雑　誌

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【V】研究成果の刊行物・別刷

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Mast Cell–Derived Tryptase Inhibits Apoptosis of Human Rheumatoid Synovial Fibroblasts via Rho-Mediated Signaling

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Objective. **An abundance of mast cells are found in the synovium of patients with rheumatoid arthritis (RA). However, the role of mast cells in the pathogenesis of RA remains unclear. This study was undertaken to elucidate a role for mast cells in RA by investigating the antiapoptotic effects of tryptase, a major product of mast cells, on RA synovial fibroblasts (RASFs).**

Methods. **RA synovial tissue was obtained from RA patients during joint replacement surgery, and histologic changes in the tissue were examined. The expression of cell surface molecules and apoptotic markers on RASFs were detected by flow cytometry. Rho activation was determined using a pull-down assay.**

Results. **Mast cells, bearing both c-Kit and tryptase, accumulated in the sublining area of proliferating synovial tissue from RA patients. Protease-activated receptor 2 (PAR-2), a receptor for tryptase, was expressed on RASFs in the lining area, close to tryptase-positive mast cells in the RA synovium. Fas-mediated apoptosis of RASFs was significantly inhibited, in a dose-dependent manner, by the addition of tryptase, and this effect correlated with increased activation of Rho kinase. Furthermore, Y27632, a Rho kinase inhibitor, reduced the antiapoptotic effect of tryptase on RASFs, suggesting that Rho was responsible for the antiapoptotic effects of tryptase.**

Conclusion. **These results demonstrate that tryptase has a strong antiapoptotic effect on RASFs through the activation of Rho. Thus, we propose that the release of tryptase by mast cells leads to the binding of tryptase to PAR-2 on RASFs and inhibits the apoptosis of RASFs via the activation of Rho. Such mechanisms could play a pivotal role in the marked proliferation of RASFs and hyperplasia of synovial tissue seen in RA synovium.**

Rheumatoid arthritis (RA) is an inflammatory disease that is characterized by persistent joint inflammation, eventually leading to destruction of the joints, which results in significant impairment of daily activity. In addition to decreased mobility, joint destruction causes tenderness and pain, and the quality of life and life expectancy of RA patients is drastically reduced compared with that of healthy subjects (1). The recent emergence of biologic drugs that target inflammatory cytokines, including tumor necrosis factor α (TNFα), has greatly improved the treatment of RA. Despite such advances, the RA remission rate still remains low, at only 30–40%. Thus, further understanding of the pathogenesis of RA in order to yield new perspectives on RA treatment is necessary in those cases in which the current therapeutic strategy is insufficient.

In the joints of RA patients, a proliferating mass in the synovium, known as pannus, covers the RA joint cartilage and contributes to joint erosion and fibrous ankylosis. Pannus consists of granulation tissue and proliferating synovial fibroblasts (SFs), accompanied by neoangiogenesis and inflammatory cell infiltrates (2). A main contributor to joint destruction is the RASF, which

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multiplies in the same manner as neoplastic cells and infiltrates into cartilage and bone, resulting in tissue destruction. However, it is puzzling that RASFs proliferate excessively in vivo despite a high level of expression of Fas and sensitivity to Fas-mediated killing in vitro (2,3). Thus, it is possible that RA joint cartilage contains a mechanism for the suppression of apoptosis of SFs, which leads to inappropriate SF hyperplasia.

We have previously reported that mast cells may be an effective therapeutic target in the treatment of chronic inflammation in RA (4). Such notions stem from the observation that an abundance of mast cells is present in the synovial tissue of RA patients compared with those with other joint diseases (5). Mast cells appear to contribute to RA pathology in mouse studies, as was shown in a mouse model of autoantibody-induced arthritis in which mast cell–deficient mice exhibited attenuated joint inflammation (6). Moreover, mast cells produce cytokines that are of great relevance in RA, including TNFα and interleukin-1 (7). Thus, although the specific details remain unclear (8), it is conceivable that mast cells also play an important role in the pathogenesis of human RA.

