The treatment is continued for 12 months after serum HCV-RNA becomes negative, which is defined as the end-of-treatment response (ETR). The response was considered to be a sustained viral response (SVR) after another 6 months of negative serologic results without antiviral treatment (Fig. 1). Serologic monitoring for HCV-RNA was consecutively performed on a monthly basis even after SVR was achieved to avoid unrecognized episodes or delayed diagnosis of relapse.

Flexible dose adjustments were made accordingly to avoid serious adverse events and to prevent any lapse in treatment. Actual levels of the given dosage at the time of data collection or final administration was recorded, represented by percentile, with 100% being the full target dose described above (i.e., PEG-IFN 0.5 μg/kg/week is represented as 33%, or RBV 200 mg/day is represented as 25%).

A fixed overall treatment period length was not defined and cessation resulting from adverse events was considered temporary unless an ETR was achieved. Poor virologic response alone was not considered an indication for discontinuation. Treatment was temporarily discontinued when there was significant leukopenia (<1500/ml), thrombocytopenia (<50 000/ml) despite administration of granulocyte colony-stimulating factor (Gran, Sankyo, Co. Ltd., Tokyo, Japan), hemolytic anemia (hemoglobin <8 g/l), renal dysfunction (serum creatinine <2 mg/dl), depressive psychologic status, or general fatigue affecting quality of life. Erythropoietin was given when recovery from anemia remained poor following cessation of antiviral treatment.

Treatment of acute cellular rejection

During the period of observation, biopsy-proven mild-to-moderate acute cellular rejections were confirmed in 27 (26%) patients and treated with a 20-mg/kg bolus of methylprednisolone intravenously with subsequent tapering of the dosage, which was decreased by 50% on each of the following days.

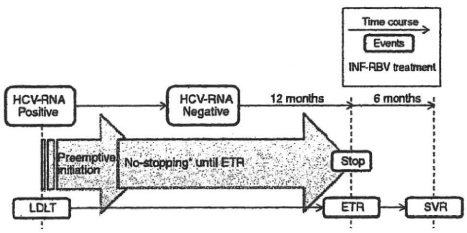

Figure 1. Diagram of combined interferon and ribavirin treatment following living donor liver transplantation at Tokyo University. HCV-RNA, status of hepatitis C virus RNA in the serum; IFN RBV, interferon and ribavirin therapy; LDLT, living donor liver transplantation; ETR, end of treatment response; SVR, sustained viral response. *Treatment with interferon and ribavirin was put on hold when serious adverse events occurred.

Statistical analysis

To clarify whether tolerability affected the outcome, tolerated rates of doses of INF and RBV were studied in accordance with the viral response and eradication. To clarify the time-dependant response, the cumulative rate of negative HCV-RNA, and of the ETR and SVR statuses were studied using the Kaplan–Meier method. The survival curves and cumulative viral response rates were compared using the log-rank test. Various clinical factors, including recipient and donor age and gender, MELD score, presence of HIV, HCV genotype, HCV-RNA viral titer prior to LDLT, occurrence of acute cellular rejection, and use of cyclosporine, were analysed for their effect on achieving an SVR and survival. A multivariate analysis was performed using the Cox proportional hazards model and a forward stepwise procedure. Continuous data were compared between groups using the Mann–Whitney *U*-test. Creation of figures including Kaplan–Meier curves, density-contour plots, box-and-whisker plots, and statistical calculations were performed using SAS software (SAS Institute, Cary, NC, USA). A P value of <0.05 was considered statistically significant.

Results

Applicability of IFN-based treatment using a pre-emptive approach

Among the 105 recipients with HCV who underwent LDLT during the observation period, 95 patients (90%) received IFN-based combination therapy with RBV, according to our early treatment regimen. Ten patients were not eligible for our pre-emptive approach; in the case of two patients, the reason was attributable to early death, in case of six attributable to lack of consent, and in case of one patient, attributable to negative HCV-RNA after transplantation. One other patient was excluded because of poor condition, including multi-organ failure, during the immediate post-transplant period, which resulted in subsequent renal failure necessitating maintenance hemodialysis. This patient eventually received IFN monotherapy for recurrent HCV, resulting in viral eradication 23 months after LDLT, but died from the progression of pulmonary hypertension before achieving the ETR. For the remaining 95 patients, the median period from LDLT to IFN/RBV initiation was 26 days (range 10 days–6 months). The median follow-up period was 45 months (range 1–122 months).

Episode of biopsy-proven acute cellular rejection was confirmed in 21 of the 95 patients. Episode of rejection

took place prior to, or after the initiation of IFN-based combination therapy in nine (9%), and 12 (13%) patients respectively. The median period from LDLT to the initiation of antiviral treatment in the nine patients was 28 days (range 21–59 days), whereas the median interval from LDLT to rejection episodes was 11 days (range 5–29 days). In the 12 patients in whom rejection took place after the initiation of IFN-based combination therapy, the median period from LDLT to the initiation of antiviral treatment was 17 days (range 12–52 days), and the median period to rejection episode from LDLT was 44 days (range 18–577 days).

Viral response

Among the 95 recipients that underwent IFN-based therapy, 51 (54%) patients had negative HCV-RNA results at least once, among whom 43 patients experienced a sustained response for 12 months (ETR). Six patients who reached the ETR eventually presented with a viral relapse, and did not achieve an SVR. At the time of data collection, 32 (34%) achieved an SVR. None of the recipients that achieved SVR have presented with a viral relapse during the observed period. The median time to achieve a negative HCV-RNA, ETR, and SVR under the treatment regimen was 12 months (range 2–63 months), 25 months (13–79 months), and 28 months (19–67 months) respectively.

Consistent with the nature of a treatment protocol without a defined time endpoint, the response rate tended to increase over time. At 3 years, negative HCV-RNA status was obtained in 63%, ETR in 48%, and SVR in 34%. By the fifth year, negative HCV-RNA status was obtained in 70%, ETR in 68%, and SVR in 53% (Fig. 2a).

Hepatitis C virus infection genotype 1b, use of cyclosporine, and a lower rate of tolerated RBV dose presented with significantly poorer outcomes (Table 1). Multivariate analysis revealed HCV genotype 1b as the only independent factor resulting in a significantly poorer viral

Table 1. Sustained viral response in patients with combined treatment and clinical factors.

	Factors	No.	%SVR at 5 years	*P*
R-age	≤55	50	58	0.85
	>	45	47	
R-gender	Male	68	57	0.39
	Female	27	40	
MELD	<15	49	54	0.75
	≥	46	51	
HIV	Positive	4	67	0.17
	Negative	91	52	
HCC	Positive	55	41	0.08
	Negative	40	66	
Genotype	1b	78	43	<0.0001
	Non-1b	17	85	
HCV-RNA titer	≤250 K IU/ml (5.4 log)*	40	49	0.29
	>	55	53	
ACR	Yes	21	37	0.27
	No	74	56	
D-age	≤35	46	47	0.35
	>35	49	61	
D-gender	Male	59	45	0.59
	Female	36	71	
CyA	Yes	64	35	0.02
	No	31	61	
INF dosage†	≥60%	48	55	0.36
	<60%	47	58	
RBV dosage†	≥50%	54	69	0.02
	<50%	41	33	

No., number of patients; %SVR, percentage of patients achieving sustained viral response; R-age, age of the recipient at the time of transplantation; R-gender, gender of the recipient; MELD, Model for end-stage liver disease score; HIV, human immunodeficiency virus; HCC, hepatocellular carcinoma; HCV-RNA, hepatitis C viral ribonucleic acid; ACR, acute cellular rejection; D-age, age of the donor at the time of transplantation; D-gender, gender of the donor; CyA, cyclosporine A.
*During the study period, quantification of real-time RT-PCR introduced for linear quantification and detection of HCV-RNA.
†Actual levels of the given dosage at the time of data collection or final administration was recorded represented by means of percentile, 100% being the per-protocol full target dose.

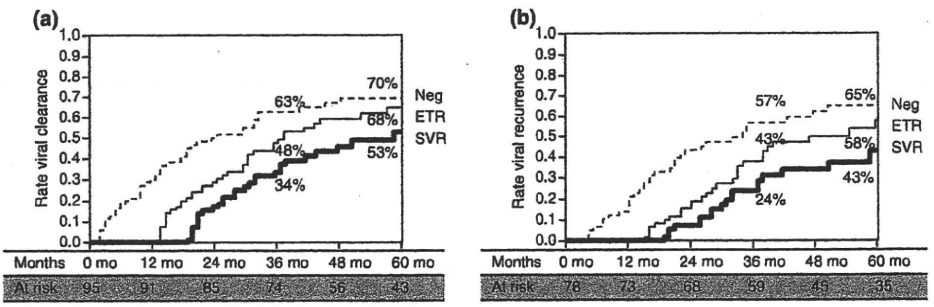

Figure 2. (a) Cumulative overall viral response depicted by Kaplan-Meier method. (b) Cumulative overall viral response of recipients with HCV genotype 1b depicted by Kaplan-Meier method. Neg, negative HCV-RNA; ETR, end of treatment response; SVR, sustained viral resposne; mo, months.

response (hazard ratio 0.263, 95% confidence interval 0.127–0.545, $P = 0.0003$). Of the recipients with an HCV genotype 1b, negative HCV-RNA status was obtained in 57%, ETR in 43%, and SVR in 24% at 3 years. By the fifth year, negative HCV-RNA status was obtained in 65%, ETR in 58%, and SVR in 43% (Fig. 2b).

Tolerability of IFN-based treatment

At the time of final data collection, a total of 24 (25%) patients had tolerated the full dose of INF, and eight (8%) patients had tolerated the planned full dose of RBV. The average dosage of INF tolerated among the 95 patients was 68% (SD 26%) of the full dose, and that of RBV was 41% (SD 24%, Fig. 3).

Tolerability in terms of rates of dosage of INF or RBV did not differ significantly between those with a viral response and those without (Fig. 4a–c). Lack of adherence to the planned target dose was common, but had no significant impact on the viral response within the observation period.

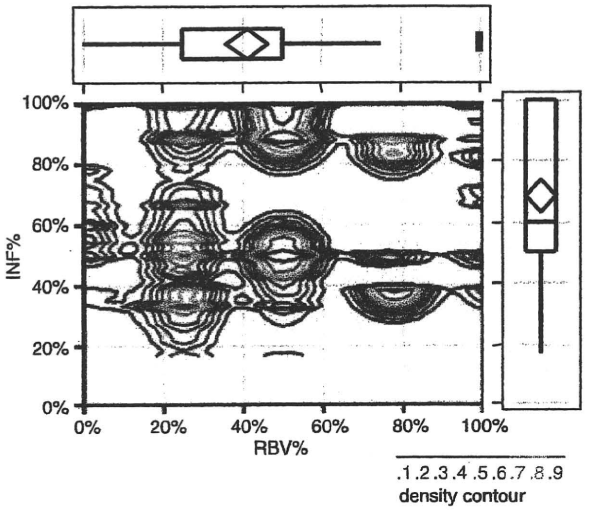

Figure 3. Actual tolerated rates of dosages of interferon (IFN) and ribavirin (RBV) by density contour plot. Box-and-whisker plots accompanying the vertical and horizontal axis represent the summery of IFN and RBV tolerated rates of dosages.

