Table 1.
Evaluable Patients from the Literature with Acquired Factor VIII Inhibitor Who Were Treated with Intravenous Immunoglobulin (IVIG)*

No.	Reference	Sex/Age, y	Associated Disease	IVIG Dosage, g/kg per d	Inhibitor Titer, Bethesda U		Response	Clinical Outcome
					Before	Nadir (d†)		
1	Hudak et al [29]	F/40	Postpartum	0.5 × 5 d	16	<1 (105)	CR	Sustained remission
2	Schwartz et al [25]	M/68	CLL	1 × 2 d	1	0 (14)	CR	Sustained remission
3	Schwartz et al [25]	F/83	Diabetes	1 × 2 d	0.9	0 (61)	CR	Sustained remission
4	Sultan et al [12]	M/62	Idiopathic	0.4 × 5 d	25,000	550 (3)	PR	No clinical benefit‡
5	Sultan et al [12]	F/29	Postpartum	0.4 × 5 d	10,500	1000 (3)	PR	No clinical benefit
6	Zimmermann et al [30]	F/64	Idiopathic	0.5 × 8 d	75	10 (25)	PR	Clinical benefit
7	Zimmermann et al [30]	F/70	Idiopathic	0.5 × 8 d	51	3.8 (9)	PR	Clinical benefit
8	Newland et al [22]	F/71	Diabetes	0.4 × 5 d	50	20 (45)	PR	Clinical benefit
9	Heyman et al [31]	M/64	Idiopathic	0.4 × 5 d	47	28 (17)	PR	No clinical benefit
10	Nishida et al [23]	F/39	Idiopathic	0.4 × 5d	115	17 (3)	PR	No clinical benefit
11	Schwerdtfeger et al [32]	F/31	Postpartum	0.5 × 5 d	420	104 (6)	PR	No clinical benefit
12	Sultan et al [33]	M/78	NA	0.4 × 5 d	42	20 (30)	PR	No clinical benefit
13	Sultan et al [33]	M/72	Carcinoma	0.4 × 5 d	38	10 (5)	PR	Transient benefit
14	Schwartz et al [25]	M/54	Alcoholism	1 × 2 d	1228	208 (7)	PR	No clinical benefit
15	Schwartz et al [25]	F/72	Idiopathic	1 × 2 d	880	570 (48)	PR	No clinical benefit
16	Schwartz et al [25]	F/25	Idiopathic	1 × 2 d	280	1.9 (57)	PR	Clinical benefit
17	Schwartz et al [25]	F/38	Postpartum	1 × 2 d	102	56 (22)	PR	Clinical benefit
18	Schwartz et al [25]	M/77	Carcinoma	0.4 × 5 d	39	24 (3)	PR	No clinical benefit
19	Schwartz et al [25]	M/60	Griseofulvin	0.4 × 5 d	29	18 (19)	PR	No clinical benefit
20	Crenier et al [28]	M/65	Cardiomyopathy	0.4 × 5 d	120	72 (30)	PR	No clinical benefit
21	Crenier et al [28]	M/74	Bronchitis	0.4 × 5 d	24	12 (7)	PR	No clinical benefit
22	Michiels et al [24]	F/31	Postpartum	0.5 × 5 d	12	1 (11)	PR	Clinical benefit
23	Lafferty et al [34]	F/42	SLE	0.4 × 5 d	500	185 (NA)	PR	Clinical benefit
24	Walsh et al [35]	F/72	Cholecystitis	30 g × 1 d	6	NA	PR	Clinical benefit
25	Hiller et al [36]	M/57	Surgery	30 g × 5 d	24	20 (2)	F	Transient benefit
26	Casas et al [37]	M/70	Lymphoma	0.4 × 7 d	8.6	35 (NA)	F	Transient benefit
27	Sultan et al [33]	M/45	Vasculitis	0.4 × 5 d	25	28 (NA)	F	NA
28	Pignone et al [38]	F/66	RA	0.4 × 6 d	13	26 (7)	F	NA
29	Hauser et al [39]	F/29	Postpartum	0.4 × 5 d	10	110 (NA)	F	NA
30	Mateo et al [40]	F/82	CLL	0.4 × 5 d	9.5	10 (30)	F	NA
31	Schwartz et al [25]	M/64	Diabetes	1 × 2 d	452	340 (6)	F	No clinical benefit
32	Schwartz et al [25]	F/83	LA	0.4 × 5 d	102	96 (5)	F	No clinical benefit
33	Schwartz et al [25]	F/48	Idiopathic	1 × 2 d	59	46 (2)	F	No clinical benefit
34	Schwartz et al [25]	M/73	Carcinoma	0.4 × 5 d	42	108 (5)	F	No clinical benefit
35	Schwartz et al [25]	M/62	Idiopathic	1 × 2 d	1.4	1.4 (11)	F	No clinical benefit

*CR indicates complete remission; CLL, chronic lymphocytic leukemia; PR, partial response; NA, not available; SLE, systemic lupus erythematosus; F, treatment failure; RA, rheumatoid arthritis; LA, lupus anticoagulant.
†Number of days after starting IVIG treatment.
‡Subjective evaluation by the doctors in charge.

cyclophosphamide reached CR. Only 2 cases of treatment with IVIG plus cyclophosphamide were reported, and these patients achieved CR [52]. Conversely, 18 (75%) of 24 patients treated with steroid plus cyclophosphamide instead of IVIG achieved CR. This degree of efficacy is consistent with the report by Green et al [45]. In these reports, however, the evaluation of efficacy depended on the patients' symptoms (ie, improvement of bleeding tendency), because the disappearance of inhibitors was not followed up.

Thus, the overall efficacy of IVIG therapy alone is almost 30%, whereas that of a combination therapy with IVIG plus steroid and/or cyclophosphamide is approximately 70%.

Recent reports have described patients with acquired factor VIII inhibitors who rapidly responded to immunosuppressive regimens including rituximab, a monoclonal antibody against $CD20^+$ B-cells [53,54]. These data suggest that immunosuppressive therapy using rituximab could become a powerful tool against coagulation inhibitors.

4.2. Acquired von Willebrand Syndrome

Acquired von Willebrand syndrome is a rare bleeding disorder with laboratory findings similar to those of congenital von Willebrand disease. According to an international registry, acquired von Willebrand syndrome is primarily associated with lymphoproliferative diseases, immunologic and cardiovascular disorders, and solid tumors. The prevalence of acquired von Willebrand syndrome in these underlying disorders is still unknown.

IVIG was also effective in stopping bleeding in acquired von Willebrand syndrome [55]. Several groups reported that acquired von Willebrand syndrome associated with systemic lupus erythematosus [56], monoclonal gammopathy [57-60], malignant lymphoma [61], and prostatomegaly [62], and of undefined origin [63,64] responded well to IVIG therapy. Some patients were successfully treated with the combination of IVIG and desmopressin, but the effect was transient

Table 2.
Responses of Patients with Acquired Factor VIII Inhibitor to Immunosuppressive Agents with or without Intravenous Immunoglobulin (IVIG) Therapy

	IVIG + Pr (26 Cases)			IVIG + Pr + Cy (19 Cases)			Pr + Cy (24 cases)		
Reference	CR	PR	F	CR	PR	F	CR	PR	F
Green et al [41]	1								
Carreras et al [21]	1								
Heyman et al [31]			1†						
OíSullivan et al [42]					1				
Pirner et al [43]					1				
Lionett et al [44]	1								
Pignone et al [38]							1		
Green et al [45]							5		5
Hauser et al [39]							1		
Mateo et al [40]	1								
Schwartz et al [25]	1	1							
Crenier et al [28]	1			1					
Lafferty et al [34]					1				
Sohngen et al [46]							2		
Bossi et al [47]	4		1	8		1	3		
Gandini et al [48]	1								
Dykes et al [49]	4	1	2						
Grunewald et al [50]				2			4		
Mazzucconi et al [51]	3	1							
Delgado et al [52]	1			3	1		2		1
Total	19	3	4	14	4	1	18		6

*Pr indicates prednisolone or dexamethasone; Cy, cyclophosphamide; CR, complete remission; PR, partial response; F, treatment failure.
†IVIG dosage: 0.4 g/kg per d for 2 d.

in most cases. According to data from an international registry, the efficacy of IVIG therapy in acquired von Willebrand syndrome was estimated to be 30% (21/63 patients) [65,66]. Of note, however, is that in most cases the efficacy of IVIG was subjectively evaluated (ie, a good response means to stop bleeding) by the doctors in charge. This efficacy is similar to that for treatment with desmopressin (38/119) or with immunosuppressive agents (23/66), but corticosteroids alone were effective in only 19% of patients (12/63).

