baboon models treated with an arteriovenous shunt. DX-9065a has also been reported to have no effect on bleeding time, while it effectively reduced the severity in acute DIC rat models induced by lipopolysaccharide and thromboplastin [7,8]. Further, Murayama [20] reported that bleeding time was not prolonged when DX-9065a was intravenously administered to healthy male volunteers, even at the highest plasma concentration of 1640 ng mL^{-1} (2.87 μM). Intravenous infusion of JTV-803 at 1–10 mg kg^{-1} h^{-1} also had less of an effect on bleeding time in rats in another study [5]. Therefore, our results regarding the effects of synthetic FXa inhibitors on T$_{50}$ and platelet aggregation induced by TF may explain the phenomena described in previous reports.

The process by which these FXa inhibitors do not prolong bleeding time in spite of a strong inhibition of thrombin formation remains poorly understood. We consider that the initial immediate formation of a small yet adequate amount of thrombin to activate platelets is important for hemostasis. Since the affinity of thrombin for platelets is hundreds of times higher than that for fibrinogen [21,22], a minimal amount of thrombin, though insufficient to convert fibrinogen to fibrin, may effectively activate platelets to induce primary hemostasis. Namely, in patients given an FXa inhibitor, a small amount of thrombin might immediately be formed when any trigger for bleeding is stimulated, similar to that in healthy subjects. The formed thrombin may be enough to activate platelets for early hemostasis, though not adequate to cause the insoluble fibrin to be related with thrombus. Tanabe [23] also speculated that the competitive and reversible inhibition of FXa by DX-9065a might result in thrombin generation sufficient to induce hemostatic plug formation, thought it would be insufficient to facilitate thrombus formation.

In the present study, we showed the formation of initial thrombin with and without the presence of a FXa inhibitor by measuring T$_{50}$, and also found that the immediate aggregation of platelets induced by the initial thrombin was not disturbed by its presence. The fact that primary hemostasis is conserved may not mitigate hemorrhaging seen, for example, in a serious surgical situation. However, we are convinced that the tested FXa inhibitors may have a role with initial thrombin forming time by preserving local hemostasis often seen during the treatment of thrombosis. Therefore, DX-9065a and JTV-803 are considered unique antithrombotic agents without hemorrhagic effects.

Acknowledgements

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Antiprothombin antibodies and the diagnosis of antiphospholipid syndrome

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Abstract

The preliminary classification criteria for definite antiphospholipid syndrome (APS) include the presence of anticardiolipin antibodies (aCL) and/or lupus anticoagulant (LA) as laboratory criteria. However, antiphospholipid antibodies (aPL) are a heterogeneous group of antibodies comprising also antibodies against phospholipid-binding proteins or their complexes with phospholipids. Prothrombin is one of the antigen recognized by aPL. In the last decade, there has been increasing interest in antibodies against prothrombin alone and those against phosphatidylserine–prothrombin complex. The latter, phosphatidylserine-dependent antiprothrombin antibodies (aPT), have been closely associated with APS and LA. In this paper, we review the properties of antiprothrombin antibodies.
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Markers for the diagnosis of antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a clinical condition characterized by recurrent thrombotic events/pregnancy morbidity associated to the persistence of antiphospholipid antibodies (aPL).

Antiphospholipid antibodies are classically classified according to their in vitro method of detection in anticardiolipin antibodies (aCL) measured by enzyme-immunosorbent assay (ELISA) and lupus anticoagulant (LA) detected by clotting assays. However, the current concept of aPL includes antibodies directed against phospholipid-binding proteins involved in the coagulation system or their complexes with phospholipids.

Anticardiolipin antibodies

Two decades have passed since the first description by Hughes et al [1] of the association of aPL with thrombotic events, neurological disease, pulmonary hyper-

tension, livedo, thrombocytopenia, and recurrent miscarriages. The syndrome was named initially "anticardiolipin syndrome" and later "antiphospholipid syndrome", emphasizing that aPL group rather than aCL alone was present in those patients.

Anticardiolipin antibodies can be detected by immunological assays (radioimmunoassay and ELISA) and were originally devised by Harris et al. [2] and Koike et al. [3]. In 1990, Harris [4] proposed the first criteria for the classification of APS and they were applied in many clinical studies of APS. In 1998, an International Consensus defined the preliminary classification criteria for definite APS [5]. In the laboratory features of those two criteria, aPL (aCL and/or LA) must be positive on at least two occasions more than 6 weeks apart to avoid false-positive aPL associated with acute infectious diseases.

Many studies indicated that antibodies against β2Gly-2Glycoprotein I (β2GPI) are one of the predominant antibodies detected as aCL in APS patients [6–8]. APS-related aCL recognize cryptic epitope(s) on the β2GPI molecule that appear when β2GPI interacts with a lipid membrane composed of negatively charged phospholipids (β2GPI-dependent aCL or antibodies against cardiolipin/β2GPI complex: aCL/β2GPI).

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Lupus anticoagulant and its cofactors

LA are immunoglobulins (IgG, IgM, IgA, or their combination) that interfere with in vitro phospholipid- dependent tests of coagulation (prothrombin time [PT], activated partial thromboplastin time [APTT], kaolin clotting time [KCT], dilute Russell's viper venom time [dRVVT]) [9]. LA was first described in a patient with systemic lupus erythematosus (SLE) bleeding tendency [10], thus called lupus anticoagulant, but this term is a misnomer because the vast majority of patients with LA does not have SLE.

Some subtypes of LA according their clotting inhibitory behavior have been reported. In 1978, Exner et al. [11] showed that there are three types of LA mixing pattern by KCT. After the identification of β2GPI as a cofactor of solid-phase aCL assay [6–8], Oosting et al. [12] and Roubey et al. [13] demonstrated that many LA plasmas depended on the presence of β2GPI in their anticoagulant activity; thus, β2GPI was considered as one of the major cofactors of LA.

In 1991, Bevers et al. [14] highlighted the importance of antiprothrombin antibodies (aPT) in causing LA activity. Later, Oosting et al. [15] showed that LA inhibited endothelial cell-mediated prothrombinase activity and the IgG fraction containing LA activity bound to phospholipid–prothrombin complex. Therefore, prothrombin was identified as another cofactor of LA.

Recently, it has been shown that neutralization process enables to differentiate between LA activity caused by anti-β2GPI and that by aPT. The addition of cardiolipin neutralized aCL/β2GPI LA but not prothrombin-dependent LA in an APTT-based assay [16]. These observations are reasonable since β2GPI reacts with cardiolipin but the phospholipid binding site of prothrombin is specific for phosphatidylserine.

Prothrombin structure and in vitro function of antiprothrombin antibodies

Prothrombin (factor II) is a vitamin K-dependent glycoprotein present at a concentration of approximately 100 μg/ml in normal plasma. Mature human prothrombin consists of a single-chain glycoprotein with a molecular weight of 72 kDa [17]. During its biosynthesis in the liver, prothrombin undergoes γ-carboxylation. These γ-carboxyglutamic residues, known as the Gla-domain, are located on fragment 1 of the prothrombin molecule. Gla-domain is essential for the calcium-dependent binding of phosphatidylserine to prothombin. A kringle domain containing two kringle structures and a carboxyl-terminal serine protease follows the Gla-domain.

Prothrombin is physiologically activated by the prothrombinase complex (activated factor X, factor V, calcium, and phospholipids). Once negatively charged phospholipids bind prothrombin, prothrombinase complex converts prothrombin to thrombin, which triggers fibrinogen polymerization into fibrin [18]. In addition, thrombin binds thrombomodulin on the surface of endothelial cells and activates protein C, then exerts its anticoagulant activity by digesting factor V and depriving the prothrombinase complex of its most important cofactor. Because of this negative feedback pathway, prothrombin/thrombin behaves as "indirect" anticoagulant.