Survival

The overall mid-term rates of survival were not statistically different between HCV and non-HCV recipients (Fig. 5a). The short-term outcomes, however, were poorer in HCV recipients. At 2 years after transplantation, recipients with HCV presented with a significantly lower survival rate compared to non-HCV recipients (82% vs.

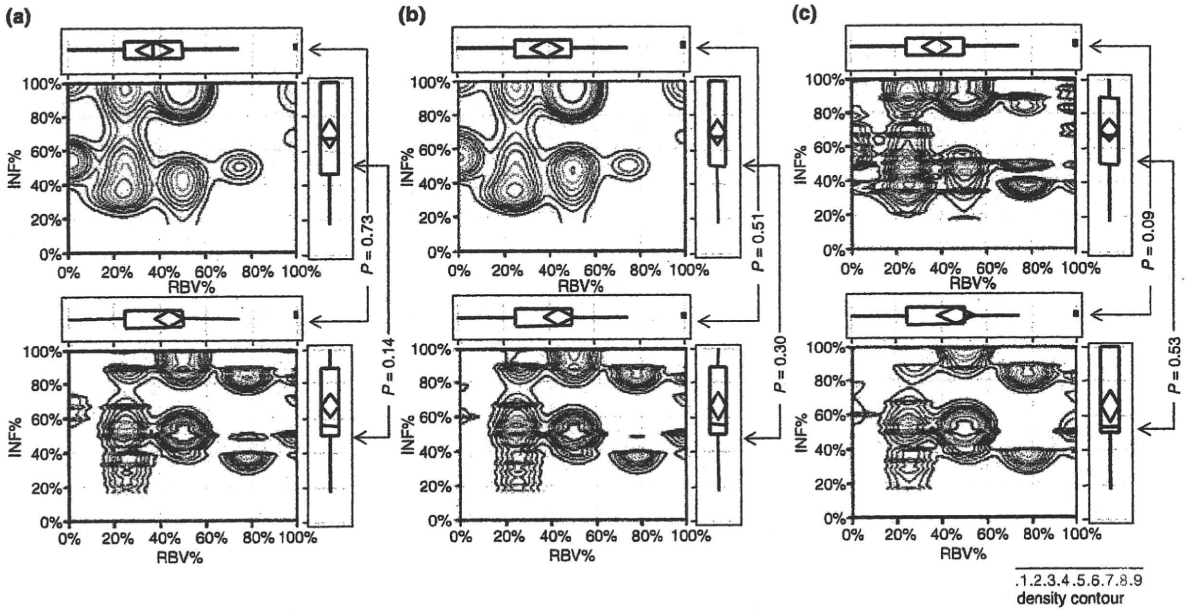

Figure 4. Actual tolerated rates of dosages of interferon (IFN) and ribavirin (RBV) by density contour plot according to viral response. Box-and-whisker plots accompanying vertical and horizontal axis represent the summery of IFN and RBV tolerated rates of dosages, respectively. Diamond in the box-plot represents the mean and 95% confidence interval. (a) Above: outcomes of patients that remained positive for HCV-RNA during the studied period. Below: outcomes of patients that demonstrated negative HCV-RNA results at least once during the studied period. (b) Above: outcomes of patients that did not achieve end-of-treatment (ETR). Below: outcomes of patients that achieved ETR. (c) Above: outcomes of patients that did not achieve sustained viral response (SVR). Below: outcomes of patients that achieved SVR (*P*-values by Mann-Whitney *U*-test).

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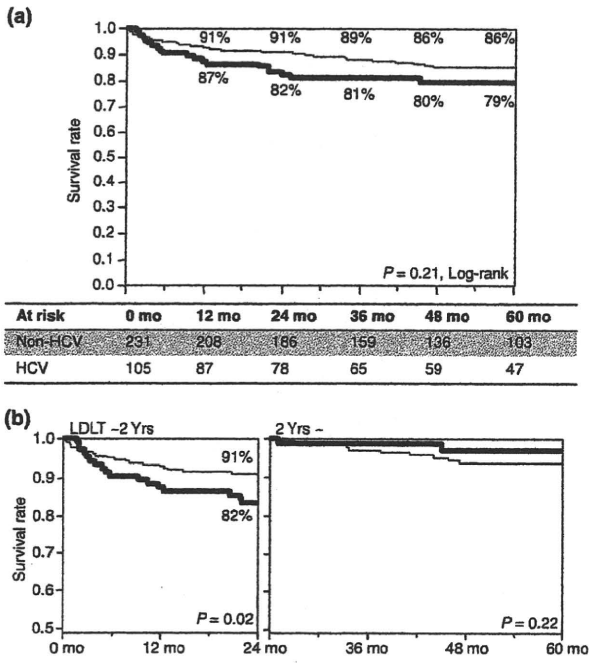

Figure 5. (a) Comparison of overall survival between hepatitis C virus infection (HCV) (n = 105) and non-HCV (n = 231) adult-to-adult LDLT recipients. Median follow up period of HCV, and non-HCV patients were 45 and 55 months, respectively. (b) Comparison of overall survival between HCV and non-HCV adult-to-adult LDLT recipients at 2 years post-LDLT and thereafter (bold lines indicate HCV patients). LDLT, living donor liver transplantation; mo, months.

91%, P = 0.02). Survival rate after the second year did not differ between HCV and non-HCV recipients (Fig. 5b).

Analysis of factors affecting the short-term survival rates indicated that viral titer prior to transplantation, viral response to treatment, acute cellular rejection, donor age, and donor gender were significant factors affecting the survival at 2 years (Table 2). Multivariate analysis revealed that a higher viral titer prior to transplantation, poor response to antiviral treatment, occurrence of acute cellular rejection, and older donor age were independently significant factors associated with poor survival (Table 3).

Discussion

In this study, as also considering the experience gained in the above-mentioned pilot series, data of a total of 105 adult patients with HCV that underwent LDLT at our institution over the past decade were collected and evaluated to validate our approach of pre-emptive treatment. Ninety-five patients were eligible and received pre-emptive antiviral therapy. The rate of complete viral eradication identified by an SVR within the observed follow-up period was comparable to a reported series of DDLT recipients with responsive treatment approaches (32 of 95 recipients, 34%). Unlike the outcome in the previously reported series with a fixed treatment period, however,

Table 2. Survival at 2-year after living donor liver transplantation and clinical factors.

	Factors	No.	%OS at 24 months	P
R-age	≤55	54	79	0.35
	>	51	86	
R-gender	Male	76	82	0.98
	Female	29	82	
MELD	<15	56	80	0.52
	≥	49	85	
HIV	Positive	6	67	0.23
	Negative	99	83	
HCC	Positive	60	79	0.39
	Negative	45	86	
Genotype	1b	84	82	0.85
	Non-1b	21	81	
HCV-RNA titer	≤250 K IU/ml (5.4 log)	49	92	0.02
	>	56	73	
Response to INF-RBV Tx	Yes	51	94	0.0005
	No	44	69	
ACR	Yes	27	63	0.0009
	No	78	89	
D-age	≤35	53	96	0.0002
	>35	52	67	
D-gender	Male	67	89	0.009
	Female	38	69	
CyA	Yes	34	76	0.29
	No	71	85	

No., number of patients; %OS, percentage of overall survival of patients; R-age, age of the recipient at the time of transplantation; R-gender, gender of the recipient; MELD, Model for end-stage liver disease score; HIV, human immune deficiency virus; HCC, hepatocellular carcinoma; HCV-RNA, hepatitis C viral ribonucleic acid; Response to INF-RBV Tx, Response to interferon ribavirin combination therapy indicated by negative serum HCV-RNA at one point or more; ACR, acute cellular rejection; D-age, age of the donor at the time of transplantation; D-gender, gender of the donor; CyA, cyclosporine A.

Table 3. Factors affecting survival at 2 years after living donor liver transplantation: a multivariate analysis.

Factors	Ratio	95% CI	P
Response to Tx	0.12	0.04–0.44	0.001
ACR	3.63	1.40–9.43	0.008
Age of the donor	8.20	1.84–36.6	0.006
HCV-RNA titer	3.30	1.04–10.5	0.04

Response to Tx, response to interferon combination therapy indicated by negative serum HCV-RNA at one point or more; ACR, acute cellular rejection; CI, confidence interval.

our current series indicates the possibility of improvement in the rate of viral eradication over a period of time with continued, non-stop application. Viral responses based on the Kaplan–Meier method demonstrate that a continued treatment is related to higher rates of viral response, as high as an expected rate of 70% for clearance of viremia, and 53% for SVR at 5 years post-LDLT (Fig. 2).

Another interesting implication of the results from our approach is the improvement in survival over the longer term. Extensive data on the outcomes of HCV patients after DDLT indicates that outcomes become poorer in later years when compared with non-HCV patients [16,17]. So far, this has not been the case for our LDLT series. The overall rate of survival after the second year following LDLT remains equivalent for HCV and non-HCV recipients (Fig. 5). In contrast to the acceptable mid-to-long term outcomes, however, our current series demonstrated poorer survival rates as compared with non-HCV recipients for the immediate short-term in HCV recipients. Analysis revealed higher viral titer prior to transplantation, poor response to antiviral treatment, occurrence of acute cellular rejection, and older donor age were significant risk factors for poorer short-term survival. This offers important insights for the management during this period.

Finally, our series demonstrated that adherence to the full target dose of INF or RBV is not mandatory. Patients who tolerated the full target dose were in the minority. The majority tolerated <70% of the intended dose of INF and less than half that of RBV (Fig. 3). The low tolerability for the target dose, however, did not have apparent disadvantage (Fig. 4). Reports in the recent literature suggest the benefits of sustained application of antiviral therapy at a lower dosage for normalizing liver function and preventing recurrent HCC in non transplant patients [47–49]. In the most recent report from the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C) study, a randomized controlled trial of PEG-IFN alpha-2a at a dosage of 90 μg/week for 3.5 years in the treatment arm indicated that there was no significant difference between groups in the rate of progression of liver disease, defined as death, HCC, or hepatic decompensation [50]. The studied population, however, was predominantly patients with advanced fibrosis who had not had any response to previous therapy with PEG-IFN and RBV. On the other hand, most interestingly, the report described significantly improved serum aminotransferase levels, decreased serum HCV-RNA levels, and improved histologic necroinflammatory scores. Kuo *et al.* [51] reported a reduced risk of fibrosis progression, even among virologic nonresponders who underwent pre-emptive treatment that was limited to 48 weeks. These outcomes may support, in part, the application of prolonged treatment initiated pre-emptively in liver transplant recipients with un-injured liver grafts, and are encouraging to our approach.

