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Apoptosis of Breast Cancer MCF-7 Cells *In Vitro* is Induced Specifically by Yeast and Not by Fungal Mycelia

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Abstract. *It was recently demonstrated that breast cancer cell lines undergo apoptosis following phagocytosis of S. cerevisiae. The present study was undertaken to determine whether other strains of fungi also induce apoptosis in cancer cells. Eight strains of yeast: C. albicans , C. krusei, C. glabrata, C. kefyr, C. neoformans, Y. lipolytica, S. cerevisiae and R. rubra, were evaluated at different developmental stages (small yeast cells, large yeast cells and pseudohyphae). In addition, conidia from four strains of fungal mycelia: Aspergillus, Aspergillus sp., T. rubrum and T. tonsurans were evaluated. Breast cancer MCF-7 cells in monolayer were cultured with fungi at a ratio of 1:10, respectively, after which phagocytosis and fungi-induced apoptosis of MCF-7 cells were examined. The MCF-7 cells were found to phagocytize all strains of heat-killed yeast, regardless of their developmental stage. Phagocytosis followed a graduated series: C. krusei > C. glabrata > R. rubra > S. cerevisiae > C. kefyr > C. neoformans > C. albicans > Y. lipolytica. Yeast-induced apoptosis also followed a graded pattern: S. cerevisiae > C. kefyr > C. krusei > C. neoformans > R. rubra > C. albicans > C. glabrata > Y. lipolytica, as examined by flow cytometry. In contrast, MCF-7 did not phagocytize or undergo apoptosis post-culture with conidia. This data may have clinical implications for the treatment of breast cancer.*

Despite recent advances in the understanding and treatment of cancer, the development of alternative therapies remains a high priority. The study of apoptosis has provided promising mechanisms for cancer treatment. Apoptosis, or programmed cell death, is a mechanism used by cells to control proliferation or to respond to DNA damage. Many anticancer therapeutic agents aim to induce apoptosis in cancer cells (1-4). The mechanisms involved in such treatments involve the Fas/FasL system (2), mitochondria (3) and DNA damage (4). These treatments fall short of perfection as they incur severe side-effects due to their lack of targeting specificity. Breakthroughs using microbes to induce apoptosis in cancer cells are promising because of their highly specific targeting capabilities (5-8).

Previously, we revealed the ability of the non-pathogenic yeast, *S. cerevisiae*, to induce apoptosis in several tumor cell lines – breast (9, 10), tongue (11) and colon (11) – without inducing a significant effect in normal cells (12). In the present study, it was of particular interest to examine: a) whether other strains of yeast and strains of conidia from fungal mycelia are also taken-up by cancer cells; and b) whether these microorganisms can induce apoptosis in cancer cells. MCF-7 breast cancer cells were found to undergo apoptosis following attachment/ingestion of all eight heat-killed strains of tested yeast. In contrast, the MCF-7 cells did not phagocytize the four conidia strains tested. This suggests that induction of apoptosis in cancer cells is yeast-specific. This observation may have great therapeutic potential for the treatment of breast cancer..

Materials and Methods

Cell line. The human breast cancer MCF-7 cell line, purchased from the American Tissue and Culture Collection (ATCC), Manassas, VA, USA, was used. The cells were maintained in our laboratory in complete medium (CM) consisting of RPMI-1640, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 μg/ml of streptomycin and penicillin.

Preparation of fungi. Eight strains of yeast at different developmental stages were used. These included small yeast cells: *Candida albicans* (*C. albicans*), *Candida glabrata* (*C. glabrata*), *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Yarrowia lipolytica* (*Y. lipolytica*); large yeast cells: *Cryptococcus neoformans* (*C. neoformans*) and *Rhodotorula rubra* (*R. rubra*); and pseudohyphae: *Candida kefyr* (*C. kefyr*) and *Candida krusei* (*C. krusei*). Conidia from four strains of fungal mycelia:

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Key Words: MCF-7 cells, apoptosis, phagocytosis, *C. glabrata*, *C. krusei*, *Aspergillus.*