4.3. Other Coagulation Inhibitors (Factor V or IX Inhibitor)

Patients with inhibitors against factor V or IX are extremely rare. Only one report described acquired factor IX inhibitor developing in a patient with autoimmune polymyositis [67]. Single-agent therapy with IVIG was effective in suppressing inhibitor synthesis and in stopping bleeding. Another report described acquired factor V inhibitor developing in an 82-year-old female patient following abdominal surgery [68]. Nine-day treatment with IVIG (0.4 g/kg per day) was partially effective in suppressing the inhibitor titer and improving the patient's hemorrhagic diathesis.

5. Safety

Adverse reactions to IVIG therapy are usually mild and self-limited: headache, back pain, low-grade fever, myalgia, and chills. The IVIG preparations currently in clinical use are also assumed to carry virtually no risk of transmitting infectious agents. Rarely, however, serious complications can occur. In recent years, thromboembolic complications have occasionally been reported in patients who received IVIG. Stroke, acute myocardial infarction, and deep vein thrombosis were estimated to occur at an incidence of 3% to 5% [69]. Thromboembolism appeared to develop mainly in patients who had other risk factors, such as an advanced age, being bedridden, and a history of thromboembolism. What triggers thromboembolic complications? During 5 courses of treatment with IVIG (24-54 g/day), the plasma IgG concentration was noted to increase 4-fold, and plasma viscosity increased to beyond the normal range [70]. It appears that increased blood viscosity after high-dose IVIG infusion is responsible for thromboembolism. Slow infusion of IVIG (a daily dose of 0.4 g/kg in not less than 8 hours) has been recommended to prevent thromboembolism [71].

Interestingly, our own review of the literature revealed no thromboembolic complications in 80 patients with acquired factor VIII inhibitor who had received IVIG. It is tempting to speculate that the presence of a coagulation inhibitor may counteract thrombosis formation.

6. Discussion

In general, treatments of acquired coagulation inhibitors are divided into 2 approaches: One is to stop the present bleeding events, and the other is to remove inhibitors by immunomodulative therapy. In cases of acute bleeding in patients with factor VIII inhibitors, conventional management consists of human factor VIII concentrate or desmopressin for low inhibitor levels (<5 Bethesda U) and porcine factor VIII or bypass therapy (eg, recombinant activated

factor VII, activated prothrombin complex concentrates) for high inhibitor levels (>5 Besthesda U). On the other hand, immunosuppressive agents (eg, corticosteroid, cyclophosphamide, azathioprine, rituximab) or IVIG has been used to suppress the generation of coagulation inhibitors. Other approaches are plasmapheresis and immunoadsorption using a protein A–Sepharose column to remove coagulation inhibitors, but the indications for these therapies are limited.

Evaluation of the response to one therapeutic modality in the management of coagulation inhibitors is not always easy, for a number of reasons. First, there are only a few inhibitor patients, and thus it is almost impossible to conduct a randomized clinical trial. There have been only a few such trials on acquired coagulation inhibitors [25,45]. This situation influences the evaluation of efficacy because cases of unsuccessful treatment with IVIG may not have been reported, with only successful cases having been evaluated. Second, most patients present with life-threatening bleeding and are treated with several different therapies simultaneously or sequentially. It is difficult, therefore, to assess the outcome of any single modality. Third, it is known that spontaneous fluctuation or disappearance of the inhibitor may occur [72].

As is shown in Table 1, the efficacy of IVIG therapy alone is not very high (ie, 30%). Moreover, the CR rates for combination therapy with IVIG plus glucocorticoid and/or cyclophosphamide (IVIG plus prednisolone/dexamethasone, 73%; IVIG plus prednisolone/dexamethasone and cyclophosphamide, 74%) did not differ from those of immunosuppressive agents without IVIG (prednisolone/dexamethasone plus cyclophosphamide, 75%) (Table 2). However, the clinical benefits of IVIG include a rapid response and fewer adverse effects, which are frequently observed with the chronic administration of glucocorticoid or other immunosuppressive agents. Regarding the use of cyclophosphamide in particular, it is possible for cytotoxicity to induce myelosuppression and secondary malignancy. Thus, IVIG therapy should be considered for acute massive bleeding in patients with acquired coagulation inhibitors because of its faster action. On the other hand, IVIG therapy costs approximately US $10,000 for a 5-day infusion, which is much more costly than other treatments except rituximab. These considerations taken together suggest that the use of IVIG for the management of acquired coagulation inhibitors might be limited, because whether a given treatment is used depends on the balance between cost and benefit.

7. Conclusion

For patients with acquired coagulation inhibitors against factor VIII, the efficacy of IVIG therapy alone was estimated to be 30% in 35 cases. On the other hand, the response to combination therapy with IVIG plus immunosuppressive agents (eg, corticosteroid, cyclophosphamide) seems to be better (ie, 70% in 45 cases) than IVIG as single-agent therapy. IVIG may be considered as one choice of treatment for acquired coagulation inhibitors, especially when a rapid response is required without myelosuppression, but its use alone would be limited because of its lower efficacy and high cost.

References

1. NIH Consensus Conference. Intravenous immunoglobulin: prevention and treatment of disease. *JAMA*. 1990;264:3189-3193.
2. Knezevic-Maramica I, Kruskall MS. Intravenous immune globulins: an update for clinicians. *Transfusion*. 2003;43:1460-1480.
3. Imbach P, Barandun S, d'Apuzzo V, et al. High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura in childhood. *Lancet*. 1981;1:1228-1231.
4. Fehr J, Hofmann V, Kappeler U. Transient reversal of throm bocytopenia in idiopathic thrombocytopenic purpura by high-dose intravenous gamma globulin. *N Engl J Med*. 1982;306:1254-1258.
5. Sullivan KM, Kopecky KJ, Jocom J, et al. Immunomodulatory and antimicrobial efficacy of intravenous immunoglobulin in bone marrow transplantation. *N Engl J Med*. 1990;323:705-712.
6. Gajdos P, Chevret S, Clair B, Tranchant C, Chastang C. Clinical trial of plasma exchange and high-dose intravenous immunoglobulin in myasthenia gravis. *Ann Neurol*. 1997;41:789-796.
7. van der Meche FGA, Schmitz PIM, for the Dutch Guillain-Barré Study Group. A randomized trial comparing intravenous immune globulin and plasma exchange in Guillain-Barré syndrome. *N Engl J Med*. 1992;326:1123-1129.
8. Newburger JW, Takahashi M, Burns JC, et al. The treatment of Kawasaki syndrome with intravenous gamma globulin. *N Engl J Med*. 1986;315:341-347.
9. Hahn AF, Bolton CF, Zochodne D, Feasby TE. Intravenous immunoglobulin treatment in chronic inflammatory demyelinating polyneuropathy: a double-blind, placebo-controlled, cross-over study. *Brain*. 1996;119:1067-1077.
10. Pollack S, Cunningham-Rundles C, Smithwick EM, Barandun S, Good RA. High dose intravenous gamma globulin for autoimmune neutropenia. *N Engl J Med*. 1982;307:253.
11. Nilsson IM, Sundqvist SB, Ljung R, Holmberg L, Freiburghaus C, Bjorlin G. Suppression of secondary antibody response by intravenous immunoglobulin in a patient with haemophilia B and antibodies. *Scand J Haematol*. 1983;30:458-464.
12. Sultan Y, Kazatchkine MD, Maisonneuve P, Nydegger UE. Anti-idiotypic suppression of autoantibodies to factor VIII (anti-haemophilic factor) by high-dose intravenous gammaglobulin. *Lancet*. 1984;2:765-768.
13. Green D, Lechner K. A survey of 215 non-hemophilic patients with inhibitors to factor VIII. *Thromb Haemost*. 1981;45:200-203.
14. Hauser I, Schneider B, Lechner K. Post-partum factor VIII inhibitors: a review of the literature with special reference to the value of steroid and immunosuppressive treatment. *Thromb Haemost*. 1995;73:1-5.
15. Hay CR, Colvin BT, Ludlam CA, Hill FG, Preston FE. Recommendations for the treatment of factor VIII inhibitors: from the UK Haemophilia Centre Directors' Organisation Inhibitor Working Party. *Blood Coagul Fibrinolysis*. 1996;7:134-138.
16. Gianella-Borradori A, Hirt A, Luthy A, Wagner HP, Imbach P. Haemophilia due to factor VIII inhibitors in a patient suffering from an autoimmune disease: treatment with intravenous immunoglobulin. A case report. *Blut*. 1984;48:403-407.
17. Seifried E, Gaedicke G, Pindur G, Rasche H. The treatment of haemophilia A inhibitor with high dose intravenous immunoglobulin. *Blut*. 1984;48:397-401.
18. Kazatchkine MD, Kaveri SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med*. 2001;345:747-755.
19. Rossi F, Sultan Y, Kazatchkine MD. Anti-idiotypes against autoantibodies and alloantibodies to VIII:C (anti-haemophilic factor) are present in therapeutic polyspecific normal immunoglobulins. *Clin Exp Immunol*. 1988;74:311-316.
20. Dietrich G, Algiman M, Sultan Y, Nydegger UE, Kazatchkine MD. Origin of anti-idiotypic activity against anti-factor VIII autoantibodies in pools of normal human immunoglobulin G (IVIg). *Blood*. 1992;79:2946-2951.