In conclusion, pre-emptive antiviral treatment with combined IFN-based therapy is feasible and effective in LDLT for HCV. The application of a non-stopping, flexible dose adjustment approach for further improvement in the outcomes is warranted in the LDLT setting.

Authorship

ST, YS and MM: designed study. ST, YS, JK, NK, and MM: performed study. ST, YS, NY, JK: collected data. ST and YS: wrote the paper.

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References

1. Poynard T, Bedossa P, Opolon I. Natural history of liver fibrosis progression in patients with chronic hepatitis C. *Lancet* 1997; **349**: 825.
2. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41.
3. Armstrong GL, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med* 2006; **144**: 705.
4. Kiyosawa K, Umemura T, Ichijo T, *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127**: S17.
5. Kuo A, Terrault NA. Management of hepatitis C in liver transplant recipients. *Am J Transplant* 2006; **6**: 449.
6. Hoofnagle JH, Seeff LB. Peginterferon and ribavirin for chronic hepatitis C. *N Engl J Med* 2006; **355**: 2444.
7. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* 2006; **55**: 1350.
8. Dienstag JL, McHutchison JG. American Gastroenterological Association technical review on the management of hepatitis C. *Gastroenterology* 2006; **130**: 231.
9. Dienstag JL, McHutchison JG. American Gastroenterological Association medical position statement on the management of hepatitis C. *Gastroenterology* 2006; **130**: 225.

10. Berenguer M, Lopez-LabradBor FX, Wright TL. Hepatitis C and liver transplantation. *J Hepatol* 2001; **35**: 666.
11. Powers KA, Ribeiro RM, Patel K, *et al.* Kinetics of hepatitis C virus reinfection after liver transplantation. *Liver Transpl* 2006; **12**: 207.
12. Garcia-Retortillo M, Forns X, Feliu A, *et al.* Hepatitis C virus kinetics during and immediately after liver transplantation. *Hepatology* 2002; **35**: 680.
13. Berenguer M, Ferrell L, Watson J, *et al.* HCV-related fibrosis progression following liver transplantation: increase in recent years. *J Hepatol* 2000; **32**: 673.
14. Yilmaz N, Shiffman ML, Stravitz RT, *et al.* A prospective evaluation of fibrosis progression in patients with recurrent hepatitis C virus following liver transplantation. *Liver Transpl* 2007; **13**: 975.
15. Kalambokis G, Manousou P, Samonakis D, *et al.* Clinical outcome of HCV-related graft cirrhosis and prognostic value of hepatic venous pressure gradient. *Transpl Int* 2009; **22**: 172.
16. Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; **122**: 889.
17. Thuluvath PJ, Krok KL, Segev DL, Yoo HY. Trends in post-liver transplant survival in patients with hepatitis C between 1991 and 2001 in the United States. *Liver Transpl* 2007; **13**: 719.
18. Mutimer DJ, Gunson B, Chen J, *et al.* Impact of donor age and year of transplantation on graft and patient survival following liver transplantation for hepatitis C virus. *Transplantation* 2006; **81**: 7.
19. Rowe IA, Webb K, Gunson BK, Mehta N, Haque S, Neuberger J. The impact of disease recurrence on graft survival following liver transplantation: a single centre experience. *Transpl Int* 2008; **21**: 459.
20. Schmitt TM, Kumer SC, Pruett TL, Argo CK, Northup PG. Advanced recipient age (>60 years) alone should not be a contraindication to liver retransplantation. *Transpl Int* 2009; **22**: 601.
21. Samuel D, Forns X, Berenguer M, *et al.* Report of the monothematic EASL conference on liver transplantation for viral hepatitis (Paris, France, January 12–14, 2006). *J Hepatol* 2006; **45**: 127.
22. Carrión JA, Navasa M, García-Retortillo M, *et al.* Efficacy of antiviral therapy on hepatitis C recurrence after liver transplantation: a randomized controlled study. *Gastroenterology* 2007; **132**: 1746.
23. Wang CS, Ko HH, Yoshida EM, Marra CA, Richardson K. Interferon-based combination anti-viral therapy for hepatitis C virus after liver transplantation: a review and quantitative analysis. *Am J Transplant* 2006; **6**: 1586.
24. Troppmann C, Rossaro L, Perez RV, McVicar JP. Early, rapidly progressive cholestatic hepatitis C reinfection and graft loss after adult living donor liver transplantation. *Am J Transplant* 2003; **3**: 239.
25. Ghobrial RM, Amersi F, Douglas GF, *et al.* Rapid and early HCV recurrence following adult living donor liver transplantation. *Am J Transplant* 2002; **2**: 163.
26. Schiano TD, Gutierrez JA, Walewski JL, *et al.* Accelerated hepatitis C virus kinetics but similar survival rates in recipients of liver grafts from living versus deceased donors. *Hepatology* 2005; **42**: 1420.
27. Garcia-Retortillo M, Forns X, Llovet JM, *et al.* Hepatitis C recurrence is more severe after living donor compared to cadaveric liver transplantation. *Hepatology* 2004; **40**: 699.
28. Russo MW, Galanko J, Beavers K, Fried MW, Shrestha R. Patient and graft survival in hepatitis C recipients after adult living donor liver transplantation in the United States. *Liver Transpl* 2004; **10**: 340.
29. Van Vlierberghe H, Troisi R, Colle I, Ricciardi S, Praet M, de Hemptinne B. Hepatitis C infection-related liver disease: patterns of recurrence and outcome in cadaveric and living-donor liver transplantation in adults. *Transplantation* 2004; **77**: 210.
30. Sugawara Y, Makuuchi M. Should living donor liver transplantation be offered to patients with hepatitis C virus cirrhosis? *J Hepatol* 2005; **42**: 472.
31. Mazzaferro V, Tagger A, Schiavo M, *et al.* Prevention of recurrent hepatitis C after liver transplantation with early interferon and ribavirin treatment. *Transplant Proc* 2001; **33**: 1355.
32. Shergill AK, Khalili M, Straley S, *et al.* Applicability, tolerability and efficacy of preemptive antiviral therapy in hepatitis C-infected patients undergoing liver transplantation. *Am J Transplant* 2005; **5**: 118.
33. Wiesner RH, Sorrell M, Villamil F. Report of the first international liver transplant society consensus conference on liver transplantation and hepatitis C. *Liver Transpl* 2003; **9**: S1.
34. Sugawara Y, Makuuchi M. Living donor liver transplantation for patients with hepatitis C virus cirrhosis: Tokyo experience. *Clin Gastroenterol Hepatol* 2005; **3**: S122.
35. Takada Y, Haga H, Ito T, *et al.* Clinical outcomes of living donor liver transplantation for hepatitis C virus (HCV)-positive patients. *Transplantation* 2006; **81**: 350.
36. Sugawara Y, Makuuchi M, Sano K, *et al.* Vein reconstruction in modified right liver graft for living donor liver transplantation. *Ann Surg* 2003; **237**: 180.
37. Kokudo N, Sugawara Y, Imamura H, Sano K, Makuuchi M. Tailoring the type of donor hepatectomy for adult living donor liver transplantation. *Am J Transplant* 2005; **5**: 1694.
38. Tamura S, Sugawara Y, Kokudo N. Donor evaluation and hepatectomy for living-donor liver transplantation. *J Hepatobiliary Pancreat Surg* 2008; **15**: 79.
39. Yamashiki N, Sugawara Y, Tamura S, *et al.* Selection of liver-transplant candidates for adult-to-adult living donor liver transplantation as the only surgical option for end-stage liver disease. *Liver Transpl* 2006; **12**: 1077.

40. Kishi Y, Sugawara Y, Akamatsu N, *et al.* Splenectomy and preemptive interferon therapy for hepatitis C patients after living-donor liver transplantation. *Clin Transplant* 2005; **19**: 769.
41. Gedaly R, Clifford TM, McHugh PP, Jeon H, Johnston TD, Ranjan D. Prevalent immunosuppressive strategies in liver transplantation for hepatitis C: results of a multi-center international survey. *Transpl Int* 2008; **21**: 867.
42. Sugawara Y, Makuuchi M, Kaneko J, Ohkubo T, Imamura H, Kawarasaki H. Correlation between optimal tacrolimus doses and the graft weight in living donor liver transplantation. *Clin Transplant* 2002; **16**: 102.
43. Tamura S, Sugawara Y, Kishi Y, *et al.* Conversion to cyclosporine provides valuable rescue therapy for living donor adult liver transplant patients intolerant to tacrolimus: a single-center experience at the University of Tokyo. *Transplant Proc* 2004; **36**: 3242.
44. Tamura S, Sugawara Y, Matsui Y, *et al.* Thrombotic microangiopathy in living-donor liver transplantation. *Transplantation* 2005; **80**: 169.
45. Simmonds P, Bukh J, Combet C, *et al.* Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962.
46. Sugawara Y, Makuuchi M, Matsui Y, *et al.* Preemptive therapy for hepatitis C virus after living-donor liver transplantation. *Transplantation* 2004; **78**: 1308.
47. Akuta N, Suzuki F, Kawamura Y, *et al.* Efficacy of low-dose intermittent interferon-alpha monotherapy in patients infected with hepatitis C virus genotype 1b who were predicted or failed to respond to pegylated interferon plus ribavirin combination therapy. *J Med Virol* 2008; **80**: 1363.
48. Kudo M, Sakaguchi Y, Chung H, *et al.* Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case–control study. *Oncology* 2007; **72**: 132.
49. Mazzaferro V, Romito R, Schiavo M, *et al.* Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. *Hepatology* 2006; **44**: 1543.
50. Di Bisceglie AM, Shiffman ML, Everson GT, *et al.* Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. *N Engl J Med* 2008; **359**: 2429.
51. Kuo A, Tan V, Lan B, *et al.* Long-term histological effects of preemptive antiviral therapy in liver transplant recipients with hepatitis C virus infection. *Liver Transpl* 2008; **14**: 1491.

