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EFFECTS OF THE NEWER ANTIFUNGAL AGENTS (BIFONAZOLE, ICI 195, 739 AND AMOROLFIN) ON *IN VITRO* PHAGOCYTIC, LYMPHOCYTIC AND NATURAL-KILLER CELL RESPONSES

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Abstract — Amphotericin B and some of the imidazole drugs have been shown to suppress certain neutrophil and lymphocyte functions both *in vitro* and *in vivo*. We present here the *in vitro* effects of: amorolfin, a morpholine derivative; the imidazoles clotrimazole and ketoconazole; the *N*-substituted imidazole bifonazole and a triazole (ICE 195, 739), on neutrophil and lymphocyte function. All of these drugs inhibited neutrophil random migration, chemotaxis and hexose monophosphate shunt activity. The effects of the drugs on neutrophil adherence, deoxyglucose transport and beta-glucuronidase release were variable while lysozyme release was unaffected. Natural Killer cell cytoxicity was depressed by all drugs tested except for amorolfin. Mitogen-induced lymphocyte blastogenesis was suppressed by all the antifungal drugs tested. Similar results were obtained using the mitogens phytohaemagglutinin, concanavalin A and pokeweed mitogen. The mechanism of action of these drugs on these cell functions remains unknown, there may be a correlation between their effects on fungi and their effects on leukocytes. Clearance of systemic fungal infection is heavily dependent on integrity of the cellular immune system and it is clearly undesirable that antifungal drugs have immunosuppressive properties. Further studies are required to determine the *in vivo* and clinical relevance of our observations.

Systemic mycoses are notoriously difficult to treat because of the propensity for these infections to occur in immunocompromised individuals (Terrell & Hermans, 1987). Since recovery from infection generally requires the participation of a reasonably intact immune system, concern has been expressed by some authors on the possible adverse effects on the clinical outcome where antimicrobial agents with immunosuppressive potential are selected for use (Eichenburg, Hahn & Opferkuch, 1982; Targett, 1985; Jeljaszewicz & Pulverer, 1986).

Host resistance to fungal infection is mediated by both phagocytic cells and cell-medicated immunity (Mason & Kirkpatrick, 1983; Ashman & Papadimitriou, 1987). In this regard, there is evidence to suggest that some established antifungal drugs may have immunosuppressive properties. Amphotericin B has been shown to inhibit neutrophil function (Bjorksten, Ray & Quie, 1976; Marmer, Fields, France & Steele, 1981) and suppress lymphocyte transformation (Ferrante, Rowan-Kelly, & Thong, 1979; Walls & Kay, 1982; Alford & Cartwright, 1983). Immunosuppression has been observed in mice (Ferrante *et al.*, 1979; Ferrante & Thong, 1979) and guinea pigs (Roselle & Kauffman, 1980) treated with amphotericin B. Some imidazole derivatives have also been shown to be inhibitory for neutrophils and lymphocytes *in vitro* (Thong & Rowan-Kelly, 1978; Rowan-Kelly, Ferrante & Thong, 1984), and *in vivo* (Grove, Mahmoud & Warren, 1977; Thong & Ferrante, 1979b).

In this report, we investigated the effects of some newer antifungal agents on human neutrophil, lymphocyte and Natural Killer (NK) cell responses. These included bifonazole, a new class of *N*-substituted imidazoles (Barug & Bastiaanse, 1983; Plempel, Regel & Buchel, 1983); ICI 195,739 which is a triazole compound (Van Cutsem, Van Gerven, Van de Ven, Borgers & Janssen, 1984; Richardson, Brammer, Marriott & Troke, 1985); and amorolfin,

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a morpholine derivative (Kerkenaar, Uchiyama & Versluis, 1981; Polak, 1983). We included two older imidazole derivatives, clotrimazole and ketoconazole, for comparison purposes.

EXPERIMENTAL PROCEDURES

Antifungal drugs

Bifonazole was kindly provided by Bayer (Sydney); ICI 195,739 was a gift from Imperial Chemical Industries (Sydney); amorolfin was obtained from Roche (Sydney); ketoconazole was provided by Janssen (Sydney); and clotrimazole was purchased from Sigma (St. Louis).

Amorolfin was dissolved in distilled water; bifonazole, clotrimazole and ketoconazole in absolute ethanol (ETOH); and ICI 195,739 in dimethyl-sulfoxide (DMSO). Each drug was further diluted in either Dulbecco's phosphate buffered saline (DPBS) or medium-199 for neutrophil experiments, or RPMI-1640 medium for lymphocyte experiments. Appropriate controls containing the maximum concentration of the relevant solvents (0.6% ETOH or 0.4% DMSO) were included in the experiments.

These drugs were studied at 10 μg/ml concentrations because their *in vitro* activity against susceptible fungi are generally in the concentration range of 10 μg/ml, and because 10 μg/ml is a readily achievable blood level during therapy (Barug & Bastiaanse, 1983; Plempel *et al.*, 1983; Van Cutsem *et al.*, 1984; Richardson *et al.*, 1985; Kerkenaar *et al.*, 1981; Polak, 1983).