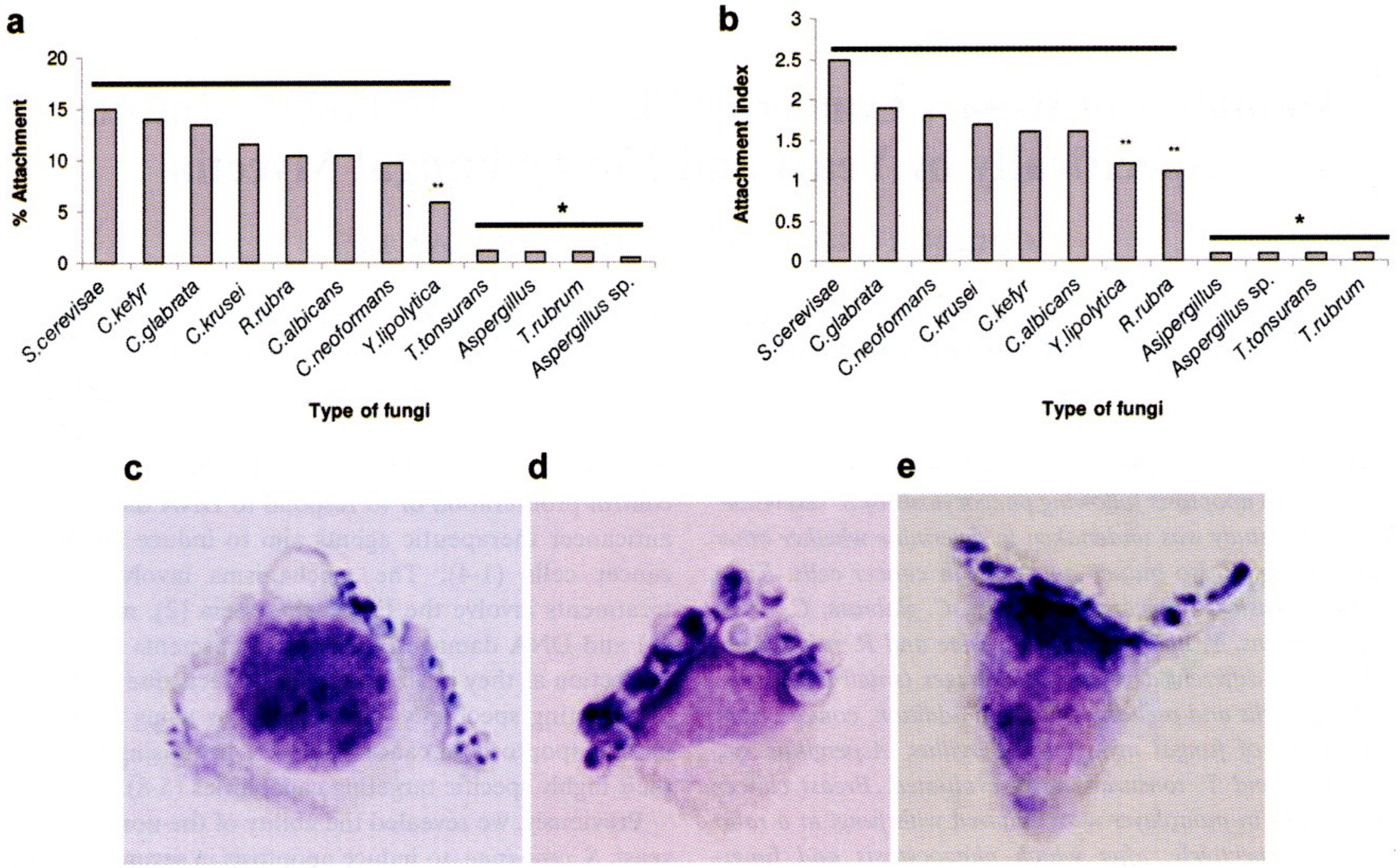

Figure 1. *The attachment of monolayer MCF-7 cells to different types of fungi. Tumor cells were cultured with fungi at a ratio of 1:10. At 2 h, non-adherent and adherent cells were stained with Giemsa and the percentages (%) of attachment (a) and attachment indices (b) were calculated. (c-e) Cytocentrifuge preparations showing attachment of non-adherent MCF-7 cells to yeast of different developmental stages; small yeast cells (C. glabrata), large yeast cells (R. rubra) and pseudohyphae (C. krusei) Giemsa x 400. *$p<0.001$ as compared to mean of mycelia as a group. **$p<0.01$ as compared to S. cerevisiae.*

Aspergillus, *Aspergillus* species (*Aspergillus* sp.) *Trichophyton rubrum* (*T. rubrum*) and *Trichophyton tonsurans* (*T. tonsurans*), were also used. Fungi were obtained from unidentified patient specimens and the ATCC. Yeast were obtained from cultures grown for 2 days and conidia from cultures grown for 3-14 days at 37°C on Sabourad dextrose agar, Emmons. Yeast and conidia suspensions containing single yeast or conidia were gently homogenized in Hank's balanced salt solution (HBSS), heat-killed by incubation for 30 min at 100°C, washed once and resuspended with HBSS. Quantification was carried out using a hemocytometer and cell suspensions were adjusted to $1x10^7$ cells/ml.

Growth of monolayer MCF-7 cells in 8-well plates. Our recent model assay system was followed to examine phagocytosis and apoptosis of monolayer MCF-7 cells (12). For this purpose, MCF-7 cells were grown in multiplate-8 wells (26X33 mm each) (LUX Scientific Corp., Thousand Oaks, CA, USA). A cover glass was placed at the bottom of each well. MCF-7 cells ($1x10^5$ cells/mL) were pipetted into each well and allowed to adhere for 2 h. The cancer cells were then washed once with 1 mL CM followed by the addition of fungi ($1x10^6$ cells/mL). At 2- and 4-h time-points post fungi application, both non-adherent and adherent cancer cells were examined as follows.

(A) Non-adherent cells: The medium containing non-adherent tumor cells (1 mL) was transferred to glass tubes. From the cell suspension, 200 μL were used to make cytospin preparations (Shandon Southern Instruments, Sewickly, PA, USA). Preparations were fixed in 100% methanol, air-dried, stained with 4% Giemsa for 15 min (Sigma-Aldrich Corp., St. Louis, MO, USA) and examined using oil immersion and a light microscope fitted with a 100x objective (Nikon, Tokyo, Japan). The number of apoptotic cells in 200 μL of the transferred medium was counted and multiplied by 5 to get the total number of apoptotic non-adherent cells in the suspension (Z). The percentage of apoptotic cells = Z/ total number of cells [100,000] x 100.

(B) Adherent cells: Cover glasses containing adherent cells were carefully removed, air-dried, mounted on slides and treated as outlined for non-adherent cells. The data collected were analyzed for percent attachment, phagocytosis and apoptosis.

Assessment of attachment and phagocytic assay. The adherent monolayer MCF-7 cells cultured with fungi (yeast or conidia) were used to measure for percent attachment. Assessment of the attachment of fungi to tumor cells was calculated as the percentage of 200 tumor cells that attached to one or more fungi. With respect to phagocytosis, a previously reported assay was employed with slight modifications (13, 14). In brief, the adherent monolayer MCF-7 cells cultured with yeast/conidia and treated as above were used to examine for percent phagocytosis. Similarly, the assessment of uptake of fungi by tumor cells was calculated as the percentage of 200 tumor cells that ingested one or more fungi.

