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Table 2　Laboratory parameters

	Test	Baseline[1)]	3months	6months	12months	18months	24months
Coagulation	FVIII：C-inhibitor（Bethesda)[2)]	○	○	○	○	○	○
	FVIII：C	○					
	FVIII：Ag	○					
ELISA test	anti-rFVIII Ab(IgG, IgM), anti-rVWF Ab, anti-CHO Ab, anti-mouse IgG Ab, and anti-BSA Ab	○		○	○	○	○
Hematology	RBC, Ht, PLT, WBC, and WBC differential	○		○	○	○	○
Biochemistry	ALT, ASP, γ-GTP, ALP, LDH, Total bilirubin, BUN, and Creatinine	○		○	○	○	○
Virus	HBsAg, HBsAb, HBcAb, HIV-Ab, and HCV-Ab	○					○

1) Pre-infusion after the patient's enrollment
2) to be measured after the infusions and at a month after the infusions when high dose is infused.

FVIII：C ; factor VIII activity, FVIII：Ag ; factor VIII antigen, VWF ; von Willebrand factor,
CHO ; Chinese hamster ovary cell-derived protein, BSA ; bovine serum albumin

用性なし」の4段階および，「判定不能」で，本調査終了時に担当医が判定した．

成　績

1. 対象症例

134症例が登録されたが，5例では本剤が投与されなかったため解析対象から除外した．

有効性および安全性の解析対象となった129例には，1歳未満の乳児3例および65歳以上の高齢者2例が含まれ，年齢は中央値22歳（生後10カ月～74歳），体重は中央値54 kg（8.2～106 kg）であった（**Table 3**）．血友病重症度は，重症（FⅧ：C＜1%）80例（62.0%），中等症（FⅧ：C 1～5%）37例（28.7%），軽症（FⅧ：C＞5%）12例（9.3%）であった．調査期間は中央値2.0年（1日～3.0年）であり，110例（85.3%）で2年以上の成績を収集し得た．有効性解析対象症例129例における総投与回数は13,736投与であり，内訳は出血時投与が6,448投与（46.9%），手術のための投与および定期的補充療法等が7,288投与（53.1%）であった．また，129例における総出血エピソード数は4,171出血であった（**Table 4**）．

2. 有効性

1) 出血時投与

総計4,171回の出血に対し出血エピソードごとの止血に要した本剤の投与回数は，中央値1回（1～72回）で，うち，1回投与が2,957出血，2回投与が778出血で，全体の89.5%（3,735/4,171出血）が2回までの投与で止血に至っていた（**Table 4**）．

出血エピソードに対する本剤の総投与回数は6,448投与で，1回当たりの投与量は，平均19.2 ± 7.4 U/kg（0.6～124.2 U/kg）であった．全体の83.1%（5,356/6,448投与）は1回当たりの投与量が10～30 U/kgの範囲内であった（**Table 5**）．1出血エピソードごとの止血効果は，著効42.4%（1,769/4,171出血），有効48.7%（2,031/4,171出血）で，有効以上の有効率は91.1%（3,800/4,171出血）であった（**Table 6**）．1出血エピソード当たりの総投与量は中央値20.0 U/kg（5.3～1,265.4 U/kg）であり，全体の85.7%（3,576/4,171出血）が50 U/kg未満の総投与量で止血に至っていた．

無効と判定された出血が2例3出血エピソードで認められた（**Table 6**）．これらは，中等度～重度の関節出血でそれぞれ約13 U/kgが，

Table 3 Patient characteristics

Characteristics	Stratum	No. of patients
Gender	Male	126
	Female	3
Age (yrs)	−6	14
	7−14	27
	15−30	50
	30−40	24
	40−	14
Mean±SD (median, range)	23.0±14.7	(22yrs, 10mos−74yrs)
Body weight (kg)	−9	1
	10−19	9
	20−29	9
	30−59	65
	60−79	38
	80−	6
	Unknown	1
Mean±SD (median, range)	50.8±18.7	(54, 8.2 ～ 106)
Severity of hemophilia A [FVIII：C (%)]	Severe [<1]	80
	Moderate [1−5]	37
	Mild [5<]	12
Mean±SD (median, range)	2.0±2.7	(<1, <1 ～ 17.3)
Complications		
Liver disease	yes	81
	no	48
Hepatitis C	yes	79
	no	50
FVIII：C-inhibitor	yes	2
	no	125
	Unknown	2
Follow-up period (yrs)	−0.9	9
	1−1.9	5
	2−	110
	Unknown	5
Mean±SD (median, range)	1.9±0.5	(2.0yrs, 1day ～ 3.0yrs)
Mean dose per infusion(U/kg)	−9	9
	10−19	62
	20−29	32
	30−39	3
	40−49	1
	50−	1
	Unknown	21
Mean±SD (median, range)	19.0±6.8	(18.2, 6.4 ～ 51.6)
Total dose(U/kg)	−499	21
	500−999	18
	1,000−1,999	23
	2,000−2,999	13
	3,000−4,999	21
	5,000−	12
	Unknown	21
Mean±SD (median, range)	2,148.2±1,871.7	(1,588.9, 9.0 ～ 8,963.2)
Exposure-days	−49	47
	50−99	22
	100−199	27
	200−299	19
	300−	6
	Unknown	8
Mean±SD (median, range)	110.1±96.6	(83, 1 ～ 368)
Cumulative no. of bleeding episodes	−9	26
	10−49	67
	50−99	17
	100−	8
	Unknown	11
Mean±SD (median, range)	35.3±33.8	(26, 1 ～ 150)

Table 4　Relationship of bleeding site to number of infusions per bleeding episode

Bleeding site	Number of infusions						Total (%)
	1	2	3	4	5≦	Unknown	
Joint	2336	594	176	72	46	2	3226 (77.3)
Muscle	188	88	24	21	12	0	333 (8.0)
Subcutaneous	208	48	13	6	9	0	284 (6.8)
Oral	79	10	2	1	1	0	93 (2.2)
Hematuria	9	4	3	3	1	0	20 (0.5)
Two or more sites	32	15	5	18	6	0	76 (1.8)
Others*	105	19	4	3	8	0	139 (3.3)
Total	2957	778	227	124	83	2	4171 (100)
(%)	(70.9)	(18.7)	(5.4)	(3.0)	(2.0)	(0.05)	

*: Epistaxis(79), traumatic(17), odontorrhagia(7), lip(5), otic(4), gastrointestinal(3), etc.

Table 5　Relationship of bleeding site to dose per infusion

Bleeding site	Dose(U/kg)							Total (%)
	−9	10−19	20−29	30−39	40−49	50≦	Unknown	
Joint	336	2781	1204	197	95	12	108	4733 (73.4)
Muscle	34	349	191	32	9	2	15	632 (9.8)
Subcutaneous	44	234	110	14	7	6	13	428 (6.6)
Oral	12	46	14	34	4	0	4	114 (0.7)
Hematuria	0	30	0	15	0	1	0	46 (1.8)
Two or more sites	12	191	35	4	1	7	9	259 (4.0)
Others	7	144	27	14	3	4	37	236 (3.7)
Total	445	3775	1581	310	119	32	186	6448 (100)
(%)	(6.9)	(58.5)	(24.5)	(4.8)	(1.8)	(0.5)	(2.9)	

Table 6　Relationship of hemostatic efficacy to bleeding site

Bleeding site	Hemostatic efficacy per bleeding episode							Total	Efficacy rate (%)
	Excellent	Good	Fair	None	Worse	Unevaluable	Unknown		[Excellent+Good]
Joint	1375	1574	253	3	0	20	1	3226	91.4
Muscle	116	181	35	0	0	1	0	333	89.2
Subcutaneous	141	120	23	0	0	0	0	284	91.9
Oral	45	41	7	0	0	0	0	93	92.5
Hematuria	7	10	2	0	0	0	1	20	85.0
Two or more sites	17	41	18	0	0	0	0	76	76.3
Others	68	64	4	0	0	3	0	139	95.0
Total	1769	2031	342	3	0	24	2	4171	91.1
(%)	(42.4)	(48.7)	(8.2)	(0.1)		(0.6)	(0.05)	(100)	

1～3回投与されたが出血状況の改善が認められず無効と判定された．しかし，悪化することなくその後止血に至っている．悪化と判定された出血は認められなかった．

2）定期補充療法等および手術のための投与

105例に対して総計7,288回の投与が行われた．目的別にみると，定期補充療法等7,128投与（97.8%），手術時等74投与（1.0%），抜歯時43投与（0.6%），ならびに，その他43投与（0.6%）であった．定期補充療法等は，運動等の前および再出血予防のための投与を含んでおり，手術時等は生検および眼科処置時の投与を含んでいる．

重症例で週1回以上の定期補充療法が行われた症例と出血時投与のみの症例における出血頻度を**Table 7**に示した．主治医により合併症として血友病性関節症等の記載のない例では，定期補充療法をしている21例と出血時のみの

