Rev Iberoam Micol 2001; 18: 147-149

Usefulness of optical brighteners in medical mycology

Reinhard Rüchel[1], Martin Behe[2], Björn Torp[3] & Hartmut Laatsch[3]

[1]Mycology Unit, Dept. of Bacteriology, [2]Dept. of Nuclear Medicine and [3]Dept. of Organic Chemistry, University of Göttingen, Germany

www.reviberoammicol.com/2001-18/147149.pdf

Besides the remarkable progress which has been made in the laboratory diagnosis of mycoses, there is still a need for an affordable diagnostic procedure which would allow the rapid diagnosis of deep seated mycoses which may be life threatening in compromised patients and are often difficult to treat. A step forward to alleviate this situation was the introduction of the optical brighteners into diagnostic mycology, which was initially suggested in 1984 almost simultaneously by three different groups [1-3]. Typical optical brighteners like Calcofluor white / Tinopal (Sigma, USA) or Blankophor (Bayer, Leverkusen, Germany) are diaminostilbene compounds (Figure 1) which by intercalation non-covalently bind to ß-glycosidically linked polysaccharides.

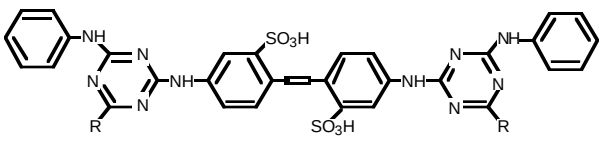

Figure 1. Blankophor®, the first optical brightener of the diaminostilbene disulfonic acid type.

They are fluorescent dyes which upon irradiation below 400 nm wavelength in aqueous media, shed a bright bluish to yellowish fluorescence above 420 nm wavelength. As the first compound of this sort, Blankophor has already been introduced in 1941 as an additive to washing agents and in paper manufacture [4]. Binding of the "optical brighteners" to fungal cells was first described by Darken [5] and the mechanism of binding was analyzed by Maeda & Ishida [6]. Ever since, optical brighteners have been used in experimental mycology for quantitation of fungal cells [e.g. 7] or studying cell wall architecture [8,9] but most important, brighteners are used in histopathology of mycosis [10,11], in dermatology [12,13], and diagnosing pneumocystosis [14].

Favourably for the use in diagnostic mycology, the major targets of the brighteners such as cellulose, glucans and chitin are not present in healthy warm-blooded animals but the latter are major constituents of the fungal cell wall. Likewise favourably, in aqueous media the fluorescence of unbound molecules of the brighteners rapidly undergo irreversible bleaching upon irradiation by ultraviolet light which is due to trans-cis isomerization, whereas the fluorescence of the bound brighteners is very stable. This favourable property allows for a "rinsing by irradiation": freshly stained microscopic mounts may be directly examined under the epifluorescence microscope without rinsing or counterstaining, e.g. by Evans blue, as has sometimes been recommended [15]. The U.V. irradiation during microscopy therefore generates a high contrast of the fluorescent fungi against a virtually black background.

Ready-to-use kits of brighteners for application in the laboratory diagnosis of mycoses are offered for instance by Polyscience Inc. (Warrington, PA, USA, Fax +1 800 343 3291) and by Hain Diagnostika (Nehren, Germany, Fax +49-7473-945199). Appropriate filter kits for fluorescence microscopy of these brighteners are available from all major manufacturers. We use filter kit # 2 from the Carl Zeiss Corp. (Germany) affording an excitation wavelength of 365-395 nm and an emission above 420 nm (barrier filter).

Since the brighteners used are stable in strongly alkaline solution, the dye, at a concentration of $> 10^{-5}$ M can be made up in approximately 15% (w/v) potassium hydroxide solution and will be stable under such conditions over longer periods of time when kept in polyethylene vials in the refrigerator under protection from light. This working solution can directly be applied at a ratio of approximately 2:1 (v/v) to cell sediments or solid tissue specimens. Vials containing biopsies immersed in the alkaline dye solution can be incubated at 56 °C even overnight if necessary. However, with a cell suspension, e.g. the sediment of bronchoalveolar lavage, the treatment takes less than five minutes. The procedure will cause maceration of the tissue and simultaneous staining of the liberated fungal elements, which morphologically resist the strong alkalinity for extended periods of time (> 1 week at room temperature). Such digests can be stored at room temperature, or smears thereof may be stored in the freezer in closed polyethylene containers.