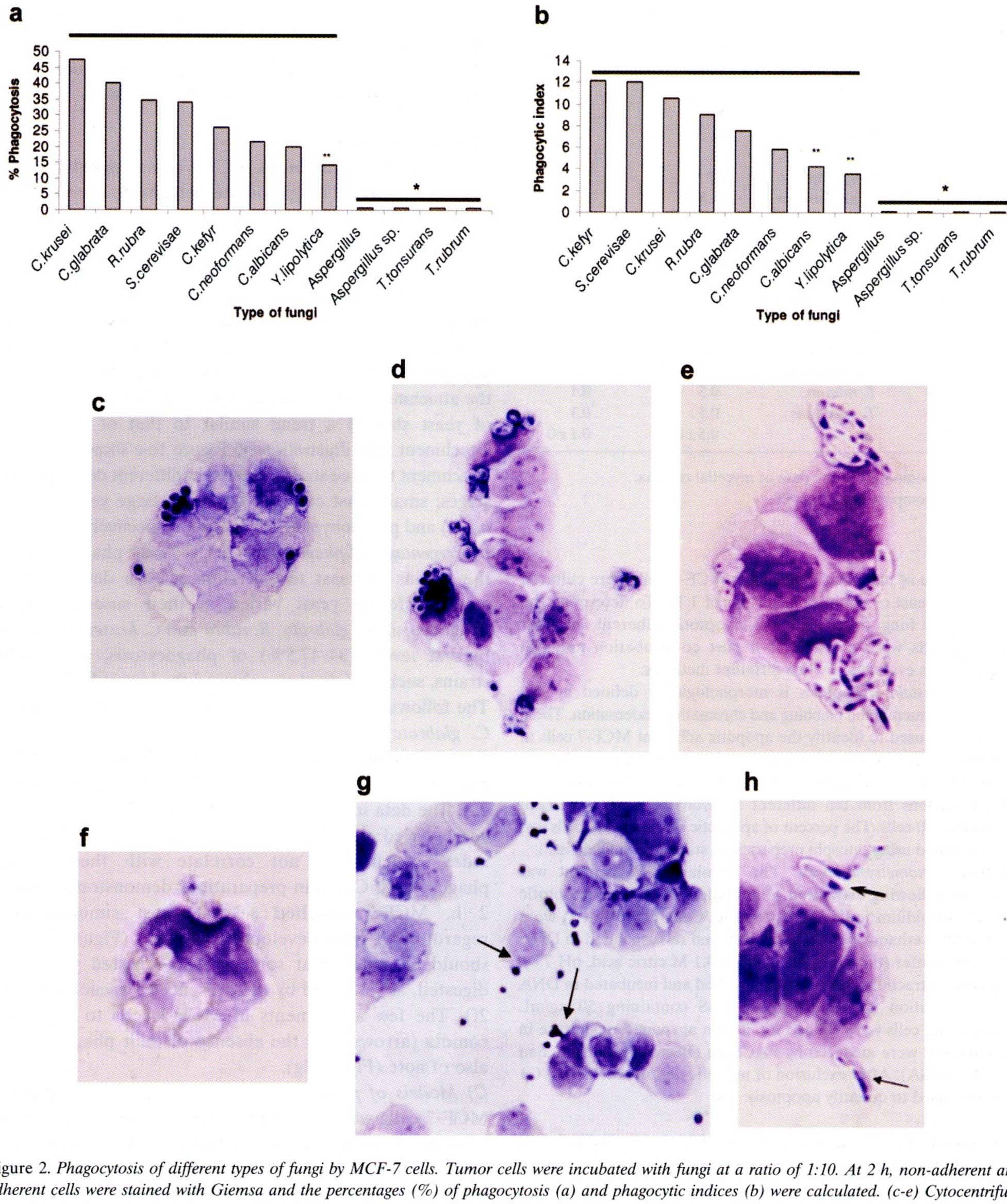

Figure 2. *Phagocytosis of different types of fungi by MCF-7 cells. Tumor cells were incubated with fungi at a ratio of 1:10. At 2 h, non-adherent and adherent cells were stained with Giemsa and the percentages (%) of phagocytosis (a) and phagocytic indices (b) were calculated. (c-e) Cytocentrifuge preparations showing non-adherent MCF-7 cells phagocytizing yeast at different developmental stages; small yeast cells (C. glabrata) (c), large yeast cells (R. rubra) (d) and pseudohyphae (C. krusei) (e). Some of the ingested yeast have been digested as indicated by the presence of vacuoles (f). The attachment of MCF-7 cells to Aspergillus conidia (arrows) and the absence of their phagocytosis by cancer cells (g). Preparation showing blue-stained C. krusei attached to extension from MCF-7 cell ← and yeast inside invagination developed by tumor cell ← (h). (c-h). Giemsa x 400. *$p<0.001$ as compared to mean of mycelia as a group. (a) **$p<0.01$ as compared to C. krusei. (b) **$p<0.01$ as compared to C. kefyr and S. cerevisiae.*

Table I. *Percent phagocytosis and phagocytic index classified according to the developmental stages of different fungi.*

Developmental stage	Name of fungi	% phagocytosis	Phagocytic index
	Yeast		
Small yeast cells	*C. albicans*	20	4.2
	C. glabrata	40	7.5
	S. cerevisiae	34	12
	Y. lipolytica	14**	3.6
Large yeast cells	*C. neoformans*	21.5	5.8
	R. rubra	34.6	9
Pseudohyphae	*C. kefyr*	25.9	12.1
	C. krusei	47.5	10.5
Mean±SD		29.7±11.3*	8.1±3.4*
	Mycelia		
	Aspergillus	0.5	0.1
	Aspergillus sp.	0.5	0.1
	T. rubrum	0.5	0.1
	T. tonsurans	0.5	0.1
Mean±SD		0.5±0	0.1±0

*$p<0.001$ as compared to the data of mycelial conidia.
**$p<0.01$ as compared to *C. krusei*.

Measurements of apoptosis. Monolayer MCF-7 cells were cultured with fungi (yeast or conidia) at a ratio of 1:10. To determine the effects of the fungi on cell survival, apoptotic adherent and non-adherent cells were collected 4 h post co-incubation and cell apoptosis was evaluated by two different methods:
(A) Giemsa stain: Apoptosis is morphologically defined by cell enlargement, membrane blebbing and chromatin condensation. These criteria were used to identify the apopotic adherent MCF-7 cells in cytospin preparations stained with Giemsa on the slides prepared above. The percent of apoptotic adherent cells was calculated based on observations from ten different sites on the cover slip, each containing 100 cells. The percent of apoptotic non-adherent cells was also calculated using cytospin preparations stained with Giemsa.
(B) Flow cytometry analysis: The cellular DNA content was measured following extraction of degraded DNA from apoptotic cells by propidium iodide (PI) staining. Cells ($1x10^6$ ml^{-1}) were fixed in 70% ethanol, washed with PBS and re-suspended in DNA extraction buffer (0.2 M Na_2HPO_4 with 0.1 M citric acid, pH 7.8). Following extraction, the cells were washed and incubated in DNA staining solution (20 μg/mL PI in PBS containing 50 μg/mL RNase). The cells were stained for 30 min at room temperature in the dark and were analyzed by FACScan (Becton Dickinson, San Jose, CA, USA). After exclusion of necrotic debris, the sub G0/G1 peak was used to quantify apoptosis.

Percent of apoptosis and survival of the MCF-7 cells. MCF-7 cells were cultured with *S. cerevisiae* for 4 and 24 h. The percent of apoptosis and survival of the MCF-7 cells were determined in Giemsa-stained cytospin preparations as above.

Statistical analysis. Using the Student's *t*-test, a significant difference in the percent changes of apoptotic MCF-7 cancer cells post culture with fungi as compared to cancer cells alone was tested for.

Results

In the present study, the phagocytic activity of MCF-7 cells against different fungi (eight strains of yeast and four strains of conidia from fungal mycelia) and the subsequent fungi-induced apoptosis of cancer cells was examined.