Isolation of lymphocytes and neutrophils

About 30 – 50 ml of blood was obtained from each healthy donor and layered into Mon-Poly Resolving Medium (Flow Laboratories, Virginia). Centrifugation at 1000 **g** for 30 min resulted in the formation of two leukocyte bands at the interface. Mononuclear cells from the top band were washed twice and resuspended in RPMI-1640 for the experiments. The second band containing neutrophils of >97% purity (Ferrante & Thong, 1980) was removed, washed twice and resuspended in either DPBS or medium-199 for the experiments.

Neutrophil adherence

The dacron fibre microcolumn assay for neutrophil adherence was performed as previously described (Thong & Currell, 1983). Briefly, dacron fibre microcolumns were prepared by carefully weighing out 10 mg lots of teased dacron fibre (Olympic Products, Queensland, Australia). These were placed into 100 μl disposable pipette tips (Stockwell Scientific, Monterey Park, California) so as to occupy the centre 2 cm portion of the 5 cm pipette tip. Neutrophil suspensions, pre-incubated for 15 min with antifungal drugs were adjusted to concentrations of between $4 - 6 \times 10^6$ cells/ml, and 100 μl was delivered into each dacron fibre microcolumn. After incubation for 10 min at 37°C and high humidity in order to allow for contact between cells and fibre, the microcolumns were placed in a specially designed apparatus, and the fluid extracted by a vacuum suction pressure of 2.5×10^4 Pa applied for 1 min into disposable test-tubes. The concentrations of cells were determined in Neubauer Haemocytometers and the results calculated as follows:

$$\text{percent adherence} = 100 - (CE/CO \times 100)$$

where CE is the neutrophil concentration in the effluent and CO is the neutrophil concentration in the original suspension.

All experiments were performed in triplicate.

Neutrophil locomotion

The movement of neutrophils under agarose was performed as previously described (Ferrante, Rowan-Kelly, Seow & Thong, 1986). Briefly, 3 ml of 2 × N medium-199 containing 10% heat-inactivated fetal calf serum were mixed with 3 ml of 2% agarose solution and poured into 60 × 15 mm tissue culture plates. Wells of 2 mm diameter and 2.5 mm apart were punched in sets of three. The middle well received 5 μl neutrophil suspension at a concentration of 4×10^7/ml, the outer well received a chemoattractant, and the inner well received medium-199 only. The chemoattractant was made by incubating 1×10^7 *Candida albicans* with 2 ml fresh human serum for 30 min at 37°C, centrifuged at 1,000 **g** for 10 min, and the activated serum removed for use.

The agarose plates were kept in a 5% CO_2/air atmosphere and high humidity for 2 h, and the distance moved towards the inner well (random movement) and the outer well (chemotaxis) measured with the aid of an eye-piece grid in an inverted microscope. Results are expressed as mean mm/2h ± S.E.M. Neutrophils were incubated with a final concentration of 10 μg/ml of antifungal agent for 15 min prior to the experiments. Also, antifungal

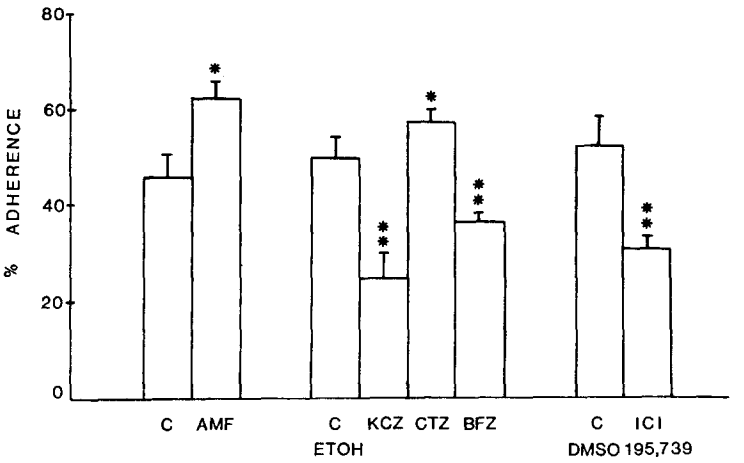

Fig. 1. Effect of five antifungal agents on neutrophil adherence. The results represent mean ± S.E.M. of three separate experiments, each performed in triplicate, (AMF, amorolfin; KCZ, ketoconazole; CTZ, Clotrimazole; BFZ, Bifonazole). Results are compared with the relevant controls (C, median; ETOH, ethanol diluent; DMSO, dimethylsulfoxide diluent). $*P<0.05$; $**P<0.01$.

agents at the same concentration were incorporated in the agarose plates to provide a uniform concentration of drug.

Deoxyglucose uptake by neutrophils

We used a newly developed ^{3}H-deoxyglucose uptake microassay for these experiments (Seow, McCormack, Smith & Thong, 1987). Approximately 1×10^6 cells in 0.1 ml of DPBS were delivered to each well of a round-bottom microtitre plate. Another 0.05 ml of DPBS containing an antifungal drug was added to test wells. Control wells received DPBS without antifungal drug. A further 0.05 ml of DPBS containing 2-D-[H^3] deoxyglucose (Amersham International, Amersham, U.K.) was added to each well to give a final concentration of 0.78 μCi/ml. The microtitre plate was incubated at 37°C in a humidified air atmosphere for 30 min, centrifuged at 4°C and 800 **g** for 5 min, and 0.05 ml of supernatant removed for determination of radioactivity in a liquid-scintillation counter. The uptake of ^{3}H-deoxyglucose is calculated from this formula:

^{3}H-deoxglucose uptake = total disintegrations/min added − in supernatant

All experiments were performed in triplicate.