Table7 Regular replacement therapy and on-demand treatment in severe PTPs

Hemophilic arthropathy	Treatment		Age (year)	Frequency of infusions (per week)		Mean dose per infusion (U/kg)		Treatment period (mos.)	Frequency of bleeding episodes per month	Frequency of joint bleeding episodes per month
				Regular replacement therapy	Regular replacement therapy + on demand	Regular replacement therapy	Regular replacement therapy + on demand			
Without hemophilic arthropathy	Regular replacement therapy (n = 21)	Mean	15.2	2.0	2.4	17.5	17.9	19.1	0.8	0.6
		SD	11.8	0.6	0.7	7.5	7.7	9.2	0.7	0.6
		Median	9.0	1.9	2.6	15.9	16.0	24.7	0.9	0.5
		Range	0.9−39	1.0−3.0	1.1−3.5	8.3−42.9	7.9−43.5	2.3−28.8	0.0−2.5	0.0−2.2
	On-demand (n = 32)	Mean	20.1	0.1	0.9	20.8	20.1	22.3	1.8	1.3
		SD	12.7	0.1	0.5	7.8	8.6	6.8	1.3	1.3
		Median	18.0	0.1	0.8	18.9	18.3	24.3	1.4	0.9
		Range	1.0−62	0.0−0.4	0.1−2.3	8.0−42.4	6.4−51.6	3.0−29.3	0.2−5.9	0.0−5.8
With hemophilic arthropathy	Regular replacement therapy (n = 6)	Mean	18.5	1.5	2.0	18.1	18.1	19.7	1.4	1.1
		SD	10.7	0.3	0.6	1.6	1.6	7.6	1.2	0.9
		Median	14.0	1.5	2.1	18.3	18.5	21.5	0.9	0.9
		Range	8−33	1.1−1.9	1.1−2.6	15.2−20.2	15.4−19.6	6.7−26.9	0.1−3.1	0.1−2.6
	On-demand (n = 9)	Mean	40.3	0.2	1.1	21.8	22.6	26.0	2.4	2.1
		SD	12.5	0.1	0.8	9.2	9.3	4.1	1.6	1.3
		Median	40.0	0.1	0.9	23.5	22.0	24.3	1.8	1.7
		Range	25−62	0.0−0.3	0.4−2.9	7.8−36.4	7.8−36.5	24.3−36.8	0.2−5.6	0.2−4.6

Table 8 Prophylactic infusions in the surgical procedures (Tooth extractions excluded)

Pt. No.	Surgical procedure	Age (yrs)	Body weight(kg)	Treatment period (Exposure-days)	Total no. of infusions	Total dose (U)	Details (Mean dose per infusion[U/kg] x Total no. of infusions)	Other
PTP-016	Retrenchment of superior react muscle and exterior react muscle for esotropia	5	18	11	18	11750	Pre-op: 55.4 x 1 Post-op: 35.0 x 17	
PTP-041	(Before op.) Infusion test	29	58	1	1	3000		
	Arthroscopic synovectomy of R knee	29	58	7	11	31000	Pre-op: 51.7 x 1 Intra～Post-op: 206.9 x 1(2days)* Post-op: 30.7 x 9	*Continuous infusion: 2.5ml/h
	(After op.) Prophylaxis after operation	29	58	15	16	17000		
PTP-080	Thoracoscopic partial resection(lobectomy) of R lung	23	46	2	2	3000	Pre-op: 43.5 x 1 Post-op: 21.7 x 1	
	(After op. ; on-demand) Hemopneumothorax	23	46	4	4	7000		
PTP-114	Debridement of R joint after total knee replacement	34	57	8	13	28000	Pre-op: 52.6 x 1 Intra～Post-op: 70.2 x 4(4days)* Post-op: 19.7 x 8	*Continuous infusion: 1.67ml/h
	Arthrocentesis	34	57	2	2	3000		
	Arthrocentesis	34	57	2	2	2000		
PTP-003	Liver biopsy	11	45	6	8	11500	Pre-op: 44.4 x 1 Post-op: 30.1 x 7	
	Treatmet of lateral angle of R eye	13	49	1	1	2500		

Table 9 Hemostatic efficacy by patient characteristics

Characteristic	Stratum	No. of patients	No. of bleeding episodes	Efficacy rate (%)
Gender	Male	126	4,130	91.4
	Female	3	41	58.5
Age (yrs)	−6	14	320	95.3
	7−14	27	854	90.4
	15−30	50	1,561	88.0
	30−40	24	891	94.4
	40−	14	545	93.4
Body weight (kg)	−9	1	11	100
	10−19	9	235	97.9
	20−29	9	208	92.3
	30−59	65	2,417	91.6
	60−79	38	1,099	89.8
	80−	6	201	82.6
	Unknown	1	0	-
Severity of hemophilia A	severe	80	2,720	90.8
	moderate	37	1,365	91.3
	mild	12	86	97.7
Complicated liver disease	yes	81	2,920	91.6
	no	48	1,251	89.8
FVIII : C-inhibitor	yes	2	63	95.2
	no	125	4,102	91.0
	Unknown	2	6	100
Follow-up period (yrs)	−0.9	9	48	97.9
	1−1.9	5	16	87.5
	2−	110	4,107	91.0
	Unknown	5	0	-
Mean dose per infusion(U/kg)	−9	9	354	94.1
	10−19	62	2,432	92.8
	20−29	32	986	91.8
	30−39	3	173	72.3
	40−49	1	12	100
	50−	1	24	87.5
	Unknown	21	190	77.4
Total dose(U/kg)	−499	21	183	89.1
	500−999	18	499	92.6
	1,000−1,999	23	1,013	93.7
	2,000−2,999	13	703	86.6
	3,000−4,999	21	953	94.4
	5,000−	12	630	90.5
	Unknown	21	190	77.4
Exposure-days	−49	47	646	91.0
	50−99	22	967	87.3
	100−199	27	1,580	93.5
	200−299	19	676	94.1
	300−	6	278	88.8
	Unknown	8	24	29.2
Cummulative no. of bleeding episodes	−9	26	109	90.8
	10−49	67	1,822	89.4
	50−99	17	1234	90.8
	100−	8	1006	94.6
	Unknown	11	0	-

投与32例を比較すると1カ月当たりの出血頻度は，定期補充療法群の平均0.8±0.7回/月に対し，出血時投与群では平均1.8±1.3回/月（$p<0.01$），関節への出血頻度は，定期補充療法群の平均0.6±0.6回/月に対し，出血時投与群では平均1.3±1.3回/月であった（$p<0.02$）．また，主治医により合併症として血友病性関節症等の記載のある症例で同様に，定期補充療法を行っている6例と定期補充療法を行っていない9例を比較すると1カ月当たりの出血頻度は，定期補充療法群の1.4±1.2回/月に対し，出血時投与群では平均2.4±1.6回/月（NS），関節への出血頻度は，定期補充療法群の平均1.1±0.9回/月に対し，出血時投与群では平均2.1±1.3回/月であった（NS）．

外科的処置時の止血管理状況を**Table 8**に示した．内斜視のため外直筋および上直筋短絡術（症例16），右膝関節鏡下滑膜切除術（症例41），胸腔鏡下右肺部分切除術（症例80）ならびに右膝人工関節置換術後感染病巣掻爬術（症例114）が各1例各1回の計4回の手術が施行された．また，関節穿刺が1例2回施行され（症例114），肝生検が1回および右眼角部処置が1回施行された（症例3）．

抜歯は13例で31回行われ，抜歯前に平均21.2±8.3 U/kg（8.2～43.5 U/kg）が投与された．このうち9例での12回の抜歯では抜歯後に追加投与され，平均19.3±9.2 U/kg（8.2～40.0 U/kg）が1～3回投与された．1回の抜歯に要した総投与量は平均58.1±30.7 U/kg（16.3～108.7 U/kg）であった．

その他は歯科および耳鼻科処置のため4例8投与，内視鏡検査のため2例3投与および頭部打撲のため2例3投与であった．歯科および耳鼻科処置のための投与量は平均18.1±2.0 U/kg（15.2～22.3 U/kg），内視鏡検査の投与量は平均17.2±2.3 U/kg（14.6～18.5 U/kg）であった．また，頭部打撲のための1回投与量は平均27.9±21.9 U/kg（12.6～52.9 U/kg）で，投与回数は全て1回であった．

3）止血効果に影響を及ぼす要因

患者背景因子別有効率を**Table 9**に示した．有効性に影響を与えると考えられる患者背景因子は特に認められなかった．

なお，女性で有効率58.5％と低値となったが，女性例3例のうち，1例は投与データが入手できず，有効性の判定から除外したため，関節症を有する1例と他の1例の2例のみで集計したものである．

また，調査開始時にFⅧインヒビターを保有していた2例は本剤で良好に止血管理された．1例（症例21）のFⅧインヒビターは調査期間中1～2 BU/mlで，本剤は1回当たり平均28.6 U/kgを1～2回投与され，有効率は100％（39/39出血）であった．他の1例（症例73）のFⅧインヒビターは調査期間中2～13 BU/mlであったが，本剤を1回当たり平均51.6 U/kg，1～4回投与され，有効率は87.5％（21/24出血）であった．

3. FⅧインヒビター

本調査期間中，新規にFⅧインヒビターが発生した症例はなかった．

4. 夾雑たん白およびrFⅧに対する抗体

夾雑たん白およびrFⅧに対する抗体の新規産生率を**Table 10**に，各抗体の新規産生症例での調査期間中の検査結果を**Table 11**に示した．

抗BSA抗体，抗rVWF抗体および抗CHO抗体の新規産生症例率は，それぞれ3.5％（4/114例），0.9％（1/117例）および0.9％（1/115例）であった．いずれの症例でも抗体産生に伴うと考えられる臨床症状は全く認められなかった．抗マウスIgG抗体の新規産生症例は認められなかった．