In the present study, we hypothesized that mast cells may contribute to the pathogenesis of RA by inhibiting the apoptosis of RASFs. The results demonstrate that mast cells are found in close proximity to RASFs in the synovium of RA patients. Furthermore, RASFs express the receptor for mast cell tryptase, known as protease-activated receptor 2 (PAR-2), and are protected from Fas-mediated apoptosis by tryptase in a Rho GTPase–dependent manner. We propose that such mechanisms could play a pivotal role in the marked proliferation of RASFs and hyperplasia seen in RA synovium.

PATIENTS AND METHODS

Human studies. The study protocol was approved by the Human Ethics Review Committee of the University of Occupational and Environmental Health in Japan. Signed informed consent was obtained from each subject involved in this study.

Synovial tissue and culture of SFs. Synovial tissue was obtained from 5 women (ages 47–60 years) with active RA, whose disease had been diagnosed according to the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (9) and who had undergone joint replacement surgery. All enrolled patients had >6 swollen joints, >3 tender joints, and an erythrocyte sedimentation rate (Westergren) of >28 mm/hour.

Synovial tissue samples were dissected under sterile conditions in phosphate buffered saline, and fibroblast-like synovial cells were isolated and cultured. Briefly, the tissue samples were minced into small pieces and digested with collagenase (Sigma-Aldrich, Tokyo, Japan) in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY). The cells were filtered through a nylon mesh, washed extensively, and suspended in DMEM supplemented with 10% fetal calf serum (FCS; Bio-Pro, Karlsruhe, Germany) and streptomycin/penicillin (10 units/ml; Sigma-Aldrich). Finally, isolated cells were seeded in tissue culture flasks (Falcon, Lincoln Park, NJ), and nonadherent cells were removed. The medium was changed biweekly, and the cells were used after 5 passages.

The resulting synovial cells were spindle-shaped and grew in a cobblestone pattern. Flow cytometric analysis of these cells indicated that they lacked macrophage markers, such as class II major histocompatibility complex, CD14, and CD11b (results not shown). Thus, the RA synovial cells obtained appeared to represent type B synovial fibroblast-like cells.

Reagents. Human β-tryptase was purchased from Promega (Madison, WI). The following monoclonal antibodies (mAb) were used: fluorescein isothiocyanate–conjugated control mAb anti-Thy1.2 (Becton Dickinson, San Jose, CA) and anti-human PAR-2 mAb (R&D Systems, Minneapolis, MN). A Rho activation kit containing glutathione S-transferase–Rhotekin-Rho binding domain (GST-RBD) beads was purchased from Cytoskeleton (Denver, CO).

Immunohistochemistry. Synovial tissue was stained as previously described (10). Briefly, sections (6 μm) were fixed in ice-cold acetone, and endogenous peroxidase was quenched with 3% H_2O_2/methanol. Sections were incubated with blocking buffer and then with the SAM-11 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 μg/ml for 12 hours at 4°C. SAM-11 is a previously characterized (11) highly specific mAb to human PAR-2 that binds the membrane-bound part of the receptor, in both cleaved and uncleaved states. Endogenous biotin was blocked using an avidin–biotin kit (Vector, Peterborough, UK), and biotinylated secondary antibody (Autogen Bioclear, Wiltshire, UK) was then applied, followed by the addition of peroxidase-conjugated streptavidin. Antigen–antibody complexes were visualized utilizing 3,3′-diaminobenzidine. Sections were also probed with an antibody specific for mast cell tryptase (Dako, Ely, UK).

Flow cytometry. Staining and flow cytometric analyses of RASFs were performed using a FACScan (BD PharMingen, San Diego, CA) and standard procedures as described elsewhere (12). The RASFs (2×10^5 cells) were incubated with a negative control antibody (mAb anti-Thy1.2; Becton Dickinson) or phycoerythrin-conjugated anti–PAR-2 mAb (Mouse-Mono 344222; R&D Systems) in fluorescence-activated cell sorting (FACS) medium consisting of Hanks' balanced salt solution (Nissui, Tokyo, Japan), 0.5% human serum albumin (Yoshitomi, Osaka, Japan), and 0.2% NaN_3 (Sigma, St. Louis, MO) for 30 minutes at 4°C. After washing the cells 3 times with FACS medium, the fluorescence intensity was detected using a FACScan.

