ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2003, p. 3979–3981 Vol. 47, No. 12
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.12.3979–3981.2003

Genotypes of *Pneumocystis jiroveci* Isolates Obtained in Harare, Zimbabwe, and London, United Kingdom

Robert F. Miller,[1]* Austin R. Lindley,[2] Helen E. Ambrose,[2] Adam S. Malin,[3]† and Ann E. Wakefield[2]‡

Department of Sexually Transmitted Diseases, Windeyer Institute of Medical Sciences, Royal Free and University College Medical School, University College London, London WC1E 6AU,[1] and Molecular Infectious Diseases Group, Department of Paediatrics, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DU,[2] United Kingdom, and Department of Clinical Pharmacology, University of Zimbabwe Clinical School, Parirenyatwa Hospital, Harare, Zimbabwe[3]

Received 12 May 2003/Returned for modification 17 June 2003/Accepted 8 September 2003

Isolates of *Pneumocystis jiroveci* from sulfa-exposed and nonexposed patients from London, United Kingdom, and Harare, Zimbabwe, were genotyped. At the dihydropteroate synthase (DHPS) locus, there was evidence of selection pressure from sulfa drug exposure, and reversal of DHPS genotype ratios occurred when selection pressure was absent or was removed.

Pneumocystis pneumonia (PCP) in humans is caused by the opportunistic fungus *Pneumocystis jiroveci* (16, 17). Several studies have demonstrated a significant association between sulfa drug prophylaxis of PCP in human immunodeficiency virus (HIV)-infected patients and the presence of dihydropteroate synthase (DHPS) mutations in samples of *P. jiroveci* (4, 7, 8). Mutations in the DHPS gene of *P. jiroveci* also correlate with geographical location, suggesting that transmission occurs either directly from person to person or through a common environmental source (2, 3, 15).

This study determined whether sulfa drug prophylaxis in HIV-infected patients with PCP was associated with the occurrence of mutant DHPS genotypes and examined the impacts of the withdrawal of sulfa drug prophylaxis in this patient group and of the use of sulfa drugs as treatment for common community-acquired infections in the general population on the occurrence of mutant DHPS genotypes.

Fifty-one HIV type 1 (HIV-1) antibody-positive adults with PCP confirmed by Grocott's staining of bronchoalveolar lavage (BAL) fluid were studied. Thirty-seven patients were admitted to Middlesex Hospital (part of University College London Hospitals, London, United Kingdom): 25 patients were admitted in the 12 months from May 1992 to May 1993 and 12 patients were admitted in the 12 months from May 2000 to May 2001). Fourteen patients were admitted to University Hospital, Harare, Zimbabwe, in the 12 months from May 1992 to May 1993; their clinical details have been described in detail elsewhere (9). Patients gave informed consent for bronchoscopy. The guidelines of the Middlesex Hospital Research Ethics Committee were followed.

We recorded whether each patient was known to be HIV-1 antibody positive before diagnosis of PCP, had received sulfa drugs as treatment or prophylaxis for PCP, or had toxoplasmosis either in the 3 months immediately prior to diagnosis of PCP or at any time since being infected with HIV. An aliquot of BAL fluid was frozen immediately at −20°C until analysis. Sample analyses were carried out in a blind fashion with regard to patients' clinical details. DNA was extracted from the BAL samples as previously described (10).

DNA amplification at the mitochondrial large-subunit rRNA (mt LSU rRNA) locus was done using single-round PCR with primers pAZ102-H and pAZ102-E (13, 18, 20), and that at the DHPS locus was done using single-round PCR with primers DHPS 3 and DHPS 4 (2, 7). Samples from Zimbabwe underwent DNA amplification at the mitochondrial small subunit rRNA (mt SSU rRNA); nested PCR was done using primer pair pAZ112-10F and pAZ112-10R, followed by pAZ112-13 and pAZ112-14, as previously described (18, 19). *Taq* DNA polymerase (Promega, Southampton, United Kingdom) was used.