抗rFⅧ抗体（IgG）および抗rFⅧ抗体（IgM）の新規産生症例率は，それぞれ0.9％（1/117例）および1.7％（2/116例）であった．いずれの症例でも調査期間中，抗体産生に伴う臨床症状およびFⅧインヒビター発生は認められなかった．抗rFⅧ抗体（IgG）の陽転化は一過性であり，抗rFⅧ抗体（IgM）の新規産生症例2

Table 10 Serologic responses

Antibody	No. of evaluable patients	Newly development of antibody	
		No. of patients	Rate(%)
anti-BSA Ab	114	4	3.5
anti-rVWF Ab	117	1	0.9
anti-CHO Ab	115	1	0.9
anti-mouse IgG Ab	116	0	0.0
anti-rFVIII Ab(IgG)	117	1	0.9
anti-rFVIII Ab(IgM)	116	2	1.7

Tabel 11 Test results in the patients with positive serologic responses

Antibody	Pt. no.	Baseline	6mos	12mos	18mos	24mos	Other
anti-BSA Ab	48	−	+	N.T.	N.T.	−	
	49	−	−	−	−	+	Negative after the study close
	58	−	−	−	+	−	
	113	−	−	−	+	−	
anti-rVWF Ab	48	−	+	N.T.	N.T.	−	
anti-CHO Ab	125	−	−	−	−	+	Negative after the study close
anti-rFVIII Ab(IgG)	93	−	+	−	N.T.	−	
anti-rFVIII Ab(IgM)	40	−	−	−	−	+	Negative after the study close
	60	−	−	−	−	+	

例では調査終了時（24カ月後）に抗体が陽転化し，1例（症例40）は担当医により有害事象と判定され，副作用症例として報告したが，調査終了後の追跡調査で陰性化し，臨床症状は認められなかった．

5. 副作用

安全性解析対象129例中3例（2.3%）に3件の副作用が認められた．内訳は頭痛（症例43），蕁麻疹（症例113）および前述の抗rF Ⅷ抗体（IgM）産生（症例40）が各1例1件であった．頭痛および蕁麻疹は軽度かつ一過性で無処置にて早期に回復した．また，抗rF Ⅷ抗体（IgM）は力価も低値で，調査終了後の追跡調査にて陰性が確認された．

なお，他の臨床検査所見に関しては本剤に起因すると考えられる検査値異常変動は認められなかった．

6. 有用性

129例中57例（44.2%）が極めて有用，62例（48.1%）が有用，1例（0.8%）がやや有用，および4例（3.1%）が判定不能と判定され，5例（3.9%）が不明であり，有用性なしと判定された症例は認められなかった．極めて有用と有用を合わせた有用以上は92.2%であった．

考　察

今回，われわれはバクスター社からの依頼に基づき，わが国において生後10カ月から74歳までのPTPs症例129例を対象とした市販後使用成績調査を多施設共同研究（43施設）として前方視的に実施し，本剤の市販後の日常使用実態下における補充療法剤としての長期の有効性および安全性を検討した．

有効以上の有効率91.1%（3,800/4,171出血）で，患者背景因子別有効率の検討において有効性に影響を与えると考えられる注目すべき患者背景因子は認められなかった．この成績は本剤の第Ⅲ相臨床試験での有効率95.8%（574/599出血）[4]と同程度であることより，本剤は市販後の長期使用においても有効性に変化はなく，十分に有効であると考えられた．また，本剤の海外におけるPTPsを対象とした長期臨床試験での有効率は91.2%であり[1]，他の遺伝子

組換え第Ⅷ因子製剤および従来のヒト血漿由来第Ⅷ因子製剤の臨床試験での有効率98.8%[9]および92.9%[10]と比較しても本剤は，同程度の有効性があると考えられた．

近年，北欧において，Nilsson[11]やAstermark[12]らは，2歳以下の小児期早期に定期補充療法を開始した症例では，1年間当たりの関節出血回数は少なく，整形外科的関節スコアやX線評価による関節スコアもOであったことを報告し，1次定期補充療法が血友病患者のQOLを改善することを指摘している．その一方で，定期的補充療法によるコストの増加，血管確保の難しさや心理的負担などの問題点が指摘され，MRIを含む関節初期変化の評価方法や適切なプロトコールなど[13][14]について検討が進められている．国内においても瀧ら[15]は日本小児血液学会を中心にプロトコールの検討をすすめ，2004年11月の第46回日本小児血液学会血友病委員会セッションにおいて試験開始が報告されている．

本調査において，重症例80例のうち，投与回数が週1回以上で定期補充療法が実施されていたと考えられた症例が27例認められた．定期補充療法症例と考えられる症例における出血頻度は，出血時投与が主な症例に比し少ない傾向が認められ，関節障害のない21例では有意に低く，定期的補充療法の有効性が示唆された．

安全性の検討では解析対象症例129例において新規にFⅧインヒビターが発生した症例はなかった．1999年国際血栓止血学会（ISTH）科学的標準化委員会（SSC）のFVIIIおよびFIXに関する小委員会は治療歴のない症例（PUPs）を対象とする臨床試験はウイルスに対する安全性評価やインヒビター発生のnatural historyの評価に重要であるが，凝固因子製剤の免疫原性，すなわち，第Ⅷ因子製剤の変更により新たな抗原性が生じFⅧインヒビターが発生する可能性を評価するためには，凝固因子製剤の実投与日数（EDs）が150日を超えたPTPs症例で検証すべきとしている[16]．本調査は1996年に市販後使用成績調査として計画されたものであるため登録時までのそれぞれの患者の実投与日数は確認されていない．しかしながら，本調査期間中，新規にFⅧインヒビターが発生した症例はなく，本剤はPTPsにおけるFⅧインヒビターの発生のリスクが，極めて低いと考えられた．

夾雑たん白およびrFⅧに対する抗体産生について検討した結果では，新規抗体出現症例率は抗BSA抗体3.5%，抗rVWF抗体0.9%，抗CHO抗体0.9%，抗rFⅧ抗体（IgG）0.9%および抗rFⅧ抗体（IgM）1.7%であり，抗マウスIgG抗体産生症例は認められなかった．海外で，血友病PTPsの68例および健常成人57名および小児100名での夾雑たん白に対する抗体産生の検討[17]が行われている．抗BSA抗体，抗CHO抗体および抗マウスIgG抗体の産生症例率はPTPsでそれぞれ7.9%，4.4%および6.8%であり，また，健常人での抗体陽性率は，成人ではそれぞれ14.0%，1.8%および19.3%，小児ではそれぞれ71.0%，10.0%および53.0%であった．国内で実施されたリコネイトの治療歴のない症例（PUPs）において抗BSA抗体出現率は15%（3/20）[18]であり，また，本調査における検査法と同じ方法で行った健常小児36名での抗BSA抗体を検討した結果は15名（41.7%）で陽性で，28から58歳の健常成人30名での抗BSA抗体を検討した結果は5名（16.7%）で陽性であったことを確認している[6]．新規に抗体が産生された症例においても，これらによる臨床症状は認められなかった．Kazukawaら[19]は健常小児87名の抗BSA抗体を測定し，特に4歳未満で抗体価が高い傾向を報告しており，食物等の環境因子の持続的な抗原暴露による自然経過である可能性を指摘している．なお，欧米でのPTPsにおける産生率がわが国での成績より全体的に高値であったのは，両者の検査法において判定方法が異なるためと考えられる．すなわち，わが国では調査開

始前後のペア検体の吸光度差が陰性基準値を超えたものにつき抗体中和確認試験を実施した上で陽性と判定しているのに対し，欧米では各検体と同時測定した正常プール人血清の吸光度と比較して陽性と判定したためと考えられた．

