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Evaluation of phagolysosome fusion in acridine orange stained macrophages infected with *Histoplasma capsulatum*

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SUMMARY

A phagosome-lysosome (PL) fusion was performed *in vitro* using peritoneal cells from normal BALB/c mice and the J774.2 macrophage cell line infected with the yeast phase of the fungus *Histoplasma capsulatum* at ratios of 5×10^5, 5×10^6 or 1×10^7 yeasts per 1×10^6 macrophages, and phagocytosis was allowed to proceed for 5, 30 and 60 min. Macrophage lysosomes were pre-labelled with acridine orange and the cells were challenged with the parasite. Fusion was evaluated by fluorescence microscopy and the number of macrophages with stained yeast cells was scored. The phagolysosome fusion frequency (PLFF) was calculated by subtracting the specific fusions of infected macrophages from the non-specific fusions of uninfected macrophages and normalizing the total number of bound yeasts. The PLFF was determined using different doses and strains of *H. capsulatum*. Results showed that PLFF in infected macrophages depends on the infection dose. Inhibition of PL fusion was detected mainly at a high infection ratio (1×10^7 yeasts/1×10^6 macrophages), and PL fusion varied with phagocytosis time. No significant differences were observed in the fusions when different *Histoplasma* strains were used. Results with J774.2 cells were similar to peritoneal cells, indicating that both methodology and fusion calculations employed were useful, in spite of the heterogeneity of macrophage populations used.

Keywords macrophages phagosome lysosome *Histoplasma capsulatum*

INTRODUCTION

The mycotic disease produced by the intracellular fungus *Histoplasma capsulatum* frequently has a favourable resolution, but in some cases survival and multiplication of this parasite occurs within the macrophage and infection progresses.

The activation of macrophages by T cells promoting parasite elimination is well documented for histoplasmosis (Howard & Otto, 1977; Tewari, Sharma & Mathur, 1978; 1980; Wu-Hsieh, Zlotnik & Howard, 1984). The host mechanisms by which mononuclear and polymorphonuclear cells promote fungal destruction have been studied, although many questions remain unsolved (Howard, 1973, 1975). Under some conditions the fungus is able to escape intracellular killing. This can be explained by alterations either in the phagocytic system or in the optimal expression of the immune response. Other factors which could be microorganism-dependent, such as high infection doses or virulence, may play an important role in the escape process. Just exactly how the fungus persists and proliferates inside the macrophages is not known. Strategies against phagocytic mechanisms have been described for several microorganisms and one of them is the inhibition of PL fusion (Friis, 1972; Jones & Hirsch, 1972; Goren *et al.*, 1976; Davis-Scibienski & Beaman, 1980; Beaman & Pappagianis, 1981; Beaman, Benjamini & Pappagianis, 1983; Frehel *et al.*, 1986).

Dumont & Robert (1970), studying hamster peritoneal macrophages *in vivo*, observed PL fusion by electron microscopy. They found yeasts in two types of phagosomes ('tight' and 'loose' phagosomes), one of them presenting acid phosphatase activity. The authors suggested that this enzyme originated from modified phagocytes which had been phagocytized together with the fungus, since most of the yeast cells that multiplied inside the phagosome were phagocytized again with macrophage debris. This situation could cast some questions on the role of PL fusion in histoplasmal infection. A study of PL fusions in mice macrophages infected with *H. capsulatum* yeast is presented, by using acridine orange macrophage labelled lysosomes and applying a formula to express the frequency of PL fusions.

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MATERIALS AND METHODS

Animals

Syngeneic female BALB/c mice (4·5 months) were used and fed with mouse chow (Purina de México, SA) and sterile water *ad libitum*.

Microorganisms
5037 and 'León Pérez' *H. capsulatum* strains were obtained from the Depto. de Ecología Humana collection. The latter was isolated from a fatal human histoplasmosis (acute form). The fungal yeast phase was maintained at 37°C in brain–heart infusion broth (BHI) (Bioxón de México, SA) supplemented with 0·1% L-cysteine and 1% glucose. *Mycobacterium tuberculosis* H37 Rv was cultured in PBY (Proskawer, Beck and Youmans) medium and used in its logarithmic growth phase. Differences in infection doses between the *Histoplasma* strains, were determined by the lethal dose 50 (LD_{50}), according to Reed & Muench (1938) as has been described previously (Taylor *et al.*, 1982).