Phagocytosis studies
A) Percentages of attachment: MCF-7 cells were cultured with different strains of fungi and the percentage of attachment between MCF-7 cells and the fungi was examined at 2 h. MCF-7 cells attached to the eight strains of yeast (Figure 1a). The amount of attachment did not significantly change among strains at different developmental stages (10-15%), with the exception of *Y. lipolytica*, which showed the lowest values (5.8%). In contrast, the attachment of MCF-7 cells to all the tested mycelial conidia was very low (~ 1%). With respect to the attachment index, Figure 1b reveals that different strains of yeast showed a trend similar to that of the percent attachment. The illustrations in Figure 1c-e show MCF-7 cell attachment to three strains of yeast at different developmental stages; small yeast cells (*C. glabrata*), large yeast cells (*R. rubra*) and pseudohyphae (*C. krusei*), respectively.
B) Percentages of phagocytosis: MCF-7 cells phagocytized all the strains of yeast tested. Among each developmental stage, different yeast varied in their susceptibility to phagocytosis. *C. glabrata*, *R. rubra* and *C. krusei* showed the highest levels (34-47.5%) of phagocytosis, while other strains, such as *Y. lipolytica* showed the lowest levels (14%). The following phagocytosis pattern was noted: *C. krusei* > *C. glabrata* > *R. rubra* > *S. cerevisiae* > *C. kefyr* > *C. neoformans* > *C. albicans* > *Y. lipolytica*. MCF-7 phagocytized almost none (≤ 1%) of the conidia (Figure 2a). The data in Figure 2b indicate that different strains of yeast varied significantly with respect to the phagocytic index, which does not correlate with their percent phagocytosis. Cytospin preparations demonstrated that, at 2 h, MCF-7 engulfed several yeast simultaneously, regardless of their developmental stage (Figure 2c-e). It should be noted that some of the ingested yeast were digested, as indicated by the presence of vacuoles (Figure 2f). The few attachments of MCF-7 cells to *Aspergillus* conidia (arrows) and the absence of their phagocytosis is also of note (Figure 2g).
C) Models of phagocytosis: The attachment of yeast to MCF-7 cells was followed by phagocytosis for all strains. Cytospin preparations showed two models of phagocytosis. The first model, illustrated in Figure 2h, showed cytoplasmic invagination (cup shape) of the tumor cell surface, which continued until the yeast was completely encircled by the cancer cell. The second model showed the cancer cell extending cytoplasmic folds, eventually encircling the entire yeast.

The data in Table I demonstrate the percent phagocytosis and phagocytic indices categorized according to the developmental stages of different yeast as well as the mycelial conidia. At 2 h, significant levels of phagocytosis of yeast by MCF-7 cells occurred, regardless of the developmental stage as young yeast cells, large yeast cells and pseudohyphae which were all susceptible to phagocytosis. In contrast, insignificant levels of phagocytosis of conidia, as compared with the background, were observed.

Apoptosis studies

A) Morphological changes of yeast-induced apoptosis of cancer cells: The morphological changes associated with the induction of apoptosis in cancer cells by the different strains of yeast are illustrated in Figure 3. Apoptotic cells were noted at 4 h post-culture of the MCF-7 cells with yeast. The yeast strains demonstrated typical characteristics of apoptosis in MCF-7 cells, including cell swelling (Figure 3a, b) and nuclear fragmentation (Figure 3c,d). Finally, the nucleus disappeared and the MCF-7 cells acquired the Trypan blue stain, indicating cell death (Figure 3e-h). In all cases, the yeast remained inside the MCF-7 cells throughout the apoptotic process. The developmental stage of the yeast was not a factor in the apoptotic process, since small cells *(C. glabrata),* large cells (*R. rubra*) and pseudohyphae (*C. krusei*) all led to cell death.

B) Measurements of apoptosis: The MCF-7 cells were cultured with fungi at a ratio of 1:10 and the apoptotic effects of yeast and conidia were examined at 4 h using two different methods:

(i) Giemsa stain. The percent apoptosis was examined in non-adherent and adherent cells. The data presented in Figure 4 indicate that MCF-7 cells undergo apoptosis post-culture with yeast. When the data of apoptotic adherent cells was combined with non-adherent cells, significant levels of apoptosis were detected (21-46%) as compared to background (13.4%), with the exception of the yeast-like species *Y. lipolytica*. In contrast, insignificant changes in apoptotic MCF-7 cells post-culture with mycelial conidia were observed as compared with the background.

(ii) Flow cytometry analysis. The survival of MCF-7 cells post-treatment with different fungi was examined by flow cytometry. The results depicted in Figure 5 show a significant increase in apoptotic, non-adherent, MCF-7 cells upon treatment with different yeast. The apoptotic effect of yeast followed a graded manner: *S. cerevisiae* > *C. kefyr* > *C. krusei* > *C. neoformans* > *R. rubra* > *C. albicans* > *C. glabrata* > *Y. lipolytica*. In contrast, the MCF-7 cells did not undergo significant levels of apoptosis post-culture with any of the mycelial conidia (Figure 6).

The data presented in Table II illustrate the percent apoptosis of MCF-7 cells categorized according to the developmental stages of the different yeast as well as mycelial conidia. Yeast-induced apoptosis of MCF-7 cells occured as early as 2 h post-culture with yeast, regardless of the developmental stage. The apoptotic effect of yeast increased by 2- to 4-fold at 4 h. In contrast, conidia induced an insignificant change in the percent apoptosis as compared with the background.

Table II. *Percent apoptosis of MCF-7 cells categorized according to the developmental stage of different fungi.*

Developmental stage	Name of fungi	% Apoptosis		
		Giemsa (2 h)	Giemsa (4 h)	Flow cytometry (4 h)
	Yeast			
Small yeast cells	*C. albicans*	12	25.4	37
	C. glabrata	11	24.3	27
	S. cerevisiae	12	45.3	93
	Y. lipolytica	5	14.1	12**
Large yeast cells	*C. neoformans*	16	21.5	47
	R. rubra	9	34.1	42
Pseudohyphae	*C. kefyr*	15	46.1	56
	C. krusei	10.5	35.1	55
Mean±SD		11.3±3.4*	30.7±11.4*	46.1±23.9*
	Mycelia			
	Aspergillus sp.	5.6	8.1	12
	T. rubrum	5.5	5.5	9
	Aspergillus	7.1	8.2	8
	T. tonsurans	7.3	11.9	10.2
Mean±SD		6.4±1.0	8.4±2.6	9.8±1.7
	Control MCF-7 cells	7.5	11.4	8

*$p<0.001$ as compared to the data of mycelia.
**$p<0.001$ as compared to data of *S. cerevisiae*.