これらから，本剤の投与による夾雑たん白およびrF Ⅷに対する抗体産生率は低く，臨床的にも特に問題となり得るものではないと考えられた．

6年間の市販後使用成績調査より，重篤な副作用は報告されず，リコネイトは血友病A患者の治療製剤として長期の使用において安全で有効な製剤であると考えられた．

文　献

1) White GC II , Courter S, Bray GL, Lee M, Gomperts ED, the Recombinant Previously Treated Patient Study Group : A multicenter study of recombinant factor Ⅷ (Recombinate™) in previously treated patients with hemophilia A. Thromb Haemost **77** : 660-667, 1997.
2) Scharrer I, Ehrlich HJ : Lack of evidence for increased inhibitor incidence in patients switched from plasma-derived to recombinant factor Ⅷ. Haemophilia **7** : 346-348, 2001.
3) 藤巻道男，田中朝志，福武勝幸，浜英永，松田重三，嶋緑倫，福井弘，西田恭治，垣下榮三，白幡聡：血友病A患者に対する遺伝子組換え第Ⅷ因子製剤BL-160の臨床評価．血栓止血誌 **4**：179-188，1993.
4) 藤巻道男，武田武夫，畑江芳郎，豊田隆謙，石川正明，柴田昭，高橋芳右，松田重三，浜英永，福武勝幸，田中朝志，稲垣稔，花房秀次，本多康次郎，三間屋純一，斎藤英彦，高松純樹，緒方完治，島田司巳，太田茂，吉岡章，嶋緑倫，辻野儀一，木下清二，垣下榮三，西田恭治，白幡聡：血友病A患者に対する遺伝子組換え血液凝固第Ⅷ因子製剤(BL-160)の多施設臨床評価．血栓止血誌 **4**：430-440，1993.
5) Kamisue S, Shima M, Nishimura T, Tanaka I, Nakai H, Morichika S, Takata N, Kuramoto A, Yoshioka A : Abnormal factor Ⅷ Hiroshima:defect in crucial proteolytic cleavage by thrombin at Arg^{1689} detected by a novel ELISA. Br J Hematol **86** : 106-111, 1994.
6) 稲葉浩，新井盛夫，福武勝幸，金東賢，花房秀次，三間屋純一，高松純樹，嶋緑倫，吉岡章，白幡聡，藤巻道男：遺伝子組換え型第Ⅷ因子製剤（リコネイト）の市販後調査－抗体検査を中心に－．血栓止血誌 **14**：480，2003.
7) 臼井敏明：正常値計算法：反復切断補正法における切断係数の選択．臨床病理 **24**：319-322，1981.
8) Edwards I.R.,: Harmonisation in Pharmacovigilance. Drug Safety **10** : 93-102, 1994.
9) 福井弘，森和夫，石川正明，柴田昭，高橋芳右，藤巻道男，福武勝幸，腰原公人，木下忠俊，中山明子，浜英永，稲垣稔，花房秀次，三間屋純一，斎藤英彦，神谷忠，高松純樹，白川茂，出口克巳，吉岡章，嶋緑倫，安永幸ニ郎，大久保進，吉岡慶一郎，木下清二，垣下榮三，西田恭治，白幡聡：遺伝子組換えヒト第Ⅷ因子製剤BAYw6240の多施設共同による臨床的検討（長期投与試験）．日輸血会誌 **37**：593-604，1991.
10) 藤巻道男，後藤三雄，宮崎保，櫻田恵右，森和夫，石川正明，木下忠俊，福武勝幸，稲垣稔，花房秀次，長尾大，高橋芳右，松田保，塩原信太郎，斎藤英彦，吉岡慶一郎，木下清二，安永幸ニ郎，大久保進，垣下榮三，西田恭治，上田一博，小林正夫，白幡聡：モノクローナル抗体精製第Ⅷ因子濃縮製剤（RCG-11）の臨床試験成績．基礎と臨床 **26**：299-319，1992.
11) Nilsson IM, Berntorp E, Lofqvist T, Pettersson H : Twenty-five years' experience of prophylactic treatment in severe haemophilia A and B. J Intern Med **232** : 25-32, 1992.
12) Astermark J, Petrine P, Tengborn L, Schulman S, Ljung R, Berntorp E,: Primary prophylaxis in severe haemophilia should be started at an early age but can be individualized. Br. J Haematol **105** : 1109-1113, 1999.
13) Shapiro A.D.:A global view on prophylaxis: possibilities and consequences. Haemophilia 9. Suppl.1 10-18, 2003.
14) Manco-Johnson M.J., Pettersson H., Petrini P., Babyn P.S., Bergstrom B-M., Bradley C-S., Doria A.S., Feldman B.M., Funk S., Hillirad P., Kilcoyne R., Lundin B., Nuss R., Rivard G., Schoenmakers M.A.G.C., van den Berg M., Wiedel J.: Physical therapy and imaging outcome measures in a haemophilia population treated with factor prophylaxis: current status and future directions. Haemophilia **10** : Suppl.4 88-93, 2004.
15) 瀧正志：血友病予防投与の現状．血栓止血誌 **13**：109-113，2002.
16) White G.C., DiMechele D., Mertens K., Negrier C., Peake I.R., Prowse C., Schwaab R., Yoshioka A., Ingerslev J.: Utilization of previously treated patients (PTPs), noninfected patietns (NIPs), and previously untreated patients (PUPs) in the evaluation of new factor VIII and factor IX concentrates. Recommendation of the Scientific Subcommittee on Factor VIII and Factor IX of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost. **81** : 462, 1999.
17) Ingerslev J., Christiansen K., Ravn H.B., Bray G.L., Gomperts E.D., The Recombinant Study Group : Antibodies to heterologous proteins in hemophilia A patients receiving recombinant factor Ⅷ (RecombinateTM). Thromb Haemost **87** : 626-634, 2002.
18) 吉岡章，福武勝幸，新井盛夫，稲葉浩，花房秀次，三間屋純一，高松純樹，嶋緑倫，白幡聡，藤巻道男，リコネイト（PUPs）研究会：過去に治療歴のない血友病A患者に対する遺伝子組換え型血液凝固第Ⅷ因子製剤（リコネイト）の市販後の多施設臨床評価（特別調査）．血栓止血誌，**15**：522-534，2004.
19) Kazukawa M., Sugihara S., Sakamaki T., Konda S., Murata A., Wataki K., Kobayashi Y., Minamitani K., Miyamoto S., Sasaki N., Niimi H.: Antibodies to bovine serum albumin in Japanese IDDM children; No association with GAD antibodies and HLA risk genotypes. Clin Pediatr Endocrinol. **6** : 23-31, 1997.

A multi-center post-marketing surveillance study of recombinant factor Ⅷ (Recombinate) in previously treated patients with hemophilia A

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Key words: recombinant factor Ⅷ, post-marketing surveillance, hemophilia A, previously treated patient

A multi-center post-marketing surveillance study was conducted in previously treated patients with hemophilia A in order to evaluate the safety and efficacy of a recombinant factor Ⅷ (Recombinate) in the long term (2 years) management and prevention of bleeding episodes. One hundred thirty-four patients were enrolled into this study and 129 patients were found to be evaluable. In total, 4,171 bleeding episodes were assessed during the study period and, hemostatic efficacy was judged to be excellent in 1,769 and good in 2,031 episodes with an overall efficacy rate of 91.1% . Twenty-one patients received at least one regular replacement therapy per week with the recombinant factor Ⅷ during this study. These patients manifested much less spontaneous bleeding episodes than the patients who had received on-demand treatments. Three adverse reactions were reported in three patients (2.3%): i.e., urticaria in one, and headache in two patients, each being only mild and transient. One patient developed an IgM antibody to recombinant the factor Ⅷ, which was not associated with any clinical symptoms. No patients developed the inhibitor to factor Ⅷ in Bethesda assay. These results indicate that the recombinant factor Ⅷ, Recombinate, is safe and efficacious for the long-term management and prevention of bleeding episodes in patients with hemophilia A, who had previously received replacement treatments with factor Ⅷ concentrates.

ELSEVIER

Evaluation of Intraoperative Infusion Solution Using a Complete Anhepatic Model in Baby Pigs

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ABSTRACT

Compared to cadaveric liver transplantation, living-related liver transplantation (LRLT) has the physiological advantage of avoiding hemodynamic changes due to the nonsystemic clamping of the inferior vena cava (IVC). However, metabolic changes in the level of blood glucose and lactate usually occur during the anhepatic phase in LRLT. For pediatric patients, intraoperative infusions have the potential to maintain immature homeostasis during LRLT. In the present study, a complete anhepatic model of baby pigs with nonsystemic clamping of IVC, which mimics the procedure of pediatric LRLT, was established using a heparin-coated tube as an internal shunt lactate Ringer solution (LR, Lactec), acetate Ringer solution (AR, VeenF), and a solution comprising acetate Ringer with 1% glucose (AR-G, Phisio140) were tested using piglets. Hemodynamic and metabolic (blood gas analysis, electrolytes, blood lactate, and glucose) changes were observed during the anhepatic phase. Although no major difference was observed in hemodynamic parameters, arterial blood gas data, or concentration of electrolytes among the three solution groups, significant progressive hyperlactatemia was observed in the LR group. Also, though severe hypoglycemia was found in the LR and AR groups, the AR-G group maintained blood glucose levels throughout the anhepatic phase. To conclude, using the simplified pig anhepatic model, we evaluated various solutions for pediatric LRLT.

BECAUSE CHILDREN are immature in their cardiovascular system and homeostasis, close attention must be paid to control hemodynamic and metabolic conditions during major surgery.[1] In pediatric orthotopic liver transplantation (OLT), a size-matched hepatic graft is traditionally used for replacement of the inferior vena cava (IVC) from a cadaveric donor. This procedure requires venous circulatory clamping, often leading to severe ischemic-reperfusion injury and hemodynamic changes in pediatric OLT.[2,3] Cut-liver grafting—living related liver transplantation (LRLT) with a reduced size liver—has recently been introduced in response to the critical shortage of cadaveric donor organs for pediatric liver transplantation.[4,5] While this procedure is capable of avoiding systemic clamping of the IVC, lactate levels have been known to increase continuously until the graft liver is reperfused.[6] Metabolic changes such as hypoglycemia and acidosis also need to be corrected during the anhepatic phase.[7] Intraoperative infusion solutions, therefore, have the potential to maintain unstable homeostasis during LRLT.

In the present study, we developed a complete anhepatic model using baby pigs with nonsystemic clamping of the IVC, which mimics the procedure of pediatric LRLT. Using this model, we tested three different solutions—lactate Ringer (LR, Lactec), acetate Ringer solution (AR, VeenF), and a solution of acetate Ringer with 1% glucose (AR-G, Phisio140)—to evaluate metabolic changes during the anhepatic phase.

