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Detection of *Pneumocystis carinii* and Characterization of Mutations Associated with Sulfa Resistance in Bronchoalveolar Lavage Samples from Human Immunodeficiency Virus-Infected Subjects

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One hundred ninety-four bronchoalveolar specimens were evaluated by microscopic examination and by amplification of a sequence of a *Pneumocystis carinii* dihidropteroate synthase gene for identification of mutations linked to sulfa resistance. PCR sensitivity and specificity were 100 and 86.7%, respectively, compared to results of microscopic examination. However, 7 out of 19 microscopy-negative, PCR-positive samples were collected from subjects with a clinically high probability of *P. carinii* pneumonia, suggesting that PCR may be more sensitive than microscopic examination, although the absolute performance of PCR cannot be determined. Mutations were identified in 28 out of 70 (40%) PCR-positive specimens and were significantly more common in patients exposed to sulfa drugs (21 out of 29 [72.4%]) than in those not exposed to sulfa drugs (4 out of 35 [11.4%]).

Pneumocystis carinii pneumonia (PCP) is still an important cause of morbidity and mortality in human immunodeficiency virus (HIV)-infected patients, in spite of specific chemoprophylaxis and aggressive antiretroviral therapy (23). Cotrimoxazole (TMP-SMZ), a synergistic combination of sulfamethoxazole and the dihydrofolato-reductase inhibitor trimethoprim, is the first choice for treatment and prophylaxis of PCP (3). In animals the efficacy of TMP-SMZ is almost entirely due to sulfamethoxazole (22) that acts by inhibiting dihidropteroato-synthase (DHPS), which is involved in the folate, and ultimately nucleotide, biosynthetic pathway (20). Dapsone, a sulfone agent, is another important second-line prophylactic agent that targets the DHPS. The widespread use of sulfa drugs in antimicrobial chemotherapy has induced the emergence of resistance in numerous microorganisms (2, 18, 19), raising concerns that *P. carinii* may become resistant to sulfa or sulfone, too. Indeed, recent studies have demonstrated that mutations in one of the active sites located at amino acids 55 (Thr→Ala) and 57 (Pro→Ser) in the *P. carinii* DHPS gene are associated with prior exposure to sulfa or sulfone drugs (6–9, 12–14). Since *P. carinii* cannot be cultured in artificial media, traditional methods of testing drug susceptibility to evaluate the meaning of these mutations cannot be used. Moreover, laboratory diagnosis of PCP based on direct examination lacks sensitivity, especially when the organism burden is low. Recently, PCR has been reported to be a specific and more sensitive diagnostic tool than the conventional methods for detection of *P. carinii* in human samples (5, 10, 15, 16, 21).

The aims of this study were (i) to optimize a simple and sensitive method for detection of *P. carinii* and revelation of DHPS mutations and (ii) to examine the prevalence of *P. carinii* DHPS mutations in Italy and to investigate the relationship between these mutations and prior sulfa exposure. To this end we set up a touchdown (TD) PCR assay coupled with double gradient-denaturing gradient gel electrophoresis (DG-DGGE) analysis from bronchoalveolar lavage (BAL) specimens of patients with a confirmed or highly probable diagnosis of PCP.

One hundred ninety-four BAL specimens were prospectively collected from 173 HIV-infected patients (31 females and 142 males; mean age, 40 [range, 23 to 82]) from February 1996 to February 2002 at the Department of Infectious Disease, San Raffaele Hospital, Milan, Italy. All subjects were investigated for pulmonary symptoms characterized by dyspnea, cough, and fever and also had abnormal chest radiographs. Clinical data were obtained by retrospective medical chart review in cases in which conventional staining and/or PCR yielded positive results. Criteria suggestive of PCP included the use of an antipneumocystic agent and the consequent resolution of symptoms, a $CD4^+$ lymphocyte count of <200 cells/mm^3, a serum lactate dehydrogenase level of >510 IU/liter (more than 20% higher than normal values), diffuse interstitial infiltrates at chest radiographs, partial pressure of arterial oxygen ≤ 70 mmHg, no active prophylaxis, and prior or subsequent PCP episodes (within 3 months from BAL collection). Cases with a negative staining result but with a positive PCR result were classified as improbable (1 to 3 suggestive criteria), probable (4 or 5 criteria), or highly probable (6 or 7 criteria) diagnosis of PCP. Only cases classified as highly probable diagnosis of PCP were considered true PCP in the recalculation of PCR assay specificity. PCR results were not reported to physicians; therefore, diagnosis and therapy were based only on microscopic findings and clinical presentation.