Extreme caution was taken to prevent cross-contamination of samples; DNA extraction, for example, was performed in a separate room, and PCR amplification and DNA sequencing were done in different areas of the laboratory. Handling of samples at each stage was done in a laminar flow cabinet, and a different set of micropipettes was used with disposable tips, tubes, and reagents. In each DNA extraction and PCR amplification experiment, negative controls (with no DNA and ultrapurified distilled water) were included. *P. jiroveci* DNA from a patient with PCP was used as a positive control in each experiment. PCR amplification was performed a minimum of six times on each sample. Amplification products were sequenced directly from both ends as described elsewhere (10, 18, 19). Genotypes were distinguished by identifying polymorphisms at the mt LSU rRNA locus (positions 85 and 248) (2, 5, 6, 18, 19), at the DHPS locus (positions 165 and 171) (2, 4, 7), and at the mt SSU rRNA locus (positions 216 and 299) (19).

Twenty of the London patients with PCP during 1992-1993 were experiencing their first episode of PCP; 15 of these 20 patients were not known to be HIV infected before presenting with PCP. Nine of 25 patients were receiving anti-*Pneumocystis*

* Corresponding author. Mailing address: Department of Sexually Transmitted Diseases, Windeyer Institute of Medical Sciences, Royal Free and University College Medical School, University College London, Mortimer Market Centre, London WC1E 6AU, United Kingdom. Phone: 44 207 380 9945. Fax: 44 207 380 9669. E-mail: rmiller@gum.ucl.ac.uk.

† Present address: Department of Respiratory Medicine, Royal United Hospital, Bath BA1 1NG, United Kingdom.

‡ Deceased.

TABLE 1. Genotypes of *P. jiroveci* isolates at the mt LSU rRNA and DHPS loci by year and sulfa exposure

Isolate group	No. of isolates with indicated genotype at locus[a]									
	mt LSU rRNA					DHPS				
	1	2	3	4	Mixed	1	2	3	4	Mixed
London 1992-1993 (n = 25)										
Sulfa exposed	4	1	3	0	1[b]	4	1	0	4	0
Not exposed	12[c]	3	1	0	0	12[c]	2	1	0	1[d]
Zimbabwe 1992-1993 (n = 14)										
Sulfa exposed	0	0	0	0	0	0	0	0	0	0
Not exposed	4	1	8	0	1[e]	13	1	0	0	0
London 2000-2001 (n = 12)										
Sulfa exposed	0	0	0	0	0	0	0	0	0	0
Not exposed	4	2	5[f]	1	0	10	1	0	0	1[d,f]

[a] Genotypes are numbered according to the method of Beard et al. (2).
[b] Genotypes 1 and 4.
[c] Five patients had received sulfa drugs >3 months previously.
[d] Genotypes 1 and 2.
[e] Genotypes 1 and 3.
[f] One patient had received sulfa drugs >3 months previously.

TABLE 2. Genotypes of isolates of *P. jiroveci* collected in Harare, Zimbabwe, in 1992-1993

Sample no.	Locus		
	mt LSU rRNA[a]	DHPS[a]	mt SSU rRNA
Z1	3	1	1
Z2	2	1	4
Z3	3	1	2
Z4	3	1	4
Z5	1	1	3
Z6	3	1	2
Z7	1	1	2
Z8	3	2	2
Z10	1, 3[b]	1	2, 4[b]
Z11	3	1	2
Z12	1	1	3
Z15	3	1	4
Z16	3	1	4
Z17	1	1	2

[a] Genotypes at these loci are numbered according to the method of Beard et al. (2).
[b] Mixed infection.

prophylaxis with sulfa drugs (trimethoprim-sulfamethoxazole [TMP-SMX] in three patients and dapsone in the remaining six). All 12 patients in London during 2000-2001 had first-episode PCP, eight were not previously known to be HIV infected, and none had received sulfa drugs in the previous 3 months. All 14 patients from Zimbabwe in 1992-1993 had first-episode PCP, none were known to be HIV infected before the diagnosis of PCP, and none had ever received sulfa drugs.

At the mt LSU rRNA locus, four different genotypes were distinguished (2). There was no association between patients' receipt of sulfa drugs in the 3 months prior to presentation with PCP and the mt LSU rRNA genotype (Table 1). At the DHPS locus, four different genotypes were identified (Table 1) (2). At the DHPS locus, genotype 4 was identified only in samples from London in 1992-1993. For all patients, the presence of "mutant" genotypes of DHPS (types 2, 3, and 4) was associated with exposure to a sulfa drug during the 3 months before the episode of PCP ($P < 0.01$, two-tailed Fisher's exact test). Furthermore, for all patients, genotype 4 was associated with sulfa drug exposure ($P < 0.0005$, two-tailed Fisher's exact test). Among isolates from London patients in 1992-1993, exposure to dapsone prophylaxis was particularly associated with DHPS genotype 4 ($P < 0.002$). A quarter of all isolates collected in 1992-1993 from London patients not exposed to sulfa were of mutant genotypes (types 2, 3, and 4), suggesting selection pressure (Table 1) (3). At the mt SSU rRNA locus, four genotypes were identified: type 1 (G/A), type 2 (G/G), type 3 (T/A), and type 4 (T/G) (Table 2).