21. Carreras LO, Perez GN, Xavier DL, Blanco AN, Penalva LB. Autoimmune factor VIII inhibitor responsive to gammaglobulin without in vitro neutralization. *Thromb Haemost.* 1988;60:343.
22. Newland AC, Macey MG, Moffat EH, Ainsworth M, Colvin BT. Effect of intravenous immunoglobulin on a spontaneous inhibitor to factor VIII. *Clin Lab Haematol.* 1988;10:435-442.
23. Nishida Y, Sakiyama N, Kakishita E, Nagai K. Evidence of the direct interaction between spontaneously occurring anti-factor VIII autoantibody and intravenous immunoglobulin. *Nippon Ketsueki Gakkai Zasshi.* 1989;52:134-139.
24. Michiels JJ, Hamulyak K, Nieuwenhuis HK, Novakova I, van Vliet HH. Acquired haemophilia A in women postpartum: management of bleeding episodes and natural history of the factor VIII inhibitor. *Eur J Haematol.* 1997;59:105-109.
25. Schwartz RS, Gabariel DA, Aledort LM, Green D, Kessler CM. A prospective study of treatment of acquired (auto-immune) factor VIII inhibitors with high-dose intravenous gammaglobulin. *Blood.* 1995;86:797-804.
26. Heyman B. Fc-dependent IgG-mediated suppression of the antibody response: fact or artifact? *Scand J Immunol.* 1990;31:601-607.
27. Dammacco F, Iodice G, Campobasso N. Treatment of adult patients with idiopathic thrombocytopenic purpura with intravenous immunoglobulin: effects in circulating T cell subsets and PWM-induced antibody synthesis in vitro. *Br J Haematol.* 1986;62: 125-135.
28. Crenier L, Ducobu J, des Grottes JM, Cerny J, Delaunoit C, Capel P. Low response to high-dose intravenous immunoglobulin in the treatment of acquired factor VIII inhibitor. *Br J Haematol.* 1996;95:750-753.
29. Hudak C, Spiridonidis CH, Hart AJ, Betras SA, Laufman LR. Pregnancy-associated factor VIII inhibitor: treatment with intravenous high-dose immunoglobulin. *Am J Hematol.* 1993;43:158.
30. Zimmermann R, Kommerell B, Harenberg J, Eich W, Rother K, Schimpf K. Intravenous IgG for patients with spontaneous inhibitor to factor VIII. *Lancet.* 1985;1:273-274.
31. Heyman MP, Chakravarthy A, Edelman BB, Needleman SW, Schiffer CA. Failure of high-dose i.v. gammaglobulin in the treatment of spontaneous acquired factor VIII inhibitors. *Am J Hematol.* 1988;28:191-194.
32. Schwerdtfeger R, Hintz G, Huhn D. Successful treatment of a patient with postpartum factor VIII inhibitor with recombinant human interferon α2a. *Am J Hematol.* 1991;37:190-193.
33. Sultan Y, Kazatchine MD, Nydegger U, Rossi F, Dietrich G, Algiman M. Intravenous immunoglobulin in the treatment of spontaneously acquired factor VIII:C inhibitors. *Am J Med.* 1991; 91(suppl 5A):35S-39S.
34. Lafferty TE, Smith JB, Schuster SJ, DeHoratius RJ. Treatment of acquired factor VIII inhibitor using intravenous immunoglobulin in two patients with systemic lupus erythematosus. *Arthritis Rheum.* 1997;40:775-778.
35. Walsh JD, Landercasper J, Bottner WA, Boyd WC. Cholecystectomy and acquired factor VIII inhibitor coagulopathy. *Am Surg.* 2004;70:815-817.
36. Hiller E, Holler E, Geursen RG, Riess H. Successful treatment with high-dose intravenous immunoglobulin in a patient with spontaneous inhibitor to factor VIII. *Blut.* 1986;52:123-126.
37. Casas E, Garcia Puig J, Villar A, Gonzalez Sanz A, Miranda ME, Ortiz Vazquez J. Sindrome hemorragico asociado a un inhibidor del factor VIII:C en un patiente con linfoma. *Med Clin (Barc).* 1989;93:23-25.
38. Pignone A, Matucci-Cerinic M, Morfini M, et al. Suppression of autoantibodies to factor VIII and correction of factor VIII deficiency with a combined steroid-cyclophosphamide-porcine factor VIII treatment in a patient with rheumatoid arthritis. *J Intern Med.* 1992;231:617-619.
39. Hauser I, Gisslinger H, Locker G, et al. Postpartum factor VIII inhibitors: report of two cases with special reference to the efficacy of various treatments. *Wien Klin Wochenschr.* 1993;105:355-358.
40. Mateo J, Martino R, Borrell M, Gari M, Casas F, Fontcuberta J. Acquired factor VIII inhibitor preceding chronic lymphocytic leukemia. *Ann Hematol.* 1993;67:309-311.
41. Green D, Kwaan HC. An acquired factor VIII inhibitor responsive to high-dose gamma globulin. *Thromb Haemost.* 1987;58:1005-1007.
42. O'Sullivan JL, Haedicke GJ, Bevivino J. Spontaneous postpartum factor VIII inhibitor development with bleeding into the face and neck. *Plast Reconstr Surg.* 1989;84:802-805.
43. Pirner K, Rosler W, Kalden JR, Manger B. Long-term remission after i.v. immunoglobulin treatment in acquired AHF hemophilia with SLE [in German]. *Z Rheumatol.* 1990;49:378-381.
44. Lionett F, Gouault-Heilmann M, Azoulay C, Lacorte JM, Schaeffer A. Auto-immune factor VIII:C inhibitor durably responsive to immunoglobulin: a new case. *Thromb Haemost.* 1990;64:488-489.
45. Green D, Rademaker AW, Briet E. A prospective, randomized trial of prednisone and cyclophosphamide in the treatment of patients with factor VIII autoantibodies. *Thromb Haemost.* 1993;70:753-757.
46. Sohngen D, Specker D, Bach D, et al. Acquired factor VIII inhibitors in non-hemophilic patients. *Ann Hematol.* 1997;74:89-93.
47. Bossi P, Cabane J, Ninet J, et al. Acquired hemophilia due to factor VIII inhibitors in 34 patients. *Am J Med.* 1998;105:400-408.
48. Gandini G, Franchini M, Manzato F, Lippi G, Aprili G. A combination of prednisone, high-dose intravenous immunoglobulin and desmopressin in the treatment of acquired hemophilia A with high-titer inhibitor. *Haematologica.* 1999;84:1054.
49. Dykes AC, Walker ID, Lowe GD, Tait RC. Combined prednisolone and intravenous immunoglobulin treatment for acquired factor VIII inhibitors: a 2-year review. *Haemophilia.* 2001;7:160-163.
50. Grunewald M, Beneke H, Guthner C, Germowitz A, Brommer A, Griesshammer M. Acquired hemophilia: experiences with a standardized approach. *Haemophilia.* 2001;7:164-169.
51. Mazzucconi MG, Bizzoni L, Giorgi A, et al. Postpartum inhibitor to factor VIII: treatment with high-dose immunoglobulin and dexamethasone. *Haemophilia.* 2001;7:422-427.
52. Delgado J, Villar A, Jimenez-Yuste V, Gago J, Quintana M, Hernandez-Navarro F. Acquired hemophilia: a single-center survey with emphasis on immunotherapy and treatment-related side-effects. *Eur J Haematol.* 2002;69:158-164.
53. Jy W, Gagliano-DeCesare T, Kett DH, et al. Life-threatening bleeding from refractory acquired FVIII inhibitor successfully treated with rituximab. *Acta Haematol.* 2003;109:206-208.
54. Wiestner A, Cho HJ, Asch AS, et al. Rituximab in the treatment of acquired factor VIII inhibitors. *Blood.* 2002;100:3426-3428.
55. Federici AB, Stabile F, Castaman G, Canciani MT, Mannucci PM. Treatment of acquired von Willebrand syndrome in patients with monoclonal gammopathy of uncertain significance: comparison of three different therapeutic approaches. *Blood.* 1998;92:2707-2711.
56. Viallard J-F, Pellegrin JL, Vergnes C, et al. Three cases of acquired von Willebrand disease associated with systemic lupus erythematosus. *Br J Haematol.* 1999;105:532-537.
57. Agarwal N, Klix MM, Burns CP. Successful management with intravenous immunoglobulins of acquired von Willebrand disease associated with monoclonal gammopathy of undetermined significance. *Ann Intern Med.* 2004;141:83-84.
58. Gross S, Traulie C, Capiod JC, et al. Efficacy of high-dose intravenous gammaglobulin in the management of acquired von Willebrand's disease during orthopaedic surgery. *Br J Haematol.* 1992; 82:170-171.
59. Delmer A, Horellou MH, Brechot JM, et al. Acquired von Willebrand disease: correction of hemostatic defect by high-dose intravenous immunoglobulins. *Am J Hematol.* 1992;40:151-152.
60. van Genderen PJJ, Terpstra W, Michiels JJ, Kapteijn L, van Vliet HHDM. High-dose intravenous immunoglobulin delays clearance of von Willebrand factor in acquired von Willebrand disease. *Thromb Haemost.* 1995;73:891-892.
61. van Genderen PJJ, Michiels JJ, Bakker JJ, van 't Veer MB. Effectiveness of high-dose intravenous gamma globulin therapy in acquired von Willebrand's disease. *Vox Sang.* 1994;67:14-17.
62. Sampson B, Anderson DR, Dugal M, Ing VW, Zayed E. Acquired type 2a von Willebrand's disease: response to immunoglobulin infusion. *Haemostasis.* 1997;27:286-289.
63. White LA, Chisholm M. Gastro-intestinal bleeding in acquired von Willebrand's disease: efficacy of high-dose immuno-globulin where substitution treatments failed. *Br J Haematol.* 1993;84:332-334.

