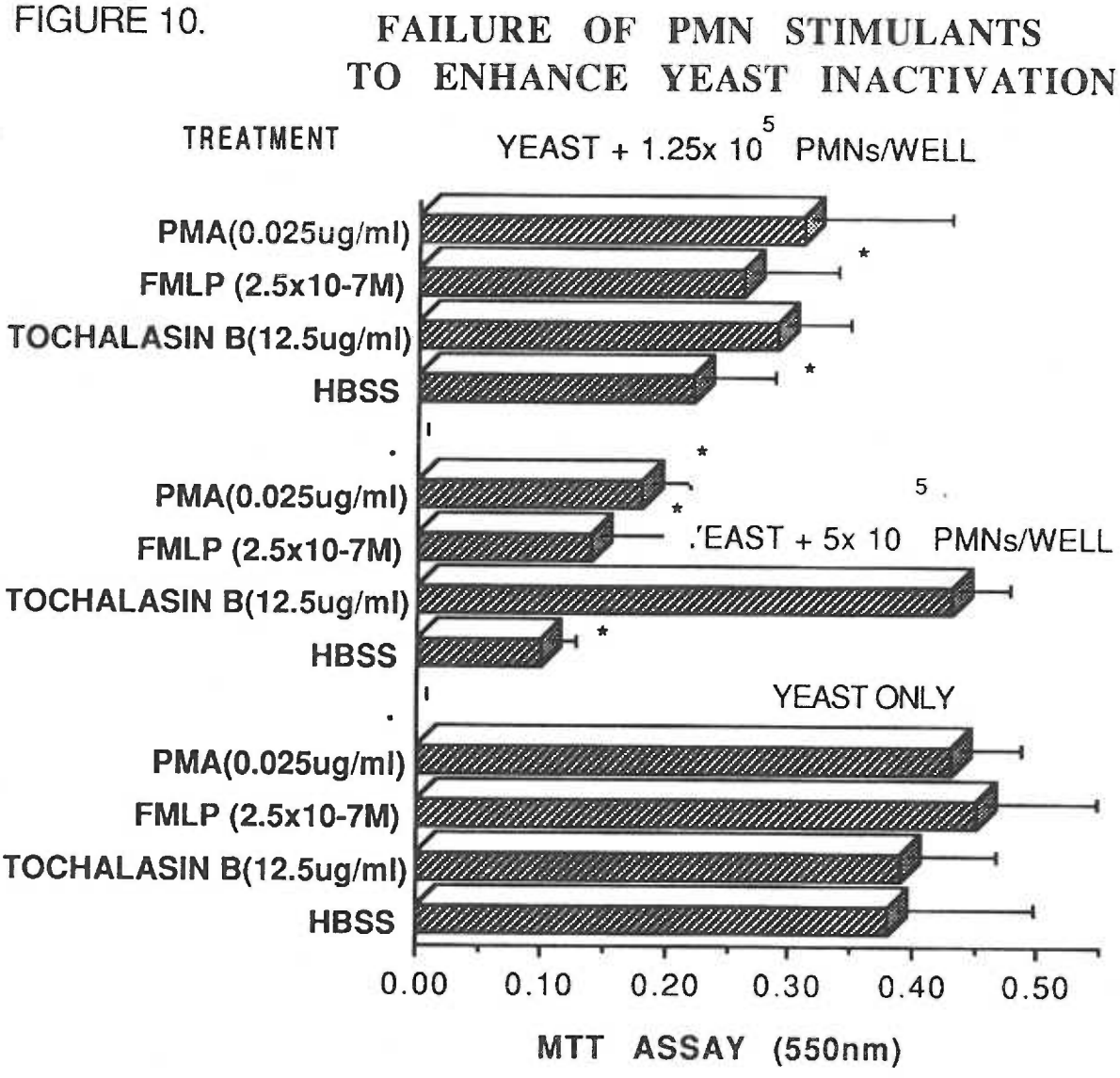

FAILURE OF PMN STIMULANTS
TO ENHANCE YEAST INACTIVATION
TREATMENT
YEAST + 1.25x 10^5 PMNs/WELL
PMA(0.025ug/ml)
FMLP (2.5x10-7M)
TOCHALASIN B(12.5ug/ml)
HBSS
PMA(0.025ug/ml)
FMLP (2.5x10-7M)
TOCHALASIN B(12.5ug/ml)
HBSS
YEAST + 5x 10^5 PMNs/WELL
YEAST ONLY
PMA(0.025ug/ml)
FMLP (2.5x10-7M)