Respiratory burst

Stimulated neutrophils metabolize glucose via the hexose-monophosphate (HMP) shunt to generate oxygen-derived free radicals. HMP shunt activity was quantitated by the conversion of ^{14}C-1-glucose to $^{14}CO_2$ (Thong & Rencis, 1980). Briefly, 2×10^6 neutrophils were treated with 0.1 μg/ml of PMA in a total volume of 1 ml at 37°C for 45 min. The 1M hydrochloric acid was added to drive out any dissolved $^{14}CO_2$ which was captured in 5M sodium hydroxide and quantitated in a scintillation spectrophotometer. Results were expressed as disintegrations/minute (dpm) ± S.E.M.

Degranulation

Two marker enzymes were used to measure neutrophil degranulation: lyzozyme, which is present in both azurophil and specific granules, and β-glucuronidase stored in azurophil granules only.

Briefly, 1×10^7 neutrophils were incubated with PMA at the final concentration of 0.1 μg/ml. The cells were centrifuged at 1000 **g** for 120 min, and the supernatant removed for assay of lyzozyme and β-glucuronidase.

Lyzozyme content was measured by the rate of lysis of *Micrococcus lysodeikticus* (West, Rosenthal, Gelb & Kimball, 1984; Gallin, Fletcher, Seligmann, Hoffstein, Cehrs & Mounessa, 1982). To each 0.8 ml of 2 mg/ml of *M. lysodeikticus* in a spectrophotometer cuvette, 0.1 ml of supernatant was added. The contents were mixed and the rate of change in absorbance at 450 nm followed immediately. β-glucuronidase content was determined by spectrophotometry using phenopthalein glucuronic acid as substrate (Gallin *et al.*, 1982).

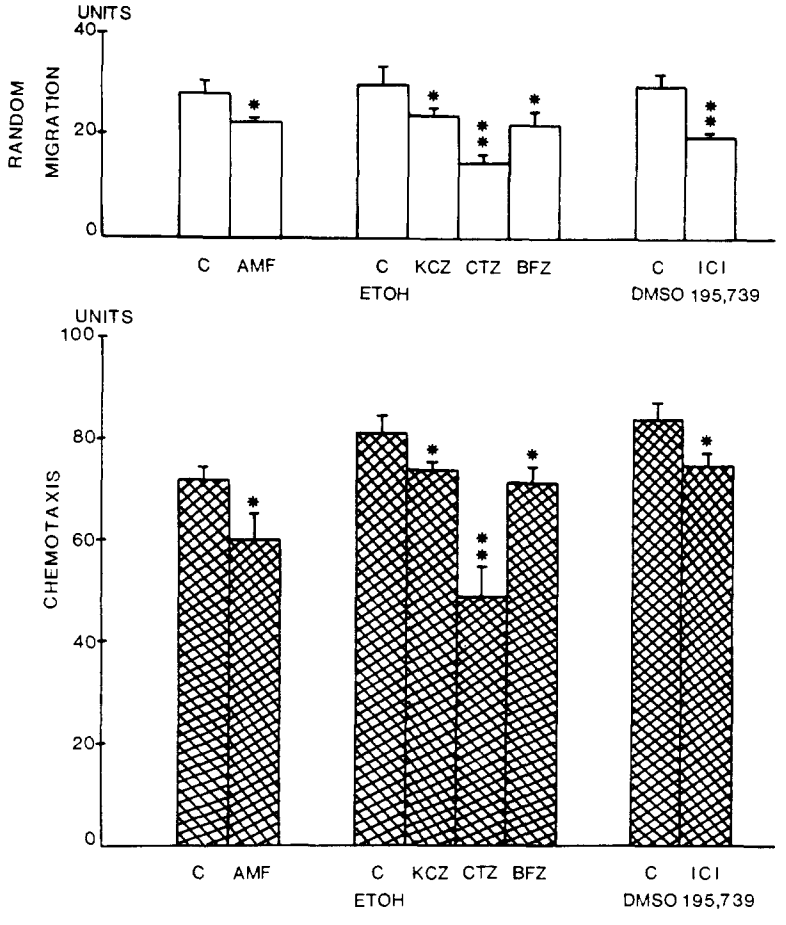

Fig. 2. Effect of five antifungal agents on neutrophil locomotion. Results are expressed as mean ± S.E.M. of five separate experiments, each performed in triplicate (NB: 1 unit of distance = 0.02 mm). Results are compared against the relevant controls containing the appropriate diluents. $*P<0.05$; $**P<0.001$.

Experiments were performed in triplicate and results expressed as percent release of lysosomal enzyme (mean ± S.E.M.). Neutrophils were incubated with varying concentrations of antifungal drugs for 15 min prior to experiments.