64. Arkel YS, Lynch J, Kamiyama M. Treatment of acquired von Willebrand syndrome with intravenous immunoglobulin. *Thromb Haemost.* 1994;72:643-644.
65. Federici AB, Rand JH, Bucciarelli P, et al. Acquired von Willebrand syndrome: data from an international registry. *Thromb Haemost.* 2000;84:345-349.
66. Federici AB. Use of intravenous immunoglobulin in patients with acquired von Willebrand syndrome. *Hum Immunol.* 2005;66:422-430.
67. Suzuki K, Murata H, Akiike T, et al. A case report of polymyositis with acquired factor IX inhibitor [in Japanese]. *Kanto Ryumachi.* 1995;29:38-47.
68. Buclin T, Schmidt PM, Hauert J, Winkel L, Chapuis G, Bachmann F. Acquired factor V inhibitor treated with intravenous immunoglobulins [in French]. *Schweiz Med Wochenschr.* 1992;122:1968-1970.
69. Stangel M, Hartung HP, Marx P, Gold R. Side effects of high-dose intravenous immunoglobulins. *Clin Neuropharmacol.* 1997;20:385-393.
70. Reinhart WH, Berchtold P. Effect of high-dose intravenous immunoglobulin therapy on blood rheology. *Lancet.* 1992;339:662-664.
71. Katz U, Shoenfeld Y. Review: intravenous immunoglobulin therapy and thromboembolic complications. *Lupus.* 2005;14:802-808.
72. Lottenberg R, Kentro TB, Kitchens CS. A natural history study of 16 patients with factor VIII inhibitors receiving little or no therapy. *Arch Intern Med.* 1987;147:1077-1081.

INTERNAL MEDICINE

ORIGINAL ARTICLE

Effectiveness of Subcutaneous Growth Hormone in HIV-1 Patients with Moderate to Severe Facial Lipoatrophy

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Abstract

Objective: To evaluate effect of recombinant human growth hormone (rhGH) among HIV-infected adults with moderate to severe facial lipoatrophy as a side effect of long-term antiretroviral treatment.

Design: A prospective open-label study

Methods: Twenty-five HIV-1 patients with moderate to severe facial lipoatrophy who had been on antiretroviral treatment for more than 18 months were enrolled. rhGH (5 mg) was given every other day for 6 months. After treatment was completed, the participants were followed up for 6 months. Facial lipoatrophy was evaluated by computed tomography at months 0, 3, 6 and 12.

Results: Nearly all participants (24 of 25) completed the study. The sum of bilateral soft tissue thickness at the level of zygomatics at months 0, 3, 6, 12 were 7.23, 8.59, 8.35, 8.60 mm, respectively. There was significant improvement from baseline in month 3 ($p=0.009$) and month 12 ($p=0.021$). In the 6 months of follow-up, the soft tissue showed no significant decrease. Several side effects including diarrhea, arthralgia, myalgia, mastalgia and hand numbness were seen, which were self-limited and transient.

Conclusion: rhGH is effective and relatively safe for moderate to severe facial lipoatrophy. Its effect was sustained at least for 6 months after the cessation of rhGH.

Key words: HIV, antiretroviral treatment, lipoatrophy, recombinant human growth hormone

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Introduction

The prognosis of HIV-1 patients has been remarkably improved by highly active antiretroviral therapies (HAART). However, many patients have suffered from long-term adverse effects including lipoatrophy, which markedly interferes with their quality of life (1, 2). Recombinant human growth hormone (rhGH) has been used for patients with fat redistribution syndrome and has shown favorable outcomes in trunk, limbs or lipid profile (3-5). Among this syndrome, a significant percentage of patients has experienced facial lipoatrophy, which has one of the strongest impacts on their quality of life (6, 7). This prospective study was undertaken to focus on the effect of subcutaneous growth hormone in HIV-1 patients with moderate to severe facial lipoatrophy.

Method Patients

The study was designed as a pilot, non-randomized prospective open-label study for HAART induced lipoatrophy. Patients who have been treated with HAART for more than 18 months with moderate to severe facial lipoatrophy were recruited from the clinic at the AIDS Clinical Center in International Medical Center of Japan, located in the center of Tokyo. All participants were screened from July 1, 2003 through Dec 31, 2003. The grade of lipoatrophy was defined based on the criteria of the facial lipoatrophy severity scale (8) (Table 1). Based on the scale, grade I is defined as mild, grades II and III as moderate and grade IV as severe facial lipoatrophy.

Other inclusion criteria are; 1) aged 20 to 65, 2) on stable antiretroviral regimen at least 6 months prior to enrollment,

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Table 1. Facial Lipoatrophy Severity Scale

Grade I: mild and focalized facial lipoatrophy
Grade II: deeper and longer atrophy, with facial muscles beginning show through
Grade III: atrophic lesion even deeper and wider, with the muscles clearly showing
Grade IV: lipoatrophy covers wide area, extending toward the eye sockets, and the facial skin lies directly on the muscles

Table 2. Characteristics of Patients at Entry

Patients (n)	25
Male	21
Female	4
Age (years)	38.6 (24-55)
Duration of antiretroviral treatment (years; mean, SD)	4.79 (1.61)
Mean CD4 (cells/mm^3) (mean, SD)	468.4 (202.2)
Number of patients with viral load less than 50 copies/ml	22 (88%)

3) have not been treated with rhGH or other anabolic steroid in the past 6 months, 4) fasting blood glucose <126 mg/dl (9), 5) no obvious current opportunistic infection.

The number of participants was set according to the conditions of the pilot study, quality control of safety and monitoring, and the amount of rhGH provided by the manufacturer; 25 patients, 21 males and 4 females, aged between 24 and 54 years were enrolled the study. The average duration of antiretroviral treatment was 4.79 years, and 22 patients were with undetectable (less than 50 copies/ml) viral load, the average CD4 was 468.4 cells/mm^3 (468.4+/- 202.2) (Table 2).

Procedures and Statistical Analysis

rhGH (5 mg) was given subcutaneously every other day for 6 months. After the completion of the rhGH injection, patients were followed up for 6 months. The observation period was a total of 12 months. Antiretroviral treatment was continued throughout the study. rhGH was provided by Serono Japan Co., Ltd.

The primary endpoint was the change in the soft tissue thickness of the face. All patients had computed tomography (CT) of the face at the level of maxillary sinus, zygomatic arch and mandibular ramus at months 0, 3, 6 and 12 (10). Preliminary, interobserver variability was evaluated. CT of the face was performed and the soft tissue thickness in each slice was measured by two independent radiologists. Kappa value was 0.754, which was considered acceptable agreement. Upon the result, the facial soft tissue in all slices was measured by one radiologist. Analysis of variance was used as the statistical method. Multiple comparison of Dunnett-Hsu analysis was used to test the difference between each soft tissue thickness in CT in comparison with their baseline.