If larger specimens such as slices of visceral organs are to be inspected for fungal invasion, the simultaneous staining and maceration process at elevated temperature in a closed vial can conveniently be completed by brief centrifugation (< 1000 x g) of the liquefied digest of the tissue in order to concentrate liberated fungal elements in a sediment, which can then be streaked on a glass slide and directly inspected microscopically as a wet mount for fungal elements as above.

The optical brighteners may be used in combination with immunohistological procedures, provided the solution of the brightener has been made up in physiological media such as PBS or saline and the antibodies employed are not directed against the polysaccharide binding sites of the brighteners [16]. In such a setting, the brightener may be used to start with, in order to allow a rapid screening of the tissue section for fungal elements. The high contrast of such mounts allows for the inspection of the specimen at low magnification, thus avoiding oil immersion at this stage of the procedure. If fungi have been spotted, the section is worth further investigation by a subsequent immunohistological procedure which may then be initiated after removal of the cover slip. Rinsing of

the mount in order to rid the tissue section of the brightener prior to the immune reaction is not necessary, provided that excitation and emission wavelength of the brightener and the subsequently used immuno-fluorophore differ sufficiently as is the case with fluorescein or rhodamin.

If mixed mycosis is suspected upon examination of tissue which has already been subjected to one type of immunohistological reaction, this in turn can also be followed by staining with the optical brightener, provided the latter has been made up in PBS or saline. Suspect areas of the tissue can then be inspected and recorded at both excitation wavelengths by simply exchanging the appropriate filter kits [17] (Figure 2) .

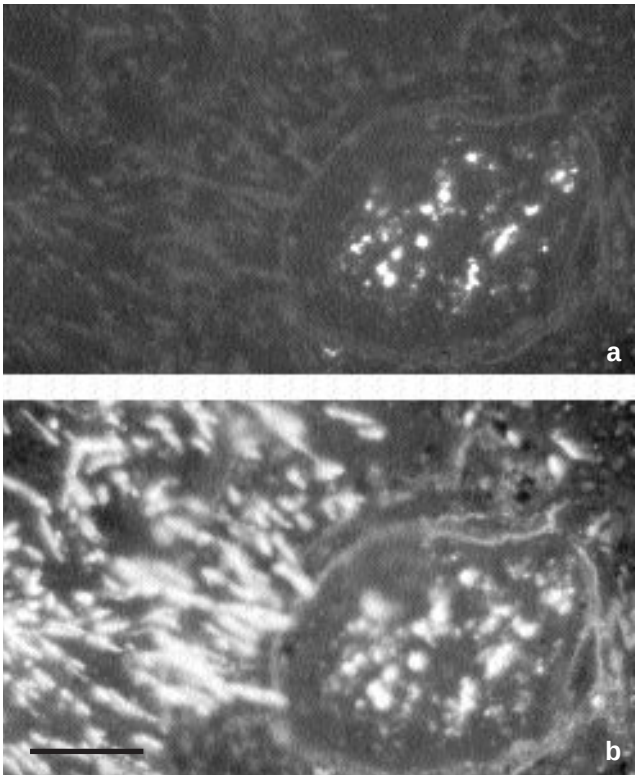

Figure 2. Kidney section from a lethal case of mycosis in a patient suffering from acute leukemia. a) Image after indirect immunohistochemical reaction for *Rhizomucor pusillus* antigens. The second antibody was labelled with rhodamin. b) Same site of the section after additional staining with Blankophor confirming the presence of a second fungus that was identified subsequently as *Aspergillus fumigatus*. The bar equals 18 µm.

In mounts, which have already been subjected to Gram stain for detection of bacteria or yeasts, filamentous fungi will usually go unrecognized. However, if such fungi are suspected in addition, optical brightener (e.g. in saline) may then be applied to the mount after removal of immersion oil by rinses with ethanol [18]. Penetration of the Gram stained mount by the brightener is somehow retarded and will take several minutes. The mount may then be inspected by fluorescence microscopy as usual, and filamentous fungal elements may then be detected (Figure 3). For any of such combined microscopic procedures which may include conventional light microscopy and/or fluorescence microscopy, recording the coordinates of the suspect areas of the tissue section under investigation is prudent. For photographic recording of microscopic images at the variable conditions outlined above, we have used Ektachrome 400 ASA film.

