An In Vitro Human Skin Model for Assaying Topical Drugs against Dermatophytic Fungi

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A shortened modification of Dittmar's in vitro human skin assay reliably predicts the efficacy of topical antifungals against ringworm fungi. Cadaver skin was treated with the test agents for 4 or 24 h. After heat separation of the epidermis for one minute at 60°C, the undersurface was point inoculated with a spore suspension of *T. mentagrophytes*. In untreated skin fluffy, 3 mm colonies develop after 4 to 5 days of incubation. Effective fungistats produce complete inhibition of growth. Commercial formulations of the imidazoles, tolnaftate and ciclopirox were highly effective. Haloprogin, undecylenate, and hydroxyquinoline in proprietary form were inactive. There was a high correspondence between in vitro and in vivo effectiveness. The method should be useful for screening new antifungals and for optimal selection of vehicles. *Key words: Antifungal; Ringworm; T. mentagrophytes.* (Received June 30, 1986.)

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Hundreds of chemicals are capable of inhibiting the growth of ringworm fungi in culture. Only a handful are in use for treating ringworm infections. Most are disqualified because they fail to achieve an inhibitory concentration at the base of the stratum corneum.

Showing that a substance inhibits dermatophytes in culture is a necessary, but only first step. Efficacy must be demonstrated in vivo. Topical treatment of guinea pig infections is often used to learn whether continued interest is warranted. However, the rapid resolution of the highly inflammatory infection is a serious limitation. This model does not mimic chronic ringworm infection. Another approach is to create human experimental infections. For example, Wallace et al. compared antifungal agents in volunteers infected with *T. mentagrophytes* (1). In this model too the infection is short lived. Besides, human infections are largely unfeasible and objectionable for many reasons. Since dermatophytes are usually confined to the horny layer, one should be able to avoid the use of living animals by growing ringworm fungi on dead skin. Attempts of this kind have been made.

Stoughton's method depends upon the capacity of the antifungal compound to attain an inhibitory level in the dermis (2). Surgical specimens from breast or leg amputations were treated for 24 hours by applying the test agents to the epidermal side. After heat separation of the epidermis, 6 mm punches of dermis were excised and placed surface side down on Miami Mycosel Medium inoculated with spores of *T. mentagrophytes*. A clear zone around the disc predicts clinical efficacy. A negative result, however, is uninterpretable. An effective compound could remain bound to the dermal disc or fail to diffuse outward because of water insolubility.

Knight devised a very swift and simple test (3). He applied the test agents to the upper arm for 15 min. Monolayers of corneocytes were then removed onto translucent adhesive tape and inoculated with *T. mentagrophytes* spores. Inhibition was estimated on a 0–3

scale at the end of 7 days. It is doubtful that the results are applicable since only the outer desquamating portion of the horny layer is sampled.

Dittmar's method overcomes these objections and comes close to mimicking in vivo realities (4, 5). He applied the test agents to pieces of excised human skin and inoculated the undersurface of the epidermis with spores of *T. mentagrophytes*. Absence of growth showed that a fungistatic concentration had been established on the internal side of the horny layer barrier. Dittmar's specifications are complicated. In our hands, growth is variable.

The object of our study was to simplify and shorten Dittmar's method, while securing greater reproducibility.

MATERIAL AND METHODS

Preparation of skin

Abdominal cadaver skin was wiped with 70% ethanol, cut into 4×5 cm sections, wrapped individually in plastic film and stored at −20°C.

After thawing, excess fat was trimmed away, and the sections cleansed with gauze pads wetted with 0.1% Triton X-100, followed by rinsing with sterile water and a final defatting with hexane. The skin was placed, surface side up, in a plastic Petri dish lined with water-moistened filter paper.

Applications of test agents

From a tuberculin syringe 50 µl was dispensed and spread evenly over the surface with a glass rod. A control sample was left untreated. The specimens were kept in the Petri dish moist chambers at room temperature for either 4 or 24 h.