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Sustained High Levels of Serum Interferon-γ During HIV-1 Infection: A Specific Trend Different from Other Cytokines

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Abstract

The expression levels of various cytokines increase with the progression of HIV-1 infection. However, the effects of antiretroviral therapy (ART) on serum cytokine levels have not been fully determined. In this study we measured serum cytokine levels of 35 HIV-1-infected Japanese adults. We first performed a cross-sectional study and observed that TNF-α, IL-6, IL-10, IL-18, and IL-7 levels all showed significant increases in those with advanced disease, and that this had a significant negative correlation with the CD4 cell count. However, IFN-γ levels did not show this relationship. A longitudinal study in 18 HIV-1-infected patients with a CD4 cell count <350/μL revealed that the introduction of ART reduced cytokine levels. Significant reductions of IL-7, IL-10, IFN-γ, and IL-18 levels were observed on days 30, 60, 90, and 90 after the initiation of ART, respectively. These results indicate a discrepancy between cross-sectional and longitudinal studies of serum levels of IFN-γ. To clarify this, we investigated serum IFN-γ levels in each patient. In 5 of the 15 patients IFN-γ levels did not decrease, even after ART initiation, and remained at 5 pg/mL or higher on day 120 after ART initiation. Higher IFN-γ levels (>5 pg/mL) were also observed in 2 of 7 asymptomatic patients, and 2 of 11 patients who underwent ART for 1 year or longer. These data demonstrate that IFN-γ levels in some patients increased and remained high even after the initiation of ART, which was a specific observation different from those of the other cytokines.

Introduction

CYTOKINES ARE INTERCELLULAR SIGNALING MOLECULES that regulate the differentiation, proliferation, and activation of immune cells (11,12). They are primarily secreted by immune cells, and they exert their biological effects by binding to specific receptors on these (autocrine) or other target cells. Cytokines serve as the immune response molecules against infective microorganisms, and also have various physiological functions in inflammation, allergy, development, and hematopoiesis. The cytokines include interferon (IFN), tumor necrosis factor (TNF), and colony-forming factor, as well as the interleukins (ILs), which all show different functions *in vivo*. Common characteristics shared by these cytokines include effectiveness, even in trace amounts, transient secretion, and a short half-life. Thus serum cytokine levels are generally low and are often undetectable in healthy individuals. Another common feature of cytokines is the presence of complicated networks or cascades, which regulate their mutual effects additively, synergistically, or antagonistically. The Th-1/Th-2 balance represents one of these networks (6). This balance determines the differentiation of naive T cells into Th-1 cells for cellular immunity, or Th-2 cells for humoral immunity.

Human immunodeficiency virus (HIV)-1 infects $CD4^+$ cells to destroy the immune system, leading to the development of acquired immunodeficiency syndrome (AIDS). After HIV-1 infection, 5–10 y will pass without symptoms in most patients. This period is called the asymptomatic carrier (AC) period. Although apparently asymptomatic in this period, HIV-1 gradually destroys the immune system and decreases the number of $CD4^+$ T lymphocytes (the CD4 cell count). When the CD4 count drops below 200/μL, various opportunistic infections, including AIDS indicator diseases, will develop. Antiretroviral therapy (ART) with multiple agents was developed in the second half of the 1990s, markedly improving the prognosis of HIV-1-infected patients. This therapy is called highly-active antiretroviral therapy (HAART), which continuously suppresses viral replication and restores the function of the immune system in HIV-1-infected patients.

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Many studies have been conducted on the importance of cytokines in the pathogenesis of HIV-1 infection because it affects the immune system (11). In the pre-HAART era, the expression levels of various cytokines reportedly increased, along with the progression of immunodeficiency (1,3). Since in previous studies researchers have reported that a Th-1/Th-2 imbalance is strongly involved in HIV pathogenesis (5,7), the cytokines responsible for the Th-1/Th-2 balance, such as IL-2 (18), IL-6 (4), IL-10 (2), and IFN-γ, have been studied in detail (1). On the other hand, in the post-HAART era, the increased cytokine expression was decreased by ART (14,16,22,23,25). However, only a few cytokines examined in the pre-HAART era that exhibit abnormal expression have been studied. It has not yet been determined whether this abnormal cytokine expression is caused by biological responses to viral proliferation, or whether it is induced secondary to other infections caused by the immunodeficiency. In fact, the abnormal cytokine expression may be caused by latent opportunistic infections due to a decreased CD4 cell count. In this study we investigated the cytokines with expression levels that differed from those of the other cytokines, by measuring their serum levels primarily in patients who underwent ART, in order to identify the cytokines directly involved in HIV-1 infection.

Materials and Methods

Patients

We first obtained written informed consent from 35 HIV-1-infected adults who regularly visited our hospital, and collected sera from these patients. The median age of the patients (34 male and 1 female) was 39 y (range 28–73 y). All of the patients were Japanese. The putative infection routes

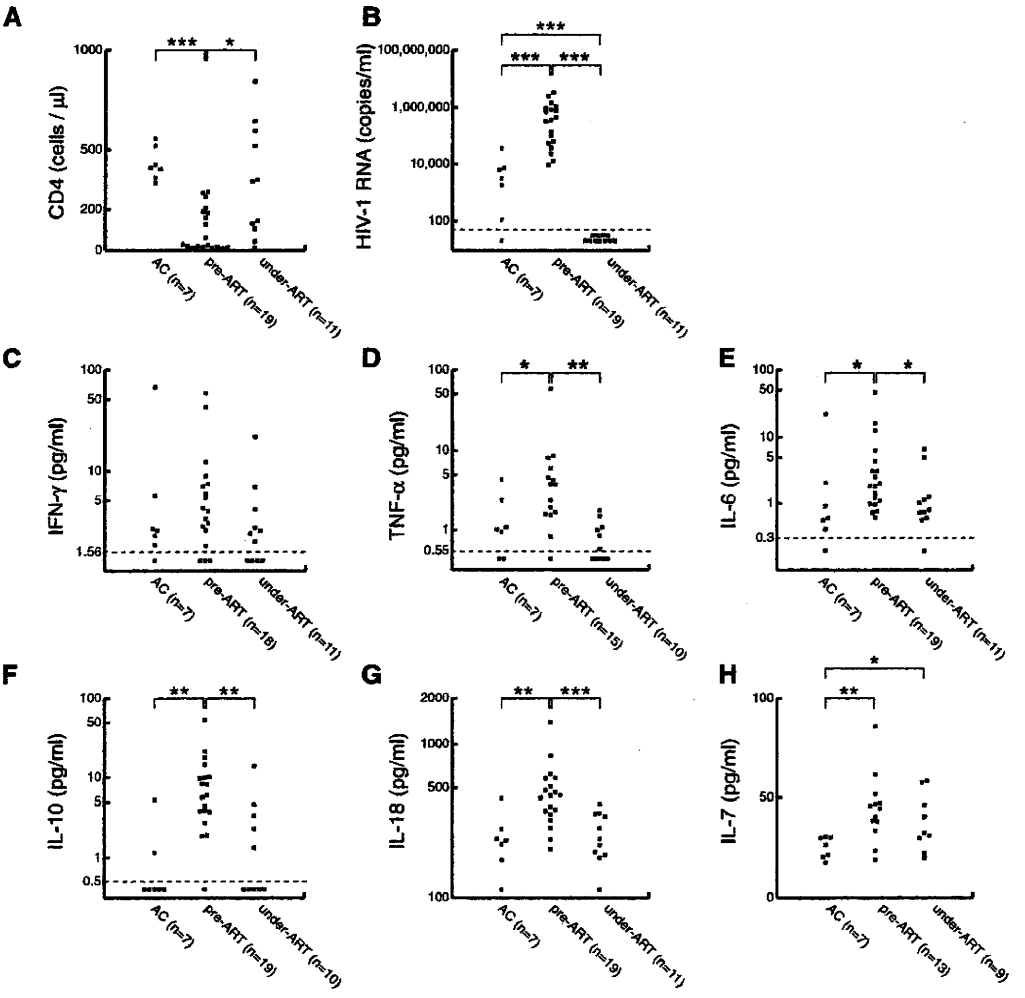

FIG. 1. Serum cytokine levels in asymptomatic carrier (AC), pre-antiretroviral therapy (pre-ART), and under-ART groups. (A and B) Shown are the CD4 cell counts and HIV-1 RNA levels of the HIV-1-infected subjects in the three study groups. (C–H) These plots show the values of the indicated cytokines in the three study groups. Statistical comparisons were made using the Kruskal-Wallis test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). ◀AU1

of these patients included homosexual contact (29 patients), heterosexual contact (5 patients), and transfusion for hemophilia (1 patient). We then measured the CD4 cell counts by flow cytometry using the whole-blood lysis method. Plasma HIV-1 RNA levels were measured using reverse-transcriptase PCR (Amplicor HIV-1 monitor test; Roche Molecular Diagnostics Corp., Indianapolis, IN). This study was reviewed and approved by the Institutional Review Board, National Hospital Organization, Osaka National Hospital (approval number 0542).

Cross-sectional and longitudinal studies

The patients were divided into the following three groups: AC, pre-ART, and under-ART. They were then subjected to a cross-sectional study to compare serum cytokine levels. The AC group included asymptomatic patients with CD4 cell counts of 350/μL or higher. The pre-ART group included untreated patients (ART naive) with CD4 cell counts less than 350/μL, from whom samples were collected after treatment for opportunistic infections. The under-ART group included patients who underwent ART for 1 year or longer, and whose plasma HIV-1 RNA levels were below the detection limit. Patients in the pre-ART group were subjected to a longitudinal study, and samples were collected from them periodically after ART introduction for comparison with the baseline.

Measurement of serum cytokines

Serum cytokine levels were measured with a sandwich enzyme-linked immunosorbent assay (ELISA). The following reagents were used: IFN-γ (human IFN-γ ELISA; Bender MedSystems, Vienna, Austria), IL-6 (Quanti Glo human IL-6 chemiluminescent immunoassay; R&D Systems, Inc., Minneapolis, MN), IL-10 (human IL-10 ultra-sensitive ELISA kit; Invitrogen, Carlsbad, CA), IL-18 (human IL-18 ELISA; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), IL-1 (Quantikine HS human IL-1 immunoassay; R&D Systems), IL-2 (Quantikine human IL-2 immunoassay; R&D Systems), IL-4 (human IL-4 ultra-sensitive immunoassay; R&D Systems), IL-12 (Quantikine HS human IL-12 immunoassay; R&D Systems), IL-7 (Quantikine HS human IL-7 immunoassay; R&D Systems), TNF-α (Quanti Glo human TNF-α chemiluminescent immunoassay; R&D Systems), IL-17 (Quantikine human IL-17 immunoassay; R&D Systems), and IL-23 (Quantikine human IL-23 immunoassay; R&D Systems). The measurements were carried out according to the manufacturers' instructions.

Statistical analysis

Multiple comparisons were carried out using the Kruskal-Wallis non-parametric analysis of variance (ANOVA) test. The Spearman rank test was used for correlation, and the Wilcoxon signed-rank test was used for paired data. The significance level was set at $p < 0.05$.