TOCHALASIN B(12.5ug/ml)
HBSS
0.00
0.10
0.20
0.30
0.40
0.50
MTT ASSAY (550nm)

FIGURE 11.

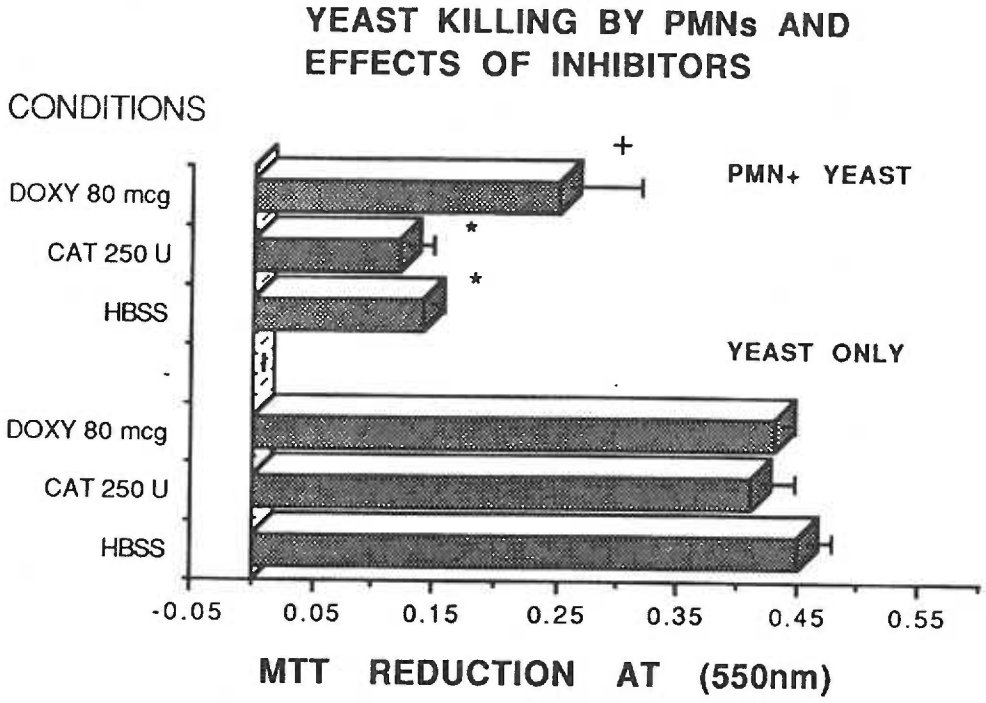

YEAST KILLING BY PMNs AND
EFFECTS OF INHIBITORS
CONDITIONS
DOXY 80 mcg
CAT 250 U
HBSS
-
DOXY 80 mcg
CAT 250 U
HBSS
+
PMN+ YEAST
YEAST ONLY
*
*
-0.05
0.05
0.15
0.25
0.35
0.45
0.55
MTT REDUCTION AT (550nm)

FIGURE 12.