The secondary endpoint includes body composition assessed by body mass index, circumflex of limbs and percentage of body fat, blood test with lipid profile, glucose and liver function test, CD4 and viral load which were measured at each visit at months 0, 3, 6 and 12. Patients were also asked to complete questionnaires on their quality of life. Facial photographs were taken at each visit.

The Ethics Committee of the International Medical Center of Japan approved the study. All participants were informed about the study and gave written consent prior to the participation.

Result

Of the 25 participants, one patient had severe diarrhea within 1 month and withdrew from the study. 24 completed the study, however, the digital CT data of 4 patients was partially lost due to technical error. Therefore, the CT scans of 20 participants were analyzed.

The sum of bilateral facial soft tissue at the level of zygomatics at months 0, 3, 6, 12 were 7.23 mm; 8.59 mm, 8.35 mm and 8.60 mm, respectively (Fig. 1). Dunnett-Hsu analysis of adjusted multiple comparison of least squares means found significant improvement of soft tissue thickness from the baseline in the month 3 ($p=0.009$) and month 12 ($p=0.021$). Even after the completion of rhGH injection at month 6, the soft tissue at the level of zygomatics showed no significant decrease for the follow-up period.

There was no significant change in the circumference of arm and thigh, and liver function, CD4 nor HIV viral load during the study. BMI and lipid profile also showed no change except for glucose between months 0 and 6, both of which were within the normal limit (Table 3).

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Table 3. **Change of Laboratory Characteristics**

	month 0	month 3	month 6	month 12	p (month 0 to 6)	p (month 0 to 12)
BMI (mean, (SD))	20.8 (2.6)	21.3(2.3)	21.1 (2.9)	21.5 (2.8)	0.188	0.009
Glucose (mean, (SD))	89.8 (15.5)	96.5 (17.7)	101.5 (17.9)	92.6 (16.3)	< 0.0005	0.592
Triglyceride (mean, (SD))	288.5 (146.5)	299.0 (157.9)	247.3 (158.5)	319.3 (254.6)	0.211	0.593
Total Cholesterol (mean, (SD))	201 (45.2)	199.9 (48.0)	191.2 (41.5)	195.5 (16.3)	0.114	0.431

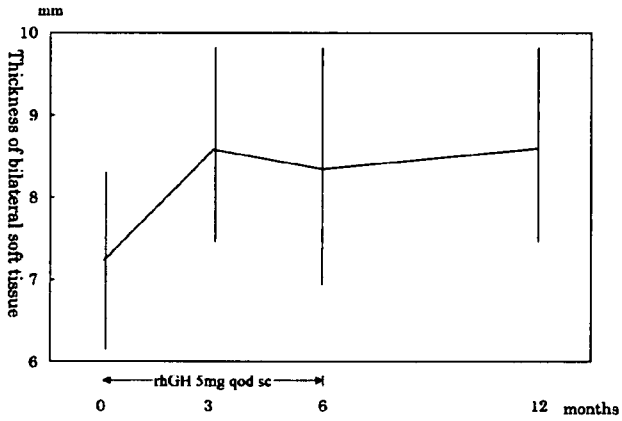

Figure 1. **Thickness of bilateral soft tissue at the level of zygomatics, 95% CIs.**

The quality of life questionnaire revealed that 19 of 25 patients had felt some improvement in their appearance and they were satisfied with the results.

Several adverse events were noted during the study. One patient withdrew due to severe diarrhea. His symptoms gradually subsided after the cessation of rhGH. Ten patients experienced transient self-limited mild arthralgia or muscle ache, 3 male patients had mild mastalgia or enlargement of breast tissue, and one had right hand numbness. All symptoms have resolved after the completion of the rhGH.

Discussion

Facial lipoatrophy is one of the long-term adverse effects of HAART for which standard treatment is not yet found and it severely interferes with the patient's quality of life. Several studies using surgical intervention have been reported to show limited transient effects (8). Some studies have reported the effect of growth hormone for lipoatrophy or lipodystrophy in relation to the total body composition or glucose metabolism (3-6, 11, 12). However, there has been no report of rhGH effect focusing on facial lipoatrophy. This prospective study was designed as a single arm pilot study to focus on the change of facial soft tissue thickness and the maintenance effect with the use and after the cessation of rhGH. All of the participants were followed and evaluated by a single institute, which had the benefit of close clinical monitoring for patient safety and quality assurance of the study. The soft tissue at the zygomatics showed significant improvement of lipoatrophy in month 3 with rhGH and in the observation period without rhGH in month 12. The effect was sustained for 6 months after the cessation of rhGH. This CT- based evaluation method is accurate and reproducible. In particular, the Kappa value of 0.754 showed that the interobserver variability is minimal.

Considering the fact that many patients who have been on long-term antiretroviral treatment in the era of HAART suffer from lipoatrophy (6, 7), this study proved that rhGH has a significant and sustained effect on the improvement of facial lipoatrophy.

Other clinical parameters, including BMI, liver function test, lipid profile, serum glucose, viral load and CD4 showed no significant change. Although severe diarrhea had led a patient to withdraw from the study, other side effects (arthralgia, myalgia, mastalgia and hand numbness) were self-limited and transient. Upon consideration of these results, rhGH can be considered relatively safe to use.

While the standard use of rhGH is 5 mg subcutouneously every day, several studies have shown that low-dose rhGH was effective in visceral adipose tissue and preventive of the change in glucose tolearanve or insulin sensitivity (11, 12). Our study protocol reduced the frequency to every other day with the standard dose, aiming to prevent changing glucose tolerance and to reduce the cost while expecting the maximal effect. The result showed that there was no significant change in glucose intolerance and lipid profile on rhGH. The outcome is quite encouraging.

A potential weakness of the study is the cost of rhGH. For this trial, rhGH was provided by the manufacturer. However, the total cost of rhGH of this study was about 37,000 USD for 6 months use for one patient. The national health insurance of Japan approves of rhGH only for the treatment of HIV-related wasting syndrome. None of the participants met the criteria at the entry of this study. The cost effectiveness of the use of rhGH for facial lipoatrophy will require further discussion.

Conclusion

rhGH is effective and relatively safe for moderate to severe facial lipoatrophy while it is in use and after the cessation. Patients were satisfied with the outcomes of subcutaneous injection of rhGH. The cost effectiveness of rhGH for facial lipoatrophy needs further discussion.

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References

1. Carr A, Samaras K, Burton S, et al. A syndrome of peripheral lipodystrophy, hyperlipidemia and insulin resistance in patients receiving HIV protease inhibitors. AIDS **12**: F51-F58, 1998.
2. Koutkia P, Canavan B, Breu J, Torriani M, Kissko J, Grinspoon S. Growth hormone releasing hormone in HIV-infected men with lipodystrophy: a randomized controlled trial. JAMA **292**: 210-218, 2004.
3. Wanke C, Gerrior J, Kantaros J, Coakley E, Albrecht M. Recombinant human growth hormone improves the fat redistributionsyndrome (lipodystrophy) in patients with HIV. AIDS **13**: 2099-2103, 1999.
4. Schwarz JM, Mulligan K, Lee J, et al. Effects of recombinant human growth hormone hepatic lipid and carbohydrate metabolism in HIV-infected patients with fat accumulation. J Clin Endocrinol Metab **87**: 942-945, 2002.
5. Kotler DP, Muurahainen N, Grunfeld C, et al. Effects of growth hormone on abnormal visceral adipose tissue accumulation and dyslipidemia in HIV-infected patients. J Acquir Immune Defic Syndr **35**: 239-252, 2004(erratum in: J Acquir Immune Defic Syndr **35**: 381, 2006).
6. Moyle GJ, Daar ES, Gertner JM, et al. Growth hormone improves lean body mass, physical performance, and quality of life in subjects with HIV-associated weight loss or wasting on highly active antiretroviral therapy. J Acquir Immune Defic Syndr **35**: 367-375, 2004.
7. Nicholas PK, Kirksey KM, Corless IB, Kemppainen J. Lipodystrophy and quality of life in HIV: symptom management issues. Appl Nurs Res **18**: 55-58, 2005.
8. James J, Carruthers A, Carruthers J. HIV-associated facial lipoatrophy. Dermatol Surg **28**: 979-986, 2002.
9. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care **29**: S43-S48, 2006.
10. Padilla S, Gallego JA, Masia M, Gutierrez F. Single-slice computed tomography and anthriopometric skinfold analysis for evaluation of facial lipoatrophy in HIV-infected patients. Clin Infect Dis **39**: 1848-1851, 2004.
11. Lo JC, Mulligan K, Noor MA, et al. The effects of low-dose growth hormone in HIV-infected men with fat accumulation: a pilot study. Clinical Infectious Diseases **39**: 732-735, 2004.
12. Andersen O, Haugaard SB, Flyvbjerg A, et al. Low-dose growth hormone and human immunodeficiency virus-associated lipodystrophy syndrome: a pilot study. Eur J Clin Invest **34**: 561-568, 2004.