Preparation of epidermal sheet

The test agent was removed by thorough cleansing with 0.1% Triton X-100, a water rinse and a final rinse in hexane. Three 1×1 cm squares were cut out and immersed in water at 60°C for one minute. The epidermis was then gently peeled off with a spatula and placed surface side down on 2% non-nutrient agar with 0.005% chloramphenicol to inhibit bacteria. Three triplicate treated pieces and one control were placed in each Petri dish.

Inoculation and incubation

Cultures of *T. mentagrophytes* were grown on Saboraud-dextrose agar and incubated for 2 weeks at room temperature. The surface was then flooded with 0.01% Tween-80, scraped with a Teflon spatula and the suspension filtered through several layers of gauze. After centrifugation, the pellet was resuspended in sterile distilled water at a density of 25–50 spores/ml, by haemocytometer count (verified for viability by quantitative plating). The suspension was stored at −50°C. A new suspension was prepared each month; we found no loss of viability after several months of storage.

One µl of the suspension was inoculated by micropipette onto the undersurface of each square at 5 equidistant sites. The plates were incubated at room temperature for either 4 or 5 days, based upon the attainment of 3 mm fluffy colonies on untreated skin.

Reading

Inhibition was graded as follows: 0=complete inhibition, 1=strong inhibition, barely perceptible colonies, 2=moderate inhibition, colonies 1 to 2 mm, 3=no inhibition, similar to control.

Suction blisters in human volunteers

One cm circular blisters were raised up on living volar forarm skin using a suction apparatus at 2 atmospheres of negative pressure. These are analogous to heat separated epidermis with the level of detachment at the dermo-epidermal junction. The undersurface was inoculated as above.