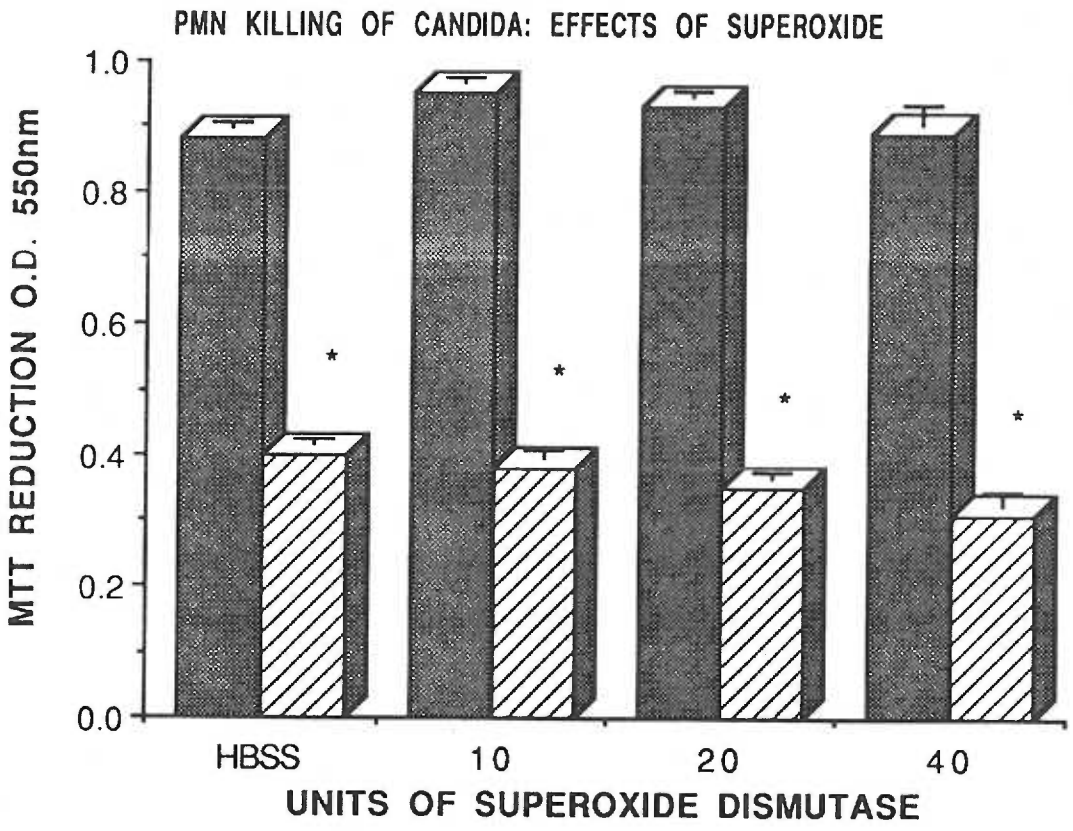

PMN KILLING OF CANDIDA: EFFECTS OF SUPEROXIDE
MTT REDUCTION O.D. 550nm
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
HBSS
10
20
40
UNITS OF SUPEROXIDE DISMUTASE

FIGURE 13.