Simmelink et al. [19] reported that addition of affinity-purified aPT from LA-positive plasma to normal plasma
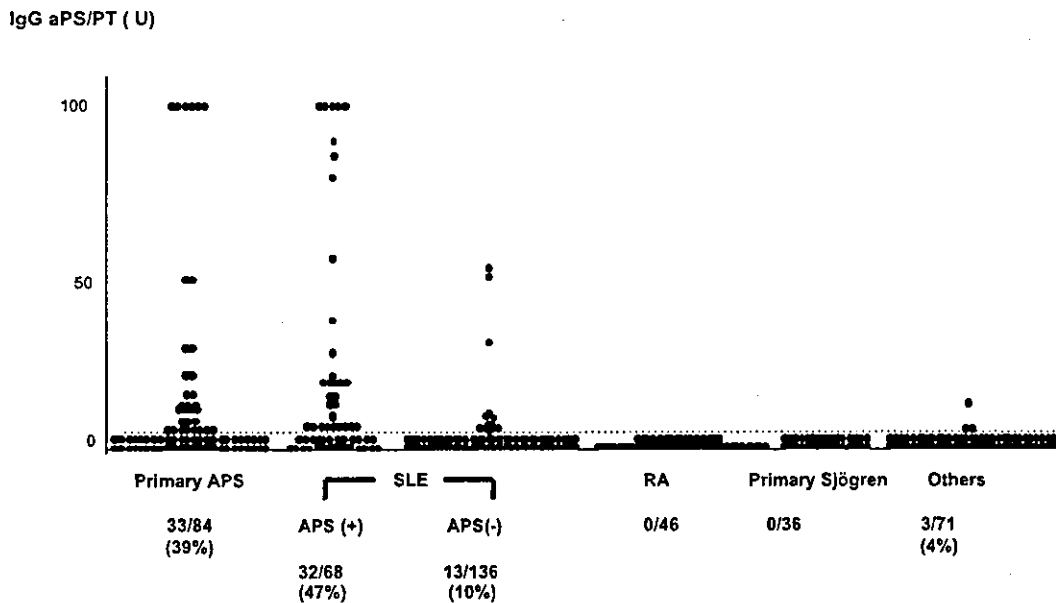

Fig. 1. Prevalence of IgG phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) in 441 patients with autoimmune diseases. IgG aPS/PT were detected by enzyme-linked immunosorbent assay. The dashed line represents the cut-off for positivity. U: units. APS: antiphospholipid syndrome, SLE: systemic lupus erythematosus, RA: rheumatoid arthritis.

induces LA activity and that LA activity was neutralized upon increasing the phospholipid concentration. They also showed that complexes of prothrombin and aPT with LA activity inhibit both prothrombinase and tenase complex. The results suggest that aPT might increase the affinity of prothrombin for negatively charged phospholipids, thereby competing with clotting factors for the available catalytic phospholipid surface. Galli et al. [20], investigating the anticoagulant activity of the protein C system, demonstrated the dominant inhibitory effect on activated protein C of aCL/β2GPI, compared with the effect of aPT; therefore, this phenomena may depend on the specificity of antibodies used in the experiments.

Detection methods and clinical associations of antiprothrombin antibodies

Double diffusion, counterimmunoelectrophoresis [21–23], and assays based on the impairment of prothrombin activation by aPT were the first techniques used for screening aPT [15,24], but they were not suitable for the routine clinical practice.

In 1995, Arvieux et al. [25] described an ELISA for detection of aPT using prothrombin as antigen coated onto irradiated plates (aPT-A). Since then, some clinical studies have investigated their clinical implications [26]. Some of these studies showed positive correlation between aPT-A and some of the clinical features of the APS including venous or arterial thrombosis [27–30], pregnancy loss [31,32], thrombocytopenia [29], or clinical features of APS in general [33,34]. Other studies, however, failed to find correlation between the presence of aPT-A and thrombosis [35–42].

Controversial results regarding aPT have also been reported in patients without autoimmune disease. In middle-aged men, high levels of aPT-A conferred high risk of myocardial infarction or cardiac death and of thrombotic events [43,44], but no correlations were found between aPT-A and thrombotic events in a large population of unselected patients with history of venous thrombosis [45].

In 1996, antibodies directed to phosphatidylserine–prothrombin complex (or phosphatidylserine-dependent antiprothrombin antibodies; aPS/PT) were described in LA-positive patients [46], and Galli et al. [39] reported that the assay using phosphatidylserine-bound prothrombin as antigen was more efficient in demonstrating the presence of aPT than the aPT-A system.

The clinical studies described above were performed using the aPT-A ELISA. Our group used the aPS/PT assay in a large population of patients with autoimmune diseases and found that aPS/PT were highly prevalent in patients with APS compared with patients with other diseases (Fig. 1). We also showed that the detection of aPS/PT strongly correlated with the clinical manifestations of APS and with the presence of LA [47]. Therefore, the aPS/PT ELISA detects a subpopulation of aPT of significant clinical relevance.

Lupus anticoagulant determination and antiprothrombin antibodies

LA is detected by functional assays according to the recommendation of the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis [48]. A tree-step approach has been proposed:

(1) Screening test to evaluate if phospholipid-dependent coagulation test are prolonged,
(2) Mixing test to demonstrate that the prolongation of the clotting time is caused by an inhibitor present in plasma,
(3) Confirmation of the phospholipid-nature of the inhibitory antibody by adding extra phospholipids or platelets neutralization.

The identification of LA is often difficult. No guidelines have been proposed on the nature and composition of the phospholipids used in those tests, and several commercial tests for LA detection are available. Some publications have shown that the correlation among these tests is very low, and in fact, some laboratories rely on poorly responsive screening assays [49]. Thus, an accurate detection of LA is
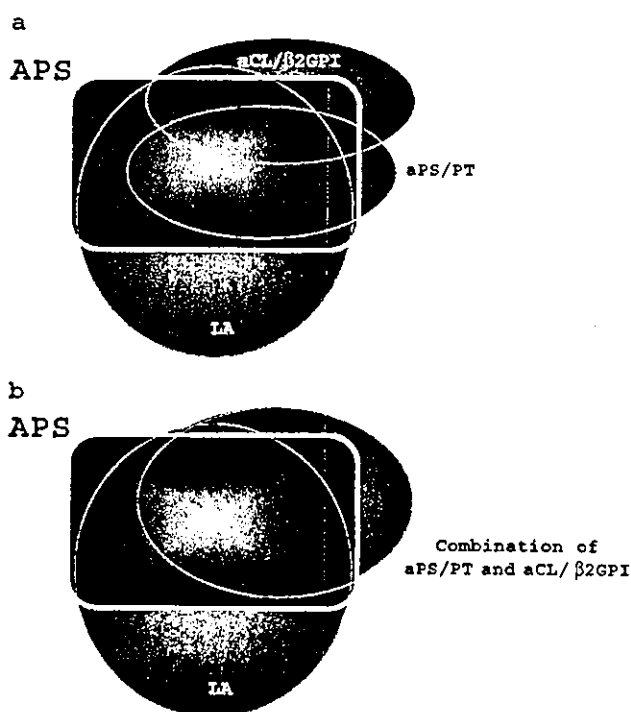
Fig. 2. Distribution of antiphospholipid antibodies in patients with antiphospholipid syndrome (APS). (a) In patients with APS, a heterogeneous population of autoantibodies can be observed including lupus anticoagulant (LA), β2Glycoprotein I-dependent anticardiolipin antibodies (aCL/β2GPI), and phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT). (b) The combination of aCL/β2GPI and aPS/PT ELISA markedly improves the sensitivity for the diagnosis of APS when compared with that observed with each independent ELISA. In addition, the ELISA combination showed higher specificity with acceptable sensitivity in comparison with LA.

essential to facilitate the diagnosis of patients with thrombotic disorders.

Two major subpopulations of specific antibodies reactive with β2GPl [13,50] or prothrombin [39] are responsible for the LA activity in phospholipid-dependent coagulation tests. We showed that 95.6% of patients with aPS/PT had LA; thus, this new assay might be a useful and easy tool to confirm the presence of LA [47].

Antiphospholipid antibodies specificity and the risk of thrombosis

LA have been reported to represent a stronger risk factor for thrombosis than aCL [51,52] or aPT [36] based on the higher relative risk of thrombotic events. Autoimmune thrombosis and pregnancy morbidity is caused by, or at least correlated with, a group of autoantibodies with similar properties that affect the phospholipid-dependent reactions. The autoantigens and the detection methods are heterogeneous. The comparison between the results of functional assays that detect activity of different aPL and one independent ELISA that evaluates one specific aPL might lead to misunderstanding of the parameters of clinical significance. Distribution of antibodies detected by those assays in our experience is shown in Fig. 2.

The detection of aCL/β2GPl and aPS/PT has significantly improved the specificity of the conventional aCL or aPT assays for the diagnosis of APS. The prevalence of the antibodies detected by those specific ELISA in patients with APS was not as high as that of LA, but the specificity of the new assays were remarkable [47,53,54]. We compared the value, for the diagnosis of APS, of testing LA with that of testing both aCL/β2GPl and aPS/PT in 172 patients with autoimmune diseases [55]. As aCL/β2GPl and aPS/PT detect different population of antigen-specific antibodies, we combined those two assays and calculated their clinical significance as "ELISA combination". We found that both ELISAs in combination lead to a higher specificity for APS than the determination of LA and consequently might further enhance their usefulness as specific marker for APS [55] (Table 1).

LA have been frequently found in patients with thrombosis. In APS, its prevalence varies widely depending on many aspects, including reagents' sensitivity or coagulation assay methods for its detection, and this heterogeneity might significantly compromise the diagnosis of LA. Because of the lack of international standardization for LA, the definition of LA may largely differ among the laboratories [56]. LA is also found in patients with infectious or malignant diseases, and other APS unrelated conditions. Few methods have been reported to discriminate those nonspecific LA phenomenons from APS-related LA. Therefore, LA definition, in routine clinical practice, is far from consensus.