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Original Article

Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan

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People with human immunodeficiency virus (HIV) infection are frequently infected with hepatitis C virus (HCV), because of the common transmission routes. Since the dissemination of hyperactive antiretrovirus therapy (HAART), the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made HCV-associated liver diseases the leading cause of mortality in Western countries. A similar situation is assumed in Japan, but the status of coinfection with HIV and HCV is unclear. We conducted a nationwide survey to determine the prevalence of coinfection with HIV and HCV by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan. Among 4877 patients reported to be HIV-positive, 935 (19.2%) were also positive for the anti-HCV antibody. Most (84.1%) of the patients coinfected with HIV and HCV were recipients of blood products. These data, for the first time, show the current status of coinfection with HIV and HCV in Japan. A detailed analysis of the progression and severity of liver diseases in the coinfected patients is expected.

Key words: coinfection, hepatitis C, HIV, liver disease

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection and human immunodeficiency virus (HIV) infection are major public health problems worldwide. In the USA, the estimated prevalence of the anti-HCV antibody is 1.8%, with 2.7 million people having HCV-RNA detected in their blood, indicative of ongoing HCV infection.[1] The prevalence of HIV is <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral ones, many people with HIV infection are also infected with HCV.[3] Before the introduction of hyperactive antiretroviral treatment (HAART) in 1996, most people with HIV infection died of HIV-associated opportunistic infections such as *Pneumocystis carinii* (currently called *P. jiroveci*) pneumonia and cytomegaloviral infection. Since the dissemination of HAART, the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made patients coinfected with HIV and HCV faced with the menace of progressive liver diseases due to HCV infection in the United States and Europe.[4,5]

Coinfection with HIV has been shown to increase the HCV load in HCV infection,[6] being a negative prognostic factor for clearance of HCV in anti-HCV therapy using interferon.[7,8] It also accelerates the development of cirrhosis and, eventually, hepatocellular carcinoma. Although still controversial, coinfection with HIV and HCV yields a more rapid progression to acquired immunodeficiency syndrome (AIDS) in some cases.[9,10] Importantly, coinfection with HIV and HCV will increase the morbidity and mortality of HIV-infected patients also in Japan, where the prevalence of HIV infection is increasing in a linear fashion, exceptionally among developed countries.[11] There are more than 10 000 HIV-positive people in Japan as of the end of 2004, according to the AIDS National Survey in Japan,[12] and approximately 1.8 million chronic HCV carriers, according to the estimation by the Ministry of Health, Labor and Welfare (MHLW) of Japan. However, unfortunately, the prevalence of coinfection with HIV and HCV in Japan has been unclarified to date. Therefore, we conducted a nationwide study by distributing an

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email-based questionnaire to the hospitals in the HIV/AIDS Network of Japan.

METHODS

IN THE QUESTIONNAIRE, the following information was obtained from hospitals regarding the number of patients who visited the hospitals at least once between January and December 2003: (1) the number of HIV-positive patients; (2) the number of anti-HCV-positive patients among (1); (3) the number of HCV-RNA-positive patients among (2); (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of anti-HCV-positive patients among (4); (6) the number of HCV-RNA-positive patients among (5); (7) the number of HIV-positive patients among men who have sex with men (MSM); (8) the number of anti-HCV-positive patients among (7); (9) the number of HCV-RNA-positive patients among (8); (10) the number of HIV-positive patients who contracted HIV through intravenous drug use; (11) the number of anti-HCV-positive patients among (10); (12) the number of HCV-RNA-positive patients among (11); (13) the number of HIV-positive patients whose transmission routes were classified as 'others'; (14) the number of anti-HCV-positive patients among (13); and (15) the number of HCV-RNA-positive patients among (14).

The questionnaire was sent to the 366 hospitals in the HIV/AIDS Network of Japan by email. When emails were returned with a failure of delivery, the questionnaire was forwarded by post. Answers were mostly returned by email, and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be browsed at: http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 366 hospitals that were on the list of hospitals in the HIV/AIDS Network of Japan in January 2004. One hundred and seventy-six hospitals (48.1%) responded within the indicated period. A collection rate of 47.8% may appear rather low, particularly considering the number of reported HIV-positive people, 10 000, in 2004 according to the statistics of the MHLW of Japan.[12] However, not all the HIV-positive cases are visiting hospitals, and answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. These factors suggest that not all but a majority of HIV-positive patients in Japan were enrolled in the study.

Table 1 Number of hospitals categorized by the number of patients infected with HIV and those coinfected with HIV and HCV

No. of HIV(+)/HCV(+)	No. of HIV(+)				
	0	1–19	20–49	50+	Total
0	43	52	5	1	101
1–9	0	45	9	3	57
10+	0	2	4	12	18
Total	43	99	18	16	176

There were one or more HIV-positive patients in 133 of 176 (75.6%) hospitals; there were no HIV-positive patients in the remaining 43 hospitals (Table 1). Eighteen of 176 (10.2%) hospitals had 20–49 HIV-positive patients, and 16 (9.1%) hospitals had 50 or more HIV-positive patients. On the other hand, there were one or more patients who were coinfected with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfected patients in 18 (10.2%) hospitals. HIV/HCV coinfected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area (Fig. 1). Of the 16 hospitals with 50 or more HIV-positive patients and of the 18 hospitals with 10 or more HIV/HCV coinfected patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfected patients, but with less than 50 HIV-positive patients had the characteristic that most HIV-positive patients contracted HIV from blood products.

In total, 4877 patients were reported to be HIV-positive. Among these, 935 (19.2%) were positive for anti-HCV (Table 2). Of these 935 patients, 780 were HCV-RNA-positive, although it should be noted that not all the patients underwent HCV-RNA testing.

HCV prevalence when fractionated by routes of transmission was as follows. Among 811 HIV-positive patients who contracted HIV from blood products such as unheated concentrated coagulation factors, 786 (96.9%) were anti-HCV-antibody-positive. Of 20 intravenous drug users, nine (45.0%) were anti-HCV-antibody-positive. Among 2730 HIV-positive patients who were MSM (men who have sex with men), 114 (4.2%) were anti-HCV positive. In the remaining 1316 HIV-positive patients whose routes of HIV transmission

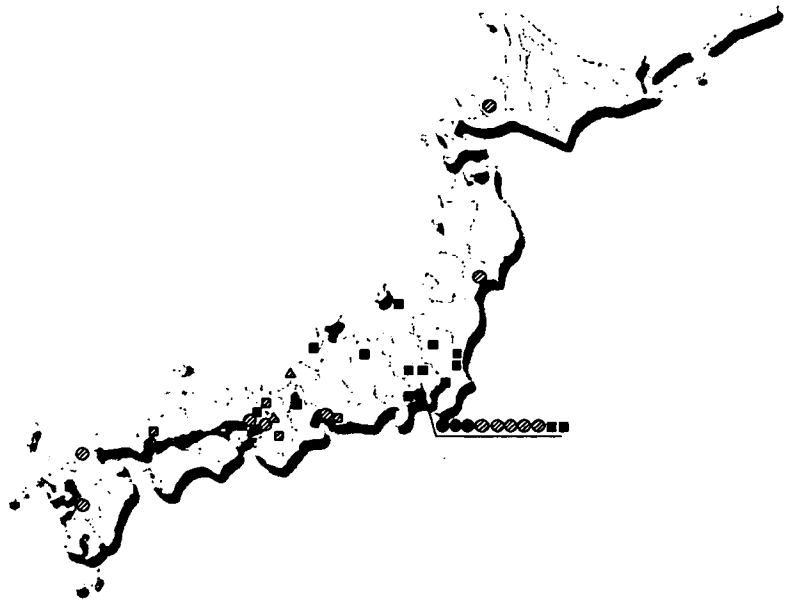

Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (△) hospitals with 1–19 HIV-positive patients; (□) hospitals with 20–49 HIV-positive patients; (○) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfected patients. Closed figures: hospitals with less than 10 HIV/HCV coinfected patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfected patients are omitted from the figure.

were classified as "others", most of whom contracted HIV heterosexually, 26 (2.0%) were anti-HCV-antibody-positive. On the other hand, in HIV/HCV coinfected patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfected patients in Japan are those who contracted HIV, and most likely also HCV, from blood products.