Results

We measured the serum cytokine levels in 35 HIV-1-infected patients. IL-1β, IL-2, IL-4, IL-12, IL-17, and IL-23 were not considered in the analysis because they were below the detection limits in the majority of the samples (60% or above) (data not shown). To examine the relationship between the serum cytokine levels and HIV-1 infection, the patients were classified into three groups on the basis of their CD4 counts and the introduction of ART: AC, pre-ART, and under-ART groups, and their serum cytokine levels were compared (cross-sectional study). The CD4 counts and plasma HIV-1 RNA levels of the groups are shown in Fig. 1A and B. As presented in Fig. 1 D–G, serum TNF-α, IL-6, IL-10, and IL-18 levels in the pre-ART group showed significant increases compared with the corresponding values in the AC and under-ART groups. The IL-7 levels were significantly higher in the pre-ART group than in the AC group, but comparable

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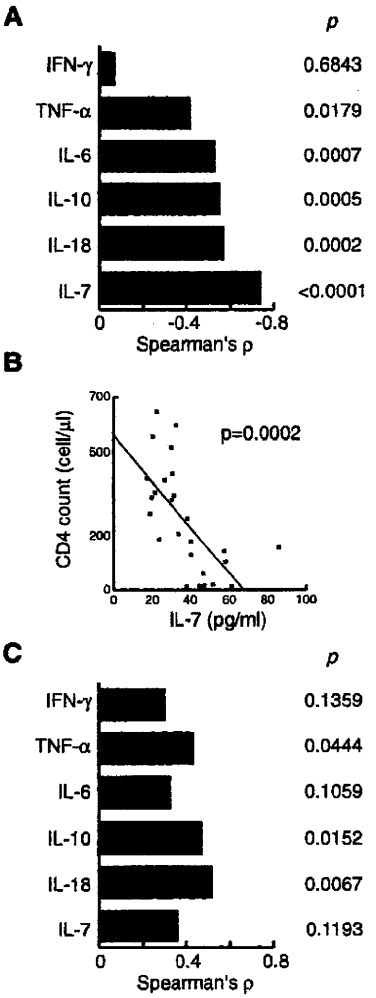

FIG. 2. Association of serum cytokine levels in HIV-1-infected patients with CD4 cell counts and plasma HIV-1 RNA levels. **(A)** The bars indicate Spearman's rank correlation coefficients (ρ). **(B)** Linear regression analysis was used to investigate the relationship of IL-7 levels with CD4 cell counts (slope = −8.3, $r^2 = 0.42$). **(C)** Correlation with plasma HIV-1 RNA levels. The bars indicate Spearman's rank correlation coefficients (ρ).

with those in the under-ART group (Fig. 1H). On the other hand, IFN-γ levels did not differ significantly among the three groups (Fig. 1C). Thus, except for IFN-γ, all of the other cytokines were found to be increased in the immunocompromised patients.

Subsequently, the correlation between the serum cytokine levels and CD4 counts was analyzed using Spearman's rank test. Except for IFN-γ, all of the other cytokines exhibited a F2 ▶ significant negative correlation with the CD4 cell counts (Fig. 2A). Among the cytokines we examined, IL-7 levels showed the strongest negative correlation (Spearman's $\rho = -0.74$); a similarly negative correlation was noted in the regression analysis (Fig. 2B). Serum cytokine and plasma HIV-1 RNA levels were also examined. Since the plasma HIV-1 RNA levels were below the detection limit in all of the patients in the under-ART group, and this might have led to bias-based errors, the under-ART group was excluded from this assay. A significant but weak correlation was noted between TNF-α/IL-18 and plasma HIV-1 RNA levels (Fig. 2C). Thus except for IFN-γ, the levels of the cytokines analyzed in this study increased with disease progression, and correlated with clinical indicators such as decreased CD4 cell counts and increased plasma HIV-1 RNA levels.

Finally, a longitudinal study was conducted on the pre-ART group to examine the effects of ART on serum cytokine levels. ART was introduced for all 19 patients in the pre-ART group. One patient in this group did not show an optimal virological response 24 wk after the introduction of ART, so this patient was excluded from the analysis. Fig. 3 shows the ◀ F3 serum cytokine levels before the introduction of ART, and on

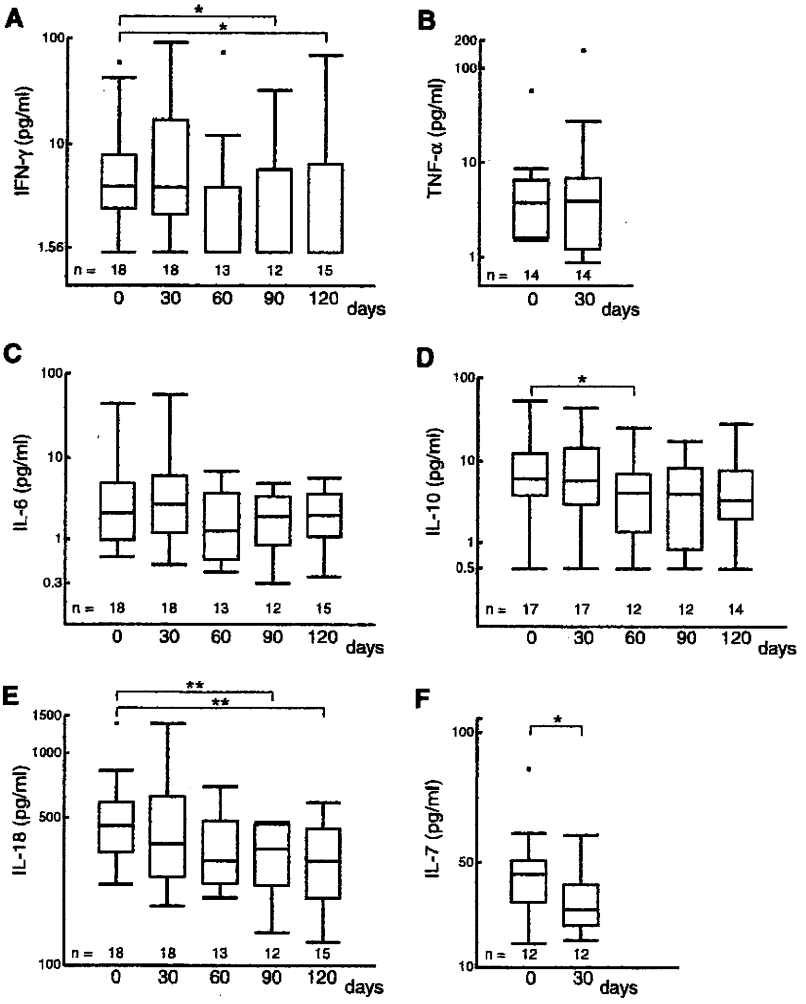

FIG. 3. Serum cytokine levels in HIV-1-infected patients before initiation of and during ART. The values shown of the indicated cytokines before, and at 30, 60, 90, and 120 d from the start of ART, are shown using box-and-whisker plots, representing the minimum, 25th percentile, median, 75th percentile, maximum, and outlying values. Statistical analyses versus baseline levels were carried out using Wilcoxon's matched-pair signed-rank test ($^*p < 0.05$, $^{**}p < 0.01$). ◀ AU2

days 30, 60, 90, and 120 after its introduction. On day 30, only IL-7 levels showed significant decreases compared with baseline levels. Some cytokines (IL-10 and IL-18) showed no change, while others (IFN-γ, IL-6, and TNF-α) showed upward trends. Four cytokines (IFN-γ, IL-6, IL-10, and IL-18), when measured over time, revealed downward trends on day 60 and beyond; IL-10 showed a significant decrease on day 60, and IFN-γ and IL-18 showed significant decreases on days 90 and 120. Thus, the IL-7 level rapidly declined after the initiation of ART, while the expression of the other cytokines decreased slightly later.

There was no correlation between the pre-ART and under-ART groups for IFN-γ (Fig. 1C). However, our longitudinal observations demonstrated a significant decrease in IFN-γ levels upon initiation of ART (Fig. 3A). To resolve this discrepancy, we examined the patients in the pre-ART group in more detail. In most of the patients, the IFN-γ levels were gradually suppressed by ART. However, in 5 of the 15 patients, IFN-γ levels did not decrease, even after the initiation of ART, and remained at 5 pg/mL or higher at day 120 after ART initiation, regardless of the ART-induced virological response. The data of two representative patients are shown in Fig. 4. In the patients shown in Fig. 4A, whose plasma HIV-1 RNA levels were maintained below the detection limit, the IFN-γ levels were above 30 pg/mL, even at 3 y after the initiation of ART. In addition, 2 of 7 patients in the AC group, and 2 of 11 patients in the under-ART group, had higher IFN-γ levels (>5 pg/mL). Thus, during the AC period or later, the IFN-γ levels in some patients increased, and remained high even after the initiation of ART.

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Discussion

Here we demonstrated changes in the serum cytokine levels in HIV-1-infected patients. The serum levels of many cytokines increased with disease progression, and were decreased by the initiation of ART. The abnormal cytokine expression patterns may be explained by two possible mechanisms: the direct effect of immune destruction by HIV-1, and the effects of opportunistic infection. In this study, 14 (74%) patients in the pre-ART group developed AIDS. However, since the samples were collected from all of the patients after the treatment of opportunistic infections, the effects of these opportunistic infections on abnormal cytokine expression patterns may be limited. On the other hand, it is important to note that there were changes in the cytokine levels after the initiation of ART. On day 30 after ART introduction in the pre-ART group, all cytokines except for IL-7 remained unchanged or increased. At this point, the HIV-1 RNA levels in the blood were decreased in all 18 patients, and the CD4 cell counts were increased in 16 (89%) patients. This indicates that the cytokine levels increased despite virological suppression and immune restoration. Immune reconstitution inflammatory syndrome (IRIS), a seemingly paradoxical pathological condition, has been extensively described (20). This is a condition in which the existing opportunistic infection is exacerbated, and/or a new opportunistic infection develops after the introduction of ART, presumably due to the restoration of immune responses against a pathogen that existed prior to ART. Of the 18 patients in the pre-ART group, only 2 developed clinically apparent IRIS. One patient experienced a relapse of an existing CMV infection, and the other patient newly developed an atypical mycobacterial infection, an AIDS-indicator disease, after the introduction of ART. In addition to these 2 patients, several other patients developed IRIS, but required no specific treatment, at 2–4 weeks after initiation of ART. In these patients, the increased cytokine levels observed on day 30 after ART introduction were associated with immune restoration, suggesting that these immune responses may have been mounted against a potential

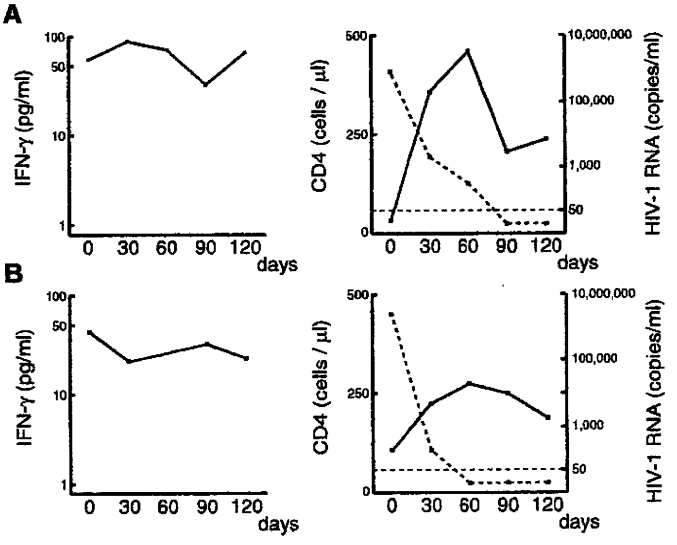

FIG. 4. Sustained elevation of serum IFN-γ levels during ART in HIV-1-infected individuals. Shown are values of serum IFN-γ (left), CD4 cell counts (right, solid lines), and levels of plasma HIV-1 RNA (right, dashed lines), of two typical patients at the indicated time points after the initiation of ART. ◀AU3

infectious agent. The direct effects of HIV-1 infection cannot be completely overlooked. However, the abnormal cytokine levels seen in immunocompromised patients may be at least partially associated with opportunistic infections.