C) Yeast source: cell line vs. infected patients. To determine whether the fungal strains obtained from unidentified patients were potentially transformed by physiological factors, thereby increasing their susceptibility for phagocytosis and induction of apoptosis, a set of experiments using *C. neoformans* and *T. tonsurans* cell lines, obtained from the ATCC, were carried out. The results revealed that *C. neoformans* was highly susceptible to phagocytosis by MCF-7 cells (21.5%). In contrast, *T. tonsurans* appeared resistant. These results are comparable to those of other patient-derived fungi.

D) Percent of apoptosis and survival of the MCF-7 cells: The data depicted in Figure 7 indicate that yeast induced 45% of the MCF-7 cells to undergo apoptosis at 4 h, which was significantly increased to 91% at 24 h post-culture of cancer cells with yeast. The percent of live cells was inversely correlated with the percent of dead cells as time passed (55% at 4 h to 9% at 24 h), suggesting that enhanced apoptosis is correlated with reduced survival of the MCF-7 cells.

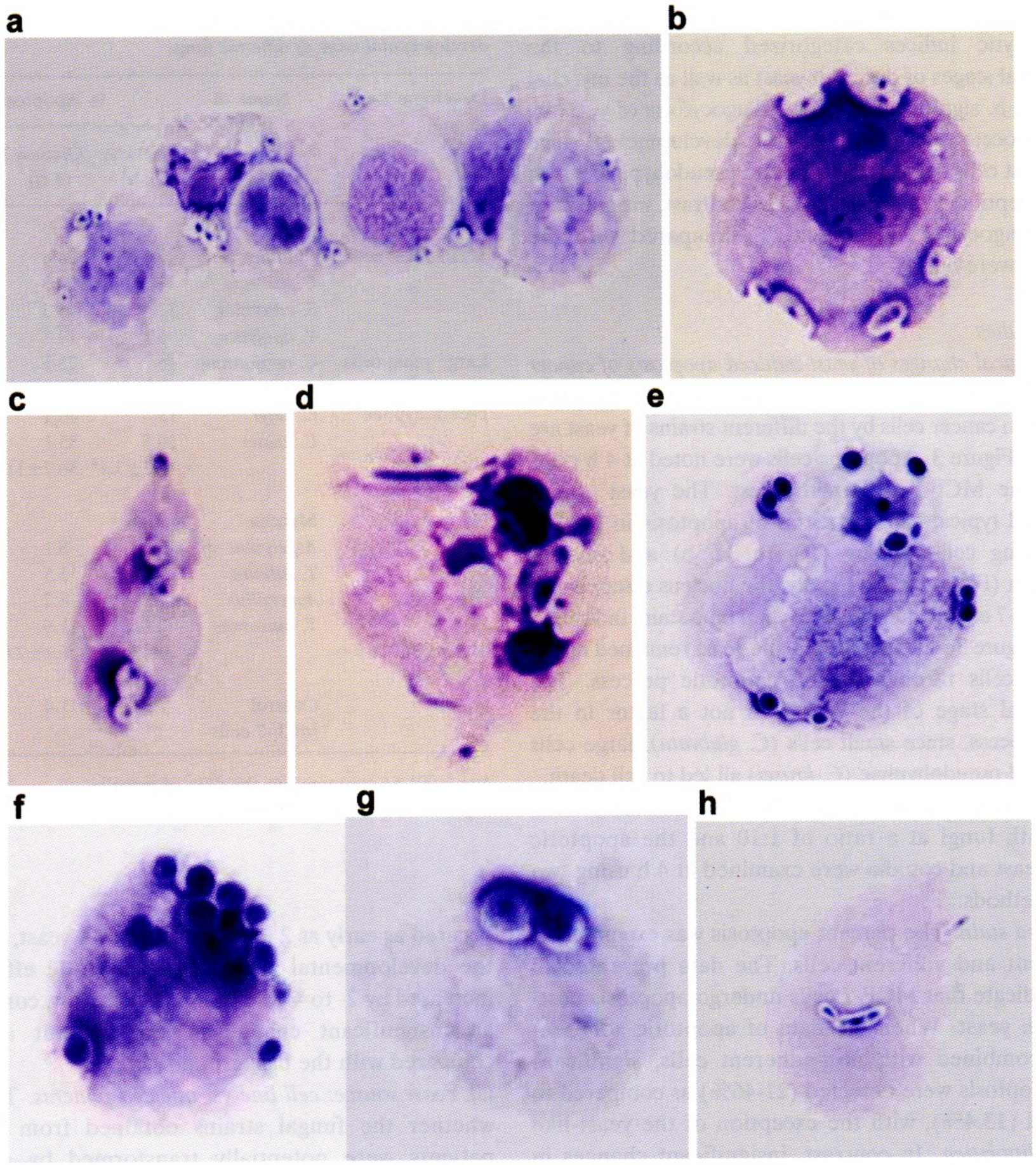

Figure 3. *Morphological examination of apoptotic non-adherent MCF-7 cells. Monolayer MCF-7 cells, grown on cover glass, were cultured with heat-killed yeast and conidia at a ratio of 1:10, respectively. At 4 h, the supernatants were collected, cytospin preparations were performed and the cells were stained with Giemsa. Preparations showing enlarged apoptotic MCF-7 cells (a-d). Preparations showing apoptotic MCF-7 cell with nuclear fragmentation post-culture with C. albicans (c) and C. krusei (d). Finally, (e-h) show dead cancer cells that have acquired the Trypan blue stain. The presence of yeast inside the apoptotic cells in (e-h) should be noted. Small yeast cells (C. albicans), (e, f) large yeast cells (R. rubra) (g) and pseudohyphae (C. krusei) (h) were all stained dark blue. Figures 3 a-h, e and f Giemsa x 400; d, g and h x 1000.*

Discussion

Our previous studies indicated that the majority of human cancer cell lines (breast, tongue, colon, leukemia) are susceptible to *S. cerevisiae*-mediated apoptosis (9-12). Subsequent *in vivo* mouse experiments, employing intra-tumorally injected yeast, revealed significant tumor regression (data not published). The results from the present study demonstrated that at least eight different strains of yeast elicited programmed cell death in breast cancer cells (MCF-7) regardless of their developmental stage. Different strains of yeast are susceptible to phagocytosis by MCF-7 cells with varying kinetics. The following graded level of phagocytic activity among the different strains of yeast was observed: *C. krusei* > *C. glabrata* > *D. rubra* > *S. cerevisiae* > *C. kefyr* > *C. neoformans* > *C. albicans* > *Y. lipolytica*. The phagocytic susceptibility of yeast could be separated into three categories; low (<15%), medium (16-30%) and high (>30%).