DISCUSSION

ACCORDING TO THE statistics of the MHLW of Japan, the number of reported HIV-positive people was just over 10 000 in 2004.[12] The total number of HIV-positive patients in the current study is approximately half of that. By a simple calculation, there would be about 1900 HIV/HCV coinfected patients in Japan. However, because HIV-positive patients who contracted HIV from blood products are almost all registered in Japan and most of them should have been enrolled in this survey, the number of HIV/HCV coinfected patients is likely smaller than 1900. It is regrettable that not all the patients underwent HCV-RNA testing, but it is unavoidable in this type of questionnaire-based study. In some cases, the existence of a positive anti-HCV antibody indicates a memory of a remote HCV infection.

Almost all of the patients who contracted HIV through blood products were also anti-HCV-antibody-positive, suggesting that both viruses were transmitted through the same route. In MSM patients who were HIV-positive, approximately 4% were anti-HCV-antibody-positive, which is about threefold higher than the prevalence of HCV in Japan.[13] In people aging from 40 to 50 years old in the general Japanese population, whose ages are similar to those of the MSM patients in the current study, the prevalence of HCV is less than 0.5%.[13] Therefore, an HCV prevalence of 4% in MSM

Table 2 Prevalence of HCV infection in HIV-positive patients

Routes of transmission	No. of patients	Anti-HCV-positive	HCV-RNA-positive†
Blood products	811	786 (96.9%)	667
MSM‡	2730	114 (4.2%)	98
Drug addicts	20	9 (45.0%)	8
Others (heterosexual etc.)	1316	26 (2.0%)	7
Total	4877	935 (19.2%)	780

†Not all patients were subjected to HCV-RNA test. ‡MSM, men who have sex with men.

HIV-positive patients is quite high, suggesting the same route of the transmission of HIV and HCV, and a more intensive exposure to HCV or more susceptibility to HCV in these HIV-positive patients. Similarly, an HCV prevalence of 1.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.

To establish measures that decrease the morbidity and mortality of HIV/HCV coinfected patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfected patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfected patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfected with HIV and HCV in Japan. A detailed analysis of the progression and severity of HCV-associated liver diseases is expected.

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REFERENCES

1 Alter MJ. Hepatitis C virus infection in the United States. *J Hepatol* 1999; 31: 88–91.
2 Weinbaum CM, Sabin KM, Santibanez SS. Hepatitis B, hepatitis C, and HIV in correctional populations: a review of epidemiology and prevention. *AIDS* 2005; 19: S41–6.
3 Sulkowski MS. Hepatitis C virus infection in HIV-infected patients. *Curr HIV/AIDS Rep* 2004; 1: 128–35.
4 Bica I, McGovern B, Dhar R *et al.* Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 32: 492–7.
5 HCV in HIV. Challenges and opportunities. *The PRN NOTEBOOK* 2001; 6: 14–18. [Cited 15 July 2004.] Available from URL: http//: www.prn.org.
6 Picchio GR, Nakatsuno M, Boggiano C *et al.* Hepatitis C (HCV) genotype and viral titer distribution among Argentinean hemophilic patients in the presence or absence of human immunodeficiency virus (HIV) co-infection. *J Med Virol* 1997; 52: 219–25.
7 Benhamou Y, Bochet M, Di Martino V *et al.* Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. *The Multivirc Group Hepatology* 1999; 30: 1054–8.
8 Marsen AH, Easterbrook PJ, Taylor C *et al.* Impact of human immunodeficiency virus (HIV) infection on the progression of liver fibrosis in hepatitis C virus infected patients. *Gut* 2003; 52: 1035–40.
9 Staples CT Jr, Rimland D, Dudas D. Hepatitis C in the HIV (human immunodeficiency virus) Atlanta V.A. (Veterans Affairs Medical Center) Cohort Study (HAVACS): the effect of coinfection on survival. *Clin Infect Dis* 1999; 29: 150–4.
10 Monga HK, Rodriguez-Barradas MC, Breaux K *et al.* Hepatitis C virus infection-related morbidity and mortality among patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 33: 240–7.
11 Tatsunami S, Taki M, Shirahata A, Mimaya J, Yamada K. Increasing incidence of critical liver disease among causes of death in Japanese hemophiliacs with HIV-1. *Acta Haematol* 2004; 111: 181–4.
12 National AIDS Survey Report. *The Ministry of Health, Welfare and Labor of Japan.* [Cited 10 July 2006.] Available from URL: http://www.wam.go.jp/wamappl/bb14GS50.nsf/vAdmPBigcategory40/73F664B9EA8D63D4492570290023D454?OpenDocument.
13 Tanaka J, Kumagai J, Katayama K *et al.* Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3 485 648 first-time blood donors during 1995–2000. *Intervirology* 2004; 47: 32–40.

HLA-Cw8 primarily associated with hypersensitivity to nevirapine

We read with interest the report by Littera *et al.* [1] about human leukocyte antigen (HLA)-dependent hypersensitivity to nevirapine in Sardinian HIV patients. The authors state that high levels of genetic homogeneity and linkage disequilibrium make the Sardinian population particularly suitable for genetic association studies, and they observed a statistically significant association between a nevirapine-hypersensitivity reaction and the HLA-Cw*0802-B*1402 haplotype. In the Sardinian population, however, HLA-Cw*0802 and B*1402 are in such strong linkage disequilibrium that they could not establish which one of these two alleles is primarily associated with the hypersensitivity reaction to nevirapine. Considering that HLA-B14(65) can not be found in the Japanese population, it might be helpful to analyse the patients in our clinic for a determination of the primarily associated HLA allele [2–5].

In our outpatient clinic, a total of 326 HIV-1-infected individuals (309 were Japanese) had given written informed consent for HLA analysis and the study of its association with HIV-1 disease progression and drug-induced adverse events. High resolution typing of the alleles at the HLA-A, HLA-B, HLA-Cw, HLA-DRB1, and HLA-DQB1 loci had been performed by polymerase chain reaction amplification using sequence-specific primers in all of them. The allele frequency of HLA-Cw8 and HLA-B14 was 13 and 0%, respectively, which is compatible with previous reports of HLA frequency in the Japanese population [2–5]. Forty-three of the analysed patients were on nevirapine treatment or had a history of nevirapine treatment. One of them died of malignant lymphoma 4 weeks after the introduction of nevirapine-containing treatment. In another patient, nevirapine-containing treatment was terminated 17 days after initiation because of granulocytopenia probably induced by co-administered zidovudine. These two patients were excluded from further analysis and the remaining 41 patients were divided into two groups; a nevirapine-hypersensitive group and a nevirapine-tolerant group (Table 1). The nevirapine-hypersensitive group included 11 patients who experienced extensive skin rash (accompanied by fever $>38^{\circ}C$ in three) and one patient with chronic hepatitis C who developed nevirapine-induced hepatotoxicity with aspartate aminotransferase/alanine aminotransferase values three times above the baseline. The nevirapine-tolerant group included 29 others who had been treated with nevirapine for a period of more than 6 months and did not develop any hypersensitive reaction [1]. There were no significant

Table 1. Demographics and immunological variables in the nevirapine-hypersensitive group and nevirapine-tolerant group.

Variable	Nevirapine hypersensitive ($n=12$)	Nevirapine tolerant ($n=29$)	P value
Mean age, years (SD)	33	40	0.07
Sex, *n* (%)			
Male	11 (92%)	26 (90%)	>0.99
Female	1 (8%)	3 (10%)	
Ethnicity, *n* (%)			
Japanese	11 (92%)	28 (97%)	0.50
Mean weight, kg (SD)	62 (13)	61 (8)	0.88
Plasma HIV-1 RNA, *n* (%)			
> 400 copies/ml	9 (75%)	14 (48%)	0.17
Immunological status, cells/μl (SD)			
CD4	306 (186)	291 (184)	0.81
CD8	587 (246)	765 (416)	0.17
HLA, *n* (%)			
Cw8	5 (42%)	3 (10%)	0.03

differences in age, sex, ethnicity, weight, HIV-1 viral load, CD4 and CD8 cell counts between the two groups (Fisher's exact test for dichotomous variables, Student's *t*-test for continuous variables). The frequency of HLA-Cw8-positive patients in the nevirapine-hypersensitive group was 42%, which was significantly higher than those of the nevirapine-tolerant group (10%) and the general Japanese population (9–14%) [2–5]. In the nevirapine-hypersensitive group, four patients including one who developed hepatotoxicity had HLA-Cw*0801 and one had HLA-Cw*0803. In the nevirapine-tolerant group, three patients had HLA-Cw*0801. HLA-Cw*0802 was not identified in the patients we analysed. There was no significant difference in the frequency of the other HLA alleles between the two groups.