After ART initiation, many cytokines showed no change or showed upward trends; on the other hand, IL-7 rapidly decreased. This appeared to be associated with the physiological actions of IL-7. Other cytokines are associated with immune responses and inflammatory reactions against microorganisms; however, the main function of IL-7 is for hematopoiesis (24). IL-7 mainly acts on hematopoietic stem cells, and induces their differentiation into T lymphocytes. The IL-7 levels were higher in the under-ART group, including those patients with poor recovery of CD4 cell counts, compared to the AC group (Fig. 1H). In addition, the IL-7 levels and CD4 counts show a marked negative correlation (Fig. 2B). These results are consistent with findings previously reported (14,15), suggesting that IL-7 is physiologically induced by decreased CD4 cell counts. Currently, IL-7 is receiving attention as a cytokine that increases the CD4 cell count (17), and is the focus of many clinical studies investigating whether IL-7 administration may induce $CD4^+$ lymphocyte expansion in HIV-1-infected patients (13,19).

In this study we demonstrated characteristic increases in IFN-γ levels due to HIV-1 infection. ART has been reported to decrease the serum levels of many of the cytokines that are elevated in patients with HIV-1 infection (16,22,23,25), and increases the levels of IL-21, which are reduced in patients with HIV-1 infection (9,10). To the best of our knowledge, ours is the first study to report that cytokine levels remain unchanged by ART, despite their abnormally high levels. IFN-γ levels were high in some patients not only during the AC period, but also in patients with sustained suppression of viral replication and immune restoration by ART. This suggests the potential induction of IFN-γ expression by HIV-1. This may be the first report on the above-mentioned phenomenon, probably because these high levels are not necessarily sustained in all cases, and thus changes occurring following the initiation of ART should be studied in more detail. Although the sustained high IFN-γ levels observed in some patients is thought to be due to individual differences in immune responses against HIV-1, or the genetic characteristics of HIV-1 (8), or both, we could find no clinical data associated with increased serum IFN-γ levels to support this. Unlike the total CD4 cell counts and viral loads presented here, the total CD8 cell counts and their kinetics after the initiation of ART were not associated with changes in IFN-γ levels (data not shown). However, the IFN-γ levels were increased in 9 of 33 patients (27%), and these patients account for a significant proportion, thus yielding important findings. IFN-γ is a cytokine used as an immunocompetence indicator in HIV-1 vaccine studies (21). It has been reported that Th-2 cell numbers tend to increase with the progression of HIV-1 infection, and that IFN-γ is one of the key cytokines for differentiation into Th-1 cells (6). Thus IFN-γ may play an essential role, different from those of other cytokines, in the pathogenesis of HIV-1 infection. One possible mechanism behind the sustained high serum IFN-γ levels seen despite ART's introduction may be that IFN-γ production by HIV-1-specific $CD8^+$ T lymphocytes is driven by HIV-1 viremia, and could even be induced by ongoing viral replication during ART. Only a small population of $CD8^+$ T lymphocytes may be involved in IFN-γ production, because there was no association between total CD8 cell counts and serum IFN-γ levels. In future studies, we intend to investigate the role of this cytokine with a focus on acute HIV-1 infection, in which HIV-1-specific $CD8^+$ T lymphocytes are preferentially expanded to control viral replication.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

References

1. Alonso K, Pontiggia P, Medenica R, and Rizzo S: Cytokine patterns in adults with AIDS. Immunol Invest 1997;26:341–350.
2. Ameglio, F., Cordiali Fei P, Solmone M, *et al.*: Serum IL-10 levels in HIV-positive subjects: correlation with CDC stages. J Biol Regul Homeost Agents 1994;8:48–52.
3. Aziz N, Nishanian P, and Fahey JL: Levels of cytokines and immune activation markers in plasma in human immunodeficiency virus infection: quality control procedures. Clin Diagn Lab Immunol 1998;5:755–761.
4. Breen EC, Rezai AR, *et al.*: Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 1990; 144:480–484.
5. Clerici M, Hakim FT, Venzon DJ, Blatt S, Hendrix CW, Wynn TA, and Shearer GM: Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. J Clin Invest 1993;91:759–765.
6. Clerici M, and Shearer GM: The Th1-Th2 hypothesis of HIV infection: new insights. Immunol Today 1994;15:575–581.
7. Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS, and Shearer GM: Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. J Clin Invest 1989; 84:1892–1899.
8. Greco G, Fujimura SH, Mourich DV, and Levy JA: Differential effects of human immunodeficiency virus isolates on beta-chemokine and gamma interferon production and on cell proliferation. J Virol 1999;73:1528–1534.
9. Iannello A, Boulassel MR, Samarani S, *et al.*: Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study. J Immunol 2010;184:114–126.
10. Iannello A, Tremblay C, Routy JP, Boulassel MR, Toma E, and Ahmad A: Decreased levels of circulating IL-21 in HIV-infected AIDS patients: correlation with CD4+ T-cell counts. Viral Immunol 2008;21:385–388.

11. Kedzierska K, and Crowe SM: Cytokines and HIV-1: interactions and clinical implications. Antivir Chem Chemother 2001;12:133–150.
12. Kishimoto T, Akira S, and Taga T: Interleukin-6 and its receptor: a paradigm for cytokines. Science 1992;258:593–597.
13. Levy Y, Lacabaratz C, Weiss L, *et al.*: Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. J Clin Invest 2009;119:997–1007.
14. Llano A, Barretina J, Gutierrez A, Blanco J, Cabrera C, Clotet B, and Este JA: Interleukin-7 in plasma correlates with CD4 T-cell depletion and may be associated with emergence of syncytium-inducing variants in human immunodeficiency virus type 1-positive individuals. J Virol 2001;75:10319–10325.
15. Napolitano LA, Grant RM, Deeks SG, *et al.*: Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med 2001; 7:73–79.
16. Orsilles MA, Pieri E, Cooke P, and Caula C: IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active antiretroviral therapy. APMIS 2006;114:55–60.
17. Rosenberg SA, Sportes C, Ahmadzadeh M, *et al.*: IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. J Immunother 2006;29:313–319.
18. Scott-Algara D, Vuillier F, Marasescu M, de Saint Martin J, and Dighiero G: Serum levels of IL-2, IL-1 alpha, TNF-alpha, and soluble receptor of IL-2 in HIV-1-infected patients. AIDS Res Hum Retroviruses 1991;7:381–386.
19. Sereti I, Dunham RM, Spritzler J, *et al.*: IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. Blood 2009;113:6304–6314.
20. Shelburne SA, Montes M, and Hamill RJ: Immune reconstitution inflammatory syndrome: more answers, more questions. J Antimicrob Chemother 2006;57:167–170.
21. Streeck H, Frahm N, and Walker BD: The role of IFN-gamma Elispot assay in HIV vaccine research. Nat Protoc 2009;4:461–469.
22. Stylianou E, Aukrust P, Kvale D, Muller F, and Froland SS: IL-10 in HIV infection: increasing serum IL-10 levels with disease progression—down-regulatory effect of potent antiretroviral therapy. Clin Exp Immunol 1999;116:115–120.
23. Stylianou E, Bjerkeli V, Yndestad A, *et al.*: Raised serum levels of interleukin-18 is associated with disease progression and may contribute to virological treatment failure in HIV-1-infected patients. Clin Exp Immunol 2003132:462–466.
24. Surh CD, and Sprent J: Homeostasis of naive and memory T cells. Immunity 2008;29:848–862.
25. Torre D, Speranza F, Martegani R, Pugliese A, Castelli F, Basilico C, and Biondi G: Circulating levels of IL-18 in adult and paediatric patients with HIV-1 infection. AIDS 2000; 14:2211–2212.

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Three Cases of Fungemia in HIV-Infected Patients Diagnosed Through the Use of Mycobacterial Blood Culture Bottles

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Abstract

We treated three cases of fungemia in HIV-infected patients. These cases were caused by *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffei*, respectively, and all were diagnosed through the use of mycobacterial blood culture bottles. Although the detection of the etiologic agents of fungal infection is difficult, it has been shown that blood culture media for mycobacteria are more effective for the detection of fungemia than media for aerobes and anaerobes. Some reports have shown that Bactec Myco/F lytic bottles were useful for the diagnosis of fungemia in clinical samples. Here, we report the successful use of BacT MB bottles.

Key words: mycobacterial blood culture bottle, BacT MB bottle, fungemia, HIV

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Introduction

The incidence of fungemia, and especially of that caused by *Candida* spp., has recently been increasing. Because diagnosis and treatment with antifungal agents tend to be delayed in such cases, the mortality rate is high (1, 2). Part of the reason for this is that the estimated sensitivity of candidemia detection methods using standard aerobic and anaerobic blood culture bottles is only about 50% (3-6). Blood culture media for mycobacteria, however, are more suitable for detecting fungi *in vitro* than these traditional media are (7). In addition, a few reports have shown that, in clinical situations, Bactec Myco/F Lytic bottles manufactured by Becton Dickinson (Franklin Lakes, NJ, USA) yield better accuracy in detecting fungemia (8, 9). In the cases reported here, we used BacT MB bottles manufactured by bioMérieux (Marcy l'Etoile, France), and showed that they, too, were useful in the diagnosis of three different fungemia cases in human immunodeficiency virus (HIV)-positive patients.