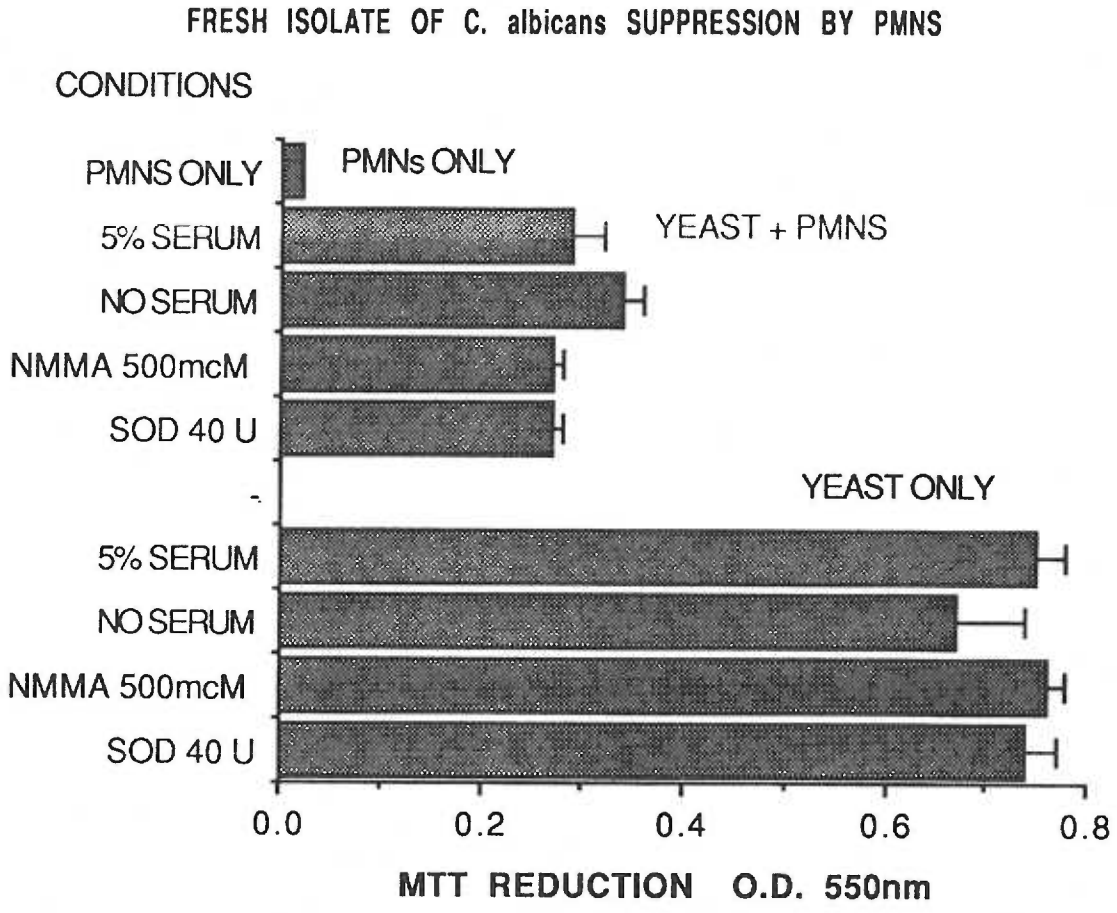

FRESH ISOLATE OF C. albicans SUPPRESSION BY PMNS
CONDITIONS
PMNS ONLY
5% SERUM
NO SERUM
NMMA 500mcM
SOD 40 U
5% SERUM
NO SERUM
NMMA 500mcM
SOD 40 U
PMNs ONLY
YEAST + PMNS
YEAST ONLY
0.0
0.2
0.4
0.6
0.8
MTT REDUCTION O.D. 550nm

FIGURE 14 A.