Cells
Two macrophage populations were used for PL fusion determination. Peritoneal cells were obtained from normal BALB/c mice by peritoneal washings with 10–15 ml of sterile BSS (balanced saline solution). After removing erythrocytes by hypotonic lysis, they were resuspended in RPMI-1640 with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulphonic acid) (Gibco NY), pH 7·0, supplemented with 20% fetal calf serum, 1% L-glutamine, and 50 μg/ml gentamycin. Macrophages adjusted to 1×10^6 cells/ml of RPMI were cultured on 12 mm diameter sterile glass coverslips in wells of 24-well microplates. Cells were allowed to adhere at 37°C, 5% CO_2 for 3 h and then rinsed to remove the non-adherent population. Adherent cells were further incubated for 24 h before infection under the same conditions. The J774.2 macrophage cell line was routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 1% L-glutamine and 20% fetal calf serum (Gibco). They were adjusted to 1×10^6 cells/ml and plated 24 h before infection.

Lysosome labelling
Monolayers of either peritoneal or J774.2 macrophages were labelled with acridine orange as described by Kielian & Cohn (1980). Briefly, cells were maintained for 20 min at 37°C in acridine orange solution (5 μg/ml in RPMI-1640 or DMEM, pH 7·0) prior to infection; cells were washed twice to eliminate colour excess.

Preparation of inocula
Viable yeasts were centrifuged twice at 300 ***g*** for 10 min in BSS, counted in a Neubauer chamber and adjusted to contain 5×10^5, 5×10^6 and 1×10^7 yeasts/150 μl of RPMI-1640 or DMEM, pH 7·0. *M. tuberculosis* bacilli were obtained from the air-water interphase of the PBY media, centrifuged at 300 ***g***, resuspended in RPMI, pH 7·0, and stirred in a vortex mixer for 10 min. They were counted and adjusted to the desired number.

Macrophage infection
Phagocytic cells were infected by adding 150 μl of the suspended microorganisms as described above at 0·5:1, 5:1 and 10:1 parasite:macrophage ratios respectively. In order to synchronize the ingestion, infected cells were incubated at least 1 h at 4°C, to allow microorganisms binding, to yield a uniform internalization for each infection dose. Macrophages were rinsed twice with BSS to remove unbound inocula and then incubated at 37°C for 0, 5, 30 or 60 min. After each phagocytosis time, cells were washed with BSS, pH 7·0, fixed with methanol and air-dried. Then coverslips were removed, mounted on slides and examined for evidence of fusions. Uninfected macrophages were used as control to subtract the cell autofluorescence, since there are some non-specific fusions due to autophagosomes or phagocytosis of membrane debris from dead macrophages. Either unbound yeasts or *M. tuberculosis* bacilli were recovered, counted and cultured in order to test viability. Heat-killed yeast phagocytosis by J774.2 macrophages were carried out to compare with viable yeast phagocytosis. Microorganisms bound at time 0 (zero) of phagocytosis were stained with Wright and counted in 100 acridine orange unlabelled macrophages.

Fusion determinations
Fusions were recorded by two different forms. One by the number of phagolysosome fusions (PLF) for both infected and uninfected macrophages in 100 phagocytes, the other by the determination of phagolysosome fusion frequency (PLFF) obtained for *Histoplasma* cells with the following formula:

$$\mathrm{PLFF}=\frac{\mathrm{A}-\mathrm{B}}{\mathrm{C}}$$

Where A is the number of fluorescent yeasts per infected macrophage stained with acridine orange at each phagocytic time, B is the number of fluorescent particles per uninfected macrophage stained with acridine orange at the same time and C is the number of bound yeasts on macrophages stained by Wright at time zero of phagocytosis.

Fluorescence microscopy
Acridine orange labelled macrophages were observed and fusions counted using epi-illumination with a Zeiss photomicroscope III, with a BG-12 primary filter and a K-44 barrier filter.

Statistical analysis
Student's *t*-test was used and P values $\leqq 0{\cdot}05$ were considered significant.

RESULTS

In order to characterize the *Histoplasma* strains' virulence, the LD_{50} was performed resulting in 2×10^7 and $>1\times10^9$ yeasts/ml for 'León Pérez' and 5037 strains respectively, suggesting differences between the strains used. Macrophage adherence and viability were calculated in the course of each experiment. Results from three assays showed that half of the macrophage population failed to adhere before infection, and this was taken into account to establish parasite:macrophage ratios. Macrophage viability averaged 80% before and after infection.