Case Report

Case 1

A 53-year-old Japanese HIV-infected man with a 1-month history of dizziness was referred to us. Four months previously, he had started trimethoprim-sulfamethoxazole for *Pneumocystis* pneumonia and anti-retroviral therapy (lopinavir/ritonavir+ tenofovir/emtricitabine). On admission, his CD4 lymphocyte count was 12/μL and his viral load was under 50 copies/mL. Brain MRI showed a 3-cm ring-enhanced tumor in his cerebellum. After open biopsy, he was diagnosed with malignant lymphoma (diffuse large B cell type). Whole-brain radiation therapy was started. One month later, he was treated with meropenem against extended-spectrum beta-lactamase (ESBL)-producing *Kleb-*

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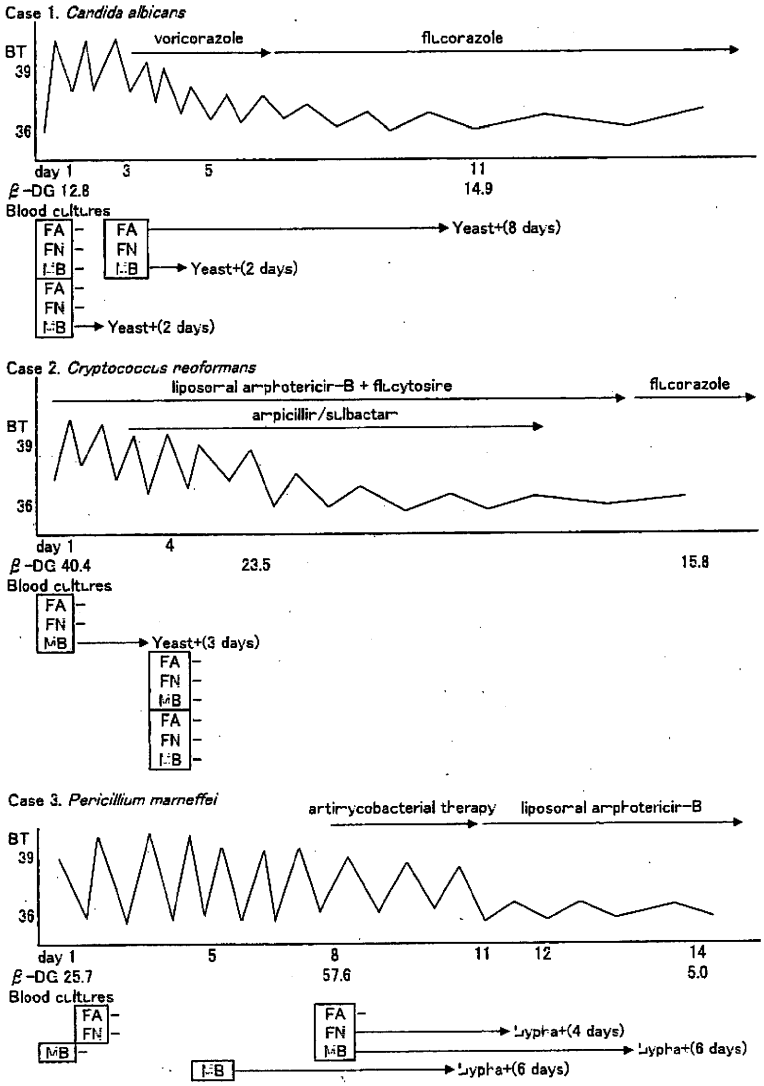

Figure 1. The timing of blood cultures and detection for the three cases. BT: body temperature (℃), β-DG: serum 1→3-β-D-glucan (pg/mL), FA, FN, and MB, BacT FA (aerobic bottle), BacT FN (anaerobic bottle), and BacT MB (mycobacterial bottle), respectively. - represents negative in culture. ( ) indicates the number of incubation days.

siella pneumoniae sepsis, and with vancomycin against methicillin-resistant *Staphylococcus epidermidis* (MRSE) sepsis. Initially his fever abated, but after antibiotic treatment he experienced another spike fever (defined hereafter as day 1). We took blood cultures in six bottles: two bottles for aerobes (BacT FA), two for anaerobes (BacT FN), and two for mycobacteria (BacT MB). Two days later (day 3), yeast was growing in one mycobacterial bottle (Fig. 1). We drew another set of blood cultures on day 3 to determine whether the yeast growth indicated a true fungemia or a contamination. Because the patient was in severe distress, we started voriconazole empirically, though it is known to interact with lopinavir/ritonavir. Yeast was found on day 5 in the mycobacterial bottle cultured on day 3, and on day 11 in the aerobic bottle cultured on day 3. All three yeasts were identified as *Candida albicans*. Since the same organism was detected in each of three bottles which had been taken on different days, we considered this case to be a true fungemia. They were sensitive to fluconazole, so we switched the patient from voriconazole to fluconazole. Serum 1→3-β-D glucan was not elevated, measuring 14.9 pg/mL at most. An ophthalmologist confirmed no endophthalmitis. The patient had no central venous catheter, and the entry point of candidemia was unknown. After treatment for candidemia, he was found to have a brain abscess, cellulitis, and a skin abscess at the site of bone marrow examination. He recovered from these serious infections and was discharged home.

Case 2

A 44-year-old Japanese HIV-infected man with a 3-week history of fever and headache was referred to us. He had chronic hepatitis B virus (HBV) infection. His CD4 lymphocyte count was 40/μL, and his viral load was 40,000 copies/

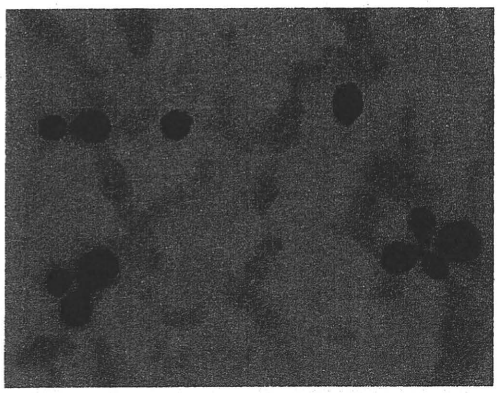

Figure 2. Gram-stained yeasts (*C. neoformans*) from a mycobacterial bottle in Case 2 (×1000).

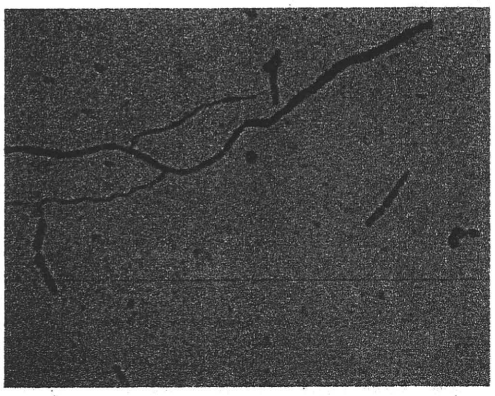

Figure 3. Gram-stained hyphae (*P. marneffei*) from a mycobacterial bottle in Case 3 (×1000).

mL. On admission (defined hereafter as day 1), we took one set of blood cultures including one bottle each for aerobes (BacT FA), anaerobes (BacT FN), and mycobacteria (BacT MB) (Fig. 1). Encapsulated yeasts were detected from the cerebrospinal fluid. We suspected *Cryptococcus* meningitis and accordingly started both liposomal amphotericin-B and flucytosine. We also used ampicillin/sulbactam for aspiration pneumonia. The patient's 1→3-β-D glucan was slightly elevated at 40.4 pg/mL. On day 4, yeasts were found growing in the only mycobacterial bottle (Fig. 2). We took two additional sets of blood cultures, which were all negative, probably because antifungal therapy had already been started. These yeasts were identified as *Cryptococcus neoformans*. After treatment for *Cryptococcus* meningitis, we started anti-retroviral therapy (atazanavir+ritonavir+ tenofovir/emtricitabine), and the patient was discharged home.

Case 3

A 30-year-old Japanese HIV-infected man with a 10-day history of fever, cervical and subclavian lymphadenopathy was referred to us. He had traveled to Thailand several months previously. His CD4 lymphocyte count was 10/μL, and his viral load was 140,000 copies/mL. On the day after admission (defined hereafter as day 1), we took a single blood culture in a mycobacterial bottle (BacT MB) to test for *Mycobacterium avium* complex (Fig. 1). On day 2, we also drew blood cultures into aerobic (BacT FA) and anaerobic (BacT FN) bottles. These three bottles were all negative. Another mycobacterial bottle was taken on day 5, and aerobic, anaerobic, and mycobacterial bottles were taken on day 8. At this point we started antimycobacterial therapy empirically. Hyphae were observed on day 11 growing from the mycobacterial bottle taken on day 5 (Fig. 3), on day 12 from the anaerobic bottle taken on day 8, and on day 14 from the mycobacterial bottle taken on day 8. All of those hyphae were identified as *Penicillium marneffei*. The culture from a subclavian lymph node biopsy tested positive for the same organism. The patient's serum 1→3-β-D glucan was elevated at 57.6 pg/mL. We started liposomal amphotericin-B and he became afebrile. After anti-retroviral therapy (fosamprenavir+tenofovir/emtricitabine), he was discharged home.

Discussion

The incidence of fungemia, especially that caused by *Candida* spp., has recently been increasing (1, 2). The diagnosis of candidemia is frequently difficult, however, because the efficacy of fungemia detection using traditional aerobic and anaerobic bottles is estimated at only 50% (3-6). In general, serum 1→3-β-D-glucan is not sufficiently sensitive or specific to serve as a diagnostic marker for fungemia (4). Delayed diagnosis leads to poor prognosis: the mortality rate is over 40 percent (1, 2, 5). *Cryptococcus* meningitis is somewhat easier to diagnose, because in most cases it can be detected in cerebrospinal fluid. One report, however, has described a case of *Cryptococcus* meningitis that was not detected in cerebrospinal fluid but only through blood culture (8). *Penicillium marneffei* infection is rare in Japan but common in Southeast Asia. In cases of delayed diagnosis, the mortality rate is about 75% (9).

We have described the detection of three different fungal species, *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffei*, through the use of BacT MB bottles. The positivity rates of *C. albicans* detection were 33% (1/3 bottles) using aerobic bottles (BacT FA), 0% (0/3 bottles) using anaerobic bottles (BacT FN), and 67% (2/3 bottles) using mycobacterial bottles (BacT MB) (Table 1). The aerobic bottles (BacT FA) required 8 days of incubation before yielding results, while the mycobacterial bottles (BacT MB) required only 2 days. Mycobacterial bottles therefore exhibited the highest sensitivity and the shortest incubation period. The positivity rates of *C. neoformans* detection were 0% (0/3 bottles) using BacT FA bottles, 0% (0/3 bottles) using BacT FN bottles, and 33% (1/3 bottles) using BacT MB bottles (Table 1); in other words, *C. neoformans* was detected only when a mycobacterial bottle was used. The positivity rates of *P. marneffei* detection were 0% (0/2 bottles) using BacT FA bottles, 50% (1/2 bottles) using BacT FN bottles, and 67% (2/3 bottles) using BacT MB bottles (Ta-

Table 1. Positivity Rates and Detection Times for Aerobic, Anaerobic, and Mycobacterial Bottles

	Case 1 *Candida albicans*	Case 2 *Cryptococcus neoformans*	Case 3 *Penicillium marneffei*
Positivity rate of aerobic bottle(BacT FA)	0.33 (1/3bottles)	0 (0/3bottles)	0 (0/2bottles)
anaerobic bottle(BacT FN)	0 (0/3bottles)	0 (0/3bottles)	0.5 (1/2bottles)
mycobacterial bottle(BacT MB)	0.67 (2/3bottles)	0.33 (1/3bottles)	0.67 (2/3bottles)
Detection time of aerobic bottle(BacT FA)	8 days	-	-
anaerobic bottle(BacT FN)	-	-	4 days
mycobacterial bottle(BacT MB)	2 days	3 days	6 days

ble 1). The mean number of days required for incubation was 4 days for BacT FN and 6 days for BacT MB. For *P. marneffei*, therefore, the mycobacterial bottle again exhibited the highest sensitivity, while the anaerobic bottle required the shortest incubation period. All three of these cases were completely cured through treatment with appropriate antifungal therapies. No other organisms were found in any other blood culture bottles.