Sulfa prophylaxis was defined as the minimum intake of TMP-SMZ or dapsone for at least 8 weeks before sample collection. Exposure to sulfa drugs was defined as continuous assumption of TMP-SMZ or dapsone for at least 2 weeks at any time after the diagnosis of HIV-1 infection (6).

BAL specimens were centrifuged at $1,500 \times g$ for 15 min.

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TABLE 1. Detection of *P. carinii* in 194 BAL specimens: comparison of two PCR protocols and direct examination

No. of samples (no. of patients)	Results for[a]:		
	Direct examination	LSU rRNA PCR	DHPS PCR
122 (111)	−	−	−
51 (48)	+	+	+
17 (17)	−	+	+
2 (2)	−	+	−
2 (2)	−	−	+
Total 194 (173)	51 (26.3%)	70 (36.1%)	70 (36.1%)

[a] LSU rRNA, large subunit rRNA; −, negative; +, positive.

Sediments were used to prepare smears and for DNA extraction. Smears were stained with Giemsa and Grocott stain and were examined by personnel experienced in *P. carinii* diagnosis. DNA was extracted with a Qiagen (Hilden, Germany) tissue kit. Two TD PCR methods were evaluated. The first protocol, consisting of the amplification of a part of the mitochondrial gene carrying the large subunit of rRNA, was carried out as previously described (5). The second PCR method amplified a sequence of 234 bp spanning the mutation sites (codons 55 and 57) of the DHPS gene of *P. carinii* by using primers DHPS 29Fw (AGCGCCTACACATATTATG) and DHPS 232Rev (GCAGGAATAACTCGAGAAAT). To guarantee the stability of the high melting domain, a GC-rich sequence of 30 bp was introduced at the DHPS 29Fw primer 5′ end. The PCR mixture was subjected to a 10-min pre-PCR heating step at 95°C, followed by denaturation at 94°C for 15 s and annealing at 58 to 48°C for 30 s with a 1°C decrement per cycle during the first 10 cycles. Each subsequent cycle (cycles 11 to 50) consisted of the following steps: 92°C for 15 s, 48°C for 30 s, and 72°C for 15 s. After the last cycle, an extension step of 72°C for 5 min was included.

In order to optimize the mutational analysis by DG-DGGE assay, we used plasmids containing wild-type or mutant sequences of the human-derived *P. carinii* DHPS gene. In the single-mutant plasmid, a G was at position 163 instead of A (resulting in the amino acid change T-55→A-55), whereas in the double mutant plasmid nucleotides G and T were present at positions 163 and 169 instead of A and C, respectively (T-55→A-55 and P-57→S-57). DG-DGGE was carried out as previously described (1, 11, 17). The PCR products from DHPS were electrophoresed in gels containing a 20 to 50% denaturant and 8 to 13% polyacrylamide. The gels were run overnight at 60°C constant temperature and at 60 V of constant voltage. The gels were then stained with a 1:5,000 dilution of GelStar (FMC Bio Products, Rockland, Maine) for 30 min and was photographed by means of a UV transilluminator.

The PCR products from the selected DHPS gene region were sequenced by using the primers described above and an

TABLE 2. Clinical characteristics of 19 out of 21 episodes with direct examination-negative and PCR-positive BAL specimens[a]