PLF
The number of PLF in 100 peritoneal macrophages is shown in Fig. 1. Infected macrophage fusions declined when the yeast inocula and the phagocytosis time increased. At the low parasite:macrophage ratio (0·5:1), maximal PL fusions were observed, even when non-specific fusions from uninfected macrophages were subtracted at each incubation period. A decrease in the fusion values was recorded for ratios of 5:1 and 10:1 often dropping below the background for uninfected macrophages. With all *Histoplasma*:macrophage ratios used, peak detection of PLF was reached between 5 and 30 min,

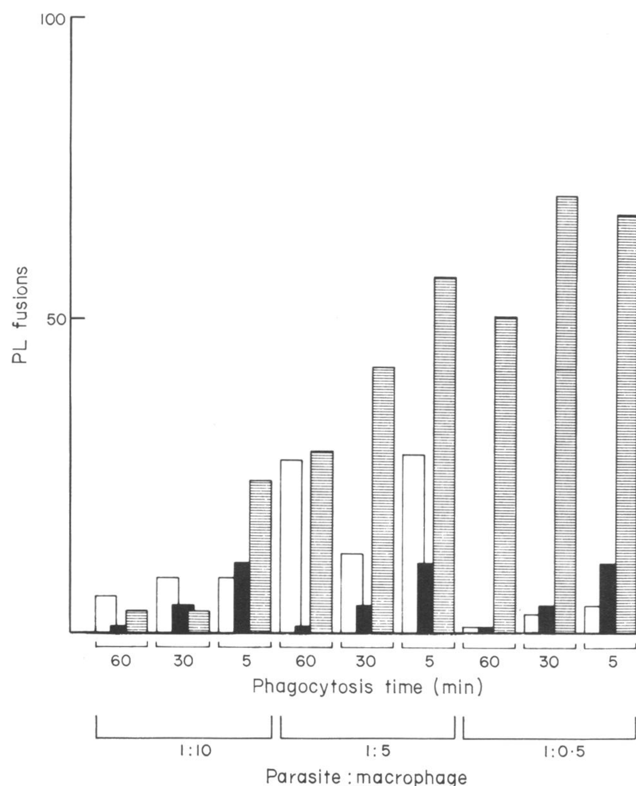

Fig 1. Effect of infection doses and phagocytosis time on PL fusions. For this assay, 100 macrophages were randomly selected. Acridine orange labelled peritoneal macrophages were infected, synchronizing the parasite inocula by their binding at 1 h, 4°C. 'León Pérez' *H. capsulatum* strain was used at $5\times10^5:1\times10^6$, $5\times10^6:1\times10^6$ and $1\times10^7:1\times10^6$ (parasites : macrophages). Figure shows the results of two experiments. ■, Macrophages infected with *H. capsulatum*; □, Macrophages infected with *M. tuberculosis*; ■ Non-infected macrophages.

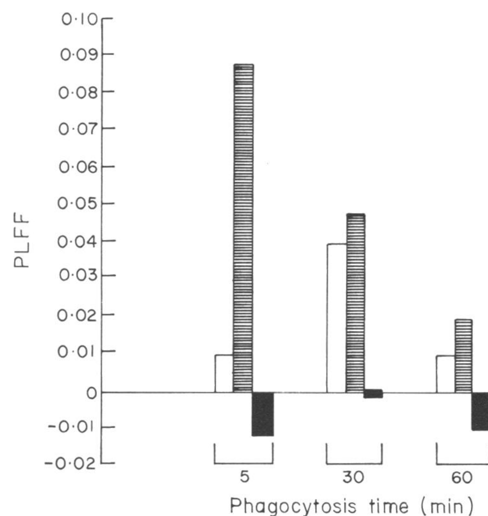

Fig. 2. PLFF for different fungal doses. Peritoneal macrophages were infected with 'León Pérez' *H. capsulatum* strain under the same conditions described earlier. The PLFF formula described in Materials and Methods was applied, taking into account only those macrophages presenting PL fusions. Results of three experiments are reported. Parasite : macrophage ratios: □, $5\times10^5:1\times10^6$; ■, $5\times10^6:1\times10^6$; ■, $1\times10^7:1\times10^6$.