In conclusion, aPS/PT, as well as aCL/β2GPl, are useful tools for a better recognition of APS. Those assays should

Table 1
Sensitivity and specificity of aPL tests for the diagnosis of APS

Assay	Sensitivity (%)	Specificity (%)
LA	94	79
aCL/β2GPl	54	86
aPS/PT	57	92
aCL/β2GPl or aPS/PT (ELISA combination)	77	92

LA: lupus anticoagulant; aCL/β2GPl: β2Glycoprotein 1-dependent anticardiolipin antibodies; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies.

be performed in conjunction with LA test. Additional and prospective studies on aPS/PT, however, are needed to establish the clinical relevance of these antibodies.

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Adult-onset Idiopathic Hypogonadotropic Hypogonadism due to Isolated Pituitary Gonadotropin Deficiency

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Abstract

A 25-year-old Japanese man with adult-onset idiopathic hypogonadotropic hypogonadism is reported. He had been delivered normally, had normal puberty, and experienced erectile dysfunction at age 24 years. Brain MRI revealed no abnormal findings and endocrinological data supported the diagnosis of isolated gonadotropin deficiency. Although most patients with idiopathic hypogonadotropic hypogonadism have a hypothalamic dysfunction, the lesion in this case may be considered to be in the pituitary since repetitive GnRH loading failed to increase serum LH and FSH.
(Internal Medicine 43: 571–574, 2004)

Key words: isolated, erectile dysfunction, gynecomastia

Introduction

Hypogonadotropic hypogonadism (HH) is classified depending on different conditions: isolated deficiency or combined deficiency with other pituitary hormones, congenital or acquired (adult onset), and hypothalamic or hypophyseal hormone defects. Most congenital cases show a type of isolated deficiency caused by genetic abnormality related to the *KAL1* gene, except for a rare case defined as congenital combined pituitary hormone deficiency (1). Most cases of the acquired form show multiple hormone deficiency induced by anatomical disorders, such as an infiltrative process or a space-occupied lesion. Psychological stress, nutritional conditions and acute illness also can cause gonadal dysfunction (2). Here, we report a rare case of adult-onset idiopathic isolated HH due to isolated pituitary gonadotropin deficiency.

Case Report

A 25-year-old Japanese man was referred to Hokkaido University Hospital with the chief complaint of erectile dysfunction and gynecomastia of a 1-year duration. He was born normally and grew up with a normal puberty; pubic hair began to grow at age 11 years and the first ejaculation at age 12 years. There was no known history of head trauma or diabetes mellitus. He had irritable bowel syndrome at the age of 17 and his parents had been treated for Graves' disease.

The patient was 168 cm tall and weighed 62 kg. Blood pressure was 122/76 mmHg. He showed no eunuchoidism, whereas, bilateral gynecomastia was present. External genitalia were grade III in Tanner stage and testis volume was 8 ml (normal: 15–25 ml) for both testes. His olfaction was normal.

Laboratory findings are shown in Table 1. Urinalysis, complete blood count, and general biochemical markers including electrolytes were within the normal limits. Serum ferritin, angiotensin-converting enzyme, and tuberculin reaction showed no abnormality. In serological examinations, anti-nuclear antibody (ANA), proteinase 3 (PR3) anti-neutrophil cytoplasmic antibody (ANCA), anti-thyroid antibody, anti-pituitary and anti-adrenal gland antibody were negative.

Regarding radiographic findings, in the pituitary MRI, the pituitary complex was normal and a space-occupying lesion in the hypothalamic-pituitary region was not detectable (data not shown).

Endocrinological examinations (Tables 2, 3, 4 and Fig. 1) revealed that thyroid function and growth hormone (GH)-insulin-like growth factor (IGF)-1 axis were normal. Noctual serum cortisol was slightly elevated. Responses of adrenocorticotropic hormone (ACTH) and cortisol was blunted on the corticotrophin releasing hormone (CRH) loading test. To further evaluate the pituitary-adrenal axis, dexamethasone

Table 1. Laboratory Data on Admission

<Urinalysis>		<Biochemistry>			
SG	1.018	TP	6.3 g/dl	T-Cho	174 mg/dl
pH	5.0	Alb	4.1 mg/dl	TG	161 mg/dl
Protein	(–)	T-bil	0.5 mg/dl	HDL-Cho	62 mg/dl
Glucose	(–)	AST	17 IU/l	Fe	116 μg/dl
Acetone	(–)	ALT	27 IU/l	TIBC	314 μg/dl
		LDH	207 IU/l	ferritin	169 ng/dl
<Complete Blood Count>		γ-GTP	39 IU/l	ESR	6 mm/h
WBC	6,300/μl	BUN	7 mg/dl	<Others>	
RBC	436×10⁴/μl	Cr	0.7 mg/dl	chromosome	46, XY
Hb	13.7 g/dl	UA	3.5 mg/dl	ACE	10.1 IU/l
Ht	39.5%	Na	138 mEq/l	ANA	(–)
Plt	21.2×10⁴/μl	K	3.9 mEq/l	PR3-ANCA	<10 U/l
		Cl	104 mEq/l	TgAb	<0.30 U/ml
		Ca	9.2 mg/dl	TPOAb	<0.15 U/ml
		P	3.2 mg/dl	anti-pituitary Ab	(–)
				anti-adrenal Ab	(–)

ACE: angiotensin-converting enzyme, ANA: anti-nuclear antibody.

Table 2. Endocrinological Examinations

fT3	3.29	pg/ml
fT4	1.42	ng/dl
TSH	2.45	μU/ml
GH	0.31	ng/ml
IGF-1	212.1	ng/ml
prolactin	26.2	ng/ml
LH	<0.2	mIU/ml
FSH	<0.5	mIU/ml
Testosterone	<0.10	ng/ml
Estradiol	<10.0	ng/ml
PRA	1.52	ng/ml/h
Aldosterone	125.1	pg/ml
DHEA-S	1,160	ng/ml

	8:00	23:00
ACTH (pg/ml)	45.4	10.9
Cortisol (μg/dl)	36.0	6.7

17-OHCS	5.5	mg/day
17-KS	7.4	mg/day
Urine-free cortisol	28	μg/day

Table 3. HCG Loading Test

	Day 1	Day 2	Day 3
Testosterone (ng/ml)	<0.10	0.66	1.0

5,000 U of HCG was given i.m. for 3 consecutive days.

Table 4. Repetitive GnRH Loading Test

		Day 1	Day 3	Day 5
LH (mIU/ml)	pre	<1.0	<1.0	<1.0
	post	<1.0	<1.0	<1.0
FSH (mIU/ml)	pre	<2.0	<2.0	<2.0
	post	<2.0	<2.0	<2.0
Testosterone (ng/ml)		<1.0	<1.0	<1.0

100 μg of GnRH was given i.v. twice a day for 5 consecutive days. On Days 1, 3, and 5, the GnRH test was done. pre: before GnRH injection, post: after GnRH injection.

suppression test gave a normal response as did urinary excretion of free cortisol and steroid metabolites. Serum leuteinising hormone (LH) and follicle stimulating hormone (FSH) were below detection limits concordant with an undetectable level of testosterone. Extremely low levels of these hormones were reproducible with different assay systems. Human chorionic gonadotropin (HCG) loading test and repetitive gonadotropin releasing hormone (GnRH) loading test were done. On the HCG loading test, testosterone weakly, but significantly responded (Table 3). Responses of LH and FSH were completely blunted on repetitive GnRH loading tests (Table 4). Based on radiological findings and

endocrinological examinations, adult-onset idiopathic HH induced by isolated pituitary gonadotropin defects was diagnosed. Neither fluorescent immunohistochemical study using normal human pituitary sections nor Western blot analysis revealed positive results for any autoantibody against pituitary cells (data not shown).