Sample	LSU rRNA/ DHPS-PCR	$CD4^+$ cell count (cells/mm^3)	Chest radiograph finding(s)	Amt of PaO_2 (mmHg)	LDH level (IU/liter)	PCP prophylaxis[b]	PCP therapy (response)	Prior or subsequent PCP[c]	PCP diagnosis classification (no. of positive criteria)[d]
2V	+/+	2	Interstitial infiltrates	55	1,095	C	No	No	Probable (5)
13V	+/+	2	Nodular infiltrates	NA[e]	497	C	No	No	Improbable (1)
14V	+/+	43	Interstitial and early focal infiltrates	88	567	P inhaled	No	No	Improbable (3)
41V	+/+	NA[a]	Focal infiltrate and pleural effusion	76	350	No	No	No	Improbable (2)
53V	+/+	1	Interstitial and early focal infiltrates	69	316	No	No	No	Probable (4)
59V	−/+	55	Focal infiltrate	NA[e]	299	No	C (NA)	No	Improbable (2)
68V	+/+	4	Interstitial infiltrates	68	895	No	P iv (yes)	No	Highly probable (7)
82V	+/+	4	Interstitial and focal infiltrates	77	556	No	C (yes)	No	Highly probable (6)
87V	+/−	54	Interstitial and focal infiltrates	NA[e]	441	P inhaled	No	No	Improbable (2)
B25	+/+	56	Nodular infiltrates	NA[e]	391	No	No	No	Improbable (2)
B30	+/+	198	Interstitial and focal infiltrates	55	1,708	No	C (yes)	No	Highly probable (7)
B38	+/+	250	Interstitial infiltrates	70	577	No	A (yes)	No	Highly probable (6)
B41	+/+	59	Interstitial infiltrates	68	818	No	No	Yes	Highly probable (6)
B54	+/+	126	Interstitial and focal infiltrates	55	1,056	No	C (yes)	No	Highly probable (6)
B72	+/+	34	Interstitial infiltrates	NA[e]	488	P inhaled	No	No	Improbable (2)
B76	+/+	55	Interstitial infiltrates	NA[e]	450	No	No	No	Improbable (3)
B91	+/+	81	Interstitial infiltrates	54	548	No	C, A (yes)	No	Highly probable (7)
B94	+/+	3	Interstitial and focal infiltrates	66	552	A, D	No	No	Probable (4)
B96	+/−	5	Focal infiltrate	100	1,557	C	No	NA	Improbable (2)

[a] LSU rRNA, large subunit rRNA; PaO_2, baseline partial pressure of arterial oxygen; LDH, serum lactate dehydrogenase; C, cotrimoxazole; P, pentamidine; A, atovaquone; D, dapsone; iv, intravenous; NA, not available.
[b] Active prophylaxis for at least 1 month before sample collection.
[c] Within 3 months since sample collection.
[d] See the text for definition of criteria suggestive of PCP and for classification of episodes.
[e] Measurement not performed because the subjects were eupnoic.

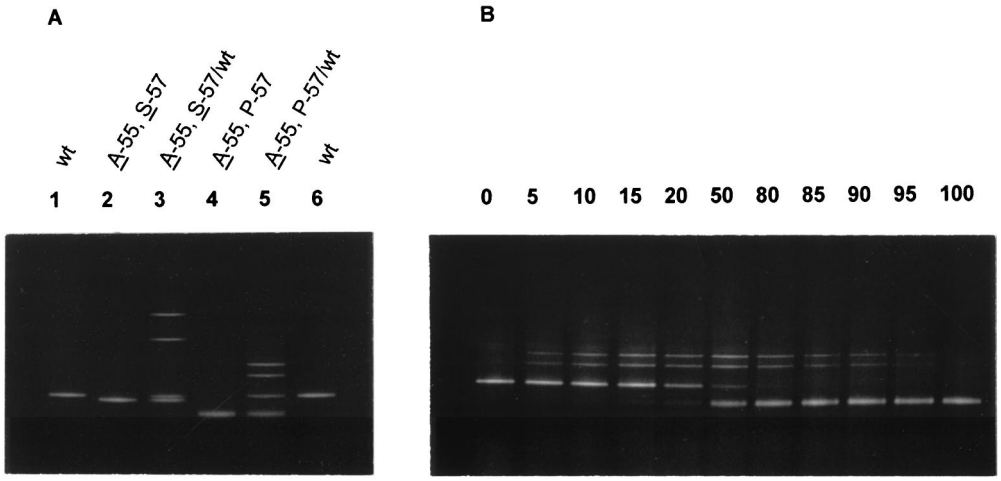

FIG. 1. DG-DGGE analysis. (A) Photograph of DG-DGGE patterns showing the electrophoretic mobility of PCR fragments from wild-type (lanes 1 and 6) and mutant (lanes 2 and 4) DHPS sequences. Heteroduplex patterns, obtained by mixing wild-type and mutant sequences, are showed in lanes 3 and 5. Amino acids that differ from those in the wild-type sequence are underlined. (B) Sensitivity of DG-DGGE analysis for detection of minority populations of the *P. carinii* DHPS gene. Plasmids containing wild-type or mutant (A-55) DHPS sequences were mixed in various concentrations and were analyzed by DG-DGGE. The percentage of mutant DNA is indicated above each lane. wt, wild type.