Considering our data together with that of Littera *et al.* [1], HLA-Cw8 antigen rather than specific alleles of other genes linked with HLA-Cw*0801 or HLA-Cw*0802 may be primarily associated with a nevirapine-hypersensitivity reaction. Nevirapine or nevirapine metabolite coupled with HLA-Cw8 antigen may be expressed on the cell surface and may induce hypersensitive reactions including skin rash and hepatotoxicities. We totally agree with Littera *et al.* [1] that a careful choice of drugs in susceptible patients identified by HLA typing would considerably reduce the risk of severe and sometimes life-threatening hypersensitive reactions.

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References

1. Littera R, Carcassi C, Masala A, Piano P, Serra P, Ortu F, *et al.* **HLA-dependent hypersensitivity to nevirapine in Sardinian HIV patients.** *AIDS* 2006; **20**:1621–1626.
2. Hashimoto M, Kinoshita T, Yamasaki M, Tanaka H, Imanishi T, Ihara H, *et al.* **Gene frequencies and haplotypic associations within the HLA region in 916 unrelated Japanese individuals.** *Tissue Antigens* 1994; **44**:166–173.
3. Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, Moriyama S, *et al.* **Sequence-based association analysis of HLA class I and II alleles in Japanese supports conversion of common haplotypes.** *Immunogenetics* 1997; **46**:199–205.
4. Tanaka T, Ohmori M, Yasunaga S, Ohshima K, Kikuchi M, Sasazuki T. **DNA typing of HLA class II genes (HLA-DR, -DQ and -DP) in Japanese patients with histiocytic necrotizing lymphadenitis (Kikuchi's disease).** *Tissue Antigens* 1999; **54**:246–253.
5. Saito S, Ota S, Yamada E, Inoko H, Ota M. **Allele frequencies and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population.** *Tissue Antigens* 2000; **56**:522–529.

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Successful Treatment of an Entecavir-Resistant Hepatitis B Virus Variant

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Emergence of a lamivudine (LAM)-resistant hepatitis B virus (HBV) with amino acid substitutions in the YMDD motif is a well-documented problem during long-term LAM therapy. Entecavir (ETV) is a new drug approved for treatment of HBV infection with or without LAM-resistant mutants. This report describes an ETV-resistant strain of HBV, which emerged after prolonged ETV therapy in a patient who did not respond to LAM therapy. Direct sequence analysis of the ETV-resistant strain showed appearance of amino acid substitution rtS202G in the reverse transcriptase (RT) domain, together with rtL180M + M204V substitution that had developed at the emergence of LAM-resistant mutant. In vitro analysis demonstrated that the rtL180M + M204V + S202G mutant strain displayed a 200-fold and a 5-fold reduction in susceptibility to ETV compared with the wild-type and the rtL180M + M204V mutant strain, respectively. Adefovir was effective against the ETV-resistant strain both in vitro and during the clinical course. In conclusion, this study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV and confirmed that the addition of rtS202G substitution to the rtL180M + M204V mutant strain is responsible for ETV resistance and we could treat the resistant mutant successfully. ***J. Med. Virol. 79:1811–1817, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: HBV; rtS202G; lamivudine; adefovir; in vitro

INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma [Bruix and Llovet, 2003; Ganem and Prince, 2004]. To date, interferon and three nucleoside and nucleotide analogs (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and improve transaminase levels and liver histology [Nevens et al., 1997; Lai et al., 1998; Suzuki et al., 1999]. However, prolonged therapy results in the emergence of drug-resistant mutants.

LAM is associated with a higher rate of emergence of drug-resistant mutants than ADV or ETV, which is 24% and 70% after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels [Lai et al., 2003]. Most LAM-resistant

Abbreviations used: HBeAg,hepatitis B e antigenHBsAg,hepatitis B surface antigenHBV,hepatitis B virusORF,open reading framePCR,polymerase chain reactionRT,reverse transcriptase

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strains show amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [Allen et al., 1998; Delaney et al., 2003].

Both in vitro and clinical studies have shown recently that ADV and ETV could suppress both wild-type and LAM-resistant strains and were confirmed as salvage therapy for LAM-refractory patients [Levine et al., 2003; Sherman et al., 2006; Rapti et al., 2007]. However, a few studies have already reported the emergence of resistant mutants to these drugs.

ADV-resistant mutations are infrequent and their appearance is delayed in treatment-naïve patients; mutation occurs at 0% after 1 year and 28% after 5 years and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [Maecellin and Asselah, 2005]. On the other hand, the emergence rate of ADV-resistant mutations in LAM-resistant patients was 18% after 48 weeks of ADV monotherapy [Lee et al., 2006]. A recent study reported patients treated with combination therapy of ADV with LAM did not develop resistance to ADV for 3 years [Rapti et al., 2007].

ETV is the most novel nucleotide analogue of the three drugs and displays greater in vitro potency than LAM or ADV against wild-type HBV. ETV-resistance is reported to be rare in treatment-naïve patients [Colonno et al., 2006]. However, ETV-resistant mutants appeared at 6–9% per year in LAM-refractory patients [Tenney et al., 2004, 2007; Sherman et al., 2006].

In the present study, an ETV-resistant strain of HBV was identified after prolonged ETV therapy in a patient who did not respond to LAM therapy. To our knowledge, this is the first report that breakthrough hepatitis was induced by emergence of an ETV-resistant strain and was successfully treated with ADV. This study checked the importance of amino acid substitutions in the HBV polymerase for resistance to ETV in vitro. Furthermore, the susceptibility of the mutant strain to ADV was analyzed.

MATERIALS AND METHODS

Antiviral Compounds

LAM [(−)-β-L-2′, 3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, UK). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and ETV {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingfold, CT).

Analysis of Virological Markers

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler (Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAA-CAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Cloning of HBV-DNA and Plasmid Construction

HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. The full-length HBV-DNA was amplified using the above HBV-DNA samples by the method of Gunther et al. [1998]. Nucleotide sequence positions were numbered from the unique *Eco*RI site. The 1.4 genome lengths HBV-DNA amplified from the serum of a patient who showed ETV resistance was cloned into a plasmid vector pcDNA3 (Invitrogen, San Diego, CA). In brief, the PCR product amplified using serum from the patient was cleaved with *Bam*HI and *Apa*I (HBV positions 1,400–2,600) and cloned into pcDNA3, which was named pcDNA3-1. Similarly, the PCR product was cleaved with *Apa*I and *Bam*HI (HBV positions 2,600–3,215, 1–1,400) and cloned into pBluerscript SK+ (Stratagene, La Jolla, CA), which was named pB-1. The *Kpn*I-*Bam*HI fragment from pB-1 and *Kpn*I-*Apa*I fragment from pcDNA3-1 were cloned into pcDNA3-1. To introduce the nucleotide substitutions into the rtL180M, M204V, and S202G, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Four plasmids with/without amino acid substitutions were created and are listed in Table IV.

Cell Culture, Transfection, and Determination of IC_{50}

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under 5% CO_2. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each anti-viral drug, various concentrations of LAM, ADV, and ETV were added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The medium containing the drugs was changed at days 1, 3, and 4. All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., San Diego, CA) was used to determine the best-fit values for individual dose–response equations.

Analysis of Replicative Intermediate of HBV by Quantitation

The cells were harvested at 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH

7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000*g*. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean ± SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

treatment		month	ALT (IU/L)	HBV-DNA (log copies/ml)
		-3	246	7.2
LAM		0	46	5.2
		5	28	3.7
		11	33	4.1
	IFN	17	72	7.5
		18	1184	5.6
		20	39	3.9
		23	34	3.4
		27	117	7.1
ETV		31	112	7.2
		39	40	2.9
		43	28	4.2
	IFN	56	140	6.8
ADV		57	313	6.8
		60	38	4
	LAM	71	24	3.3
		75	19	3.1

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

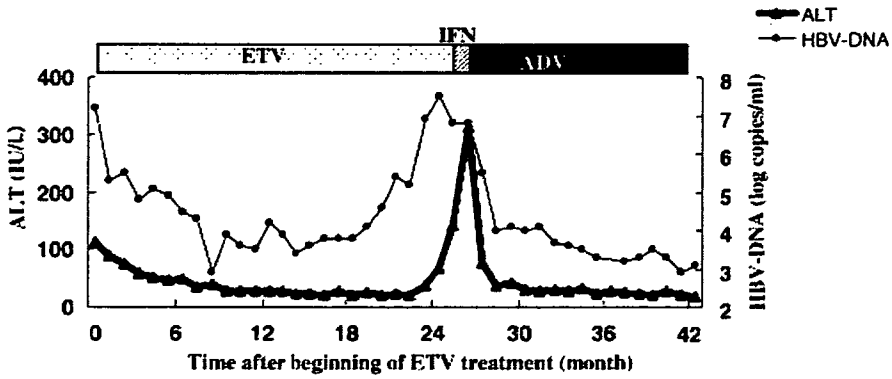

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.