Discussion

For the patient data presented here, there are three characteristic points: 1) adult-onset (acquired) type of hypogonadism, 2) isolated gonadotropin deficiency, 3) hypogonadotropic hypogonadism (HH) with pituitary defects. Adult-
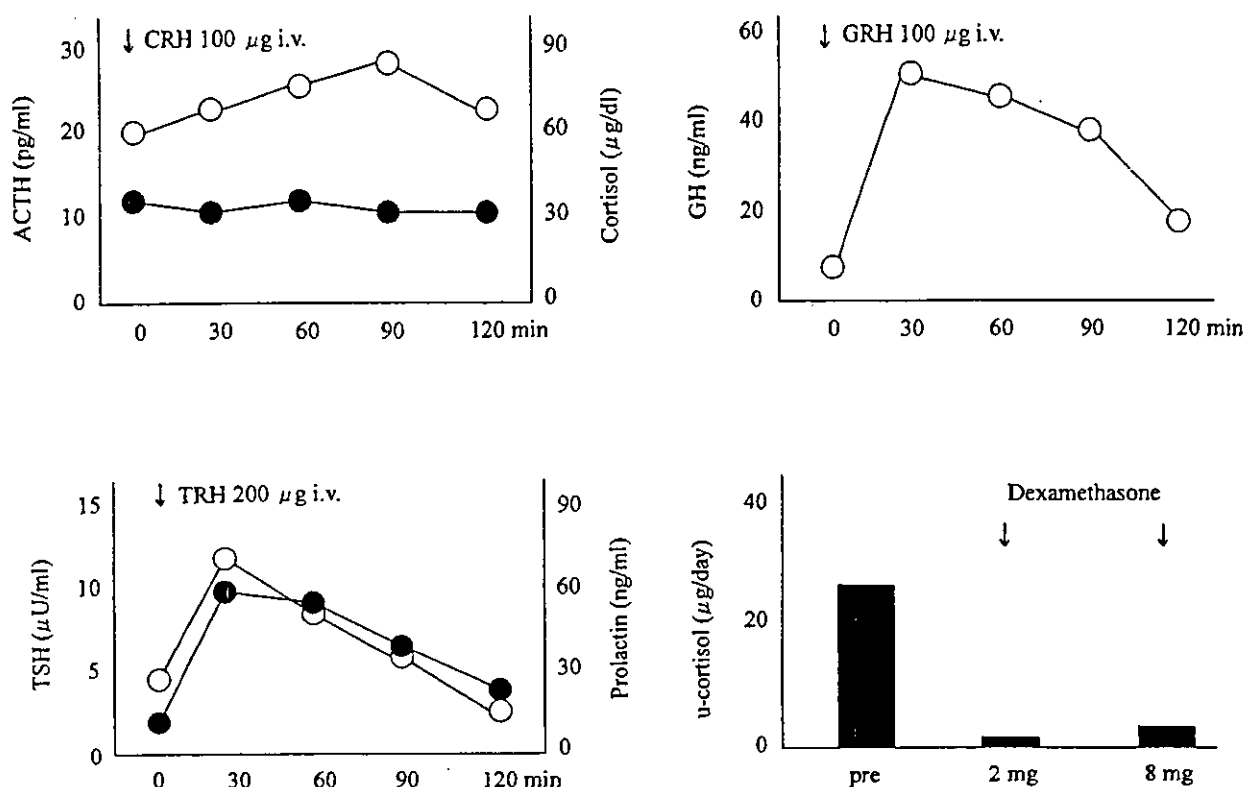
Figure 1. Response of hormones to CRH (100 μg), TRH (200 μg) and GRH (100 μg) loading test and dexamethasone suppression test. Open and closed circles indicate ACTH and cortisol in CRH test, respectively. Open and closed circles represent TSH and prolactin in TRH test, respectively.

onset HH is caused by a large range of disorders, including infiltrative processes and space-occupying lesions such as hemochromatosis, granulomatous disease, lymphocytic hypophysitis, pituitary adenoma and other tumors (2). The patient showed neither radiographic abnormality in the hypothalamic-pituitary region nor serological positive findings that support infectious and granulomatous disease, resulting in the diagnosis of idiopathic HH. Furthermore, the lesion was speculated to be in the pituitary based on repetitive GnRH loading tests. The disease most likely to cause acquired isolated gonadotropin deficiency with pituitary defect is hemochromatosis (3, 4), and was ruled out with evidence of normal ferritin levels in this case.

Psychological stress, acute illness, abnormal nutritional status with either severe emaciation or obesity, and excessive exercise cause hypothalamic amenorrhea in women (5). Although the patient had never exercised excessively or starved himself, he had a history of irritable bowel syndrome with which psychologically nervous subjects can be victims. Furthermore, he showed an abnormal response to CRH loading test that can be seen in depressed patients (6). If erectile dysfunction is the counterpart of amenorrhea in women, it is possible that psychological status in this case may cause the present condition.

A few cases of adult-onset idiopathic HH caused by genetic defects involved in genes related to gonadal function or by autoimmune process have been reported (1, 8, 9). Mutation in the gene encoding DAX-1 causes X-linked adrenal hypoplasia congenita (AHC). A subpopulation of patients with DAX-1 gene mutation was found to show a phenotype of delayed-onset HH that accompanies various extents of adrenal insufficiency (8). Whether the HH is a result of hypothalamic or pituitary dysfunction, or both, remains unclear (10). Mutation in the gene encoding the GnRH receptor also shows a wide spectrum of phenotype of HH (11). However, we found no report of a complete blunt response of gonadotropin to repetitive GnRH injection in patients with such gene mutations. Mutations of the responsible gene were not investigated, but this case does not seem to have been caused by gene mutation since there is neither family history nor adrenal hypofunction.

Autoimmune processes also should be considered as a cause of adult-onset HH, since reported cases have been associated with polyglandular autoimmune syndrome (7, 8). Regardless of the lack of positive findings with autoantibodies in addition to negative results of a fluorescent immunohistochemical study and Western blot analysis, the parents of this patient had a history of Graves' disease.

Whether or not an autoimmune process might be involved for the pathogenesis of this case would need to be excluded.

Adult-onset idiopathic HH caused by an unknown etiology has been reported (12). In the description given, regardless of the lack of causative factors including medical history and imaging studies for defects in GnRH release, the patient had low levels of testosterone and gonadotropins. Most of the patients described responded to pulsatile GnRH administration, and libido and sexual function were restored. The category of these cases seems to be a hypothalamic disorder judging from the responsiveness to GnRH. Therefore, this form of HH should be kept in mind as a possible treatable condition in subjects with male infertility.

We presented adult-onset idiopathic HH with pituitary defect of unknown etiology. Further molecular and immunological analysis will be necessary to elucidate the pathogenesis of the present case.

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Localized Relapse in Bone Marrow of Extremities After Allogeneic Stem Cell Transplantation for Acute Lymphoblastic Leukemia

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We report a patient with a relapsed in bone marrow of extremities after allogeneic peripheral blood stem cell transplantation for acute lymphoblastic leukemia (ALL). The patient complained of pain in the right upper arm and left leg 15 months after transplantation. Magnetic resonance imaging (MRI) and fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) showed abnormal findings in bone marrow of upper and lower extremities. There were no findings of relapse in aspirates from the sternum and iliac bone marrow. Biopsy specimen from the iliac bone marrow showed normocellular marrow without leukemic cells. Biopsy specimen from the right humerus revealed marked leukemic cell infiltration in the bone marrow. This is apparently the first case of localized relapse of ALL in bone marrow of extremities. Physicians should be aware of unusual relapse sites of leukemia after allogeneic stem cell transplantation. MRI and FDG-PET may be of value in detecting this type of relapse. Am. J. Hematol. 76:279–282, 2004. © 2004 Wiley-Liss, Inc.

Key words: acute lymphoblastic leukemia; localized relapse; extremities; allogeneic stem cell transplantation

INTRODUCTION

In patients with acute leukemia, it is generally assumed that leukemic cells are distributed throughout the bone marrow. Hence examinations of most patients with acute leukemia are done of the sternum or iliac bone marrow. There are reports of a localized extramedullary relapse of acute leukemia following stem cell transplantation [1–3], but a localized bone marrow relapse is rare [4,5]. We describe herein what may be the first case of a localized relapse of acute lymphoblastic leukemia (ALL) in bone marrow of extremities after allogeneic peripheral blood stem cell transplantation (PBSCT).

CASE REPORT

A 37-year-old Japanese woman was diagnosed with ALL in September 2001. The iliac bone marrow aspirate had 97% lymphoblasts. The leukemic cells expressed CD19, CD10, CD79a, and CD13 antigens. © 2004 Wiley-Liss, Inc.

Southern blot analysis showed monoclonal rearrangement of the immunoglobulin heavy chain (IgH) JH gene. Relative expression of the Wilms tumor-1 gene (WT-1) [6] mRNA to K562 was elevated to 278×10^{-4} (normal range $< 10 \times 10^{-4}$) with quantitative RT-PCR assay of the bone marrow aspirate. After achieving complete remission while on combined chemotherapy, she received an allogeneic PBSCT from her HLA-matched sister in February 2002. The conditioning regimen consisted of etoposide (15 mg/kg/day on days −7 and −6), cyclophosphamide (60 mg/kg/day

on days −5 and −4), and fractionated total body ir-radiation (4 Gy/day on days −3 to −1). Donor unma-nipulated peripheral blood stem cells were infused on day 0. As graft-versus-host disease (GVHD) prophyl-axis, she also received short-course methotrexate and cyclosporin A (CyA). Her granulocyte count was > 1.0 × 10⁹/L from day 13, and her platelet count was > 50 × 10⁹/L from day 16. Bone marrow aspirates and biopsy specimens revealed complete remission, including the disappearance of clonal rearrangement of IgH-JH. WT-1 mRNA became undetectable. based on a quan-titative RT-PCR assay. Neither acute GVHD nor chronic GVHD occurred. CyA was tapered and with-drawn in February 2003.

In May 2003. 15 months after the PBSCT. she complained of right upper arm and left leg pain. X-ray examination showed no destructive or osteolytic lesion. Her symptoms were overcome while on loxo-profen sodium, a nonsteroidal anti-inflammatory drug (NSAID). She experienced two more episodes of right upper arm pain over the ensuing 3 months. and each time she responded symptomatically to the NSAID. A repeat aspiration and biopsy of sternum or iliac bone marrow revealed no evidence of relapse during this period. except for the slight elevation of WT-1 gene expression level (49.0 × 10⁻⁴). In September 2003, she

had an episode of right upper arm and bilateral leg pain with fever. As the existence of osteomyelitis was sus-pected, magnetic resonance imaging (MRI) of extrem-ities and fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) (ECAT EXACT 47. Siemens/CTI. Knoxville, TN) were performed. MRI demonstrated an abnormal signal in the bone marrow of extremities (Fig. 1) as seen on low-intensity weighted imaging in T1 and enhancement with contrast MRI using gadolinium. There were no abnormal sig-nals detected in bone marrow of iliac and vertebral bones. FDG-PET showed a significant uptake only in the extremities (Fig. 2). Standardized uptake value (SUV) of the spot images at right upper arm and left leg were high (6.466 and 6.816. respectively).