Apoptosis assay. Apoptosis was evaluated by flow cytometry utilizing annexin V binding (Annexin V–Fluorescein Isothiocyanate Apoptosis Detection Kit I; Becton Dickinson). Briefly, RASFs were cultured under starved conditions for 24 hours with 1% DMEM, and were then incubated with or

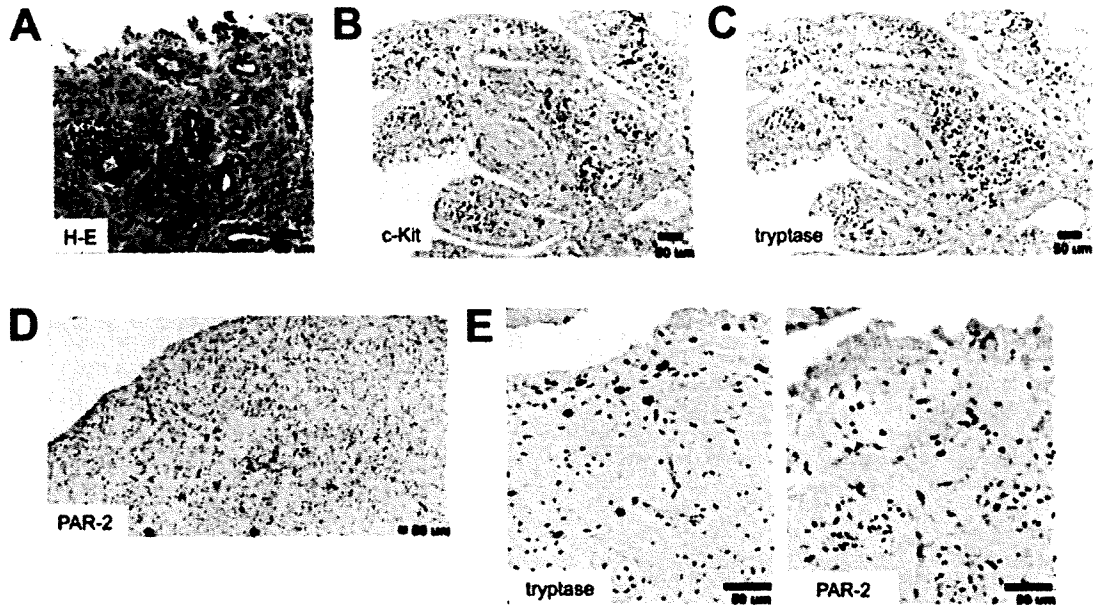

Figure 1. Detection of mast cells and protease-activated receptor 2 (PAR-2)–expressing synovial fibroblasts in rheumatoid arthritis (RA) synovial tissue. **A–D,** Synovial tissue specimens (surgically removed basal layer) from a patient with RA were stained with hematoxylin and eosin (H&E) **(A)** or subjected to immunohistochemical staining using an anti–c-Kit antibody **(B)**, antitryptase antibody **(C)**, or anti–PAR-2 antibody **(D)**. **E,** In synovial tissue from the same patient, immunohistochemical staining was performed on serial sections using antitryptase antibody **(left)** and anti–PAR-2 antibody **(right)**. Representative results from 1 of 5 RA patients are shown.

without CH11 (1 μg/ml), tryptase (1–4 μg/ml), and Y27632 (0.1–10 μ*M*; Calbiochem, La Jolla, CA) for 12 hours in DMEM containing 1% FCS. In some experiments, E11 fibroblasts (immortalized RASF cell line [13]) were treated with CH11 (1 μg/ml) with or without tryptase (2 μg/ml) and/or nafamostat mesylate (1 n*M*; Tocris Biosciences, Ellsville, MO) for 12 hours in DMEM containing 10% FCS. Cells were then stained with annexin V and propidium iodide (PI), according to the manufacturer's instructions, and analyzed using a FACScan flow cytometer (Becton Dickinson). All PI-positive cells were considered dead. PI-negative and annexin V–positive cells were considered early apoptotic cells, and the remaining double-negative cells were considered viable.