automated DNA sequencer (model 377; Applied Biosystems, Foster City, Calif.).

Table 1 summarizes the results obtained from 194 BAL specimens. Both PCR protocols produced similar results. In particular, sensitivity (100%), specificity (86.7%), and predictive negative (100%) and positive (72.9%) values were identical for both PCR protocols compared to results from direct examination. Twenty-one direct examination-negative samples were positive by one (4 samples) or both (17 samples) amplification protocols. To address the apparently low specificity of the PCR assays we conducted a retrospective chart review to identify indicators, described above, that might have contributed to the discrepant results. Clinical data were available for 19 of 21 episodes. According to those indicators, seven samples were considered true positive, because they were collected from patients with a highly probable diagnosis of PCP (Table 2). Recalculation of specificity indicated that the TD PCR assays were at least 91.2% specific and 82.9% efficient.

DG-DGGE analysis revealed altered homoduplex mobility (compared to that with wild-type migration) and heteroduplex molecule formation in the PCR products of 28 out of 70 (40%) DHPS PCR-positive samples. Two different patterns were clearly observed in these specimens, consisting of sets of four bands when the heteroduplexes were produced (Fig. 1A). Direct DNA sequencing confirmed these results and showed the following amino acid substitutions: single mutation A-55 (12 samples) and double mutation A-55 and S-57 (16 samples). The possibility of a mixture of two clones was observed in 8 of the 28 (28.6%) samples containing mutations. In these samples the simultaneous presence of mutant and wild-type DNAs was assumed, since heteroduplex formation was observed without mixing the amplification product obtained from these samples with the PCR product obtained from a wild-type clone.

In order to measure the sensitivity of DG-DGGE for detection of one allele in a mixture of two alleles, plasmids containing wild-type or mutant *P. carinii* DHPS sequence were mixed, with mutant concentrations ranging from 5 to 95%. Our method was able to detect the minority population (either wild type or mutant) when it was present in as little as 5% of the population (Fig. 1B)

Table 3 shows the DHPS characteristics according to previous or present exposure to sulfa drug. DHPS mutations were significantly more common in patients exposed to sulfa drugs: 21 out of 29 (72.4%) patients exposed to the antibiotics had DHPS mutations versus 4 out of 35 (11.4%) patients not exposed (chi square = 22.28, $P < 0.0001$).

In the present study we optimized two TD PCR protocols for amplifying sequences of the DHPS gene and the mitochondrial 5S rRNA gene of *P. carinii*. The actual prevalence of PCP in our study population was not ascertained, and thus the absolute specificity of PCR protocols cannot be determined. Nevertheless, PCR showed high sensitivity but an apparently low specificity compared to direct examination. Analysis of the patients' charts, however, demonstrated that the diagnosis of PCP may have been missed by direct examination in at least 7 out of 19 subjects, strongly suggesting that our PCR protocols may be more sensitive than microscopic examination. Moreover, *P. carinii* DNA was detected by one or both PCRs in 12 patients without clear clinical evidence of PCP. Such a discrepancy has already been reported by other investigators who used different PCR protocols and amplification targets, suggesting a status of colonization or subclinical infection (5, 15, 16).

The DG-DGGE assay may become a powerful tool for detection of the DHPS mutations. Under optimized conditions, a mixed population was detected when the minority sequence represented as little as 5% of the total population, while this level of sensitivity is unlikely to be achievable by direct DNA sequencing (12).

The prevalence of *P. carinii* DHPS mutations (40%) in this Italian patient population is higher than that reported in a previous, larger Italian study (13) but is similar to that observed in populations of other countries (20 to 69%) (6, 8,9).

