Inability of *Pneumocystis* organisms to incorporate bromodeoxyuridine suggests the absence of a salvage pathway for thymidine

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Because thymidine metabolism is a potential target for therapy of *Pneumocystis* pneumonia, it was investigated whether *Pneumocystis* organisms have a salvage pathway for thymidine by administering 5-bromo-2′-deoxyuridine (BrdU) to mice and rats with *Pneumocystis* pneumonia. Although BrdU incorporation was detected in host cells, no incorporation was seen in *Pneumocystis* organisms infecting either rats or mice. This suggests that *Pneumocystis* organisms do not have a salvage pathway for thymidine, and that inhibitors of de novo synthesis, such as thymidylate synthase inhibitors, may be effective drugs for treating *Pneumocystis* pneumonia.

INTRODUCTION

*Pneumocystis jirovecii* is an atypical fungus that infects patients suffering from a variety of immunosuppressive diseases, such as AIDS and cancer (Kovacs et al., 2001a; Roblot et al., 2002). Although there has been a decrease in morbidity among AIDS patients due to the availability of potent anti-retroviral regimens, *Pneumocystis* pneumonia (PCP) continues to be the most common life-threatening opportunistic infection among HIV-infected patients. Therapy with the combination of trimethoprim and sulfa-methoxazole (TMP/SMX) is the preferred approach for primary prophylaxis in susceptible patients (i.e. for AIDS patients with CD4+ count levels <200 mm−3) as well as for treatment of active PCP (Kovacs & Masur, 2000; Phair et al., 1990). These drugs target the enzymes dihydrofolate reductase and dihydropteroate synthase (DHPs), respectively. Other drugs including dapsone, atovaquone and pentamidine are alternatives to TMP/SMX, but they are either more toxic or less active against *P. jirovecii* (Hughes et al., 1993; Hughes, 1998; Kovacs et al., 2001a; Sattler et al., 1988; Vasconcelles et al., 2000). Unfortunately, recent studies have identified mutations in the gene encoding DHPS suggesting the emergence of resistance in *P. jirovecii* to sulfa drugs (dapsone and SMX) (Helweg-Larsen et al., 1999; Kazanjian et al., 2000; Ma et al., 1999; Navin et al., 2001). Development of new drugs to treat or prevent PCP thus remains an important priority for research.

*P. jirovecii* is closely related to *Pneumocystis* species that infect rats (*Pneumocystis carinii*) and mice (*P. carinii* f. sp. *muris*). A better understanding of the metabolic pathways of *Pneumocystis* species may lead to the identification of novel therapeutic targets. Thymidine metabolism has been partially characterized in *Pneumocystis* thymidylate synthase has been identified, isolated, cloned and crystallized (Anderson et al., 2000; Edman et al., 1989; Kovacs et al., 1990; Santi et al., 1991). Thus, *Pneumocystis* organisms possess de novo synthetic pathways for thymidine, a target of chemotherapy in other infections. Therapies that target de novo thymidylate synthesis can potentially be bypassed using thymidine salvage pathways. To see if *Pneumocystis* organisms possessed a salvage pathway for thymidine, 5-bromo-2′-deoxyuridine (BrdU) was administered for 14 days to *Pneumocystis*-infected rats and mice. BrdU is a thymidine analogue which can be incorporated into DNA during the S-phase of cell cycle if the cells have a salvage pathway. Incorporation can be readily detected histologically, since a monoclonal antibody (mAb) is available against BrdU. Incorporated BrdU in mammalian cells appears to be stable and can be detected for many weeks without affecting cell function (Dolbeare, 1995a, b; Dolbeare, 1996; Kovacs et al., 2001b). Studies were conducted in animals because of the difficulty in culturing *Pneumocystis in vitro* and concerns that lack of incorporation ex vivo may be due to lack of proliferation of *Pneumocystis*.

METHODS

PCP was induced in Sprague–Dawley rats by immunosuppression with dexamethasone placed in the drinking water (0.8 mg l−1) as described previously; immunosuppression was continued throughout the study (Kovacs et al., 1988). Uninfected BALB/c SCID mice, which are highly susceptible to PCP (Walzer et al., 1997), were co-housed with *P. carinii* f. sp. *muris*-infected animals for at least 2 months prior to administration of BrdU. Animals were given...
BrdU (Sigma), 0.8 mg ml⁻¹, in the drinking water for 14 days as described previously (Flynn et al., 1999). The BrdU solution was shielded from light and made fresh daily.

At day 14, the animals were killed and the lungs were removed. One lung was used for making impression smears that were stained with Diff-Quik (Dade Behring) to confirm the presence of infection. The other lung was inflated with Histochoice (Amresco) with 20 % ethanol and subsequently stored in the fixative until embedding in paraffin. A portion of the small intestine, processed in a similar manner, served as a positive control for BrdU incorporation (Kellett et al., 1992).