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Table 1. Components of Lactate Ringer, Acetate Ringer, and Acetate Ringer With Glucose Infusion Solutions

Components of solutions	Lactate Ringer (Lactec)	Acetate Ringer (Veen F)	Acetate Ringer With 1% Glucose (Phisio140)
Na^+ (mEq · L^{-1})	130	130	140
K^+ (mEq · L^{-1})	4	4	4
Cl^- (mEq · L^{-1})	109	109	115
Ca^{2+} (mEq · L^{-1})	3	3	3
Mg^{2+} (mEq · L^{-1})	—	—	2
$Lactate^-$ (mEq · L^{-1})	28	—	—
$Acetate^-$ (mEq · L^{-1})	—	28	25
$Gluconate^-$ (mEq · L^{-1})	—	—	3
$Citrate^{3-}$ (mEq · L^{-1})	—	—	6
Glucose (%)	—	—	1

MATERIALS AND METHODS

Animals and Experimental Group

Baby domestic pigs (2–3-months old of both sexes, weighing 15–20 kg) underwent an overnight fast immediately prior to use. Animals were assigned randomly to three different groups (n = 5 in each group). These groups were used to test LR, AR, and AR-G. The components of these infusions are described in Table 1. The three different infusion solutions were kindly donated by Otsuka Pharmaceutical (Tokyo, Japan). In the LR group, animals were continuously infused with lactate Ringer's solution. The AR group was infused with acetate Ringer's solution, and the AR-G group was infused with acetate Ringer's solution containing 1% glucose. Each fluid was continuously infused at 40 ml · kg^{-1} per hr^{-1} in each group. All experimental procedures were conducted according to the Guidelines for Animal Experimentation.

Anesthesia and Surgery

After pigs were sedated with ketamine (250 mg) and atropine (0.5 mg), the trachea was intubated and anesthesia was maintained with 2% sevoflurane and 100% oxygen.

As shown in Fig 1, the blood supply to the liver was totally occluded using a heparin-coated internal shunt tube between the splenic vein and IVC. After separation of the hepatic artery and portal vein, blood from the portal vein flooded into the splenic vein, into the shunt tube, and finally into the vena cava. The blood from the lower limb was also flooded through a side hole of the shunt tube into the vena cava. This method ensured that the liver did not receive any regurgitant flow from the vena cava to the hepatic veins. Total liver ischemia was confirmed by the absence of bleeding upon cutting a small amount of the liver.[8]

Monitoring and Sampling

Three needle electrodes were placed on the chest as a standard lead II electrocardiogram. The right femoral artery was cannulated with an 18-gauge catheter for continuous measurement of systemic arterial pressure. An 8-French Swan-Ganz pulmonary catheter (CCOmbo Catheter, Edwards Lifesciences Irvine, Calif) was inserted into the right jugular vein to continuously measure the cardiac output, pulmonary artery blood pressure, mixed venous oxygen saturation, and blood temperature by the cardiac output monitor (Baxter Vigilance Continuous Cardiac Monitor; Baxter Healthcare, Deerfield, Ill). Blood samples for metabolic analysis were taken from the arterial line before total occlusion of the blood flow to the liver and 30, 60, 90, 120, 150, and 180 minutes afterwards. Blood gas data (pH, pCO_2, pO_2, base excess [BE], and HCO_3), concentration of electrolytes (Na^+, K^+, Cl^-, and Mg^{2+}), hematocrit, glucose, and lactate were all measured using a blood gas analyzing system (Stat Profile Ulta; Nova Biomedical, Waltham, Mass).

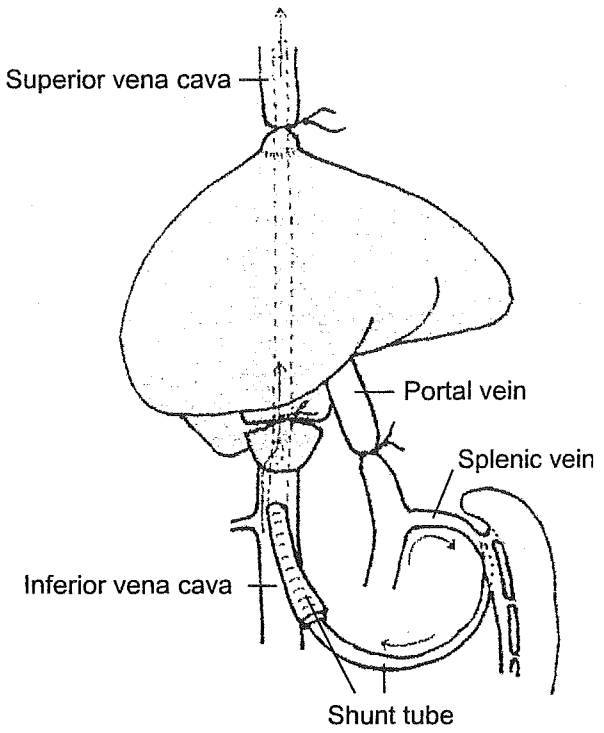

Fig 1. Schematic representation of a pig anhepatic model with nonsystemic clamping of inferior vena cava (IVC). Heparin-coated tubes were used as an internal shunt between the splenic vein and IVC.

Statistical Analysis

The values of measured variables were expressed as means ± SD. Data from the entire study were analyzed using repeated measured analysis of variance. Differences with two-tailed $P < .01$ were significant.

RESULTS

Changes in Hemodynamic Parameters, Blood Gases, and Serum Electrolytes During the Anhepatic Phase

All procedures were successfully performed without surgical complications. All pigs survived throughout the experiments. Figures 2, 3, and 4, show changes of hemodynamics, blood gas analysis data, and blood electrolytes, respectively, in the anhepatic phase of the three different solution groups.

Just after the induction of complete hepatic ischemia, cardiac output and mean arterial pressure fell drastically from their preoperative levels in all groups (Figs 2B and C). Systemic vascular resistance and heart rate gradually increased (Figs 2A and D). There was no significant difference in any hemodynamic parameter across the three

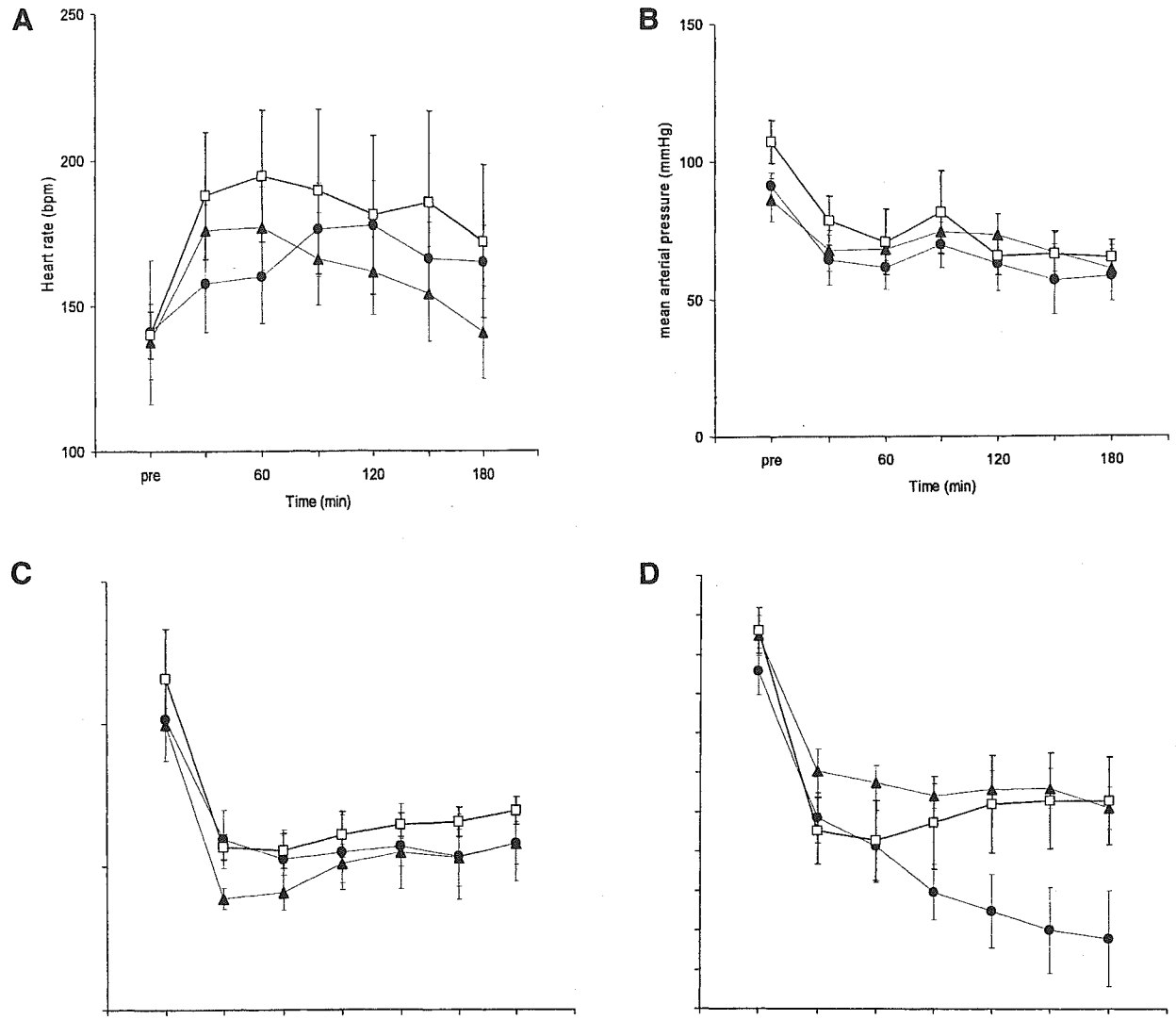

Fig 2. Hemodynamic parameters during anhepatic phase of LR (●), AR (▲), and AR-G (□) groups (n = 5 in each group). (**A**) heart rate (HR, bpm); (**B**) mean arterial blood pressure (MAP, mm Hg); (**C**) cardiac output (CO, L · min^{-1}); and (**D**) systemic vascular resistance (SVR, dyn · sec · cm^{-5}).

experimental groups (Figs 2A–D). Blood loss during the surgical procedure was negligible, and the rate of hematocrit decrease did not differ significantly between the groups (data not shown).