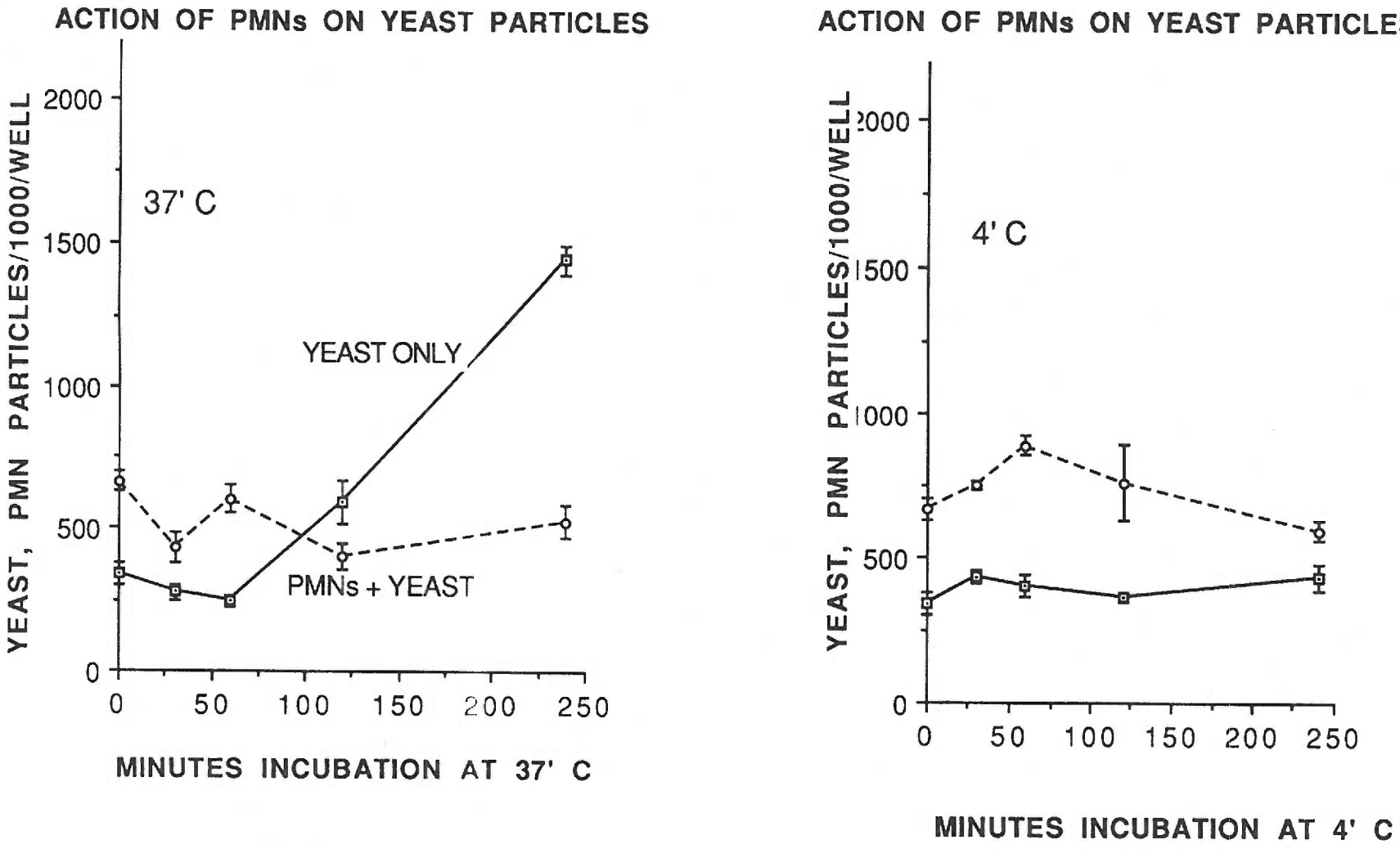

ACTION OF PMNs ON YEAST PARTICLES
YEAST, PMN PARTICLES/1000/WELL
2000
1500
1000
500
0
37' C
YEAST ONLY
PMNs + YEAST
0
50
100
150
200
250
MINUTES INCUBATION AT 37' C
ACTION OF PMNs ON YEAST PARTICLES
YEAST, PMN PARTICLES/1000/WELL
2000
1500
1000
500
0
4' C
0
50
100
150
200
250
MINUTES INCUBATION AT 4' C

FIGURE 14 B.