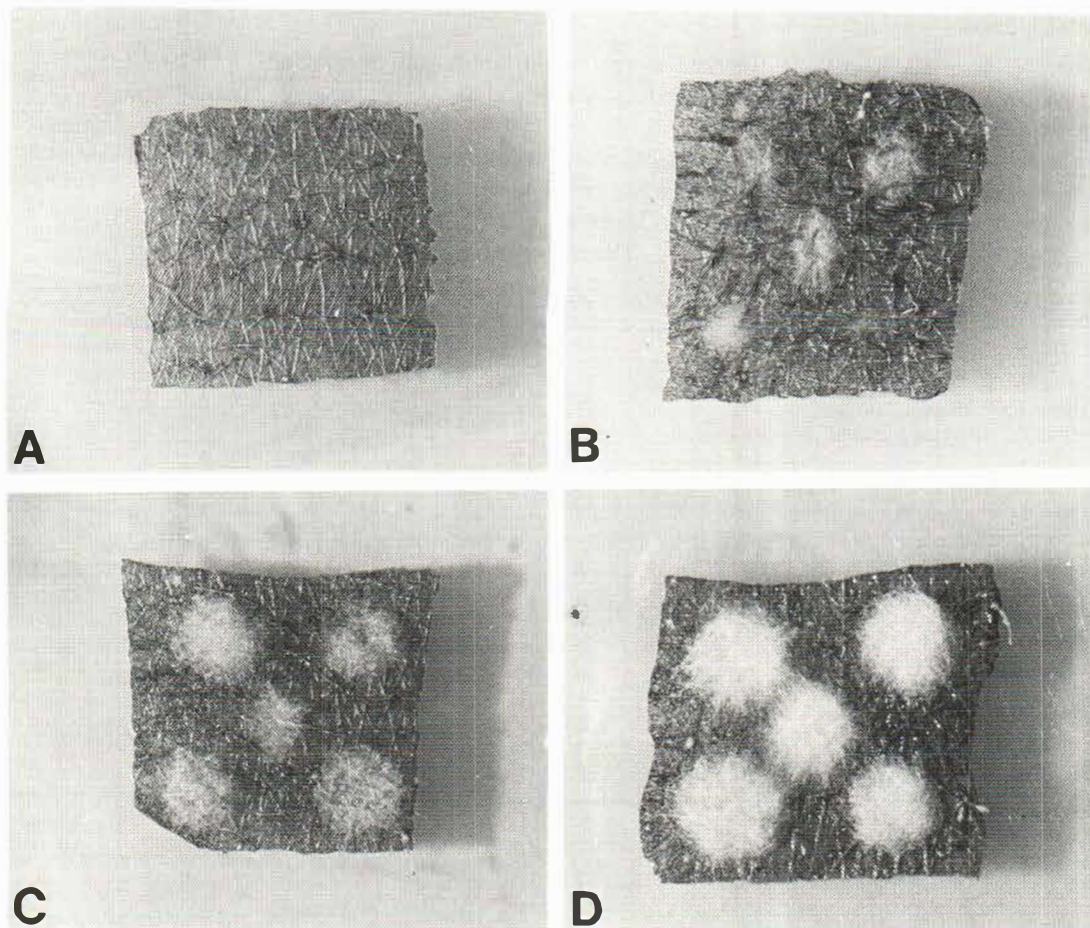

Fig. 1. Grading system. (A) complete inhibition. No growth at any of 5 inoculation points. (B) Moderate inhibition, grade 1. (C) Slight inhibition, grade II, (D) no inhibition, indistinguishable from control.

RESULTS

Pure antifungal chemicals were assayed in concentrations ranging from 0.001% to 2% in ethanol for 24 hour exposures. Wide differences in potency came to light (Table I). Five agents were completely inhibitory down to 0.1%: tolnaftate, sodium pyridinethione, chlortrimazole, ketoconazole and econazole. However, miconazole, also an imidazole, was incompletely suppressive at 2.0%. Ciclopirox was no better than miconazole.

Table II depicts the results with proprietary antifungals, for 4 and 24 hour exposure periods. Only Loprox (Hoechst) and Spectazole (Ortho) were active at 4 h. Vioform (Ciba) Halotex (Westwood) and Whitfields Ointment were completely inactive.

Simultaneous comparison was made on suction blisters and cadaver skin with Tinactin, Lotrimin, Micatin and Spectazole, for 24 h exposures. Agreement was complete, the degree of inhibition for each agent corresponding to that shown in Table II.

For these same test materials, we showed that inhibition was not altered by overlaying the inoculated skin with serum or sebum.

DISCUSSION

The arrival of the imidazoles marked a new era in antifungal therapeutics. The old standbys, Whitfield's Ointment, the undecylenates and the iodinated and chlorinated quinolines had to give way.

The results we obtained with a modification of Dittmar's technique conform to clinical experience. We found Whitfield's Ointment, undecylenate and haloprogin to be ineffective. Vioform showed marginal activity. These have virtually been crowded out of the market place because of limited efficacy. There is a curious, but ill-advised, rebirth of interest in the undecylenates which became popular almost 50 years ago. Starting with Smith et al.'s "new look at undecylenic acid" in 1977 (6) two reports have been published

Table I. *Antifungal chemicals*
Ethanolic solutions for 24 hour exposures

Surface conc. ($\mu g/cm^2$)	2 % 50	1 % 25	0.5 % 12.5	0.2 % 5	0.1 % 2.5	0.01 % 1.0	0.001 % 0.1
Sodium pyridinethione	0	0	0	0	0	1	3
Ketoconazole	0	0	0	0	0	1	2
Clotrimazole	0	0	0	0	0	2	3
Tolnaftate	0	0	0	0	0	2	3
Econazole	0	0	0	0	0	1	2
Sulconazole nitrate	0	0	1	1	1	2	3
Ciclopirox olamine	0	1	2	3	3	–	–
Miconazole nitrate	1	1	1	2	2	3	3
Thiabendazole	2	2	2	2	3	–	–