NK-cell cytotoxicity

The K562 human erythroid myeloid cell line was used for the assay of NK-cell activity as previously described (Seow, Ferrante, Goh, Chalmers & Thong, 1988). Briefly, the K562 cells were washed and resuspended in RPMI-1640 medium at a concentration of 5×10^6 cells/ml, labelled with 100 μCi of $Na_2{}^{51}CrO_4$ at 37°C for 1 h, washed three times and adjusted to a concentration of 1×10^5 cells/ml. To each round bottom well of a microtitre plate (Flow Laboratories, Virginia) was delivered: 0.1 ml of ^{51}Cr-labelled target cells; 0.05 ml of lymphocytes (1×10^7 cells/ml), effector:target ratio of 50:1) and 0.05 ml of antifungal drug. Control wells contained ETOH or DMSO diluent, or medium only. The microtitre plates were incubated at 37°C in 5% CO_2/air and high humidity for 4 h, centrifuged at 800 g and the supernatants removed for quantitation of ^{51}Cr-release in a gamma counter. NK-cell cytotoxicity was expressed as percent ^{51}Cr-release as follows:

$$\%^{51}\text{Cr-release} = \left[\frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right] \times 100$$

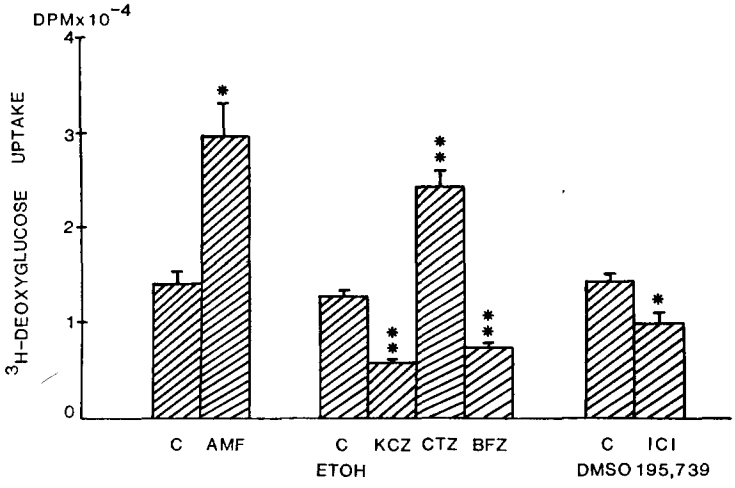

Fig. 3. Effect of five antifungal agents on deoxyglucose uptake by neutrophils. Results represent mean ± S.E.M. of four separate experiments, each performed in triplicate. Results are compared against relevant controls containing the appropriate concentrations of diluent. *P<0.01; **P<0.001.

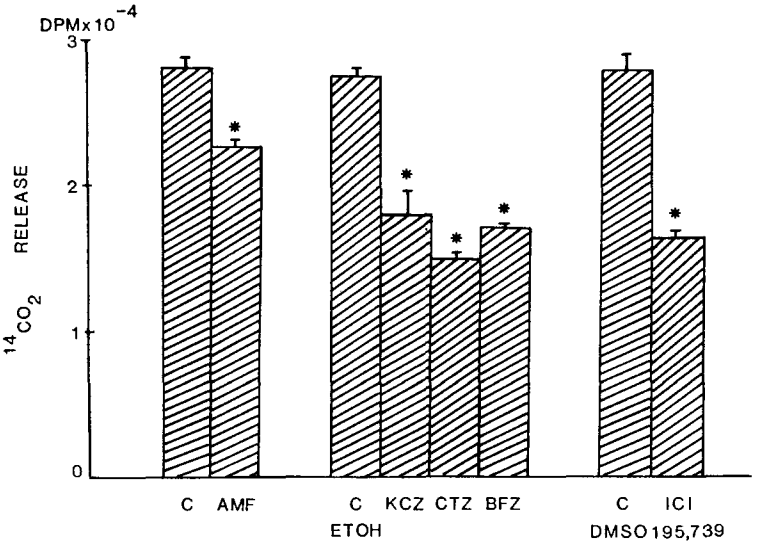

Fig. 4. Effect of five antifungal agents on the respiratory burst of neutrophils, as measured by release of $^{14}CO_2$ via the HMP-shunt. Results are shown as mean ± S.E.M. of five separate experiments, each performed in triplicate. Statistical comparisons are made against relevant controls containing the appropriate diluent. *P<0.001.

All experiments were performed in triplicate.

Mitogen-induced lymphocyte proliferation

The lectins phytohaemaggutinin A (PHA), pokeweed mitogen (PWM) and Concanavalin A (Con A) were purchased from Flow Laboratories, Sydney. These were used at optimal concentrations of PHA (0.02 μg/ml), PWM (0.312 μg/ml), and Con A (0.625 μg/ml). To each microtitre well was delivered 0.1 ml RPMI-1640 medium containing 2 × 10^6/ml lymphocytes, 25 μl of lectin, and 0.1 ml of antifungal agent or medium only, as previously described (Thong & Ferrante, 1979a).