For immunohistopathology, which was performed by contract (SAIC, Frederick, MD), 5 μm thick tissue samples were deparaffinized and stained for Pneumocystis or for BrdU. Staining for Pneumocystis was performed by using mAb 4D7, which is specific for a surface protein of Pneumocystis (Angus et al., 1996; Mei et al., 1995), together with the Mouse Elite Kit (Vector Laboratories) according to the manufacturer’s recommendations. Staining for BrdU incorporation in rat tissue used the same kit together with an anti-BrdU mAb (clone Bu20a; DAKO). For mouse tissue, BrdU staining was performed using the Animal Research Kit (DAKO). In addition, BrdU staining was performed using the tyramide signal amplification technique with the TSA-Indirect kit (NEN Life Science Products) according to the manufacturer’s recommendations. Slides prepared with no primary mAb served as negative controls. For BrdU staining, after deparaffinization the slides were placed in 4 M HCl at 37 °C for 20 min to partially denature the DNA and provide access for the anti-BrdU mAb, and then rinsed in boric acid/horae buffer, pH 7-6. Next, slides were placed in 0.01 % trypsin at 37 °C for 3 min, then rinsed in PBS, following which the manufacturer’s instructions for the individual kits were followed. The anti-BrdU mAb was used at a dilution of 1 : 500 to 1 : 1000.

The use of animals for the experimental protocol was approved by the Animal Care and Use Committee, Clinical Center, National Institutes of Health.

RESULTS

Eleven mice (housed in two cages) and seven rats (in two cages) were used in the study. All animals were infected with Pneumocystis as determined by staining with Diff-Quik and mAb 4D7. BrdU incorporation was seen in both small intestine epithelial cells and alveolar cells in both mice and rats (Fig. 1A, B). Thus, as expected, BrdU was readily available for utilization by thymidine salvage pathways, which are present in mammalian cells.

In all animals, Pneumocystis organisms were clearly visible and easily identified by microscopic examination (Fig. 1C). However, no BrdU incorporation was seen in Pneumocystis organisms, even in animals with heavy infection. Tyramide signal amplification has been used in the past to demonstrate low levels of BrdU incorporation. Although this method can substantially increase the sensitivity of immunohistochemical techniques, again no incorporation of BrdU by Pneumocystis organisms could be detected (Fig. 1B, D).

DISCUSSION

The current study strongly suggests that Pneumocystis organisms, in contrast to mammalian cells, do not have a salvage pathway for thymidine and must therefore depend solely on de novo synthesis of this pyrimidine for DNA synthesis. Our results supports earlier findings from our group, in which Pneumocystis cells failed to incorporate [³H]thymidine, which is also dependent on salvage pathways, during short-term cultures (Martine & Kovacs, 1993).

The source of thymidine for DNA synthesis varies among different micro-organisms. Two pathogens in which the thymidine pathways are well characterized are Giardia lamblia and Plasmodium falciparum. Whereas G. lamblia depends solely on salvaging thymidine for DNA synthesis (Jarroll et al., 1989), P. falciparum only utilizes de novo synthesis of this pyrimidine (Sherman, 1979). In contrast, mammalian cells possess both pathways. It is noteworthy that the two fungi most closely related to Pneumocystis, as assessed by comparing the amino acid sequences of homologous proteins, Schizosaccharomyces pombe and Saccharomyces cerevisiae, both lack thymidine kinase and the ability to efficiently take up exogenous deoxyribonucleosides, and thus cannot utilize exogenous thymidine (Hodson et al., 2003; Vernis et al., 2003). Both fungi have been genetically engineered to salvage thymidine and incorporate BrdU, which can be detected by immunofluorescent assays (Hodson et al., 2003; Vernis et al., 2003).

If Pneumocystis organisms possess only a de novo synthetic pathway for thymidine, thymidylate synthase is an especially crucial enzyme for DNA synthesis in this micro-organism. Thymidylate synthase functions in the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Inhibition of Pneumocystis thymidylate synthase activity, in the absence of salvaged thymidine, would lead to cell cycle arrest and thereby cell death. Thymidylate synthase inhibitors such as 5-fluorouracil have been used for more than 40 years as anti-neoplastic agents (Danenberg et al., 1999). Thus, targeting this enzyme is a feasible approach for developing chemotherapeutic agents.

It is possible that the sensitivity of the techniques that we utilized was too low to detect BrdU incorporated by Pneumocystis, as a result of inaccessibility of the DNA to the anti-BrdU mAb, or to the smaller size of the Pneumocystis genome. However, Pneumocystis DNA is accessible to in situ hybridization by oligonucleotide probes in fixed tissues similar to those we utilized here (Edman et al., 1988), and BrdU incorporation has been detected by immunofluorescent staining in the ciliate Colpoda inflata (Gutierrez et al., 2000), and in Schizosaccharomyces pombe and Saccharomyces cerevisiae that have been genetically engineered to salvage thymidine (Hodson et al., 2003; Vernis et al., 2003).

It is also possible that the dose of BrdU that we utilized was too low, or inaccessible to the organisms in the lung, although BrdU is a small molecule that should readily diffuse throughout the body, and DNA in cells immediately adjacent to clusters of Pneumocystis organisms incorporated BrdU (Fig. 1B).
The crystal structure of thymidylate synthase from rat-derived 

*P. carinii*, which was cloned and expressed in

*Escherichia coli*, has been published (Anderson *et al.*, 2000). Comparing the structure to the structure of human thymidylate synthase suggested the potential for designing *Pneumocystis*-specific inhibitors which would not inhibit human thymidylate synthase. If *Pneumocystis* cells lack salvage pathways, as suggested by the current study, such an approach, perhaps combined with co-administration of thymidine to rescue host cells (Jiang *et al.*, 2000), may be an effective therapy for PCP.

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