In London in 1992-1993, TMP-SMX was used as the first-line therapy for both prophylaxis and treatment of PCP; dapsone was the second-line drug for prophylaxis in patients intolerant of TMP-SMX (11). In the general population, TMP-SMX was used as the first-line therapy for urinary tract infection, acute exacerbations of chronic bronchitis, and otitis media. In July 1995 in the United Kingdom, the license for TMP-SMX use in the general population was severely restricted because of concerns about the drug's adverse-reaction profile (1). After this, it was no longer used within the general population for the indications listed above, but it remained as the first-line therapy for prophylaxis and treatment of PCP. In late 1996, the availability of protease inhibitor-based regimens of highly active antiretroviral therapy was associated with a reduction in the use of cotrimoxazole as prophylaxis for PCP (12). Thus, among the United Kingdom samples collected in 2000-2001, 2 of 12 (17%) isolates of *P. jiroveci* were mutant (genotype 2), whereas among 1992-1993 samples, 9 of 25 (36%) isolates were mutant (genotypes 2, 3, and 4).

In Zimbabwe in 1992-1993, TMP-SMX use was limited to use as second-line therapy for pneumonia in those unresponsive to penicillin and as third-line therapy for urethral discharge in males. Fansidar (sulfadoxine with pyrimethamine) was the second-line therapy for chloroquine-resistant uncomplicated malaria, and adjunctive therapy, with quinine, was used for treatment of complicated malaria (14). Among the samples from Zimbabwe, only one isolate was mutant (genotype 2) and the genotypes identified at the mt LSU rRNA, DHPS, and mt SSU rRNA loci were no different from those previously described in London over a 9-year period (Table 2) (19).

This study showed an association between exposure to sulfa drugs and mutant DHPS genotypes and demonstrated evidence of selection pressure at the DHPS locus from sulfa drug exposure among London patients in 1992-1993. Furthermore, reversal of the mutant-to-wild-type genotype ratios was seen when selection pressure was absent, as in Harare in 1992-1993, or was removed, as in London in 2000-2001. These data support the hypothesis that human *Pneumocystis* infection arises by recent transmission, since if reactivation of latent infection were the explanation, then no differences in DHPS genotypes would be observed over time or by geographical location, irrespective of patients' receipt of sulfa drugs.

This research was supported by the Royal Society (A.E.W.), the Wellcome Trust (A.E.W.), the Medical Research Council (H.E.A.), the Fifth Framework Programme of the European Commission (grant QLK2-CT-2000-01369) (A.E.W., A.R.L., and R.F.M.), and Camden Primary Care Trust (R.F.M.).