The microtitre trays were kept at 37°C in a 5% CO_2/air atmosphere and high humidity for 72 h. Six hours prior to harvesting 1 μCi of ^{3}H-thymidine was added to each culture. The cultures were aspirated onto glass-fibre filter paper by a Skatron harvester,

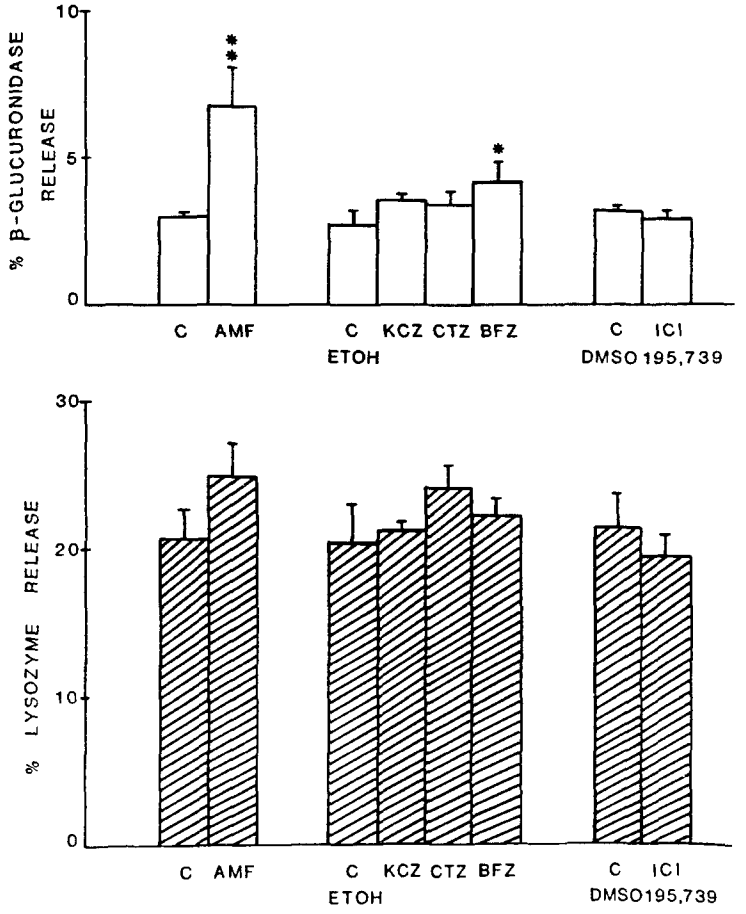

Fig. 5. Effect of five antifungal agents on neutrophil degranulation. Results are depicted as mean ± S.E.M. of three experiments, each performed in triplicate. Statistical comparisons are made against relevant controls containing the appropriate diluent. $*P<0.05$; $**P<0.01$.

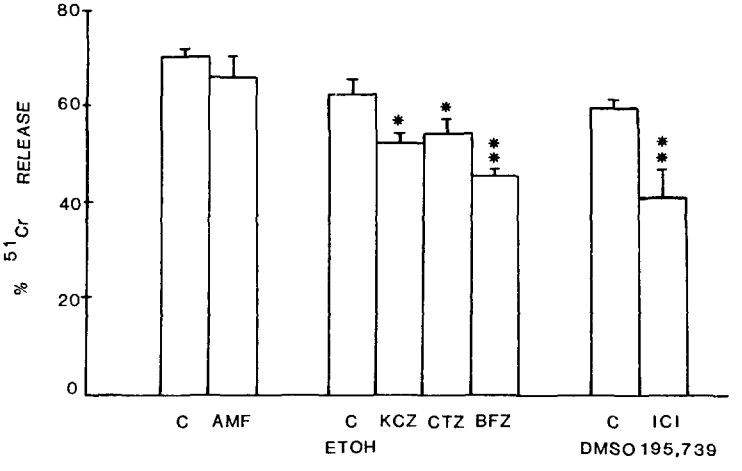

Fig. 6. Effect of five antifungal agents on NK-cell cytotoxicity. Results are shown as mean ± S.E.M. of three separate experiments, each performed in triplicate. Statistical comparisons are made against relevant controls containing the appropriate diluent. $*P<0.05$; $**P<0.01$.