TABLE 3. DHPS characteristics according to previous or present exposure to sulfa drugs in 48 out of 51 confirmed and in 16 out of 19 microbiologically unconfirmed episodes of PCP

Sample type	DHPS residue variation	No. (%) of DHPS PCR-positive samples	No. of samples with DHPS mutations			
			No sulfa exposure		Previous (present) sulfa exposure[a]	
			Confirmed PCP	Unconfirmed PCP	Confirmed PCP	Unconfirmed PCP
Wild type	T55, P57	39 (60.9)	22	9	5 (0)	3 (2)
Mutated						
Codon 55	A55, P57	6 (9.4)	1	0	4 (0)	1 (1)
Codon 55+57	A55, S57	12 (18.75)	1	1	8 (2)	2 (1)
Ambiguous						
Codon 55	T/A55, P57	4 (6.25)	1	0	3 (0)	0
Codon 55+57	T/A55, S/P57	3 (4.7)	0	0	3 (0)	0

[a] Comparison of the rate of DHPS mutations according to sulfa exposure: 21 of 29 (72.4%) patients exposed to the antibiotics versus 4 of 35 (11.4%) patients not exposed had DHPS mutations (chi square = 22.28; $P < 0.0001$).

We found that these mutations were significantly more common among patients who were exposed to sulfa or sulfone drugs than those who were not. This is consistent with previously published observations (4, 6–8, 13).

In conclusion, the protocol for TD PCR targeting of the DHPS gene that we have optimized reaches a sensitivity significantly superior to that of the protocols previously described (6, 8,13), and in our opinion it could be advantageously used to detect the presence of *P. carinii* DNA from bronchoalveolar specimens and for the rapid revelation of mutations associated with sulfa resistance.