Arterial blood gas analysis showed that pH, BE, and HCO_3^- decreased at the beginning of the anhepatic phase, then became stable (Figs 3A, D, and E). Values of pH, PaO_2, $PaCO_2$, BE, and HCO_3^- did not differ significantly between groups, except for the pH between the AR and AR-G groups (Figs 3A–E). The pH in the AR group remained significantly higher (P = .005) than that in the AR-G group, whereas the pH in the LR group gradually decreased (Fig 3A).

Analysis of the serum electrolytes revealed that Na^+, K^+, Ca^{2+}, and Mg^{2+} were relatively stable during the experiments (Figs 4A–D). Since AR-G contains 2 mEq · L^{-1} of Mg^{2+} (Table 1), the concentration of Mg^{2+} in the AR-G group was significantly higher than the LR and AR groups (P = .003 and P < .001, respectively) (Fig 4D).

Changes in Blood Lactate and Glucose During the Anhepatic Phase

Changes in blood lactate and glucose are shown in Fig 5. Although the initial lactate levels (1.6 ± 0.3 mmol · L^{-1} in LR group, 0.8 ± 0.1 mmol · L^{-1} in AR group, and 1.2 ± 0.1 mmol · L^{-1} in AR-G group) did not differ significantly across the three groups, the lactate level increased progressively after occlusion of the hepatic blood flow in the LR group due to the lactate added in the LR solution (Fig 5A). However, the increases in blood lactate in the AR and

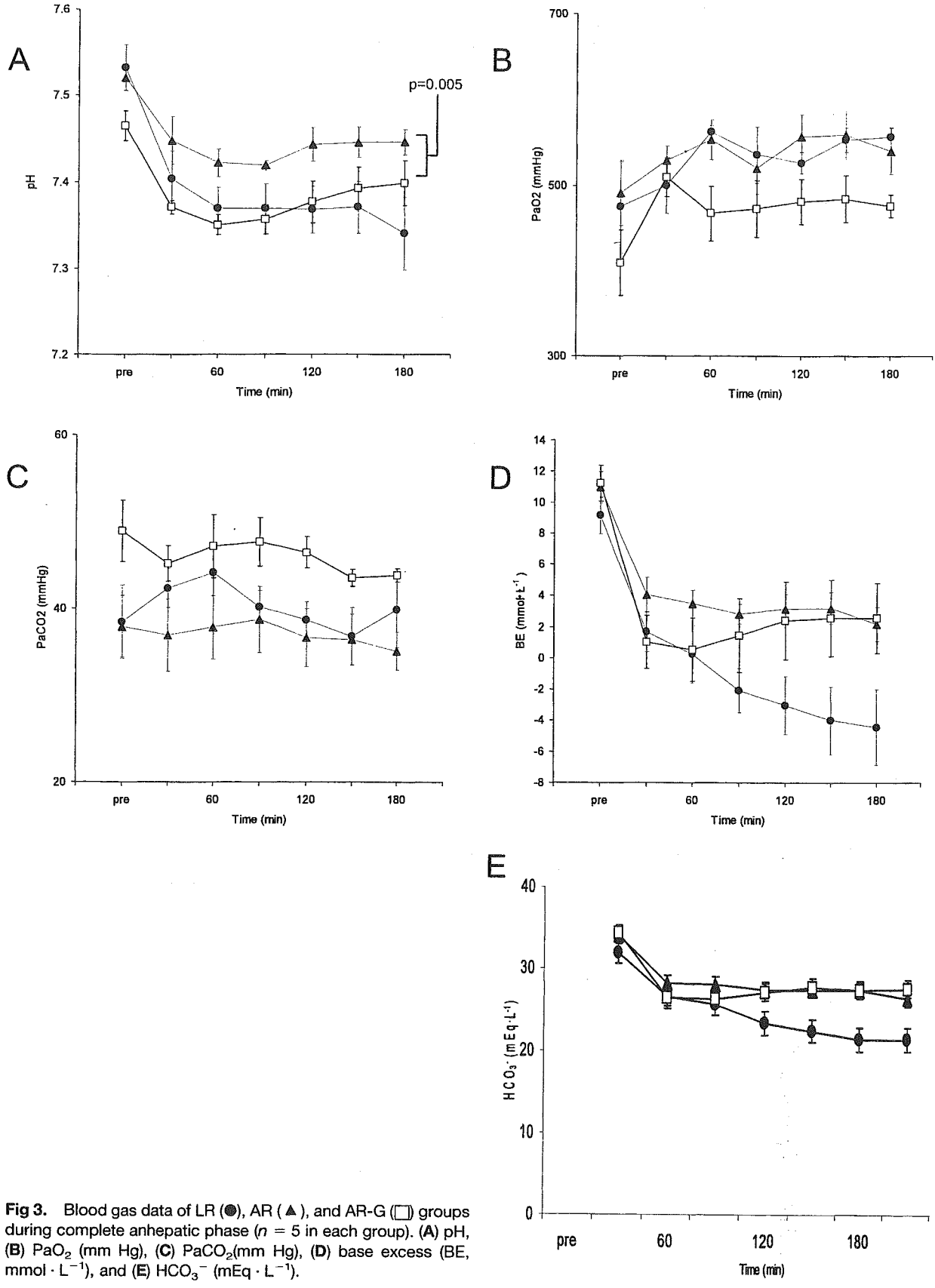

Fig 3. Blood gas data of LR (●), AR (▲), and AR-G (□) groups during complete anhepatic phase (n = 5 in each group). (**A**) pH, (**B**) PaO_2 (mm Hg), (**C**) $PaCO_2$(mm Hg), (**D**) base excess (BE, mmol · L^{-1}), and (**E**) HCO_3^- (mEq · L^{-1}).