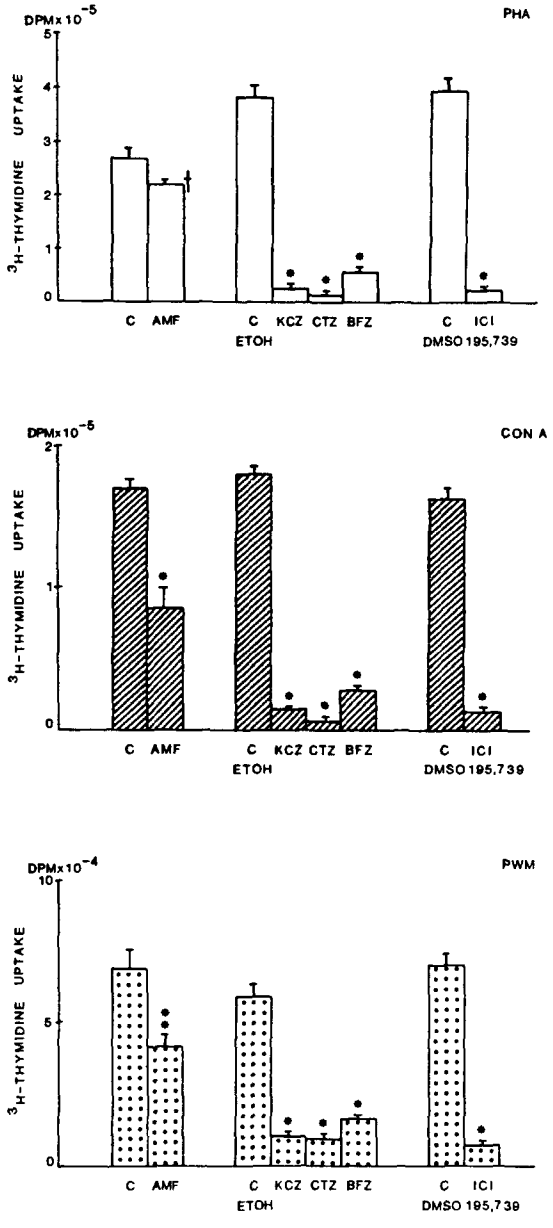

Fig. 7. Effect of five antifungal agents on mitogen-induced lymphocyte transformation. Results are expressed as mean ± S.D. of triplicate samples. Similar results were obtained in four other experiments. Statistical comparisons are made against relevant controls containing the appropriate diluents. $^{*}P<0.001$; $^{**}P<0.01$; $^{\dagger}P<0.05$.

and ^{3}H-thymidine uptake determined by liquid scintillation spectroscopy. All experiments were performed in triplicate.

Statistical analysis

The Student's *t* test was used for statistical analysis.

RESULTS

Neutrophil adherence

We first studied the effects of the five antifungal agents on neutrophil adherence, an early behavioural change during activation. As shown in Fig. 1, significant inhibition of neutrophil adherence was

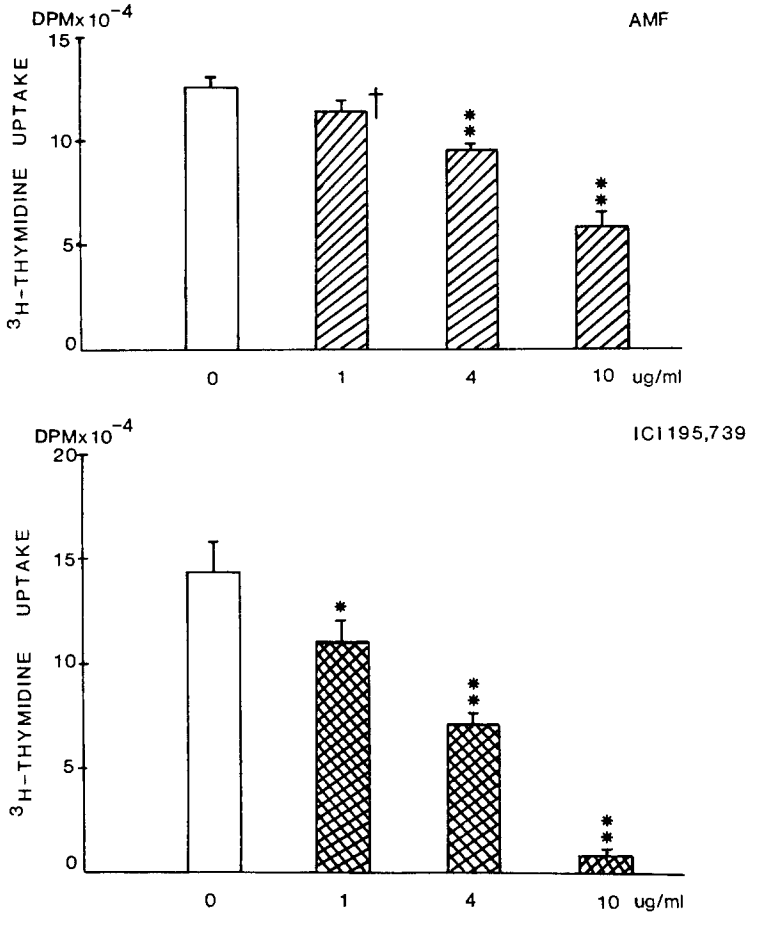

Fig. 8. Dose-dependent inhibition of PWM-induced lymphocyte transformation by amorolfin and ICI 195, 739. Results are shown as mean ± S.D. of triplicate samples. Similar results were obtained in two other experiments. *$P<0.01$; **$P<0.001$; †$P<0.05$.

observed in the presence of 10 μg/ml of ketoconazole, bifonazole and ICI 195,739. By contrast clotrimazole and amorolfin significantly enhanced neutrophil adherence.

Neutrophil locomotion

In the next set of experiments, we examined the effects of the antifungal agents on neutrophil locomotion, i.e. the mechanism by which these cells reached sites of infection. All five antifungal agents used in this study caused significant inhibition of neutrophil random movement and chemotaxis at 10 μg/ml concentration (Fig. 2).