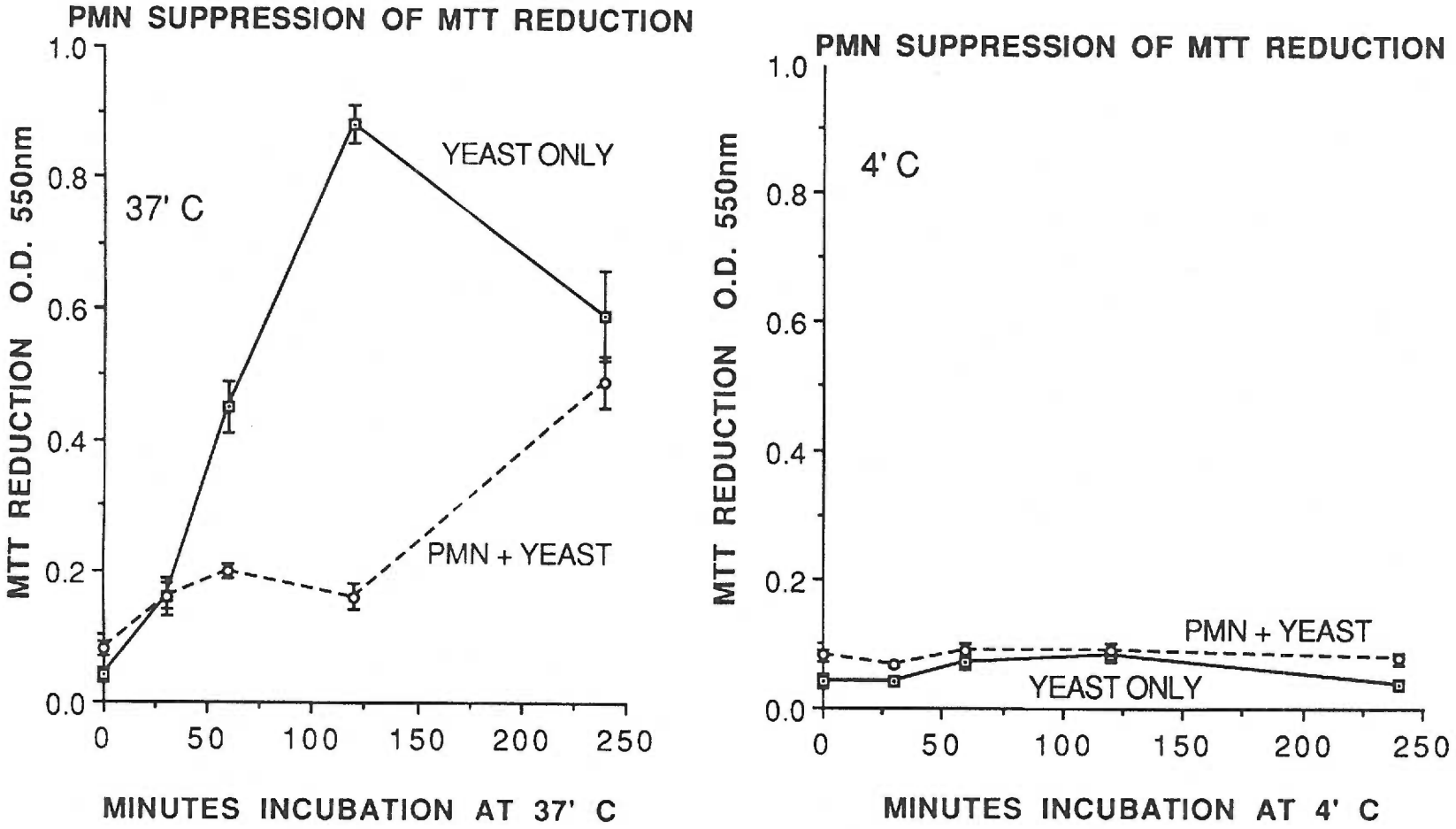
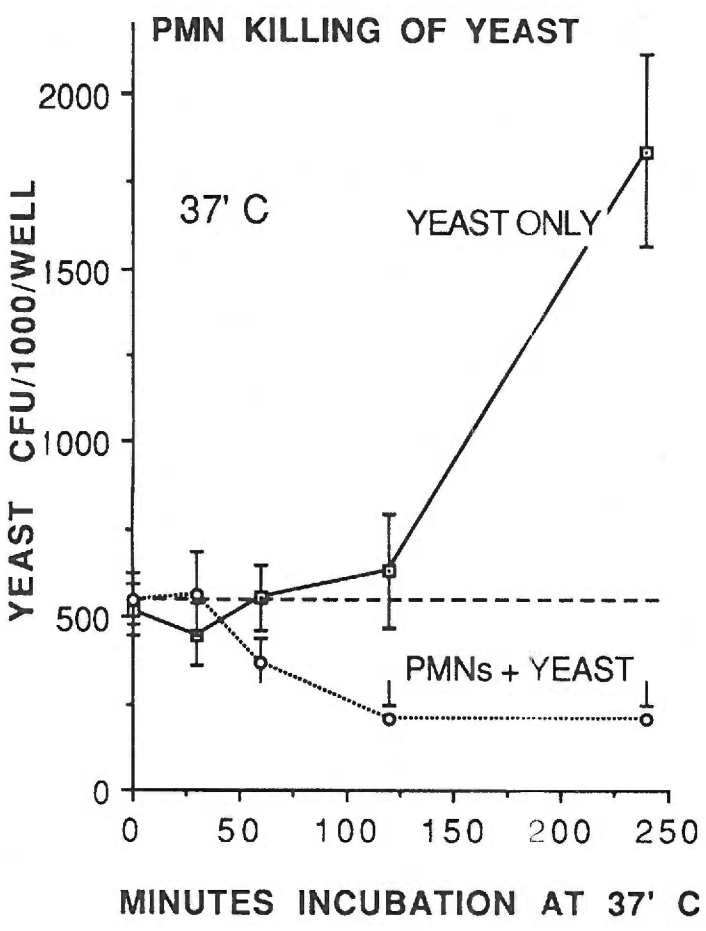
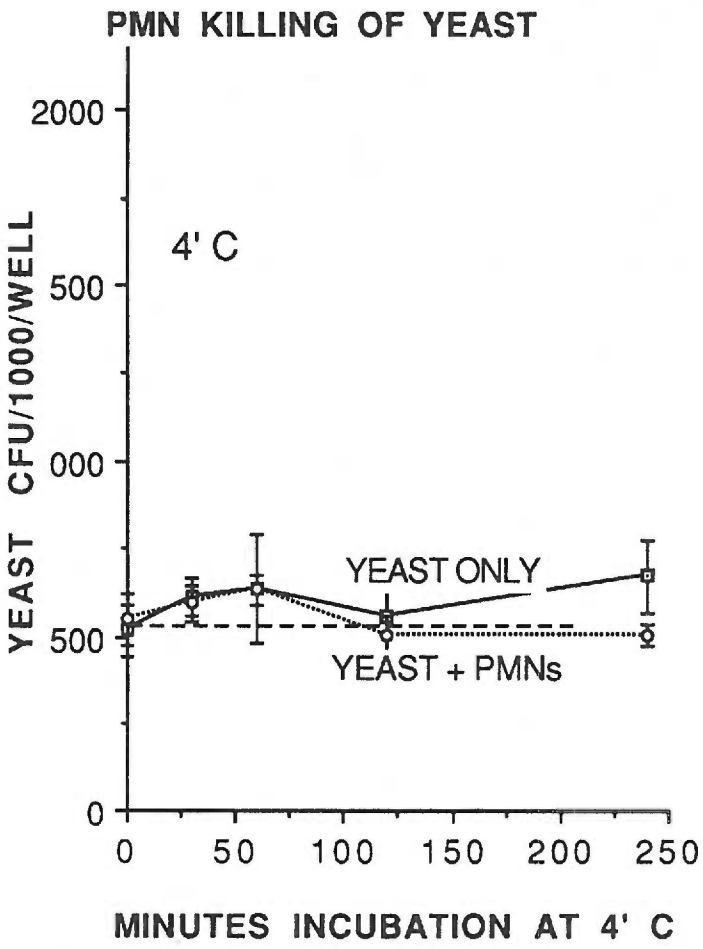

PMN SUPPRESSION OF MTT REDUCTION
MTT REDUCTION O.D. 550nm
1.0
0.8
0.6
0.4
0.2
0.0
37' C
YEAST ONLY
PMN + YEAST
0
50
100
150
200
250
MINUTES INCUBATION AT 37' C
PMN SUPPRESSION OF MTT REDUCTION
MTT REDUCTION O.D. 550nm
4' C
PMN + YEAST
YEAST ONLY
MINUTES INCUBATION AT 4' C

FIGURE 14 C.


PMN KILLING OF YEAST
2000
1500
1000
500
0
37' C
YEAST ONLY
PMNs + YEAST
YEAST CFU/1000/WELL
0
50
100
150
200
250
MINUTES INCUBATION AT 37' C


PMN KILLING OF YEAST
2000
500
000
500
0
4' C
YEAST ONLY
YEAST + PMNs
YEAST CFU/1000/WELL
0
50
100
150
200
250
MINUTES INCUBATION AT 4' C