Rho activation assay. Rho activation was determined with the use of a pull-down assay with GST-RBD beads (14,15). RASFs were stimulated with 0.1–2 μg/ml tryptase, quickly washed with ice-cold Tris buffered saline, and lysed in 500 μl of lysis buffer (50 m*M* Tris, pH 7.5, 10 m*M* $MgCl_2$, 0.5*M* NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 500 μg/ml tosyl arginine methyl ester, and 10 μg/ml each of leupeptin and aprotinin). Cell lysates were immediately centrifuged at 8,000 revolutions per minute at 4°C for 5 minutes, and equal volumes of lysates were incubated with 30 μg GST-RBD beads for 1 hour at 4°C. The beads were washed twice with wash buffer (25 m*M* Tris, pH 7.5, 30 m*M* $MgCl_2$, 40 m*M* NaCl), and bound Rho was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from the beads and total cell lysate were then electrophoresed on 12% SDS–polyacrylamide electrophoresis gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a polyclonal anti-Rho antibody.

Statistical analysis. Results are expressed as the mean ± SD. Differences in comparison with the control group were examined for statistical significance by the Mann-Whitney U test. *P* values less than 0.01 were considered statistically significant.

RESULTS

Detection of mast cells in close proximity to PAR-2–expressing SFs in RA synovial tissue. Synovial tissue specimens were surgically removed from the joints of patients with RA, and the samples were used to investigate the localization of mast cells and SFs in the synovial tissue. Histologic examination of the tissue by hematoxylin and eosin staining revealed the presence of pannus, represented by detection of SFs, inflammatory cell infiltrates, and finer vessels (Figure 1A). Moreover, immunohistochemical staining of the tissue samples demonstrated numerous c-Kit–positive cells (Figure 1B)

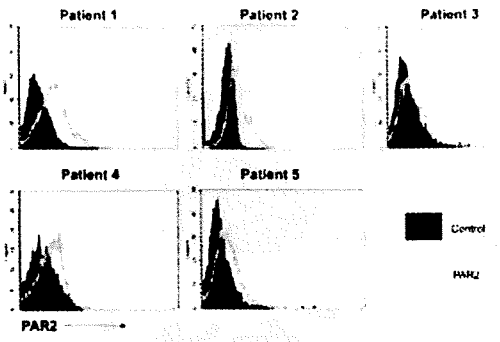

Figure 2. Expression of protease-activated receptor 2 (PAR-2) by isolated rheumatoid arthritis synovial fibroblasts (RASFs) from the synovial tissue of 5 patients with RA. RASFs were labeled with an antibody against PAR-2, in comparison with an isotype control, and the fluorescence intensity in 5 separate RA patient samples was measured by flow cytometry.

and tryptase-positive cells (Figure 1C), which are characteristic of mast cells, in the pannus and sublining area. This suggested that large numbers of mast cells are present in hyperplastic synovial tissue.

Since we hypothesized that tryptase, a mast cell–specific protease, may be involved in the pathogenesis of RA, we next determined which cells could respond to tryptase, by examining the expression of PAR-2, the receptor for tryptase. PAR-2 was expressed in spindle-shaped cells, most likely representing RASFs, which were present in the lining area (Figure 1D). Notably, in serial sections, PAR-2–expressing cells were found in close proximity to the area in which tryptase was expressed (Figure 1E). Similar results were obtained in synovial tissue samples from 5 other patients with RA (results not shown).

To verify that PAR-2 is expressed on RA fibroblasts, SFs were isolated from the RA synovial tissue samples and PAR-2 expression was detected with the use of flow cytometry. Consistent with the findings on immunohistochemical analysis, flow cytometry revealed the expression of PAR-2 on isolated RASFs obtained from 5 separate RA patients (Figure 2), thus confirming that PAR-2 is expressed on RASFs.