Fig. 1.   T1-weighted magnetic resonance imaging (MRI) of the lower leg. Abnormal low-intensity signals were seen in the bone marrow of the bilateral femora.
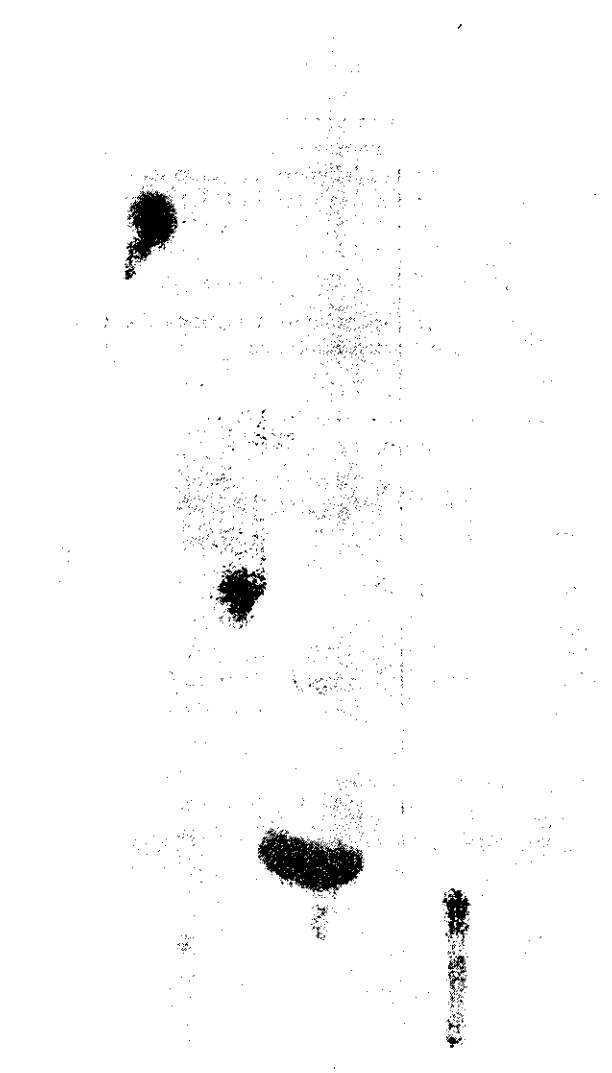

Fig. 2.   Fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET). Significant uptake was seen only in the extremities.

Open biopsy of the right humerus was done under general anesthesia on 21 September 2003. There were no findings of osteomyelitis, but the biopsy specimen revealed marked leukemic cell infiltration in the bone marrow without destruction of the bone (Fig. 3A). Immunophenotyping of these cells showed CD79a$^+$ and CD10$^+$. There were no morphological findings of relapse in aspirates from the sternum and iliac bone marrow and in the biopsy specimen from the iliac bone marrow (Fig. 3B) at that time. IgH-JH rearrangement was undetectable with Southern blot analysis, but a further elevation of WT-1 gene expression level (64.9 × 10^{-4}) was evident. ABO antigen analysis of peripheral red blood cells showed reconstitution with 100% donor cells. Computed tomography scans revealed no evidence of extramedullary relapse. Localized relapse of ALL in bone marrow of extremities was thus diagnosed. The patient was treated with radiotherapy to right upper arm and bilateral leg (8-15 Gy/site), and her symptoms were gradually overcome. She was subsequently prescribed systemic chemotherapy with cytosine arabinoside following infusion of donor lymphocytes (CD3$^+$ cell count, 1.9 × 10^8 cells/kg), including peripheral blood

stem cells (CD34$^+$ cell count, 1.8 × 10^6 cells/kg). The abnormal findings of MRI and FDG-PET improved after the therapy but still remained. Further donor lymphocyte infusions are scheduled.

DISCUSSION

ALL can often relapse even after allogeneic stem cell transplantation. The most common site of relapse is the bone marrow, and leukemic cells are then distributed throughout the bone marrow. Localized extramedullary relapses are not rare in patients with ALL after stem cell transplantation [1-3]. Localized "bone" relapses of ALL have been reported [1,7,8], but localized "bone marrow" relapses are rare [4,5]. The relapse site in our patient was pathologically not bone but bone marrow. Golembe et al. [4] and Maeda et al. [5] reported localized relapse in bone marrow in a patient of ALL and acute myeloid leukemia (AML), respectively. In both these cases, relapse sites were unilateral iliac bone marrow. The patient we have described herein is a unique case in that the leukemic cells were seen in the bone marrow of the extremities and not in the sternum or iliac bone marrow where
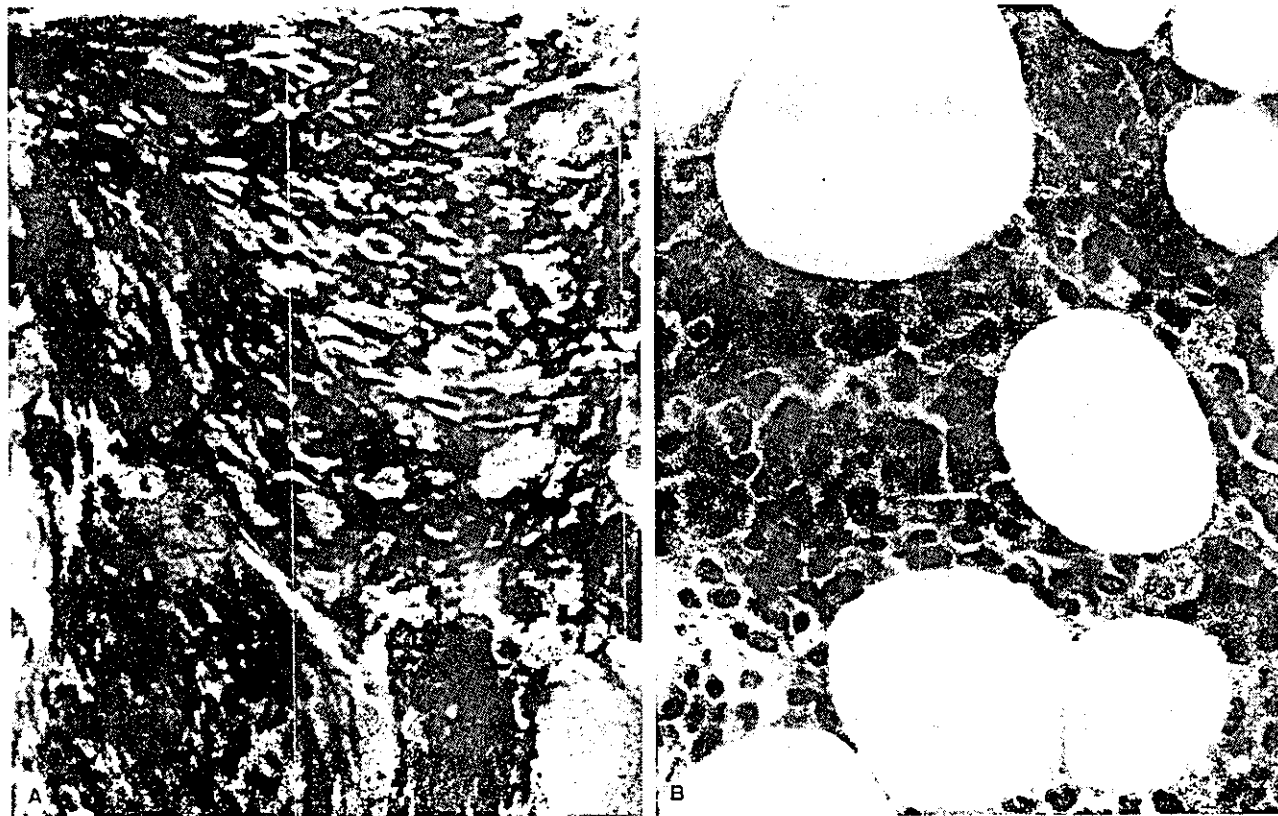

Fig. 3. (A) Trephine biopsy section from the right humerus. Marked leukemic cell infiltration is seen in the bone marrow. There is no destruction of the bone. Hematoxylin–eosin staining. (B) Trephine biopsy section from the right iliac crest. No evidence of relapse was seen at the same time of the right humerus biopsy. Hematoxylin–eosin staining.

we commonly evaluate the disease status. Thus, bone marrow aspiration and biopsy from iliac bone or sternum are not always sufficient for diagnosis of relapse of leukemia.