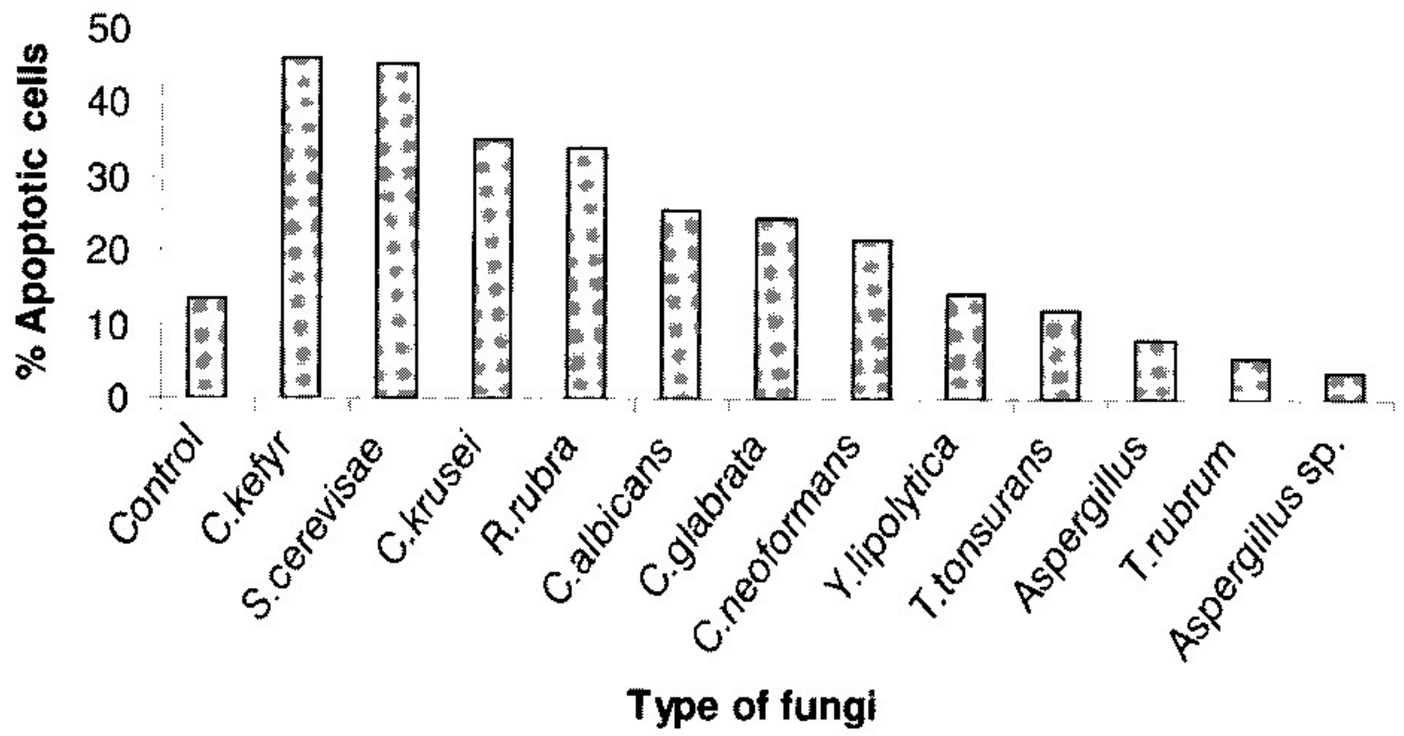

Figure 4. *Fungi-induced apoptosis of MCF-7 cells using Giemsa-stained cytospin preparations. Tumor cells were cultured with fungi at a ratio of 1:10. At 4 h. the percent of apoptotic non-adherent and adherent MCF-7 cells were calculated. Data represent the mean of three separate experiments.*