Inhibition of anti-Fas antibody–induced cell death by tryptase in RASFs. We previously reported that RASFs express Fas and are susceptible to Fas-induced cell death (3). Nevertheless, in the synovial tissue of RA patients, RASFs proliferate, rather than undergo apoptosis, suggesting that there might be a mechanism that prevents RASFs from undergoing apoptosis in situ. Given that mast cells lie in close proximity to RASFs and that RASFs express PAR-2, we questioned whether a mast cell–specific PAR-2 activator such as tryptase would suppress apoptosis induction in RASFs. To test this notion, RASFs were treated with or without anti-Fas antibody (CH11) in the presence or absence of tryptase, under starved conditions. As expected, cell death was morphologically apparent and increased 12 hours after incubation with CH11, as compared with that in cultures with untreated cells (Figure 3). In contrast, the addition of tryptase significantly inhibited such morphologic changes in the RASFs (Figure 3).

To enumerate the proportion of live cells remaining in each well, the cells were removed from the wells with the use of trypsin, and live cells were counted using trypan blue exclusion. Compared with untreated cells, a significant decrease in the proportion of live cells was found in cells treated with CH11, which was reversed by the addition of tryptase (Figure 3). Of note, cell death was also observed in ~50% of the untreated cells, which was attributable to the starved culture conditions necessary to make the cells more sensitive to CH11-induced apoptosis.

To confirm the results obtained by trypan blue exclusion, we next used a flow cytometric approach involving annexin V and PI staining to detect apoptotic cells. The fraction of PI^{low}annexin V^{high} cells (early apoptotic) and PI^{high}annexin V^{high} cells (late apoptotic) increased after treatment of RASFs with CH11 (Figure

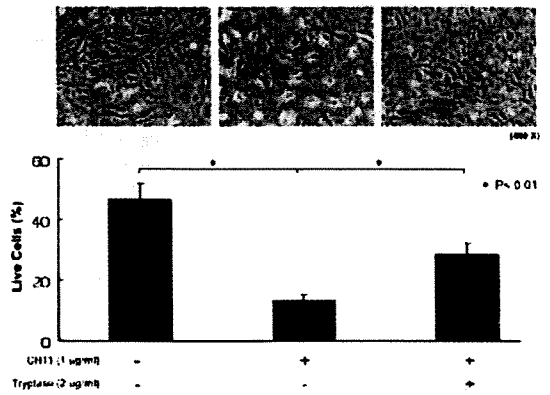

Figure 3. Inhibition of anti-Fas antibody–mediated apoptosis of rheumatoid arthritis synovial fibroblasts (RASFs) by tryptase. **Top,** RASFs were left untreated **(left)** or treated with anti-Fas antibody in the absence **(middle)** or presence **(right)** of tryptase. Cells were observed using an inverted light microscope 12 hours after treatment (original magnification × 400). **Bottom,** The total number of viable cells was determined by counting the number of live cells on trypan blue staining. Bars show the mean and SD percentage of live cells.

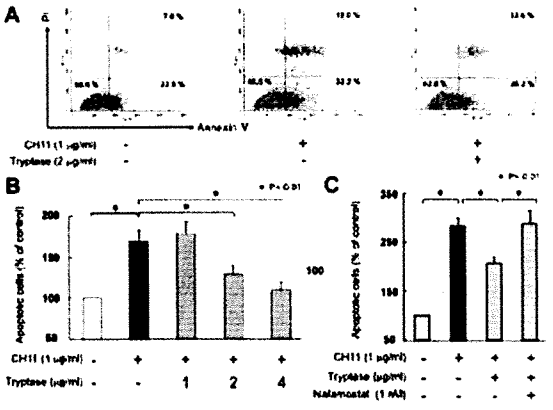

Figure 4. Inhibition of anti-Fas antibody–mediated early and late apoptosis of rheumatoid arthritis synovial fibroblasts (RASFs) by tryptase. **A,** RASFs were left untreated **(left)** or treated with anti-Fas antibody in the absence **(middle)** or presence **(right)** of tryptase. Twelve hours after treatment, RASFs were stained with annexin V/propidium iodide (PI) and analyzed for apoptosis by flow cytometry. **B,** Apoptosis of RASFs was determined in cultures of cells left untreated (controls) or treated with anti-Fas antibody in the absence or presence of various doses of tryptase, with results normalized to untreated control values (set at 100%). Bars show the mean and SD results from 5 different RA synovial tissue samples. **C,** E11 fibroblasts were left untreated or treated with anti-Fas antibody in the absence or presence of tryptase with or without nafamostat mesylate. Twelve hours after treatment, RASFs were stained with annexin V and analyzed for the percentage of apoptotic (annexin V–positive) cells by flow cytometry. Bars show the mean and SD results from triplicate determinations in each condition, with results normalized to untreated control values (set at 100%).