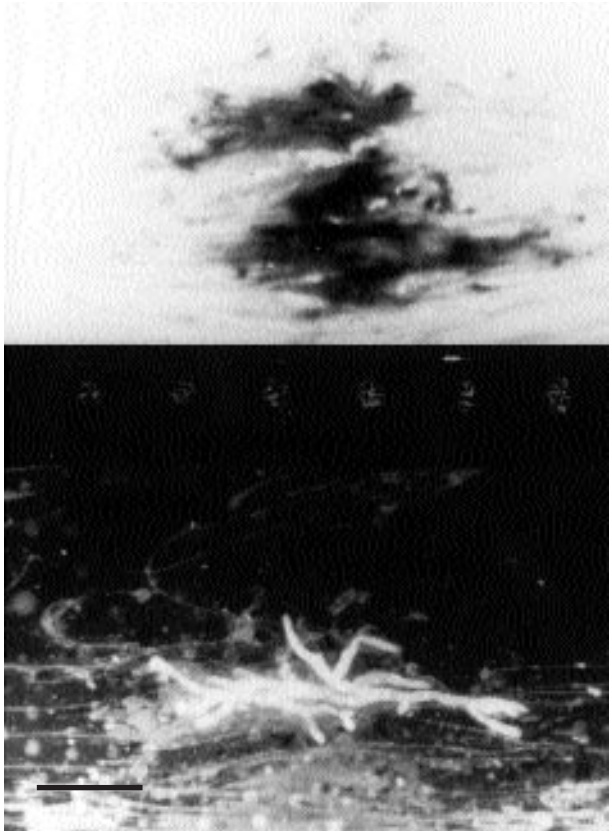

Figure 3. Sequential application of Gram stain (top) and Blankophor stain (bottom) on a smear of bronchial secretions. Conventional Gram stain hardly allows the recognition of filamentous fungi. After removal of immersion oil, the same mount was subsequently subjected to Blankophor stain, which unveiled mycelia; which by positive culture from the same specimen could be ascribed to *Aspergillus fumigatus*. The bar equals 18 µm.

Projected additional use of optical brighteners in medical mycology

Optical brighteners of the diaminostilbene type seem to be virtually non-toxic. Since they are highly water soluble and no specifically binding structures like ß-glycosides are expected to exist in the healthy warm-blooded animal, injection of the brightener Blankophor into mice suffering from deep seated mycoses was performed. Animals were sacrificed shortly afterwards and upon necropsy were inspected for suggestive lesions. Tissue from the latter were subjected directly to epifluorescence microscopy and inspected for vitally stained fungal elements. Such stained elements were consistently detected in tissue of animals suffering from aspergillosis, blastomycosis, candidosis, coccidioidomycosis, cryptococcosis and histoplasmosis [19]. These findings suggest the potential of the brighteners as parenterally applicable diagnostic tools.

Consequently, the labeling of the brightener Blankophor with I^{125} or I^{131} may yield a compound, which upon intravenous application may be suited for the scintigraphic detection of deep seated mycotic foci in the body. This has experimentally been realized by linking tyramine to Blankophor and subsequently labelling the compound with radioiodide by the chloramine-T method. The intravenously applied radiolabelled brightener was sufficiently specific upon scintigraphy to allow the distinction between deposits of heat killed bacteria (*Escherichia coli*) and

deposits of heat killed fungal cells (*Candida albicans*) that had been injected previously into murine thighs. By injection of labeled Blankophor into infected mice, pulmonary aspergillosis was thus visualized scintigraphically with an isotope camera which is routinely used in nuclear medicine (Figure 4).

Another conceivable feature of the brighteners in medical mycology is their potential use as homing agents for systemically applied antifungals. This may be particularly effective with those antifungals that act on the cell wall as is the case with the candins [20]. A corresponding potentiation of the in vitro activity of candins by the brightener Blankophor has been observed (own unpublished results). Chemical linking of the brightener to certain antifungal agents like amphotericin should also covey an increased water-solubility to the complexes, which in turn might alleviate the need of problematic solubilizers. This is presently under investigation.