REFERENCES

1. **Anonymous.** 1995. Co-trimoxazole use restricted. Drug Ther. Bull. **33**:92–93.
2. **Beard, C. B., J. L. Carter, S. P. Keely, L. Huang, N. J. Pieniazek, I. N. S. Moura, J. M. Roberts, A. W. Hightower, M. S. Bens, A. R. Freeman, S. Lee, J. R. Stringer, J. S. Duchin, C. del Rio, D. Rimland, R. P. Boughman, D. A. Levy, V. J. Dietz, P. Simon, and T. R. Navin.** 2000. Genetic variation in *Pneumocystis carinii* isolates from different geographic regions: implications for transmission. Emerg. Infect. Dis. **6:**265–272.
3. **Huang, L., J. Friedly, A. M. Morris, J. E. Carter, J. R. Turner, C. Merrifield, T. Navin, and C. B. Beard.** 2001. *Pneumocystis carinii* dihydropteroate synthase genotypes in HIV-infected persons residing in San Francisco: possible implications for disease transmission. J. Eukaryot. Microbiol. **48**(Suppl.)**:** 137S–138S.
4. **Kazanijian, P., W. Armstrong, P. A. Hossler, W. Burman, J. Richardson, C.-H. Lee, L. Crane, J. Katz, and S. R. Meshnick.** 2000. *Pneumocystis carinii* mutations are associated with duration of sulfa or sulfone prophylaxis exposure in AIDS patients. J. Infect. Dis. **182:**551–557.
5. **Keely, S. P., R. P. Baughman, A. G. Smulian, M. N. Dohn, and J. R. Stringer.** 1996. Source of *Pneumocystis carinii* in recurrent episodes of pneumonia in AIDS patients. AIDS **10:**881–888.
6. **Keely, S. P., J. R. Stringer, R. P. Baughman, M. J. Linke, P. D. Walzer, and A. G. Smulian.** 1995. Genetic variation among *Pneumocystis carinii hominis* isolates in recurrent pneumocystosis. J. Infect. Dis. **172:**595–598.
7. **Lane, B. R., J. C. Ast, P. A. Hossler, D. P. Mindell, M. S. Bartlett, J. W. Smith, and S. R. Meshnick.** 1997. Dihydropteroate synthase polymorphisms in *Pneumocystis carinii*. J. Infect. Dis. **177:**482–485.
8. **Ma, L., L. Borio, H. Masur, and J. A. Kovacs.** 1999. *Pneumocystis carinii* dihydropteroate synthase but not dihydrofolate reductase gene mutations correlate with prior trimethoprim-sulfamethoxazole or dapsone use. J. Infect. Dis. **180:**1969–1978.
9. **Malin, A. S., L. K. Z. Gwanzura, S. Klein, V. J. Robertson, P. Musvaire, and P. R. Mason.** 1995. *Pneumocystis carinii* pneumonia in Zimbabwe. Lancet **346:**1258–1261.
10. **Maskell, N. A., D. J. Waine, A. Lindley, J. C. T. Peperall, A. E. Wakefield, R. F. Miller, and R. J. O. Davies.** 2003. Asymptomatic carriage of *Pneumocystis jiroveci* in subjects undergoing bronchoscopy. Thorax **58:**594–597.
11. **Miller, R. F.** 1994. Prevention and treatment of *Pneumocystis carinii* pneumonia in patients infected with HIV. Drug Ther. Bull. **32:**12–15.
12. **Miller, R. F.** 2000. Prophylaxis of *Pneumocystis carinii* pneumonia: too much of a good thing? Thorax **55**(Suppl. 1)**:**S15–S22.
13. **Miller, R. F., H. E. Ambrose, and A. E. Wakefield.** 2001. *Pneumocystis carinii* f. sp. *hominis* DNA in immunocompetent health care workers in contact with patients with *P. carinii* pneumonia. J. Clin. Microbiol. **39:**3877–3882.
14. **Ministry of Health and Child Welfare.** 1994. Essential drugs list for Zimbabwe (EDLIZ), 3rd ed. Ministry of Health and Child Welfare, Harare, Zimbabwe.
15. **Morris, A. M., M. Swanson, H. Ha, and L. Huang.** 2000. Geographic distribution of human immunodeficiency virus-associated *Pneumocystis carinii* pneumonia in San Francisco. Am. J. Respir. Crit. Care Med. **162**:1622–1626.
16. **Stringer, J. R., C. B. Beard, R. F. Miller, and A. E. Wakefield.** 2002. A new name (*Pneumocystis jiroveci*) for pneumocystis from humans. Emerg. Infect. Dis. **8:**891–896.
17. **Stringer, J. R., M. T. Cushion, and A. E. Wakefield.** 2001. New nomenclature for the genus *Pneumocystis*. J. Eukaryot. Microbiol. **49** (Suppl.)**:**184S–189S.
18. **Tsolaki, A. G., P. Beckers, and A. E. Wakefield.** 1998. Pre-AIDS era isolates of *Pneumocystis carinii* f. sp. *hominis*: high genotypic similarity with contemporary isolates. J. Clin. Microbiol. **36:**90–93.
19. **Wakefield, A. E., A. R. Lindley, H. E. Ambrose, C.-M. Denis, and R. F. Miller.** 2003. Limited asymptomatic carriage of *Pneumocystis jiroveci* in human immunodeficiency virus-infected patients. J. Infect. Dis. **187:**901–908.
20. **Wakefield, A. E., F. J. Pixley, S. Banerji, K. Sinclair, R. F. Miller, E. R. Moxon, and J. M. Hopkin.** 1990. Detection of *Pneumocystis carinii* with DNA amplification. Lancet **336:**451–453.