Table II. *Proprietary antifungal agents*

	Surface conc. ($\mu g/cm^2$)	Time 4 h	Time 24 h
Loprox[1] (1 % ciclopirox olamine)	25	0	0
Spectazole[2] (1 % econazole nitrate)	25	0	0
Tinactin[3] (1 % tolnaftate)	25	2	1
Lotrimin[3] (1 % clotrimazole)	25	2	1
Monistat-Derm[2] (2 % miconazole nitrate)	50	2	2
Vioform[4] (1 % iodochlorhydroxyquin)	25	3	2
Halotex[5] (1 % haloprogin)	25	3	3
Akrinol[3] (0.2 % acrisorcin)	5	3	3
Whitfield's Ointment[6] (6 % benzoic acid)	150	3	3
Hydrophilic ointment U.S.P.	–	3	3

Loprox[1] (Hoechst AG).
Spectazole[2] (Ortho).
Tinactin[3] (Schering Corporation).
Lotrimin Cream[3] (Schering).
Monistat-Derm[2] (Ortho Pharmaceutical Corp.).
Vioform[4] (CIBA Pharmaceutical Inc.).
Halotex Cream[5] (Westwood Pharmaceutical Inc.).
Akrinol Cream[3] (Schering Corporation).
Whitfield's Ointment[6] (Eli Lilly & Co.).

comparing Desenex Ointment (zinc undecylenate and undecylenic acid) to 1% Tinactin Cream (tolnaftate) (7, 8). Therapeutic equivalence was found. This is at variance with our findings. Tinactin cream is a highly effective topical antifungal while Desenex has marginal clinical value. The FDA Dermatology Panel III has expressed an opinion which is shared by most dermatologists. "The panel feels that this product (Desenex) is at best weakly fungistatic." The newest introductions Loprox (ciclopirox) and Spectazole (econazole) are probably more effective than Micatin (miconazole) and Lotrimin (chlortrimazole). The first two were the only ones which gave complete inhibition at 4 h, indicating rapid diffusion into the horny layer. Likewise, they were alone in completely suppressing growth after a 24 h exposure.

The importance of the vehicle was evident in the comparison of the same agent in ethanol and in its proprietary cream form. At a 0.2% concentration in ethanol ciclopirox gave no inhibition while chlortrimazole was partially suppressive down to 0.01%. In ethanol miconazole was not impressive and fared only moderately well in its 2% commercial form. It was barely suppressive (grade 2) at 24 h. Tolnaftate, on the other hand, registered strongly in ethanol and likewise in its 1% cream base.

The superiority of ethanol probably relates to its rapid evaporation, leaving a high concentration in the surface. We found that dipping the heat separated epidermis in ethanol for up to 2 h had no effect on permeability.

For screening new compounds a range of concentrations in ethanol (or other volatile solvents such as acetone) will almost certainly identify effective fungistats. Complete inactivity probably justifies elimination from further study. Proper formulation is decisive. An inappropriate vehicle may weaken or nullify efficacy. Selecting the optimal vehicle by clinical trials is a formidable undertaking. The cadaver skin model enables swift assessment of many formulations.

We have evaluated the methods described by Knight and by Stoughton and abandoned them after encountering sharp discrepancies. As an example, Knight found complete inhibition with Vioform and Haloprogin and no inhibition with 1% Tinactin. Res ipsa loquitur!

Similarly, in Stoughton's dermal disc technique, neither 1% tolnaftate in ethanol nor 1% Tinactin cream showed any inhibition. We found 2% Micatin cream completely inactive and Lotrimin only marginally effective.

We judge that our model reliably predicts clinical efficacy; no serious discrepancies with clinical experience have turned up. Actually, the procedure is an in vivo–in vitro blend which eliminates the numerous variables of clinical trials. Much time and money can be saved by determining the most efficacious vehicle and concentration before embarking on onerous clinical trials. As an example of optimization, we found 2.0% ethanolic thiabendazole only weakly fungistatic. However, a 5.0% concentration in a polyethylene glycol base was as effective as any of the proprietary drugs. Battistine et al. have demonstrated that thiabendazole, when properly formulated in sufficient concentration (10%) is highly effective in various tineas (9). Our unpublished studies are in agreement with this conclusion.

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