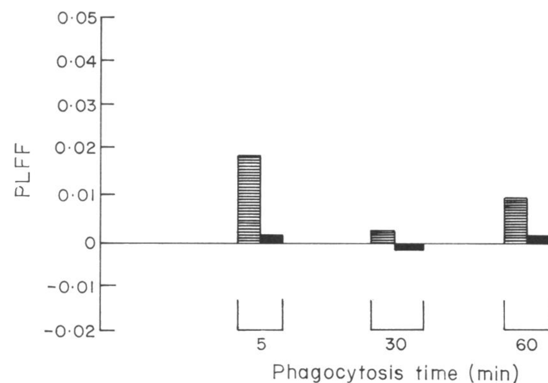

Fig. 3. PLFF in macrophages infected with different strains of *H. capsulatum*. Peritoneal macrophages were infected with 5×10^6 yeasts/ 1×10^6 macrophages. Three experiments were performed to apply the PLFF formula. For this calculation 100 macrophages were randomly selected. ■, Macrophages infected with 5037 strain; ■, macrophages infected with 'León Pérez' strain.

decreasing after 60 min of infection. For *M. tuberculosis*, infected macrophage fusions were observed only for the intermediate ratio (5:1), these values are very close to those obtained in uninfected controls and the application of the PLFF formula in this point gives a negative fusion value (data not shown).

Fluorescent particles in uninfected macrophages were used as references at each period of incubation, in order to account for non-specific fusions occurring within the cells as a consequence of membrane recycling and degradation. Most of these background values were below those found for the *H. capsulatum* infected cells.

PLFF

To determine the frequency of PL fusions for *Histoplasma* yeasts, the formula described in Materials and Methods was applied. Figure 2 shows the PLFF obtained, taking into account only those macrophages presenting fusions. A variation in the positive values for ratios of 0·5:1 and 5:1 during the different periods of phagocytosis, as well as very low fusion values for the 10:1 infection ratio were detected. Figure 3 shows the results of PLFF for different strains of *H. capsulatum* at the 5:1 yeast : macrophage ratio, for a random sample of 100 macrophages from the infected population. Very low positive values for the PLFF were obtained with strain 5037, and values within background levels were detected for the 'León Pérez' strain. These differences were not statistically significant.

In order to express graphically the PLFF strain-dependence, PLFF 5037/PLFF 'León Pérez' ratios were plotted (Fig. 4). Maximal strain-dependent differences in the PLFF were found immediately following infection, and these decreased throughout the experiment.

To observe the PL fusion mechanism for this parasite in another type of macrophage population, the PLFF determination for *Histoplasma* yeasts was also performed in the J774.2

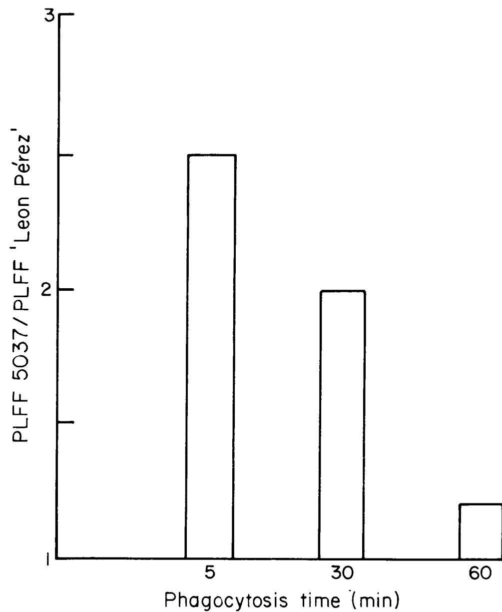

Fig. 4. Relationship between PLFF values of 'León Pérez' and 5037 *H. capsulatum* strains. To obtain comparative values, 100 randomly selected peritoneal macrophages were considered and PLFF data calculated. The PLFF values of strain 5037 were divided by those of 'León Pérez' to give a quotient of one strain with respect to the other.