Deoxyglucose uptake

We next studied the effects of the five antifungal agents on deoxyglucose uptake, an important indicator of metabolic activity. Significant inhibition of deoxyglucose uptake was seen with 10 μg/ml of ketoconazole bifonazole and ICI 195,739 (Fig. 3). By contrast, clotrimazole and amorolfin induced an increase in uptake.

Neutrophil respiratory burst

The effect of the five antifungal agents on the respiratory burst which is responsible for the production of oxygen-derived free radicals, was investigated. The results (Fig. 4) show that all five antifungal drugs caused significant inhibition of HMP-shunt activity at 10 μg/ml concentrations.

Neutrophil degranulation

We further studied the effect of the five antifungal agents on degranulation. None of the antifungal agents caused significant release of enzyme from

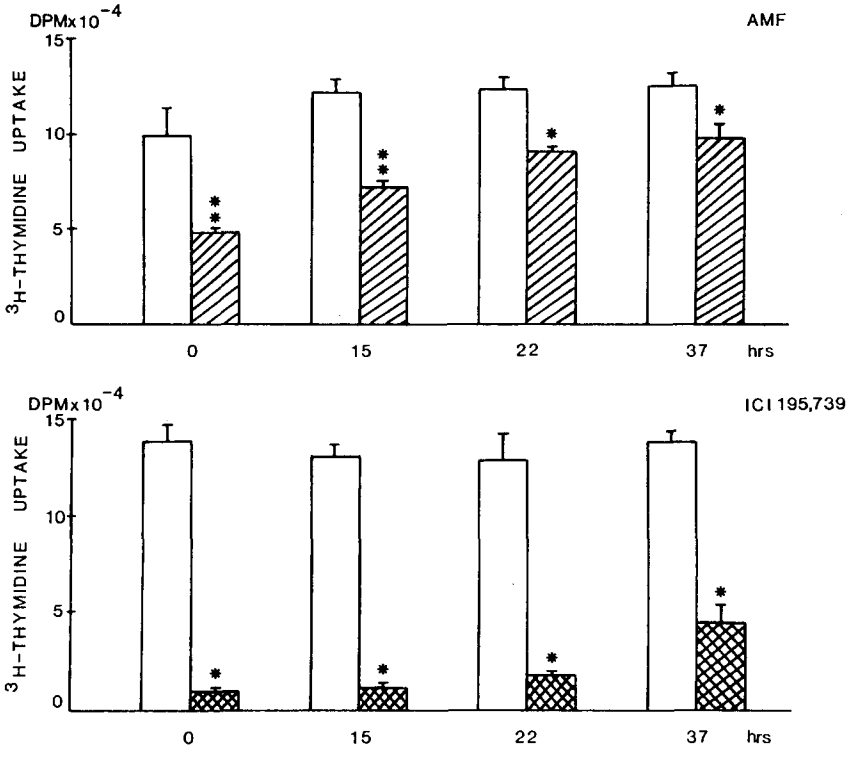

Fig. 9. Effect of delayed addition of amorolfin and ICI 195,739 on PWM-induced lymphocyte transformation. The antifungal agents were added at 0, 15, 22 and 37 h after the start of cultures. Results represent mean ± S.D. of triplicate cultures. Similar results were obtained in two other experiments. *$P<0.001$; **$P<0.01$.

specific granules (Fig. 5). However, significant release of azurophil granules was observed in the presence of bifonazole and amorolfin.

Natural Killer cytotoxicity

In other experiments, we investigated the effects of the five antifungal agents on NK-cell cytotoxicity. Significant inhibition of NK-cell cytotoxicity was observed in the presence of 10 μg/ml of ketoconazole, clotrimazole, bifonazole and ICI 195,739. No inhibition was shown by amorolfin (Fig. 6).

Mitogen-induced lymphocyte transformation

Finally, we examined the effects of the five antifungal drugs on lymphocyte transformation. All five antifungal drugs demonstrated the ability to significantly suppress lymphocyte transformation induced by all three mitogens of a concentration of 10 μg/ml (Fig. 7).

The two experimental antifungal drugs amorolfin and ICI 195,739 were further studied to determine the kinetics of this inhibitory activity. Their inhibitory effects on PWM-induced lymphocyte transformation were shown to be dose-dependent in the range of 1 – 10 μg/ml (Fig. 8).

Using the concentration of 10 μg/ml, it was further shown that their inhibitory effects remained significant even when addition of the drugs was delayed for up to 37 h after the initiation of cultures (Fig. 9).

In order to determine whether amorolfin and ICI 195,739 bind tightly and universally to cell structures, we performed washing experiments where the drugs are incubated with the cells for 60 min, then washed twice prior to culture with PWM. The results (Fig. 10) show that the inhibitory effects of these two drugs can be reversed by washing.