REFERENCES

1. **Cremonesi, L., S. Firpo, M. Ferrari, P. G. Righetti, and C. Gelfi.** 1997. Double-gradient DGGE for optimization detection of DNA point mutations. BioTechniques **22:**326–330.
2. **Dallas, W. S., J. E. Gowen, P. H. Ray, M. J. Cox, and I. K. Dev.** 1992. Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of *Escherichia coli* MC4100. J. Bacteriol. **174:**5961–5970.
3. **Fishman, J. A.** 1998. Prevention of infection due to *Pneumocystis carinii*. Antimicrob. Agents Chemother. **42:**995–1004.
4. **Hauser, M. H., P. Sudre, A. Nahimana, P. Francioli, and the Study Group.** 2001. Prophylaxis failure is associated with a specific *Pneumocystis carinii* genotype. Clin. Infect. Dis. **33:**1080–1082.
5. **Helweg-Larsen, J., J. S. Jensen, T. Benfield, U. G. Svendsen, J. D. Lundgren, and B. Lundgren.** 1998. Diagnostic use of PCR for detection of *Pneumocystis carinii* in oral wash samples. J. Clin. Microbiol. **36:**2068–2072.
6. **Helweg-Larsen, J., T. L. Benfield, J. Eugen-Olsen, J. D. Lundgren, and B. Lundgren.** 1999. Effects of mutations in *Pneumocystis carinii* dihydropteroate synthase gene on outcome of AIDS-associated *P. carinii* pneumonia. Lancet **354:**1347–1351.
7. **Kazanjian, P., A. B. Locke, P. A. Hossler, B. R. Lane, M. S. Bartlett, J. W. Smith, M. Cannon, and S. R. Meshnick.** 1998. *Pneumocystis carinii* mutations associated with sulfa and sulfone prophylaxis failure in AIDS patients. AIDS **12:**873–878.
8. **Kazanjian, P., W. Armstrong, P. A. Hossler, W. Burgman, J. Richardson, C. H. Lee, L. Creane, 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.
9. **Lane, B. R., J. C. Ast, P. A. Hossler, D. P. Mindell, M. S. Bartlett, J. W. Smith, and S. R. Meshnick.** 1997. Dihydropteroato synthase polymorphisms in *Pneumocystis carinii*. J. Infect. Dis. **175:**482–485.
10. **Leibovitz, E., H. Pollack, T. Moore, J. Papellas, L. Gallo, K. Krasinski, and W. Borkowsky.** 1995. Comparison of PCR and standard cytological staining for detection of *Pneumocystis carinii* from respiratory specimens from patients with or at high risk for infection by human immunodeficiency virus. J. Clin. Microbiol. **33:**3004–3007.
11. **Lerman, L. S., and K. Silverstain.** 1987. Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis. Methods Enzymol. **155:**482–501.
12. **Ma, L., and J. A. Kovaks.** 2001. Rapid detection of mutations in the human-derived *Pneumocystis carinii* dihydropteroate synthase gene associated with sulfa resistance. Antimicrob. Agents Chemother. **45:**776–780.
13. **Ma, L., J. A. Kovaks, A. Cargnel, A. Valerio, G. Fantoni, and C. Atzori.** 2002. Mutations in the dihydropteroate synthase gene of human-derived *Pneumocystis carinii* isolates from Italy are infrequent but correlate with prior sulfa prophylaxis. J. Infect. Dis. **185:**1530–1532.
14. **Mai, Q., S. Gurunthan, H. Masur, and J. A. Kovacs.** 1998. Failure of cotrimoxazole in *Pneumocystis carinii* infection and mutations in dihydropteroato synthase gene. Lancet **351:**1631–1632.
15. **Olsson, M., K. Elvin, S. Löfdhal, and E. Linder.** 1993. Detection of *Pneumocystis carinii* DNA in sputum and bronchoalveolar lavage samples by polymerase chain reaction. J. Clin. Microbiol. **31:**221–226.
16. **Rabodonirina, M., D. Raffenot, L. Cotte, A. Boibieux, M. Mayecon, G. Bayle, F. Persat, F. Rabatel, C. Trepo, D. Payramond, and M. A. Piens.** 1997. Rapid detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens from human immunodeficiency virus-infected patients: use of a simple DNA extraction procedure and nested PCR. J. Clin. Microbiol. **35:**2748–2751.
17. **Scarpellini, P., S. Braglia, P. Carrera, M. Cedri, P. Cichero, A. Colombo, R. Crucianelli, A. Gori, M. Ferrari, and A. Lazzarin.** 1999. Detection of rifampin resistance in *Mycobacterium tuberculosis* by double gradient-denaturing gradient gel electrophoresis. Antimicrob. Agents Chemother. **43:**2550–2554.
18. **Swedberg, G., S. Ringertz, and O. Skold.** 1998. Sulfonamide resistance in *Streptococcus pyogenes* is associated with differences in the amino acid sequence of its chromosomal dihydropteroate synthase. Antimicrob. Agents Chemother. **42:**1062–1067.
19. **Triglia, T., J. G. Menting, C. Wilson, and A. F. Cowman.** 1997. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **94:**13944–13949.
20. **Volpe, F., S. P. Ballantine, and C. J. Delves.** 1993. The multifunctional folic acid synthesis fas gene of *Pneumocystis carinii* encodes dihydroneopterin aldolase, hydroxymethyldihydropterin pyrophosphokinase and dihydropteroato synthase. Eur. J. Biochem. **216:**449–458.
21. **Wakefield, A. E., F. J. Pixley, S. Banerji, K. Sinclair, R. F. Millar, E. R. Maxon, and J. M. Hopkin.** 1990. Detection of *Pneumocystis carinii* with DNA amplification. Lancet **336:**451–453.
22. **Walzer, P. D., J. Foy, P. Steele, C. K. Kim, M. White, R. S. Klein, B. A. Otter, and C. Allegra.** 1992. Activities of antifolate, antiviral, and other drugs in an immunosuppressed rat model of *Pneumocystis carinii* pneumonia. Antimicrob. Agents Chemother. **36:**1935–1942.
23. **Weverling, G. J., A. Mocroft, B. Ledergerber, O. Kirk, J. Gonzales-Lahoz, A. d'Arminio Monforte, R. Proenca, A. N. Phillips, J. D. Lundgren, and P. Reiss.** 1999. Discontinuation of *Pneumocystis carinii* pneumonia prophylaxis after start of highly active antiretroviral therapy in HIV-1 infection. Lancet **353:**1293–1298.