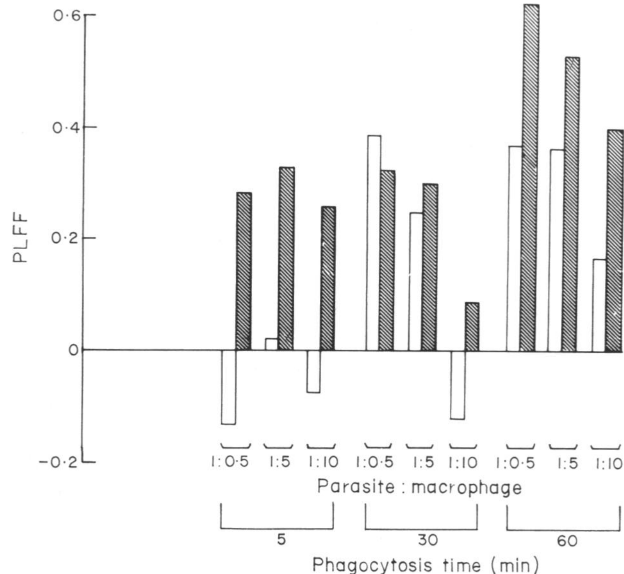

Fig. 5. PLFF in macrophages infected with live and heat-killed yeast cells. J774.2 macrophage cell line infected with different doses of live (□) and heat-killed (▨) 'León Pérez' *H. capsulatum* yeast cells were processed to obtain the PLFF as in previous experiments. Results express three assays.

macrophage cell line. Figure 5 shows the results of PLFF for viable and heat-killed *Histoplasma* yeasts, which disclosed similar behaviour to peritoneal macrophages, demonstrating that high parasite:macrophage ratios give low or negative PLFF values. Heat-killed control yeast cells induced higher PLFF positive values.

DISCUSSION

Several fungal species are able to grow inside phagocytes, and at present there are few studies suggesting how the fungus survives intracellularly. Beaman & Pappagianis (1981), found that arthroconidia and endospores from *Coccidioides immitis*, appear to inhibit PL fusion in normal macrophages. However, macrophage activation by lymphokine enhances the fusions and intracellular killing of *C. immitis* cells (Beaman, Benjamini & Pappagianis, 1983). Recently PL fusions in *H. capsulatum*-infected macrophages were reported by Patiño, Hansen & Graybill (1986), who observed *H. capsulatum* yeast cell-wall antigen released into the phagolysosome or associated with the phagolysosome membrane after in-vitro or in-vivo phagocytosis by peritoneal or alveolar mice macrophages. They induced infection with a low yeast:macrophage ratio of 1:4, which probably does not inhibit the PL fusion mechanism. Our results indicate that at high infection ratios the fungus seems to interfere with the PL fusion step of the phagocytic process, and fusion inhibition can be observed (Figs 1 and 2). This fact is further suggested by the decrease in fusion frequency when a high yeast:macrophage ratio was used.

Although the results shown in Figs 3 and 4 suggest differences in PLFF in the two strains, these were not statistically significant, which implies that either a larger macrophage sample would have to be used to detect such differences, or that these are negligible.

For PLF assays some authors (Hart & Young, 1975, 1979; Kielian & Cohn, 1980) have used 6–10-day mouse macrophage cultures because fusions increase after several days of macrophage incubation. For this purpose experiments using a macrophage-cell line were also carried out, which, besides a long term incubation period, also allowed cell sample heterogeneity and fluorescent background reduction. In spite of a short culture period, sample heterogeneity and fluorescent background found in peritoneal macrophages, results obtained with the J774.2 cell line were similar to those obtained in peritoneal macrophages.

It is unquestionable that electron microscopy (EM) with cells labelled by electron opaque markers is the most useful methodology to distinguish specific fusions, although EM sometimes produces alterations in the sample that could interfere with observation. There is discussion concerning the usefulness of the acridine orange technique to test PLF (Mor & Goren, 1987). However, by using adequate controls and taking into account non-specific fusions, this technique could be a good implement for PL fusions studies, and support the validity of our results.

Considering that an increase in the *H. capsulatum* dose induces a decrease in the frequency of PLF in infected macrophages, that PLFF values also decrease as a function of time of phagocytosis and that *Histoplasma* virulence does not modify the PLF frequency, we suggest that inhibition of PL fusions does not appear to be the major *Histoplasma* escape mechanism if a low infective dose of fungus is used.

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REFERENCES

BEAMAN, L.E. & PAPPAGIANIS, D. (1981) Role of lymphocytes in macrophage-induced killing of *Coccidioides immitis in vitro*. *Infect. Immun.* **39,** 347.

BEAMAN, L.E., BENJAMINI, E. & PAPPAGIANIS, D. (1983) Activation of macrophages by lymphokines: enhancement of phagosome-lysosome fusion and killing of *Coccidiodes immitis*. *Infect. Immun.* **39,** 1201.