4A). Both early and late apoptosis were inhibited by tryptase in a concentration-dependent manner (Figure 4B). Of note, apoptosis of primary dermal fibroblasts could not be induced under the same conditions (results not shown), suggesting that this phenomenon might be specific to RASFs. Taken together, these results suggest that tryptase inhibits Fas-induced apoptosis in RASFs.

We next tested whether tryptase inhibits Fas-mediated apoptosis of fibroblasts via the activation of PAR-2. Since PAR-2 is activated by proteolytic cleavage of the receptor, we tested whether the addition of the protease inhibitor nafamostat mesylate would reverse the antiapoptotic effects of tryptase. As expected, treatment of fibroblasts with anti-Fas antibody resulted in cell death, and this was attenuated by the addition of tryptase (Figure 4C). However, the protective effect of tryptase was lost when the cells were cotreated with tryptase and nafamostat mesylate (Figure 4C), suggesting that the proteolytic function of tryptase and subsequent cleavage of PAR-2 is responsible for the antiapoptotic effects of tryptase against Fas-mediated apoptosis of fibroblasts.

Involvement of Rho activation in the antiapoptotic effect of tryptase on RASFs. Activation of Rho, which is a low molecular weight G protein, is related to cell survival (16,17). We have previously reported that activation of Rho through the ligation of PAR-1 by thrombin promotes proliferation of RASFs (18). Since Rho also mediates downstream signaling of PAR-2 (19), the activation of Rho in tryptase-stimulated RASFs was evaluated. The activation of Rho was examined with the use of a pull-down assay for the detection of GTP-bound Rho (active form of GTPases) followed by Western blot analysis of the Rho protein. An increase in GTP-bound Rho was observed in RASFs after treatment with tryptase (Figure 5), suggesting that PAR-2 stimulation induces the activation of Rho.

We then tested whether the activation of Rho is involved in the protection of RASFs against CH11-induced apoptosis. After the addition of CH11 to the RASF cultures, an increase in annexin V^{high} cells was observed, and this was again inhibited by tryptase (Figure 6A). The protective effect of tryptase was abrogated by the addition of a Rho kinase–specific inhibitor, Y27632, in a dose-dependent manner (Figures 6A and B). Similar results were obtained in RASFs from 5 separate RA patients (results not shown). Taken together, these data suggest that tryptase inhibits Fas-induced apoptosis of RASFs through a mechanism that involves Rho kinase.

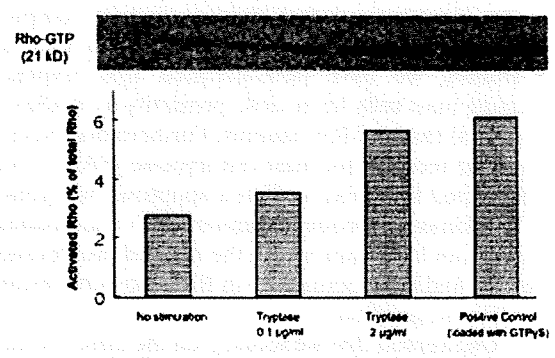

Figure 5. Activation of Rho kinase by tryptase in rheumatoid arthritis synovial fibroblasts (RASFs). RASFs were treated with tryptase (0.1 or 2 μg/ml) for 1 minute (or treated with GTPγS as a positive control). **Top,** After cell lysis, GTP-bound Rho was assessed using a pull-down assay involving the Rhotekin-Rho binding domain. **Bottom,** The ratio of activated Rho to total Rho protein was determined. Equal loading of Rho in the pull-down assay was confirmed by Western blotting. Representative results from 1 of 3 RA patients are shown.