Quantitative RT-PCR assays of WT-1 are useful for evaluating minimal residual disease (MRD) in cases of acute leukemia [6]. Because of the elevation of WT-1 mRNA levels, we suspected a relapse even when repeat aspiration and biopsy of sternum or iliac bone marrow showed a morphologically complete remission.

Takagi and Tanaka detected (by MRI) focal residual disease in some patients with acute leukemia in complete remission [9]. FDG-PET was also reported to be useful to detect the infiltration of leukemia [10]. In case of unusual sites of relapse, evaluations with MRI and FDG-PET are useful.

Although the cause of the unusual site of relapse is uncertain, the graft-versus-leukemia (GVL) effect followed by allogeneic stem cell transplantation may have affected the formation. Namely, localized relapse may occur at sites where the GVL effect does not occur. In this case, the level of WT-1 mRNA progressed slowly and symptoms were sporadic for 4 months. It may be that the GVL effect suppressed leukemic cell growth and localized the leukemic cell distribution although systemic GVHD was not overt.

Because it is rare for a patient to relapse in the focal bone marrow, there is no definite treatment strategy. In this case, the local manifestations of relapse could be controlled with radiation therapy. Although leukemic cells were localized in the bone marrow of extremities at the time of relapse, systemic relapse probably was a subsequent occurrence. Therefore, the patient was treated with additional systemic chemotherapy and donor lymphocyte infusions after local radiation therapy.

In summary, we experienced the first reported case of localized relapse of ALL in bone marrow of extremities. Physicians should be aware of unusual relapse sites of leukemia after allogeneic stem cell transplantation. MRI scanning, FDG-PET, and serial monitoring of WT-1 may be of value in detecting this type of relapse.

ACKNOWLEDGMENTS

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Rheumatoid Factors Induce Signaling from B Cells, Leading to Epstein-Barr Virus and B-Cell Activation

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B-cell antigen receptor signaling is initiated upon binding of the antigen to membrane-bound immunoglobulin (Ig), and the anti-Ig antibody (Ab) mimics this signaling. In B cells latently infected with Epstein-Barr virus (EBV), the same signals induce virus activation. We examine here whether rheumatoid factors (RFs), autoantibodies directed against the Fc portion of IgG, induce EBV and B-cell activation. As a source of RFs, RF-producing lymphoblastoid cell line (LCL) clones were isolated from peripheral blood mononuclear cells (PBMC) and synovial cells from patients with rheumatoid arthritis (RA) by EBV transformation. Burkitt's lymphoma-derived Akata cells, which are highly responsive to EBV activation by anti-Ig Abs, were used for the assay of EBV activation. Akata cells expressed IgG3 as membrane-bound Ig. RFs from a synovium-derived LCL were directed to IgG3 and induced EBV activation in 16 to 18% of Akata cells, whereas RFs from another synovium-derived LCL were directed to IgG1 and did not induce EBV activation. Pretreatment of RFs with the purified Fc fragment of human IgG completely abolished EBV activation. Furthermore, B-cell activation was assessed by incorporation of [³H]thymidine. RFs from synovium-derived LCLs efficiently induced B-cell activation, and the addition of CD40 ligand had a synergistic effect. On the other hand, RFs from PBMC-derived LCLs were polyreactive, had a lower affinity to IgG, and did not induce EBV and B-cell activation. The present findings imply a possible role for RFs as EBV and B-cell activators.

Epstein-Barr virus (EBV) is a human herpesvirus, which infects the majority of the human population and is the causative agent of infectious mononucleosis. After primary infection, EBV persists in B cells in a latent state for the life of the host (15). Various reagents have been found to induce virus activation in latently EBV-infected B cells in vitro. They include halogenated pyrimidine (13), phorbol ester (42), anti-immunoglobulin (Ig) antibody (Ab) (9, 32, 37), and butyrate (20). Although we do not know the physiological stimuli that control activation of the virus productive cycle and the switch from the latency in vivo, anti-Ig treatment, which activates B-cell antigen receptor (BCR) signaling (39), would serve as a more physiologically relevant activator.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown cause (17), and EBV has long been suggested as a causative agent. RA patients have a higher frequency and/or higher levels of antibodies to EBV in serum than do normal individuals (1, 7, 11, 30). It has been reported that the number of circulating B cells infected with EBV is increased in RA patients (36). More recently, we have reported that there is an extremely high EBV load and that EBV replication occurs in the synovial tissue of RA patients (34). Besides EBV activation, B cells are also activated in RA patients (18). Rheumatoid factors (RFs) are autoantibodies directed against the

Fc portion of IgG (14, 21) and are found in the sera of most patients with RA. It is well established that there is a direct correlation between the severity of the disease and the titer of these antibodies in patients with RA (19, 43). Although the precise pathogenic role of RF in RA has not been defined, a great deal of evidence suggests its participation in sustaining inflammatory synovitis (5, 38, 43).

Anti-Ig Abs efficiently induce lytic virus replication in some Burkitt's lymphoma (BL) cell lines, including Akata (32, 33), which expresses a limited number of EBV latent gene products, including EBV-determined nuclear antigen 1 (EBNA1), two EBV-encoded small RNAs known as EBER1 and EBER2, the rightward transcripts from the BamHI A region (BARTs), and a very small amount of latent membrane protein 2A (LMP2A) (termed type I latency) (15, 31). On the other hand, EBV-immortalized lymphoblastoid cell lines (LCLs) are unresponsive to EBV induction by anti-Ig Abs. LMP2A, which is expressed in a high amount in LCLs, is known to interfere with EBV activation after BCR cross-linking (23, 24). Therefore, low LMP2A expression is important for efficient EBV activation in anti-Ig-treated cells (16). The high-level expression of LMP2A in LCLs is caused by transactivation of the EBNA2 protein (41). On the other hand, BL cells are negative for EBNA2 expression and express little or no LMP2A. The analysis of peripheral blood lymphocyte by PCR showed that only EBNA1 and LMP2A were expressed in EBV latency in vivo (8, 25, 27, 35). Although the level of LMP2A expression in peripheral lymphocytes has not been measured quantitatively, the absence of EBNA2 expression suggests a low level LMP2A

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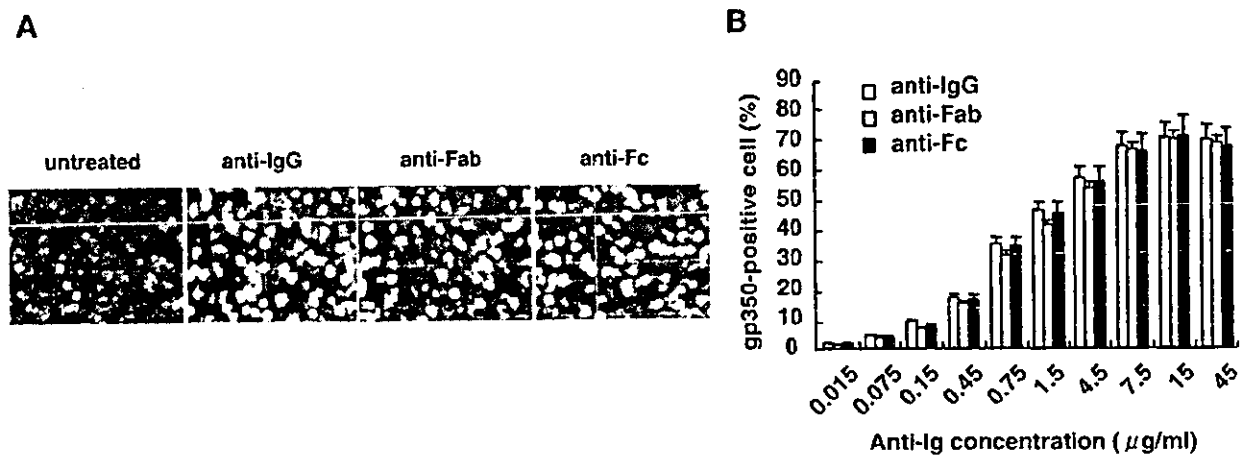
9918

A

untreated   anti-IgG   anti-Fab   anti-Fc

B

FIG. 1. Anti-Fc Ab induces EBV activation in the latently EBV-infected B-cell line Akata as efficiently as anti-Fab Ab does. (A) Immuno-fluorescence assay showing induction of an EBV lytic protein, gp350, after treatment with 7.5 μg of anti-IgG, anti-Fab, and anti-Fc Abs·ml for 24 h. (B) Dose response of gp350 expression showing that anti-Fc Ab induces gp350 expression as efficiently as anti-Fab Ab.

expression in these cells. Therefore, BL cells with type I latency are likely to represent in vivo latency. Hence, by using Akata cells, we investigated whether RF could induced virus activation. The results indicated that RFs induce EBV and B-cell activation.

MATERIALS AND METHODS

Cell culture. BL-derived EBV-positive Akata cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics at 37°C in 5% CO₂.