DAVIS-SCIBIENSKY, C. & BEAMAN, B.L. (1980) Interaction of alveolar macrophages with *Nocardia asteroides*: immunological enhancement of phagocytosis, phagosome-lysosome fusion and microbicidal activity. *Infect. Immun.* **30,** 578.

DUMONT, A. & ROBERT, A. (1970) Electron microscopic study of phagocytosis of *Histoplasma capsulatum* by hamster peritoneal macrophages. *Lab. Invest.* **23,** 278.

FREHEL, C., CHASTELLIER, C., LANG, T. & RASTOGI, N. (1986) Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic *Mycobacterium avium*. *Infect. Immun.* **52,** 252.

FRIIS, R.R. (1972) Interactions of L cells and *Chlamydia psittaci*: entry of the parasite and host response to its development. *J. Bacteriol.* **111,** 706.

GOREN, M.B., HART, P.D., YOUNG, M.R. & AMSTRONG, J.A. (1976) Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis*. *Pathology* **73,** 2510.

HART, P.D. & YOUNG, M.R. (1975) Interference with normal phagosome-lysosome fusion in macrophages, using ingested yeast cells and suramin. *Nature* **256,** 47.

HART, P.D. & YOUNG, M.R. (1979) The effect of inhibitors and enhancers of phagosome-lysosome fusion in cultured macrophages on the phagosome membranes of ingested yeasts. *Exp. Cell Res.* **118,** 365.

HOWARD, D.H. (1973) Fate of *Histoplasma capsulatum* in guinea pig polymorphonuclear leukocytes. *Infect. Immun.* **8,** 412.

HOWARD, D.H. (1975) The role of phagocytic mechanisms in defense against *Histoplasma capsulatum*. In: *Mycosis*, No. 304, pp. 50–59. Pan American Health Organization Scientific Publication, Washington.

HOWARD, D.H. & OTTO, V. (1977) Experiments of lymphocyte-mediated cellular immunity in murine histoplasmosis. *Infect. Immun.* **16,** 226.

JONES, T.C. & HIRSCH, J.G. (1972) The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusions with phagocytic vacuoles containing living parasites. *J. exp. Med.* **136,** 1173.

KIELIAN, M.C. & COHN, Z.A. (1980) Phagosome-lysosome fusion: characterization of intracellular membrane fusion in mouse macrophages. *J. Cell. Biol.* **85,** 754.

MOR, N. & GOREN, M.B. (1987) Discrepancy in assessment of phagosome-lysosome fusion with two lysosomal markers in murine macrophages infected with *Candida albicans*. *Infect. Immun.* **55,** 1663.

PATIÑO, M.M., HANSEN, J.T. & GRAYBILL, J.R. (1986) Immunocytochemical staining of *Histoplasma capsulatum* at the electron microscopic level. *Mycopathologia* **94,** 157.

REED, L.J. & MUENCH, H. (1938) A simple method of estimating fifty percent end points. *Am. J. Hygiene* **27,** 493.

TAYLOR, M.L., REYES-MONTES, M.R., GONZALEZ, G.R., CASASOLA, J. & HERNANDEZ-RAMIREZ, A. (1982) Immune response changes with age and sex as factors variations in resistance to *Histoplasma* infection. In: *Proceedings VIII Congress International Society for Human and Animal Mycology* (ed. M. Baxter) pp. 260–264. Massey University, New Zealand.

TEWARI, R.P., SHARMA, D.K. & MATHUR, A. (1978) Significance of T lymphocytes in immunity elicited by immunization of ribosomes or live yeast cells of *Histoplasma capsulatum*. *J. Infect. Dis.* **138,** 605.

TEWARI, R.P., SHARMA, D.K. & MATHUR, A. (1980) Importance of T lymphocytes in acquired immunity to histoplasmosis. In: *Proceedings VII Congress International Society for Human and Animal Mycology* (eds E. S. Kuttin & G. L. Baum) pp. 93–96. Excerpta Medica, Amsterdam.

WU-HSIEH, B., ZLOTNIK, A. & HOWARD, D.H. (1984) T-cell hibridoma produced lymphokine that activates macrophages to suppress intracellular growth of *Histoplasma capsulatum*. *Infect. Immun.* **43,** 380.