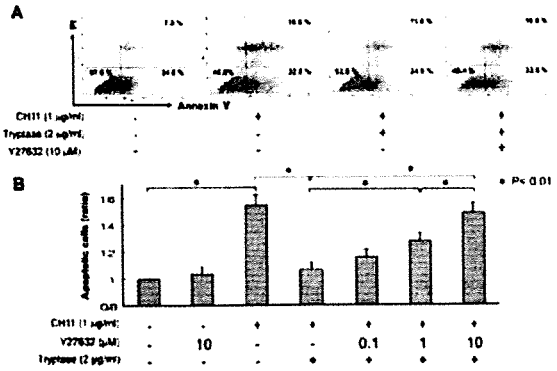

Figure 6. Rho kinase–dependent tryptase-mediated inhibition of apoptosis. **A,** Rheumatoid arthritis synovial fibroblasts (RASFs) were left untreated **(left)** or treated with anti-Fas antibody in the absence or presence of tryptase **(middle panels)** or treated with anti-Fas antibody in the presence of tryptase and the Rho kinase inhibitor Y27632 **(right)**. Twelve hours after treatment, RASFs were stained with annexin V/propidium iodide (PI) and analyzed for apoptosis by flow cytometry. **B,** The ratio of apoptotic cells to total cells was determined in experiments using RASFs from 5 different patients, with results normalized to untreated control values. Bars show the mean and SD.

DISCUSSION

Although numerous mast cells are present in RA synovial tissue, their involvement in the pathogenesis of RA remains unclear. In this report, we shed light on one potential mechanism by which mast cells may contribute to RA. Since tryptase is a protease that is specifically produced by mast cells and is considered one means by which mast cells can convey information to surrounding cells, we hypothesized that tryptase and its receptor, PAR-2, may play a role in RA pathogenesis. Through our studies, we have demonstrated that tryptase-expressing mast cells lie in close proximity to RASFs in the synovial tissue of RA patients. Furthermore, RASFs express the receptor for mast cell tryptase (PAR-2) and are protected from Fas-mediated apoptosis by tryptase in a Rho kinase–dependent manner. Such a mechanism could play an important role in the marked proliferation of RASFs and hyperplasia seen in RA synovium, leading to disease progression.

One reason that we focused on the effect of mast cell mediators on SF apoptosis was to yield insight into the apparently paradoxical finding that RASFs proliferate vigorously in vivo despite the high expression of Fas (2,3). Moreover, RASFs are readily susceptible to anti-Fas–mediated apoptosis in vitro (20). These findings suggest that a mechanism that prevents Fas-mediated cell death exists in RASFs, and that this excessive proliferation may contribute to disease pathogenesis. Indeed, we were able to demonstrate that RASFs isolated from 5 independent patients with RA exhibited apoptosis when incubated with anti-Fas antibody. However, Fas-mediated apoptosis was inhibited by a mast cell–specific protease, tryptase. Thus, we propose that the accumulation of mast cells in RA synovium creates an environment that is rich in tryptase and allows RASFs to counteract Fas-mediated killing. In fact, the interplay between RASFs and mast cells may be important for the maintenance of chronic inflammation in the RA synovium.

The PARs represent a unique family of receptors that are activated by proteolytic cleavage (21). The ligand for these receptors is encoded in the N-terminal region of the receptor itself but is unable to bind until the N-terminus is cleaved at specific sites by serine proteases, such as thrombin and tryptase (22). The proteolytic cleavage of PARs creates a new N-terminus that can now bind to this G protein–coupled receptor, and subsequently activates the small G protein Rho. In our present study, several lines of evidence suggest that tryptase protects RASFs against Fas-mediated apoptosis through PAR-2. First, PAR-2 was found to be expressed both in vivo and ex vivo on RASFs. Second, the inhibition of the protease function of tryptase by the protease inhibitor nafamostat mesylate reversed the protective effect conferred by tryptase. Third, Rho was activated upon tryptase treatment of RASFs. Finally, the anti-apoptotic effect of tryptase was abrogated by the addition of the Rho kinase inhibitor Y27632. We have previously reported that thrombin-mediated PAR-1 activation also allows RASF survival and proliferation through a similar mechanism (18), by inhibiting apoptosis through the activation of Rho (16,17). Taken together, these results suggest the possibility that a series of protease-mediated signals is important in the pathogenesis of RA.