RF-producing cell lines. RF-producing cell lines were generated by EBV transformation of peripheral blood mononuclear cells (PBMC) or single cells from the synovial tissue of RA patients. The cells were incubated in the culture supernatant of the B95-8 cell line and resuspended in GIT medium (Nihonsei-yaku Co., Ltd., Tokyo, Japan). The cells were transferred to 96-well plates (Costar Corp., Cambridge, Mass.) at 200 cells well for PBMC and 2,000 cells well for synovial cells. X-ray-irradiated (5,000 rads) allergenic PBMC were added as feeder cells at 5 × 10⁴ cells well. Cells were cultured for 4 weeks. RFs were purified from the culture supernatant by using HiTrap IgM purification columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Abs and reagents. The Abs used for EBV activation and B-cell activation were a rabbit polyclonal Ab to human IgG (γ-chain-specific) (Dako, Copenhagen, Denmark), F(ab')₂ fragment of mouse monoclonal Ab (MAb) to the Fc frag-ment of human IgG (Jackson ImmunoResearch, West Grove, Pa.), and the F(ab')₂ fragment of mouse MAb to the Fab fragment of human IgG (Jackson ImmunoResearch). Ordinarily, they were used at a concentration of 7.5 μg ml. Other Abs included mouse MAb to phosphotyrosine (Cell Signaling, Beverly, Mass.), rabbit polyclonal Abs to phospho-Syk and phospho-ERK (Cell Signal-ing), a mouse MAb to EBV BZLF1 (Dako), and an MAb to EBV gp350 (C1, kindly provided by T. Sairenji). The purified Fc fragment of human IgG and purified Fab fragment of human IgG were purchased from Jackson Immuno-Research, and CD40 ligand (CD40L) was from PEPRO Tech (Rocky Hill, N.J.).

Immunofluorescence assay. Expression of EBV lytic antigens was examined on acetone-fixed cells by the indirect immunofluorescence method with MAb C1 specific to the EBV glycoprotein gp350. The second Ab was a fluorescein iso-thiocyanate (FITC)-conjugated F(ab')₂ fragment of rabbit Ab to mouse IgG (Dako).

Determination of calcium mobilization. Akata cells (10⁶ ml) were loaded with 4 μM fluo-3AM (Molecular Probes, Eugene, Oreg.) at room temperature for 30 min. Cells were washed twice with phosphate-buffered saline and resuspended at the same concentration in 500 μl of fresh medium for each sample. Baseline calcium release was measured for 30 s and 500 μl of purified IgM RF or medium containing 15 μg of anti-Fc Abs ml was then added to the cell suspension. The intracellular calcium levels were measured by using flow cytometry (Becton Dickinson, Franklin Lakes, N.J.).

Immunoblot analysis. Twenty micrograms of cell lysate was separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was incubated overnight at 4°C with the first Ab and then reacted with horseradish peroxidase-conjugated sheep Ab to mouse IgG (diluted at 1:5,000) or horseradish peroxidase-conjugated donkey Ab to rabbit IgG (diluted at 1:2,000) (Amersham Bioscience Corp., Piscataway, N.J.).

Determination of IgG subclass in Akata cells. Akata cells were incubated with FITC-conjugated goat anti-human IgG1, IgG2, IgG3, or IgG4 at a concentration of 1:50 in 37°C for 1 h. The cells were washed with PBS containing 1% bovine serum albumin, followed by flow cytometric analysis.

Affinity determination by inhibition ELISA. To determine the affinities of RFs and the anti-Fc Ab, we used a standard competitive inhibition enzyme-linked immunosorbent assay (ELISA), in which soluble human IgG Fc from 10⁻¹⁰ M to 10⁻⁵ M were preincubated with a fixed amount of RFs at 4°C overnight. This mixture of IgG Fc-RF was then centrifuged, and the supernatant was transferred to a plate coated with human IgG Fc (MBL, Tokyo, Japan). The plates were incubated at 37°C for 2 h, followed by a wash with washing buffer. Then, 100 μl of alkaline phosphatase-conjugated goat anti-human IgM or goat anti-mouse IgG Abs was added to each well at a dilution of 1:6,000 and incubated for 30 min at 37°C. Plates were washed, and the substrate was added. The results were plotted as the percent bound versus the concentration of the competitor. The percent bound was calculated by using the optical density and taking the reading without a competitor as 100% bound.

B-cell purification and assay of B-cell activation. PBMC were separated from adult peripheral blood by using Histopaque (Sigma, St. Louis, Mo.). B cells were purified from PBMC with anti-human CD19 magnet beads (Dynal ASA). A total of 200 μl of a cell suspension (2 × 10⁵ well) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics in 96-well tissue culture plates. Cells were stimulated with 30 μg of anti-IgG, anti-Fab, or anti-Fc Abs ml or 200 μg of RFs ml for 2 h, and then 3 μg of CD40L ml was added to the culture. After 48 h of incubation, entry into the cell cycle was assessed by measurement of incorporation of [³H]thymidine (ICN Biomedicals Canada, Inc., Mississauga, Ontario, Canada) after a 16-h pulse with 0.5 μCi of [³H]thymidine.

RESULTS AND DISCUSSION

Activation of latently infected EBV by anti-Fc Ab. By using Akata cells, we investigated whether RF could induce virus activation. First, we studied whether an MAb to the Fc portion of human IgG (anti-Fc Ab) could induce EBV activation. Akata cells were treated with an anti-Fc Ab for 24 h, and the expression of viral glycoprotein gp350, which is expressed at a late stage of EBV lytic infection (15), was examined by immu-nofluorescence assay. As shown in Fig. 1A, the anti-Fc Ab
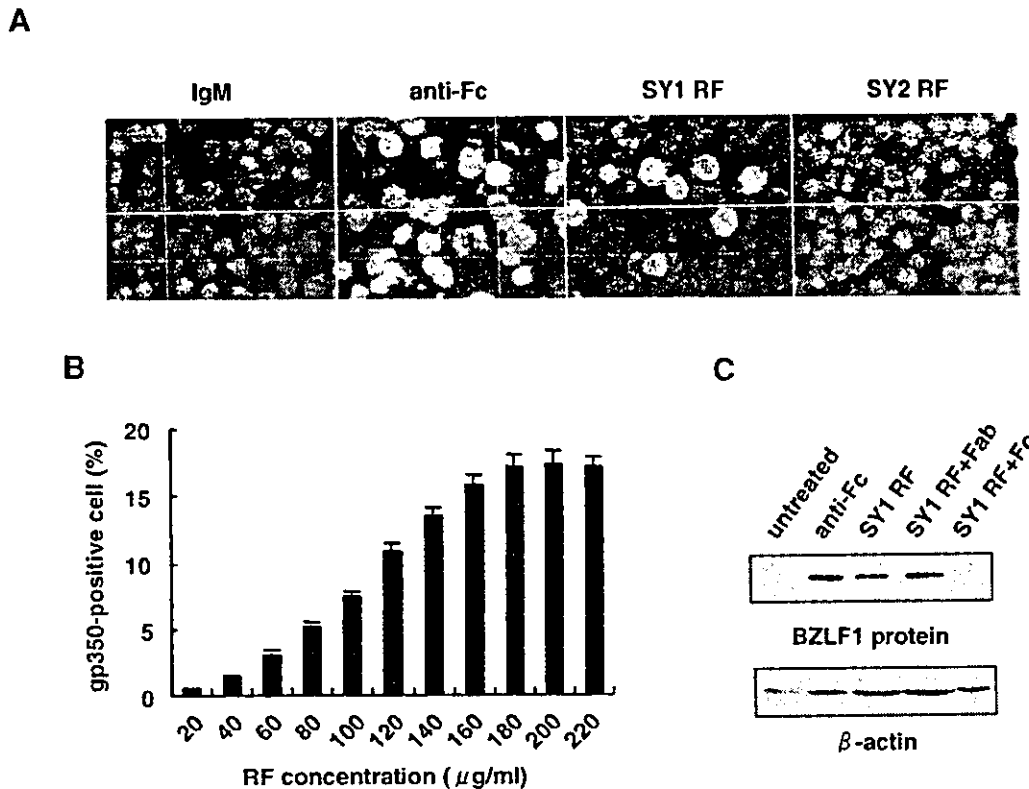
A

B

C

RF concentration (μg/ml)

FIG. 2. RFs produced from a synovium-derived LCLs (SY1) induce EBV activation in latently EBV-infected B-cell line Akata. Anti-Fc Ab is used as a positive control. (A) Immunofluorescence assay showing induction of an EBV lytic protein, gp350, after treatment with synovium-derived RFs. SY1 RFs, at 100 μg ml for 24 h; (B) dose response of gp350 expression showing that SY1 RFs induce maximum expression of gp350 at 180 to 220 μg ml; (C) immunoblot analysis showing induction of an EBV lytic protein, BZLF1, after treatment with SY1 RFs for 24 h. Pretreatment of SY1 RFs with the Fc fragment of IgG completely abolished the BZLF1-inducing ability of RFs.

induced lytic infection as efficiently as the anti-IgG polyclonal Ab and an MAb against the Fab portion of human IgG (anti-Fab Ab). Anti-Fc and anti-Fab Abs gave similar dose-response curves in their ability to induce EBV activation (Fig. 1B), suggesting that signals from the Fab and Fc portions of IgG were equally potent as EBV activators.