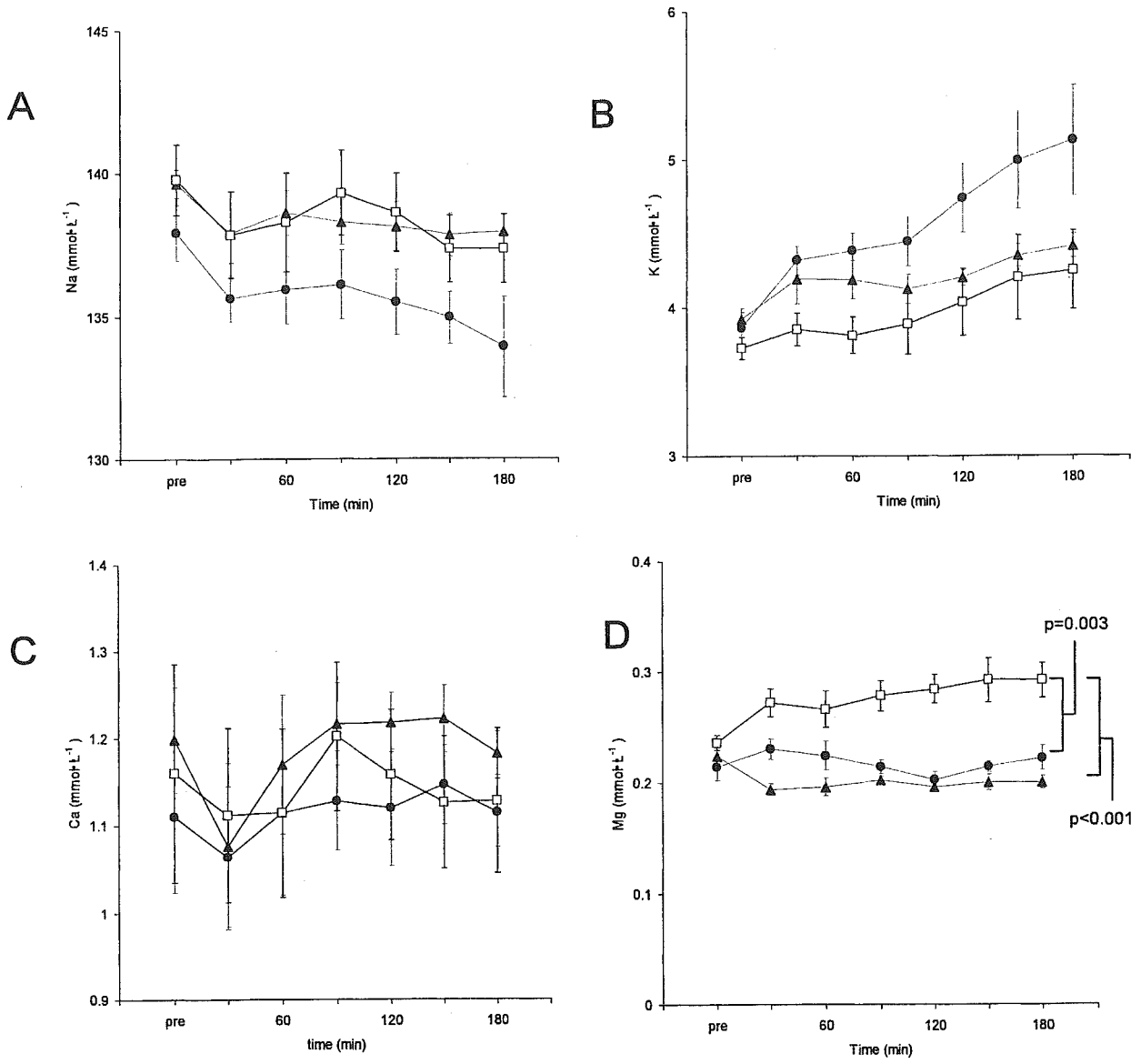

Fig 4. Serum electrolytes of LR (●), AR (▲), and AR-G (□) groups during complete anhepatic phase (n = 5 in each group). (**A**) Na^+ (mmol · L^{-1}), (**B**) K^+ (mmol · L^{-1}), (**C**) Ca^{2+} (mmol · L^{-1}), and (**D**) Mg^{2+} (mmol · L^{-1}).

AR-G groups were moderate and significantly lower than in the LR group (P = .006 and P = .004, respectively) (Fig 5A).

As shown in Fig 5B, blood glucose gradually declined to a minimum in both LR and AR groups after the complete hepatic ischemia (6 ± 4 mg · dl^{-1} and 5 ± 3 mg · dl^{-1}, respectively). However, the AR-G group maintained a stable glucose level and did not show hypoglycemia during the anhepatic phase, showing that blood glucose in the AR-G group was significantly higher than in the LR and AR groups (P = .004 and P = .003, respectively) (Fig 5B).

DISCUSSION

In Japan, LRLT is commonly performed because of the shortage of cadaveric donor organs.[6] At our institute, we anesthetized more than 50 LRLTs between May 2001 and November 2004. The recipients were all children who varied from 7 to 105 months old (mean 26.4 ± 23.2 months) and from 5.1 to 22.4 kg in weight (mean 10.5 ± 3.8 kg). As we sometimes experienced metabolic acidosis during the anhepatic phase in our LRLT program, frequent correction was required to balance immature homeostasis in pediatric recipients (such as imbalances of blood electrolytes, glucoses, metabolites, and hemodynamics). In the anhepatic phase,

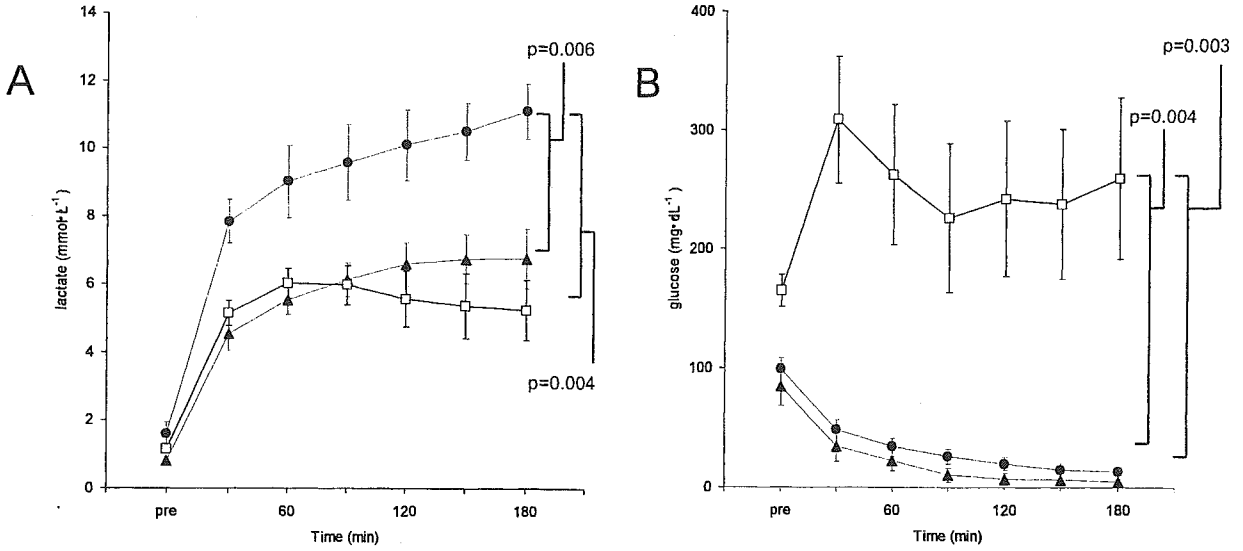

Fig 5. Blood lactate and glucose of LR (●), AR (▲), and AR-G (□) groups during complete anhepatic phase (n = 5 in each group). (**A**) blood lactate (mmol · L^{-1}) and (**B**) blood glucose (mg · dl^{-1}).

blood glucose has usually been controlled by repeated administration of glucose and insulin. As strict blood glucose monitoring and its control are essential for critically ill patients to reduce the morbidity and mortality in the surgical intensive care unit,[9,10] we are planning to develop more adequate solutions for the perioperative phase of LRLT.

In the present study, we initially developed an anhepatic model of baby pigs, with complete internal shunts of the splenic vein to the IVC, which mimics the surgical procedure of pediatric LRLT. Using this total anhepatic model, we then evaluated the effect of three different solutions, LR, AR, and AR-G, on metabolic changes in blood lactate and glucose levels. Progressive hyperlactatemia was observed in the LR group because of no lactate metabolism during the complete anhepatic phase. On the other hand, blood lactate levels remained significantly lower in both the lactate-free AR and AR-G solution groups than in the LR group. Because pH levels of the LR group did not differ significantly from the other groups, transient hyperlactatemia in the LR group might not be physiologically detrimental during the observation period. Although glucose levels need to be monitored as others have indicated,[9,10] AR-G solution prevented both hyperlactatemia and hypoglycemia during the anhepatic phase. In LRLT, it has generally been believed that increased blood lactates was not always due to reduced lactate elimination as a result of depressed liver function or increased lactate production as a result of organ hypoperfusion.[6] Our results support previous clinical reports that exogenous administration of lactates could be a cause of the elevation[3,6] and AR could suppress the progressive increase in blood lactates.[3,6]

In conclusion, we have established a complete anhepatic model of baby pigs with nonsystemic clamping of the IVC, which mimics the anhepatic phase during pediatric LRLT. This experimental model may also be a useful tool for pharmacokinetic studies of drug metabolism including anesthetics to examine the contribution of the liver.

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REFERENCES

1. Carmichael FJ, Lindop MJ, Farman JV: Anesthesia for hepatic transplantation: cardiovascular and metabolic alterations and their management. Anesth Analg 64:108, 1985
2. Mallett SV, Kang Y, Freeman JA, et al: Prognostic significance of reperfusion hyperglycemia during liver transplantation. Anesth Analg 68:182, 1989
3. Shangraw RE, Winter R, Hromco J, et al: Amelioration of lactic acidosis with dichloroacetate during liver transplantation in humans. Anesthesiology 81:1127, 1994
4. Tanaka K, Uemoto S, Tokunaga Y, et al: Living related liver transplantation in children. Am J Surg 168:41, 1994
5. Deshpande RR, Bowles MJ, Vilca-Melendez H, et al: Results of split liver transplantation in children. Ann Surg 236:248, 2002
6. Orii R, Sugawara Y, Hayashida M, et al: Peri-operative blood lactate levels in recipients of living-related liver transplantation. Transplantation 69:2124, 2000
7. Lauritsen TL, Grunnet N, Rasmussen A, et al: The effect of hepatectomy on glucose homeostasis in pig and in man. J Hepatol 36:99, 2002
8. Suzuki S, Satoh T, Yoshino H, et al: Impact of warm ischemic time on microsomal P450 isoforms in a porcine model of therapeutic liver resection. Life Sci 76:39, 2004
9. van den Berghe G, Wouters P, Weekers F, et al: Intensive insulin therapy in the critically ill patients. N Engl J Med 345:1359, 2001
10. Krinsley JS: Effect of an intensive glucose management protocol on the mortality of critically ill adult patients. Mayo Clin Proc 79:992, 2004

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RESEARCH ARTICLE

Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved

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Efficient gene transfer and regulated transgene expression in primate embryonic stem (ES) cells are highly desirable for future applications of the cells. In the present study, we have examined using the nonintegrating Sendai virus (SeV) vector to introduce the green fluorescent protein (GFP) gene into non-human primate cynomolgus ES cells. The GFP gene was vigorously and stably expressed in the cynomolgus ES cells for a year. The cells were able to form fluorescent teratomas when transplanted into immunodeficient mice. They were also able to differentiate into fluorescent embryoid bodies, neurons, and mature blood cells. In addition, the GFP expression levels were reduced dose-dependently by the addition of an anti-RNA virus drug, ribavirin, to the culture. Thus, SeV vector will be a useful tool for efficient gene transfer into primate ES cells and the method of using antiviral drugs should allow further investigation for regulated SeV-mediated gene expression.
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Keywords: *primate embryonic stem cell; Sendai virus vector; gene transfer; green fluorescent protein; pluripotency; ribavirin*