DISCUSSION

There has been much enthusiasm in recent years for the design and development of better and safer antifungal agents for the therapy of systemic mycoses. Some of the most promising classes of these novel compounds include the *N*-substituted

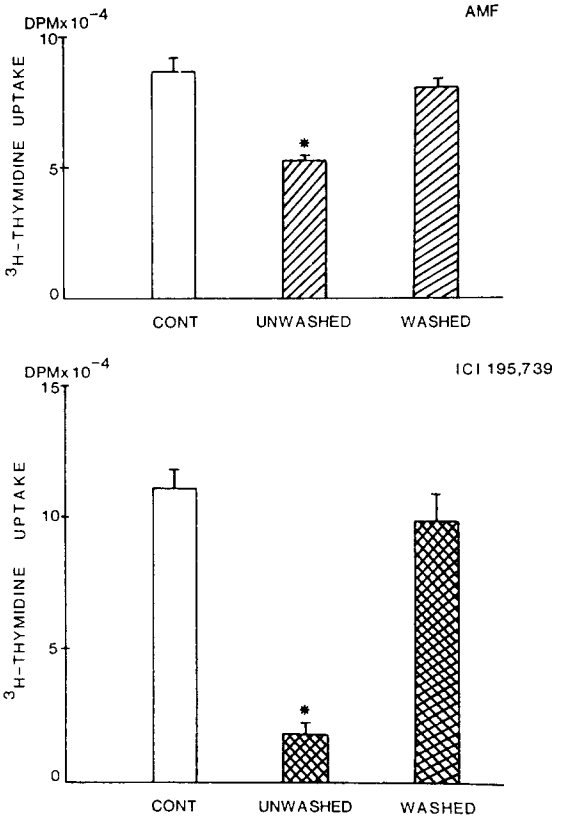

Fig. 10. Reversibility of inhibition of PWM-induced lymphocyte transformation by amorolfin and ICI 195, 739. Results represent mean ± S.D. of triplicate cultures. Similar results were obtained in two other experiments. *$P<0.001$.

imidazoles (e.g. bifonazole) which have broad spectrum activity against dermatophytes and yeasts (Plempel *et al.*, 1983; Barug & Bastiaanse, 1983); the triazoles (e.g. ICI 195,739) which have potent activity against *Candida albicans* (Van Cutsem *et al.*, 1984; Richardson *et al.*, 1985); and the phenylpropylpiperidine and morpholine derivatives (e.g. amorolfin) with activity against a wide range of fungi (Kerkenaar *et al.*, 1981; Polak, 1983). The present study shows that representatives of each of these groups of drugs can have adverse effects on neutrophil, lymphocyte and NK-cell responses *in vitro*.

Of relevance to this discussion is the finding that amorolfin has the same ability as amphotericin-B to enhance adherence and activate other neutrophil functions (Marmer *et al.*, 1981), and like amphotericin-B, may have the potential for causing neutrophil aggregation and the clinical complications of pyrexia and chills (Terrell & Hermans, 1987). The capacity to activate neutrophils is also shown by clotrimazole, but this is of less concern as this older imidazole is only used topically.

The mechanism(s) by which antifungal drugs interfere with the function of immune cells is unknown, but may be related to their action on fungal cells. In the case of the imidazoles, inhibition of ergosterol biosynthesis results in damage to the fungal cell membrane (Van den Bossche, Willensens, Cools, Lauwers, Lejeune, 1978), and similar damage to mammalian cell membranes may lead to the deleterious effects on neutrophil, lymphocyte and NK-cell responses shown by this and other studies (Tarnvik & Ansehn, 1974; Rowan-Kelly *et al.*, 1979; Marmer *et al.*, 1981; Alford & Cartwright, 1983; Buttke & Chapman, 1983; Gergely, Nekam, Lang, Kalmar, Gonzales-Cabello & Perl, 1984). The results of the present study suggest that the newly developed *N*-substituted imidazoles such as bifonazole may not be devoid of immunosuppressive properties.

The triazole antifungals such as ICI 195,739 appear to have a similar mode of action as the imidazole compounds. Data from this study demonstrate a similar propensity to suppress a broad range of neutrophil functions, inhibition of NK-cell

cytotoxicity and mitogen-induced lymphocyte transformation. Additional experiments revealed that these effects were dose-dependent; were reversible after short-term incubation suggesting that it does not bind tightly to cell constituents and that its inhibitory action was still significant when added to cultures as late as 37 h after their initiation.

Similar results were obtained with amorolfin, a morpholine antifungal agent. Its antifungal mode of action is still unclear, but may be related to its morpholine structure which it shares with the anthracycline anti-tumour drugs (Gianni, Corden & Myers, 1983). As such, it may act by inhibition of DNA or RNA synthesis, and may account for the somewhat delayed antimycotic action shown previously (Vuddhakul, McCormack, Seow, Smith & Thong, 1988), compared with the more immediate effects of polyenes and azoles which act by disrupting the integrity of the fungal cell membrane.

The clinical relevance of these and other *in vitro* findings of immuno-suppression by antifungal agents remains unclear. Certainly, additional immunosuppression imposed on already compromised patients who tend to be the ones who contract systemic mycotic infections is logically undesirable. The situation is further compounded by the possibility of immunosuppression by the fungal infection itself (Segal, Sandovsky-Losica & Vardinon, 1980). Well-designed *in vivo* and clinical studies may help to unravel this important question.

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