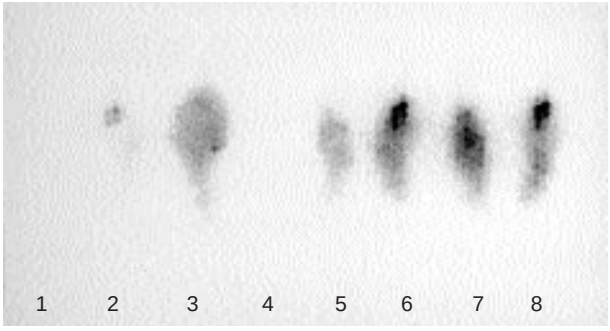

Figure 4. Scintigraphic scans of immunomodulated mice (No. 2, 3, 5, 6, 7, 8) after intranasal infection with 10^7 conidia of *Aspergillus fumigatus*. Foci of pulmonary mycosis were depicted by intravenously applied radioiodinated Blankophor. Healthy controls (No. 2 + 4) did not show any retention of the labeled brightener 48 h after injection. Postmortally, the fungus was grown from infected animals.

References

1. Monheit JE, Cowan DF, Moore DG. Rapid detection of fungi in tissues using calcofluor white and fluorescence microscopy. Arch Pathol Lab Med 1984; 108: 616-618.
2. Holländer H, Keilig W, Bauer J, Rothemund E. A reliable fluorescent stain for fungi in tissue sections and clinical specimens. Mycopathologia 1984; 88: 131-134.
3. Hageage GJ, Harrington BJ. Use of calcofluor white in clinical mycology. Lab Med 1984; 15: 109-112.
4. Anliker R. History of whitening. In: Anliker R, Müller G (Eds.) Fluorescent Whitening Agents. Stuttgart, Georg Thieme, 1975: 12-18.
5. Darken MA. Absorption and transport of fluorescent brighteners by microorganisms. Appl Microbiol 1962; 10: 387-393.
6. Maeda H, Ishida N. Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. J Biochem 1967; 62: 276-278.
7. Coleman T, Madassery JV, Kobayashi GS, Nahm MH, Little JR. New fluorescence assay for the quantitation of fungi. J Clin Microbiol 1989; 27: 2003-2007.
8. Rico H, Miragall F, Sentandreu R. Abnormal formation of *Candida albicans* walls produced by calcofluor white: an ultrastructural and stereologic study. Exp Mycol 1985; 9: 241-253.
9. Hector RF, Braun PC, Hart JT, Kamarck ME. The use of flow cytometry to monitor chitin synthesis in regenerating protoplasts of *Candida albicans*. J Med Vet Mycol 1990; 28: 51-57.
10. Green LK, Moore DG. Fluorescent compounds that nonspecifically stain fungi. Lab Med 1987; 18: 456-458.
11. Wachsmuth ED. Visualization of fungi in histological sections. Virchows Archiv B Cell Pathol 1988; 56: 1-4.
12. Monod M, Baudraz-Rosselet F, Ramelet AA, Frenk E. Direct mycological examination in dermatology: a comparison of different methods. Dermatologica 1989; 179: 183-186.
13. Midgley G, Moore KM. Onychomycosis. Rev Iberoam Micol 1998; 15: 113-115.
14. Baselski VS, Robison MK, Pifer LW, Woods DR. Rapid detection of *Pneumocystis carinii* in bronchoalveolar lavage samples by using Cellufluor staining. J Clin Microbiol 1990; 28: 393-394.
15. Koch HH, Pimsler M. Evaluation of Uvitex 2B: a nonspecific fluorescent stain for detecting and identifying fungi and algae in tissue. Lab Med 1987; 9: 603-606.
16. Rüchel R, Schaffrinski M. Versatile fluorescent staining of fungi in clinical specimens by using the optical brightener Blankophor. J Clin Microbiol 1999; 37: 2694-2696.
17. Binder C, Rüchel R. Mixed systemic mycosis with fatal outcome in a patient with acute myeloblastic leukaemia. Mycoses 2000; 43: 59-63.
18. Rüchel R, Margraf S. Rapid microscopical diagnosis of deep-seated mycoses following maceration of fresh specimens and staining with optical brighteners. Mycoses 1993; 36: 239-242.
19. Rüchel R, Schaffrinski M, Seshan KR, Cole GT. Vital staining of fungal elements in deep-seated mycotic lesions during experimental murine mycoses using the parenterally applied optical brightener Blankophor. Med Mycol 2000; 38: 231-237.
20. Denning DW. Echinocandins and pneumocandins-a new antifungal class with a novel mode of action. J Antimicrob Chemother 1997; 40: 611-614.