Introduction

Since human embryonic stem (ES) cell lines have the ability to both proliferate indefinitely and differentiate into multiple tissue cells,[1,2] they are expected to have clinical applications as well as to serve as models for basic research and drug development. Although efficient and stable gene transfer into primate ES cells would be useful for such purposes, it has been difficult and only lentiviral vectors have been successful in achieving it.[3–5] We have previously developed Sendai virus (SeV) vectors that replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and do not go through a DNA phase.[6] SeV vectors can efficiently introduce foreign genes without toxicity into airway epithelial cells,[7] vascular tissue,[8] skeletal muscle,[9] synovial cells,[10] retinal tissue,[11] and hematopoietic progenitor cells.[12] Here we report that the SeV-mediated gene transfer into primate ES cells is very efficient and stable even after the terminal differentiation of the cells. In addition, we show that SeV-mediated transgene expression levels can be reduced by the addition of a ribonucleoside analog, ribavirin, to the culture. Ribavirin is a mutagen and inhibitor of viral RNA polymerase.[13,14] It shows antiviral activity against a variety of RNA viruses and is used to treat infections of hepatitis C virus in combination with interferon-α[15,16] and of lassa fever virus.[17] The method of using antiviral drugs might offer a novel approach for regulated SeV-mediated gene expression in primate ES cells.

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Results

SeV-mediated gene transfer into ES cells

In this study, we have used an SeV vector, which is capable of self-replication but incapable of transmitting to other cells.[6] The vector does not encode the fusion (F) protein (Figure 1a), which is essential for viral entry into cells. It can be propagated only in a packaging cell line expressing the F protein. The green fluorescent protein (GFP) gene was introduced after the leader sequence of the vector genome. Cynomolgus ES cells[18] were exposed to the SeV vector for 24 h. Flow cytometric analysis at 2 days after infection showed that 15, 38, and 61% of cells fluoresced at 2, 10, and 50 transducing units (TU) per cell, respectively (Figure 1b). The gene transfer efficiency of about 60% is comparable to or even better than that for lentiviral vectors.[3] We confirmed that the undifferentiated cell fractions remained unchanged after the infection with SeV vector, as assessed by the expression of undifferentiated markers, alkaline phosphatase and SSEA-4 (data not shown). The GFP expression after infection was stable at least for a month. On the other hand, the GFP gene transfer to cynomolgus ES cells with adenovirus- and adeno-associated virus (AAV)-based vectors resulted in much lower expression levels (<20% by flow cytometry) and the levels declined to zero within a week after infection (Figure 1c).

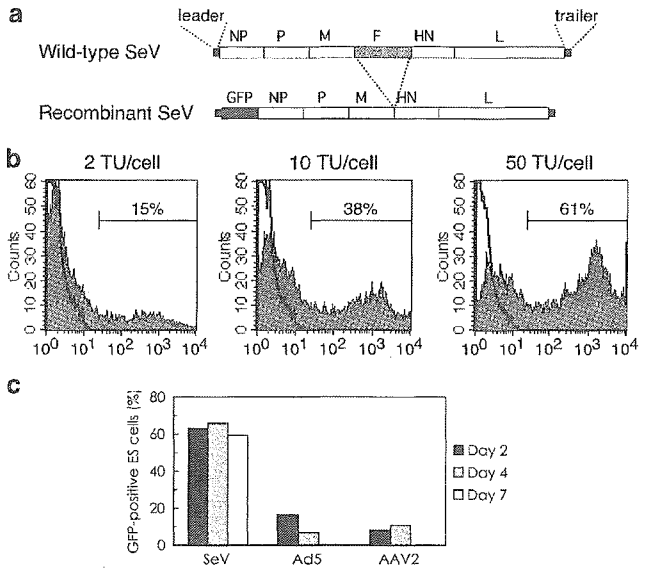

Figure 1 High-level transgene expression in cynomolgus ES cells after infection with SeV vector. (a) Schematic diagrams of the wild-type SeV genome and recombinant F-defective SeV carrying the GFP gene (SeV vector in this study). The SeV genome is 15 384 nucleotides long and its genes (NP, P, M, F, HN, and L) are in order from 3′ to 5′ in the negative-strand RNA. In the SeV vector, the entire fusion (F) gene was removed and the GFP gene was introduced at a unique NotI site between the leader sequence and NP gene. (b) The GFP expression by the SeV vector in cynomolgus ES cells. Cynomolgus ES cells were infected with the SeV vector at 2, 10, and 50 TU/cell. The flow cytometric profiles at day-2 postinfection are shown in gray. The white areas indicate uninfected ES cells. The fractions of GFP-positive cells are indicated. (c) The GFP expression levels in cynomolgus ES cells infected with the SeV (50 TU/cell), adenovirus serotype 5 (Ad5, 3.4×10^2 g.c./cell), and AAV serotype 2 (AAV2, 2.4×10^4 g.c./cell) vectors. The fractions of GFP-positive cells were examined by flow cytometry at 2, 4, and 7 days postinfection.

We plucked fluorescent ES cell colonies under a fluorescent microscope once at 1 month after infection and propagated them. After this selection procedure, approximately 90% of the ES cells expressed GFP (Figure 2a and b) and the high-level expression was stable for a year as assessed by flow cytometry (Figure 2c, upper). The mean fluorescence intensity per cell was also stable (Figure 2c, lower), indicating that the replicating vector genome was almost equally delivered to each cell of all progeny. The self-replication of the SeV vector in infected cells was confirmed by RNA-PCR that amplified the viral RNA genomic sequence (Figure 3a). The GFP cDNA sequence, however, could not be detected by DNA-PCR in the infected cells (Figure 3b), indicating that no DNA phase was involved in the GFP expression.

Pluripotency of infected ES cells

The SeV-infected, fluorescent cynomolgus ES cells were able to form fluorescent tumors when transplanted into immunodeficient mice (Figure 4a–c). The fluorescence was observed uniformly by fluorescent microcopy (Figure 4d and e). The tumors consisted of all three embryonic germ layer cells (Figure 4f–i). Thus, the SeV-infected ES cells were capable of forming teratomas and the SeV infection did not spoil the pluripotency of ES cells. The infected, fluorescent cynomolgus ES cells were also able to generate fluorescent embryoid bodies (Figure 5a and b), MAP-2-positive neurons (Figure 5c), clonogenic hematopoietic colonies (Figure 5d and e), and mature functional (NBT test-positive) neutrophils (Figure 5f and g), all of which fluoresced. In addition, the GFP expression levels were not decreased during the teratoma formation or differentiation, indicating that no 'silencing' of the transgene occurred.

Drug-inducible reduction of transgene expression

Next, we examined whether ribavirin inhibits the replication and transcription of the SeV vector resulting in a reduction of transgene expression. We first used a rhesus monkey kidney cell line (LLC-MK2) to test the effect of ribavirin on the replication and transcription of the SeV vector. LLC-MK2 is a standard control cell line for SeV infection. Ribavirin was added at various concentrations 2 days after the infection. The formation of viral particles quantified by the hemagglutination assay decreased drastically upon the addition of ribavirin (Figure 6a). The decrease was dependent on the dose of ribavirin. The GFP expression was also depressed dose-dependently (Figure 6b). Thus, ribavirin dose-dependently inhibits the replication and transcription of the SeV vector in LLC-MK2 cells. The toxicity associated with ribavirin was not observed in LLC-MK2 cells.

We then examined the effect of ribavirin on SeV-infected, fluorescent cynomolgus ES cells. The addition of ribavirin also resulted in a dose-dependent reduction of GFP expression in the cells (Figure 6c). Although the GFP expression was almost completely inhibited after a 3-day exposure with 4 mM of ribavirin, the cells could not be propagated thereafter. Ribavirin at high concentrations (>1 mM) hampered the proliferation of cynomolgus ES cells. With lower concentrations (0.5–0.75 mM) of ribavirin, the GFP expression level decreased by half. After the discontinuation of ribavirin treatment, the cells could be propagated and nearly regained the original level of GFP expression. The undifferentiated cell fractions were unchanged after the discontinuation as assessed by alkaline phosphatase and SSEA-4 staining (Figure 6d).

Discussion

There are several advantages in using SeV vectors over other vectors. (i) SeV vectors can infect nondividing, quiescent cells as well as dividing cells unlike oncoretroviral vectors.[7–11] Thus, they can be used to infect cells that are terminally differentiated as well as at various stages of differentiation, whether they are dividing or not. (ii) SeV vector-mediated gene transfer does not require a DNA phase. Thus, there is no concern about the unwanted integration of foreign sequences into the host genome unlike with oncoretroviral or lentiviral vectors. (iii) Transgene expression is stable even in dividing cells since the SeV vector replicates by itself in the cytoplasm of host cells. On the other hand, gene transfer using nonreplicating adenoviral and AAV vectors resulted in decreased levels of transgene expression in dividing cells over time, since the non-replicating transgene was