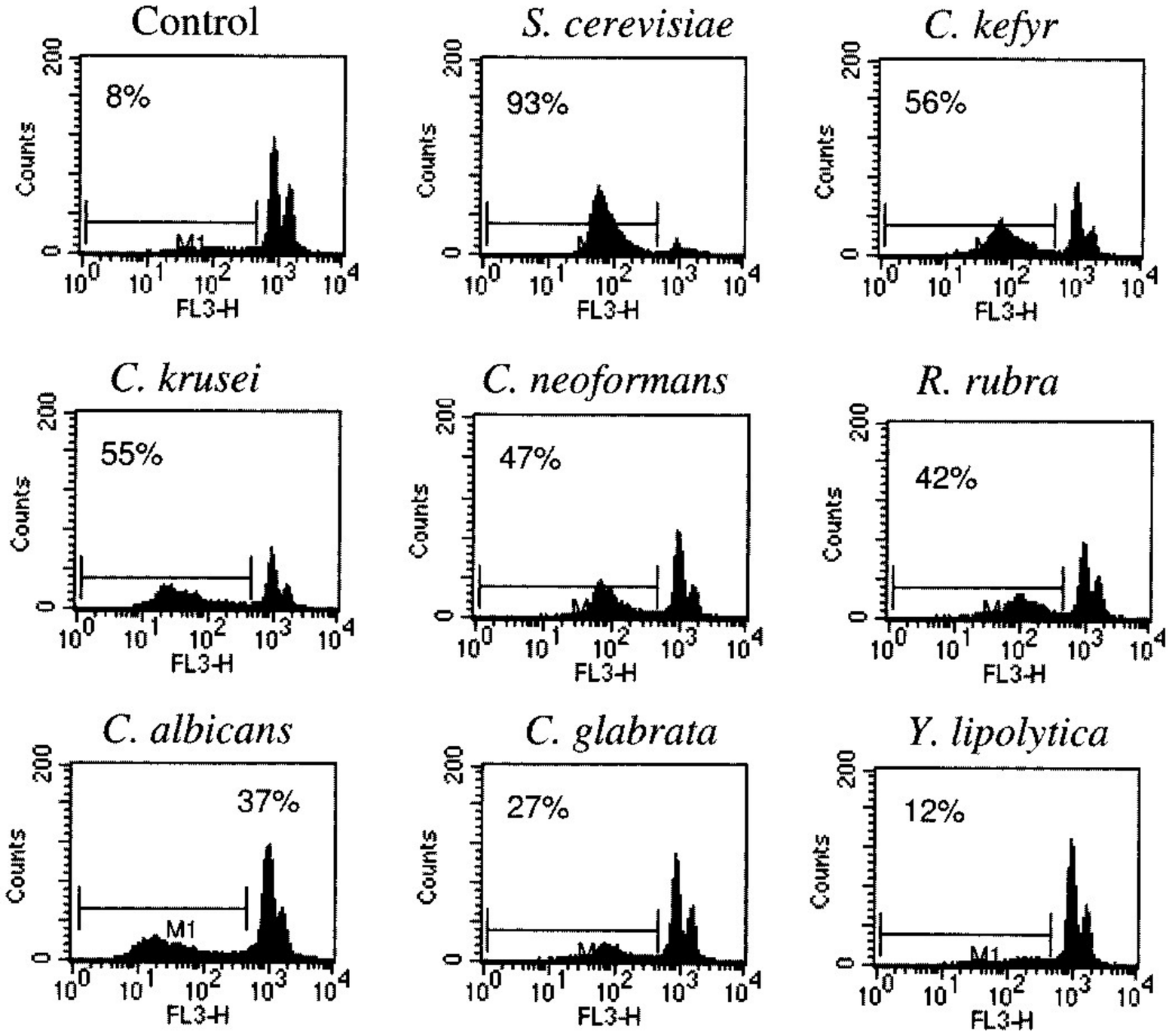

Figure 5. *Yeast-induced apoptosis of MCF-7 cells using flow cytometry. MCF-7 cells were cultured with yeast for 4 h. The supernatants were collected and the percentages of apoptotic cells were examined using flow cytometry.*

Phagocytosis occurred through cytoplasmic invagination of the tumor cell surface and by extension of cytoplasmic folds. We have recently reported both of these models of phagocytosis in studies conducted using *C. albicans* as the test organism against breast cancer cells (15). Factor(s) responsible for the graded phagocytic susceptibility among the different strains of yeast observed is/are not known but may be attributed to differential expression of poly-

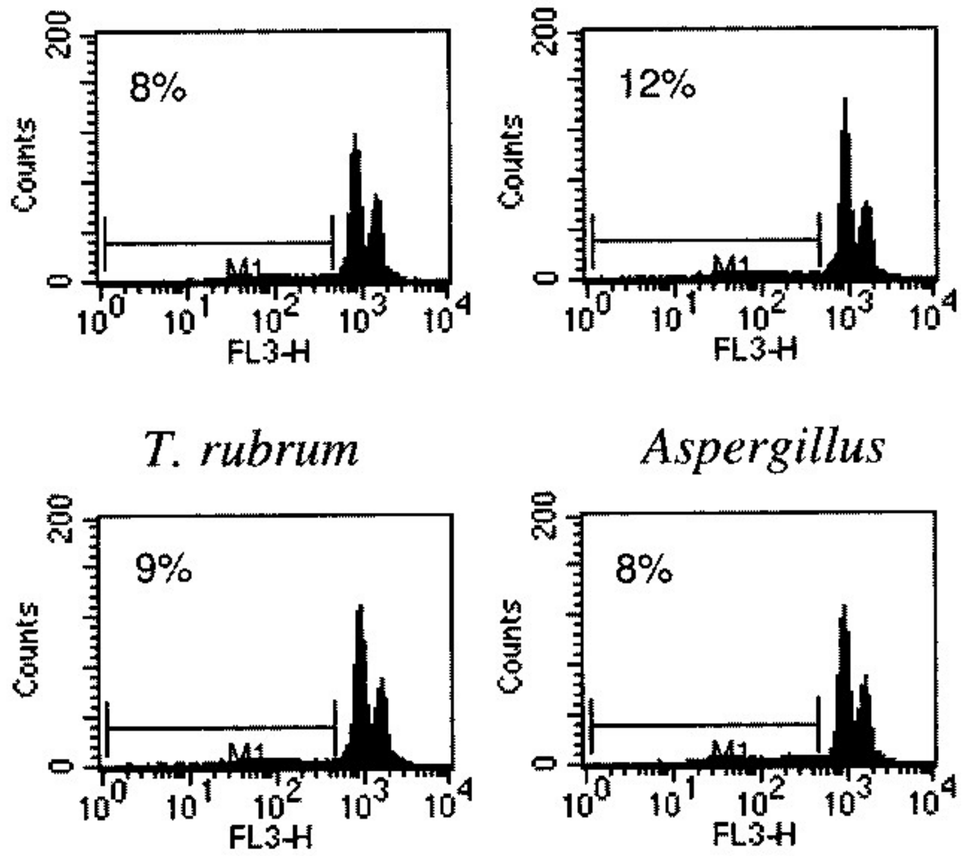

Figure 6. *Fungal mycelia-induced apoptosis of MCF-7 cells using flow cytometry. MCF-7 cells were cultured with yeast for 4 h. Supernatants were collected and the percentages of apoptotic cells were examined using flow cytometry.*