Activation of latently infected EBV by RFs. Based on these findings, we studied whether RF could induce EBV activation. To obtain RFs, B cells from PBMC and single cells prepared from the synovial tissue of patients with RA were infected with EBV (B95-8 strain) and cultured for 4 weeks in the wells of 96-well plates at 200 and 2,000 cells/well, respectively (3, 12). Two of each of the EBV-transformed LCLs producing RFs were chosen as a source of RFs. RFs were purified from the culture supernatant by using an IgM affinity column and were added to the Akata cell culture at 100 μg/ml. After 24 h of cultivation, the expression of gp350 was examined by using an immunofluorescence assay. As shown in Fig. 2A. RFs produced from a synovium-derived LCL (SY1) induced gp350 in ~8% of Akata cells, whereas RFs produced from another synovium-derived LCL (SY2) and RFs from two PBMC-derived LCLs (PBMC1 and PBMC2) had no substantial gp350 induction (<0.2% [data not shown]). The dose-response experiment indicated that SY1 RFs gave a maximum gp350 induction of ca. 16 to 18% at concentrations of 180 to 220 μg/ml

(Fig. 2B). EBV activation by SY1 RFs was further confirmed by detection of an EBV lytic protein, BZLF1 (15), by immunoblot analysis (Fig. 2C). Pretreatment of SY1 RFs with the purified Fc fragment of human IgG completely abolished BZLF1 induction, whereas pretreatment with the Fab fragment of human IgG did not (Fig. 2C), suggesting that EBV activation was induced by specific binding of RFs to the Fc portion of cell membrane Ig.

Activation of BCR signaling by RFs. It is known that cross-linking of BCR is followed by phosphorylation of tyrosine kinases and elevation of the intracellular calcium concentration (10, 29), and both are required for EBV activation (9). We therefore studied whether RFs could stimulate these pathways. Immunoblot analysis revealed that SY1 RFs induced phosphorylation of tyrosine kinases, including Syk and ERK (Fig. 3A). Calcium mobilization of SY1 RF-treated Akata cells was also examined by flow cytometry. The results showed that SY1 RFs induced enhanced calcium influx (Fig. 3B).

Lower affinity of RFs to Fc fragment of IgG than anti-Fc Ab. The flow cytometric analysis indicated that Akata cells expressed IgG3 among four subclasses of IgG: IgG1, IgG2, IgG3, and IgG4 (Fig. 4A). RFs used in the present studies were examined to determine their reactivities with IgG subclasses. The results indicated that SY1-RFs, which induced EBV activation, reacted with IgG3 and not with IgG1, whereas SY2
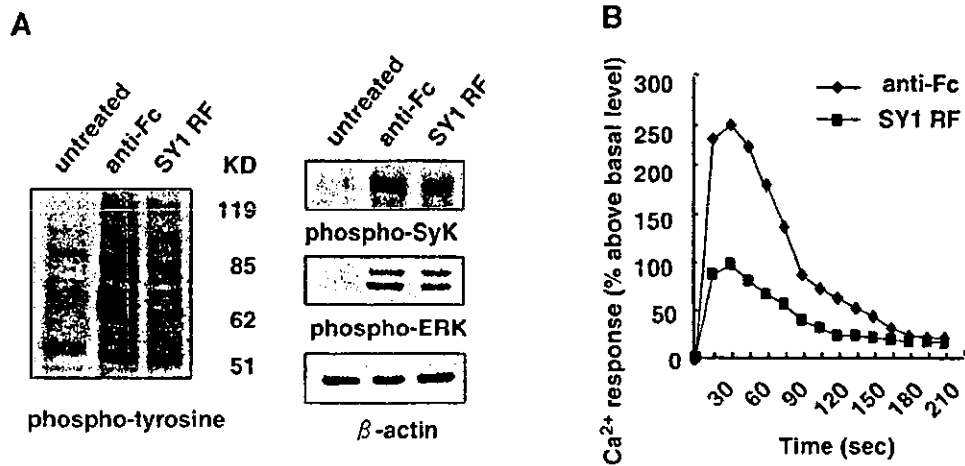
A

B

FIG. 3. RFs produced from synovium-derived LCLs (SY1) induce phosphorylation of tyrosine kinases and elevation of the intracellular calcium concentration in latently EBV-infected B-cell line Akata. Anti-Fc Ab is used as a positive control. (A) Immunoblot analysis showing phosphorylation of tyrosine kinases after treatment with SY1 RFs (100 μg ml); (B) flow cytometry showing elevation of intracellular calcium levels after treatment with SY1 RFs (100 μg/ml).

RFs, which did not induce EBV activation, reacted with IgG1 but not with IgG3 (Fig. 4B). On the other hand, PBMC-derived RFs reacted with both IgG1 and IgG3, and weakly reacted with bovine serum albumin as well. These results are consistent with previous reports that most RFs produced from PBMC are polyreactive (3, 12).

Although anti-Fc Abs induced lytic infection in ca. 70% of Akata cells, SY1 RFs induced EBV activation in 16 to 18% of the cells, and PBMC-derived RFs could not induce EBV activation in spite of their reactivity with IgG3. Furthermore, dose-response analysis indicated that the maximum EBV induction by SY1 RFs was obtained at a concentration of 180 μg/ml, whereas the maximum induction by the anti-Fc Ab was obtained at a concentration of 7.5 μg/ml. To examine the possibility that RFs had a lower affinity to Fc fragments of IgG than

the anti-Fc Ab, competitive inhibition ELISA was performed. RFs or anti-Fc Abs were first incubated with purified Fc fragment of human IgG at various concentrations. The concentration of free RFs or anti-Fc Ab was then determined by an indirect ELISA. As a result, the affinities of SY1 RF and SY2 RF were 7.1 × 10⁻⁷ M and 1.9 × 10⁻⁶ M, respectively, and were ca. 100 times lower than that of the anti-Fc Ab (8.9 × 10⁻⁹ M), and the affinities of PBMC1 RF and PBMC2 RF were 4.5 × 10⁻⁶ M and 7.0 × 10⁻⁶ M, respectively, and were ca. 1,000-times lower than that of the anti-Fc Ab (Fig. 5).

B-cell activation by RFs. Finally, we examined whether RFs could induce B-cell activation. B cells were purified from adult peripheral blood by using anti-human CD19 magnetic beads and were treated with RFs (200 μg/ml) with or without addition of CD40L, which is necessary for efficient activation of
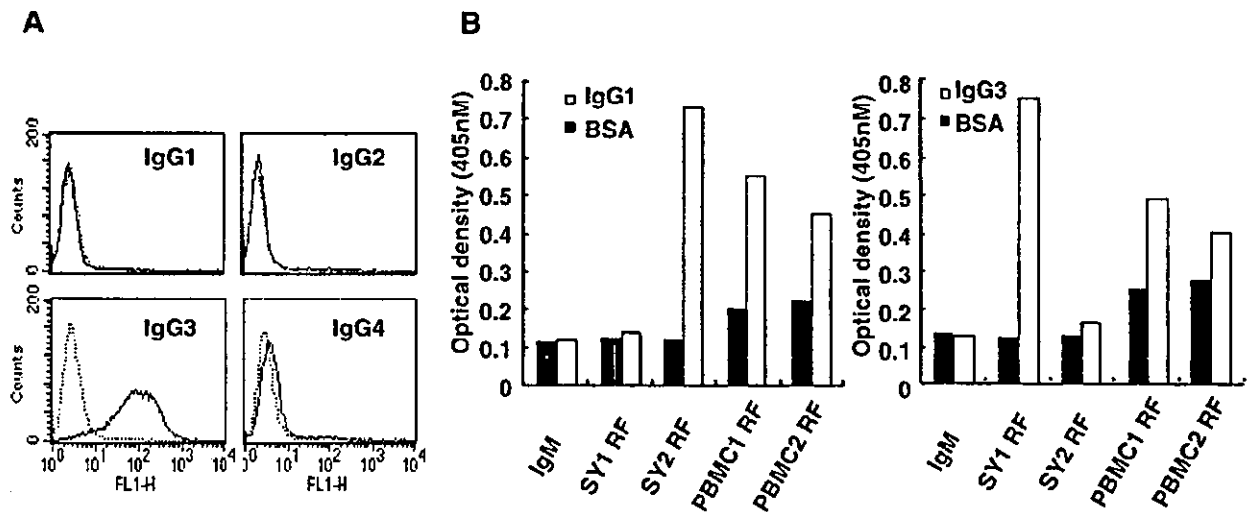
A

B

FIG. 4. Synovium-derived RFs are monoreactive and LCL-derived RFs are polyreactive for IgG subclasses. (A) Flow cytometric analysis showing that Akata cells express the IgG3 subclass on the cell membrane. (B) RFs produced by synovium-derived LCLs (SY1 and SY2) react with IgG3 or IgG1, and PBMC-derived RFs (PBMC1 and PBMC2) react with both IgG1 and IgG3.