About 200 HIV-positive patients are admitted to our hospital each year. When these HIV patients are febrile, we routinely take six bottles of blood culture, two each for the detection of aerobes, anaerobes, and mycobacteria. The required amounts of blood are 10 mL for each aerobic or anaerobic bottle and 5 mL for each mycobacterial bottle. Our laboratory uses the BacT/ALERT 3D automated blood culture system.

Between 2000 and 2005, we took 552 sets of aerobic and anaerobic blood cultures and 390 sets of mycobacterial blood cultures from 684 HIV-positive patients. The positivity rate among aerobic and anaerobic cultures was 3.81% (21/552 sets); three of the 21 positive results were considered to have been contaminations. The organisms involved in the true-positive cases were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Cryptococcus neoformans*, and *Candida guilliermondii*. The positivity rate among mycobacterial cultures was 1.74% (7/390 sets); all of these involved only *Mycobacterium avium*.

The main reason for culturing blood in mycobacterial bottles is to detect miliary tuberculosis or *Mycobacterium avium* complex. In the three cases described here, however, the routine use of mycobacterial bottles for these other purposes led to early diagnosis of fungemia, not mycobacteremia. In Case 1, the first *Candida* culture grew in only one mycobacterial bottle, but because we had suspected opportunistic infection, we did not assume that it represented a contamination. Because continuous fungemia was demonstrated in another set of blood cultures, *Candida* was determined to be the etiologic agent. Having noticed that the *Candida* grew well in the mycobacterial bottle, we were not surprised when *Cryptococcus* and *Penicillium* were also detected through the use of mycobacterial bottles in Cases 2 and 3. Before witnessing these three cases, when fungi or bacteria other than mycobacteria grew in a mycobacterial bottle, we had suspected that the bottle had been contaminated.

Two kinds of blood culture bottles for mycobacteria are available: the Bactec Myco/F Lytic bottle manufactured by Becton Dickinson, and the BacT MB bottle manufactured by bioMérieux. Bactec Myco/F Lytic bottles are designed to detect both mycobacteria and fungi, but BacT MB bottles are designed to detect mycobacteria only, and not fungi. Nevertheless, both mycobacterial bottles were superior to aerobic and anaerobic bottles for fungal detection *in vitro* (7). One report has stated that, in a clinical situation, Bactec Myco/F Lytic bottles exhibited higher sensitivity and shorter incubation periods in the detection of *Candida albicans* and *Candida glabrata* than aerobic bottles (Bactec Plus Aerobic/F) did (5). Another report has shown that the routine use of Bactec Plus Aerobic/F, Plus Anaerobic/F and Myco/F Lytic bottles for immunocompromised hosts, such as patients in the ICU, permitted highly efficient *Candida albicans* detection (6). That study examined 1,253 blood culture sets (3,759 bottles) in two years. From these sets, 62 yeasts were isolated. The positivity rates were 7.33% among Plus Aerobic/F bottles (44/600 bottles), 1.13% among Plus Anaerobic/F bottles (5/441 bottles), and 25.4% among Mycocis IC/F bottles (48/189 bottles).

Because the present report includes only three fungemia cases, it may not be appropriate to compare these results with those of their reports, but our data correspond well with those from the larger studies in showing that mycobacterial blood cultures can detect fungi with a higher sensitivity than aerobic or anaerobic cultures offer.

Nevertheless, it is very difficult to estimate the true positivity rate of fungemia detection through the use of these blood cultures. Especially among HIV-positive patients, even if serum 1→3-β-D-glucan is elevated, this is frequently caused by *Pneumocystis* pneumonia, not by fungemia. Thus, the direct detection of fungi from blood cultures is particularly important in this area.

Some antibiotics are included inside Bactec Myco/F Lytic bottles to inhibit the growth of bacteria other than mycobacteria or fungi. No antibiotics are included inside BacT MB bottles. The reason why fungi grow so well in mycobacterial bottles is unclear. Both fungi and mycobacteria grow well in aerobic environments, and fungi grow faster than mycobacteria. Accordingly it is possible that, in cases of co-infection with fungi and mycobacteria, the mycobacteremia will be

overlooked.

Each mycobacterial bottle requires an extra 5 mL of blood from the patient, as well as laboratory space for its storage. The mycobacterial bottles also cost three times as much as typical aerobic or anaerobic bottles (7, 10). The efficacy of aerobic and anaerobic detection is so low that the regular use of mycobacterial bottles is not recommended in the case of community-acquired infection. Mycobacterial bottles are suitable for patients with high risk of fungemia, including immunocompromised hosts and patients with central venous catheters in place. The prognosis of fungemia is still not very good, but early diagnosis leads to early antifungal treatment which is more likely to result in a complete cure.

We have encountered three cases of fungemia in HIV-infected patients, caused by *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffei*, respectively, all of which were diagnosed through blood culture in BacT MB bottles. Blood culture in aerobic and anaerobic bottles alone would not have been sufficient in these cases. We have found that BacT MB bottles are also useful for the isolation of fungi in clinical situations. More data are required to confirm the usefulness of these mycobacterial bottles for the detection of fungemia in immunocompromised hosts.

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References

1. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. Antimicrob Agents Chemother **49**: 3640-3645, 2005.
2. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis **29**: 239-244, 1999.
3. Jones JM. Laboratory diagnosis of invasive candidiasis. Clin Microbiol Rev **3**: 32-45, 1990.
4. Odabasi Z, Mattiuzzi G, Estey E, et al. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. Clin Infect Dis **39**: 199-205, 2004.
5. Meyer MH, Letscher-Bru V, Jaulhac B, Waller J, Candolfi E. Comparison of Mycosis IC/F and plus Aerobic/F media for diagnosis of fungemia by the bactec 9240 system. J Clin Microbiol **42**: 773-777, 2004.
6. Chiarini A, Palmeri A, Amato T, Immordino R, Distefano S, Giammanco A. Detection of bacterial and yeast species with the Bactec 9120 automated system with routine use of aerobic, anaerobic, and fungal media. J Clin Microbiol **46**: 4029-4033, 2008.
7. Horvath LL, George BJ, Murray CK, Harrison LS, Hospenthal DR. Direct comparison of the BACTEC 9240 and BacT/ALERT 3 D automated blood culture systems for candida growth detection. J Clin Microbiol **42**: 115-118, 2004.
8. Sivasangeetha K, Harish BN, Sujatha S, Parija SC, Dutta TK. Cryptococcal meningoencephalitis diagnosed by blood culture. Indian J Med Microbiol **25**: 282-284, 2007.
9. Wu TC, Chan JW, Ng CK, Tsang DN, Lee MP, Li PC. Clinical presentations and outcomes of *Penicillium marneffei* infections: a series from 1994 to 2004. Hong Kong Med J **14**: 103-109, 2008.
10. Fricker-Hidalgo H, Lebeau B, Pelloux H, Grillot R. Use of the BACTEC 9240 System with Mycosis-IC/F blood culture bottles for detection of fungemia. J Clin Microbiol **42**: 1855-1856, 2004.

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CASE REPORT

Immune reconstitution to parvovirus B19 and resolution of anemia in a patient treated with highly active antiretroviral therapy

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Abstract Immune reconstitution inflammatory syndrome (IRIS) is an unsolved problem in the treatment of human immunodeficiency virus (HIV)-1 infection. Despite the high seroprevalence of parvovirus B19 (PVB19) among HIV-1-positive patients, reports on PVB19-induced anemia, especially that associated with PVB19-related IRIS, in these patients are limited. We present the case of a man with acquired immunodeficiency syndrome who developed severe transfusion-dependent anemia and was seropositive and borderline positive for immunoglobulin-M and IgG antibodies against PVB19, respectively. PVB19-DNA was also detected in his serum. The patient was diagnosed with pure red cell anemia (PRCA) caused by a primary PVB19 infection and was treated with periodical blood transfusions. However, he subsequently tested negative for IgG antibodies and developed chronic severe anemia with high levels of PVB19 viremia. This indicated a transition from primary to persistent infection. After initiation of highly active antiretroviral therapy, the patient showed an inflammatory reaction with rapid deterioration of anemia and seroconversion of the IgG antibody to PVB19. Subsequently, PRCA was completely resolved, but the patient's serum still contained low levels of PVB19-DNA. Thus, this was a case of IRIS associated with PVB19 infection. Our report highlights the significance of seroconversion to PVB19 in the diagnosis of IRIS and reemphasizes the finding that persistently high levels of PVB19 viremia after primary infection are probably because of the lack of protective antibodies.

Keywords HIV-1 infection · Parvovirus B19 · Pure red cell anemia · Immune reconstitution inflammatory syndrome

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Introduction

Human immunodeficiency virus (HIV)-1 is known to infect $CD4^+$ T lymphocytes and cause acquired immunodeficiency syndrome (AIDS) by decreasing the number of $CD4^+$ cells. In the mid-1990s, a new and specific treatment, namely, highly active anti-retroviral therapy (HAART), was developed to treat HIV-1 infection; HAART is a combination therapy comprising administration of two or three classes of antiretroviral drugs. This therapy induces long-term suppression of viral proliferation and immunological reconstitution in HIV-1-infected patients and thus increases their survival rate. Although HAART cures opportunistic infections by restoring the immune system, it can also induce an inflammatory reaction that is characterized by the aggravation of a preexisting opportunistic infection and the emergence of other infectious diseases that were not observed before the initiation of HAART. This phenomenon, termed as immune reconstitution inflammatory syndrome (IRIS), is thought to be caused by an immunological reaction to a pathogen that was present in the host before the antiviral therapy [1]. This paradoxical syndrome poses a major problem in the patients who undergo HAART.

Human parvovirus B19 (PVB19) belongs to the genus *Erythrovirus*. PVB19 is the predominant pathogenic erythrovirus in humans and is the prototype strain for