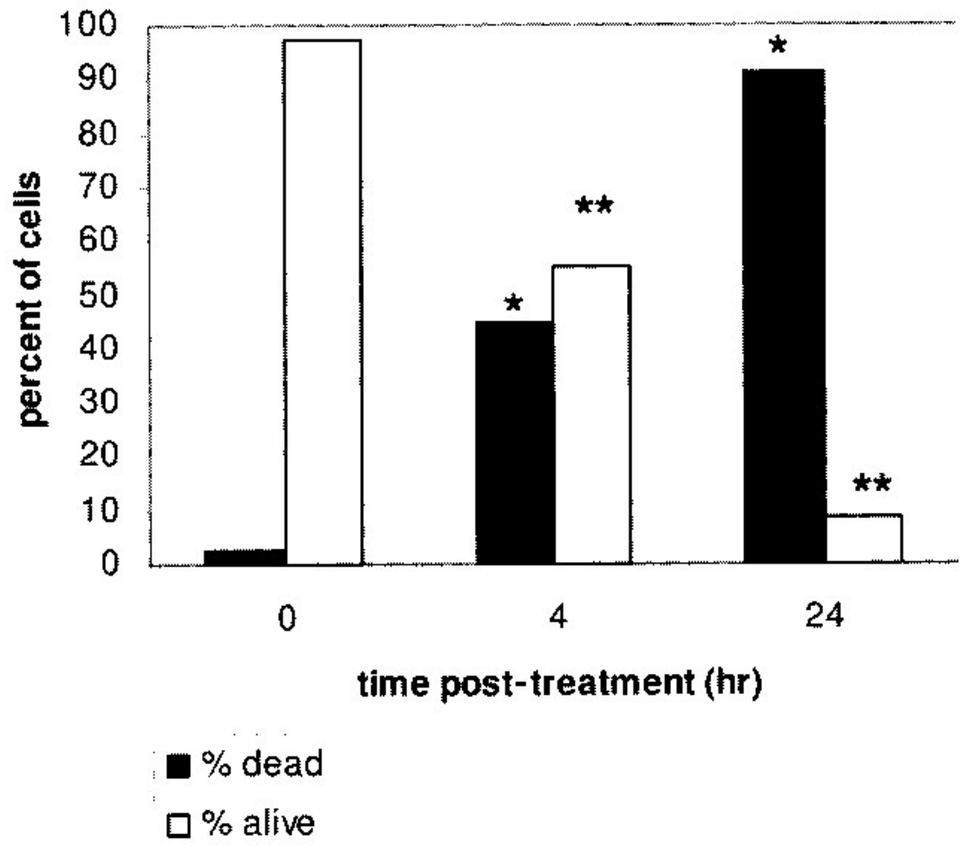

Figure 7. *Percent survival versus percent dead MCF-7 cells post-treatment with S. cerevisiae. Monolayer MCF-7 cells were cultured with S. cerevisiae at a ratio of 1:10. At different time-points (0, 4, 24 h), the percentages of live and dead cancer cells were determined in Giemsa-stained cytospin preparations. *p<0.001 as compared to 0 h time-point of dead cells, **p<0.001 as compared to 0 h time-point of live cells.*

saccharide components of the cell wall among strains. Indeed, a variety of components within the yeast cell wall have been implicated in recognition by human macrophages including β-glucan (16, 17), mannans (18, 19) and oligomannosides (20, 21). Cell-surface receptors on human macrophages recognize specific components of the yeast cell wall, such as mannose receptors for mannans (22) and Dectin-1 receptors for β-glucans (23).

We were particularly interested in whether or not the apoptotic effects previously observed were specific for yeast. Therefore, conidia from four strains of mycelia (*Aspergillus*, *Aspergillus* sp., *T. rubrum* and *T. tonsurans*) were also used as test organisms for phagocytosis by MCF-7 cells. Despite similar cell wall compositions between the different fungi (including β(1,3)-glucan, chitin and mannan), no significant level of phagocytosis of conidia by MCF-7 cells was observed. These data demonstrated that phagocytosis by MCF-7 cells is a selective process in which the cancer cells can discriminate between 'ingestible' and 'non-ingestible' pathogens in a manner resembling that of professional phagocytes. Resistance to phagocytosis and killing of conidia from different mycelial strains by macrophages and neutrophils has been previously reported (24-27).

Causal factors responsible for the distinguishable difference in the MCF-7 cell phagocytic ability of conidia compared to yeast could be attributed to differential exposure of cell surface components involved in recognition. Recently, Gantner *et al.* (28) reported that mycelia and yeast have unequal levels of β-glucan exposure due to their respective mechanisms of cell wall growth during replication. Greater amounts of β-glucan exposure found in yeast during replication (28) and after subjection to high heat (29) provide a potential means of distinguishing between yeast and mycelia with regards to cancer cell phagocytic susceptibility. This information may be relevant to elucidating the mechanism by which yeast, and not conidia, are able to induce apoptosis in cancer cells. It is also of interest to note that the structure of yeast glucans differs among different strains of yeasts. Lowman *et al.* (30) reported that *C. glucans,* derived from blastospores or hyphae, are different compared to those isolated from *S. cerevisiae* with regard to side-chain branching along the backbone and at the reducing terminus. This may explain the observed differences in the phagocytosis and apoptosis induced by different yeast.

All strains of yeast tested here were potent inducers of apoptosis in the MCF-7 cells. The morphological analysis *via* Giemsa staining revealed the presence of multiple vacuoles of ingested and digested yeast inside apoptotic cancer cells. The stereotypical series of morphological changes associated with apoptosis are well illustrated for all strains of tested yeast: cell enlargement, blebbing of the plasma membrane and DNA fragmentation. Flow cytometry analysis also demonstrated a significant increase in MCF-7

cell apoptosis upon treatment with various yeast. The apoptotic effect of yeast followed a graded pattern: *S. cerevisiae* > *C. kefyr* > *C. krusei* > *C. neoformans* > *R. rubra* > *C. albicans* > *C. glabrata* > *Y. lipolytica*. Differences between the graded pattern of phagocytosis and that of apoptosis were revealed, suggesting that the apoptotic effect of yeast may be governed by both the percentages of attachment/phagocytosis as well as by their indices. The mechanism of apoptosis of breast cancer cells post-attachment/phagocytosis of yeast was previously examined in our laboratory and was found to operate *via* a caspase-independent mechanism (9). Yeast-induced cancer cell apoptosis was highly correlated with reduced MCF-7 cell survival in a time-dependent manner. The data in Figure 7 showed that 45% of apoptotic MCF-7 cells was detected at 4 h post-culture of cancer cells with yeast. Prolonging the culture period to 24 h revealed that most of the cancer cells (over 90%) were either undergoing apoptosis or were dead.

High levels of morbidity and mortality among immunocompromised patients have been attributed to fungal infections (31-35). While *Aspergillus* and *Candida* sp. collectively account for the majority of these infections, the number of patients infected by other fungi, including *Aspergillus* sp. (33), *C. glabrata*, *C. krusei* , *C. neoformans* and *R. rubra* (34), is increasing. Studies have shown that virulence varies between the different fungal species (35). Data from the current study revealed a lack of correlation between the apoptotic effect of fungi and their virulence as indicated by: a) the absence of an apoptotic effect by the highly virulent fungal mycelia; and b) the presence of apoptosis induced by the non-pathological species *S. cerevisiae*. The data also did not indicate a correlation between the apoptotic effect and the developmental stage of the yeast, since small yeast cells, large yeast cells and pseudohyphae all induced apoptosis in MCF-7 cells. Furthermore, the current study examined whether the source of yeast affected their phagocytotic and apoptotic potential. The results revealed that MCF-7 cells underwent apoptosis following phagocytosis of yeast, regardless of whether they came from a patient or a cell line. This suggests an absence of possible "patient-induced" physiological changes in the yeast's underlying ability to be phagocytized by cancer cells and to induce apoptosis.

In conclusion, human cancer cells undergo apoptosis following phagocytosis of various heat-killed strains of yeast, with certain strains more capable than others of initiating cell death. The apoptotic effect of yeast on cancer cells also appeared to be highly specific, as fungal mycelia failed to induce similar results. Identification of the cancer cell-surface receptor(s) mediating the phagocytosis of yeast, leading to the subsequent induction of apoptosis, might present a novel therapeutic approach that can be exploited for the development of cancer drugs.

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