Obviously, RASFs are not the only cells that contribute to joint destruction and inflammation, since the pannus is a complex inflammatory granulation tissue (23) consisting of RASFs, vascular tissue, and an inflammatory cell infiltrate. However, it is noteworthy that PAR-2 is also expressed on other cells in the pannus, including vascular endothelial cells and inflammatory cells (24,25). Thus, it is tempting to speculate that mast cells also affect other cell types in the synovium of RA patients, through a tryptase/PAR-2–dependent mechanism.

Our present findings are consistent with those from previous studies in animal models, in which it has

been demonstrated that tryptase is involved in the pathogenesis of murine joint inflammation. For example, mice lacking monocyte chemotactic protein 6 (analogous to human tryptase) show resistance to antibody-mediated arthritis (26), suggesting that mouse mast cell proteases play an important role in joint inflammation. Furthermore, the injection of tryptase directly into the joints of mice results in inflammation and swelling. However, this inflammation is not observed when tryptase is injected into PAR-2–deficient mice, suggesting that tryptase can cause joint inflammation through the activation of PAR-2 (27).

Our study demonstrates a potential mechanism by which mast cells contribute to RA pathogenesis, through their communication with RASFs. Numerous mast cells reside in close proximity to PAR-2–expressing RASFs in the synovium of RA patients. We believe that the interaction of mast cell–associated tryptase and PAR-2 on RASFs inhibits the apoptosis of RASFs, causing hyperplasia of RA synovial tissue. This notion is consistent with the observation that, similar to neoplastic cells, RASFs multiply, and this occurs even though RASFs express high levels of Fas (2,3). Although further studies are required to test whether such interactions indeed occur in vivo, we propose that therapy aimed at inhibiting the mast cell/tryptase/PAR-2/Rho pathway may be a new treatment target for patients with RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sawamukai, Tanaka.

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Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis: RRR (remission induction by Remicade in RA) study

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ABSTRACT

Background Tumour necrosis factor (TNF) inhibitors enable tight control of disease activity in patients with rheumatoid arthritis (RA). Discontinuation of TNF inhibitors after acquisition of low disease activity (LDA) is important for safety and economic reasons.

Objective To determine whether infliximab might be discontinued after achievement of LDA in patients with RA and to evaluate progression of articular destruction during the discontinuation.

Methods 114 patients with RA who had received infliximab treatment, and whose Disease Activity Score, including a 28-joint count (DAS28) was <3.2 (LDA) for 24 weeks, were studied.

Results The mean disease duration of the 114 patients was 5.9 years, mean DAS28 5.5 and mean modified total Sharp score (mTSS) 63.3. After maintaining LDA for >24 weeks by infliximab treatment, the drug was discontinued and DAS28 in 102 patients was evaluated at year 1. Fifty-six patients (55%) continued to have DAS28<3.2 and 43% reached DAS<2.6 at 1 year after discontinuing infliximab. For 46 patients remission induction by Remicade in RA (RRR) failed: disease in 29 patients flared within 1 year and DAS28 was >3.2 at year 1 in 17 patients. Yearly progression of mTSS (ΔTSS) remained <0.5 in 67% and 44% of the RRR-achieved and RRR-failed groups, respectively. The estimated ΔmTSS was 0.3 and 1.6 and Health Assessment Questionnaire-Disability Index was 0.174 and 0.614 in the RRR-achieved and RRR-failed groups, respectively, 1 year after the discontinuation.

Conclusion After attaining LDA by infliximab, 56 (55%) of the 102 patients with RA were able to discontinue infliximab for >1 year without progression of radiological articular destruction.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that causes significant morbidity and mortality. The combined use of biological agents targeting tumour necrosis factor (TNF) and methotrexate (MTX) has produced significant improvements in clinical, radiographic and functional outcomes that were not previously seen and has revolutionised the treatment goal of RA to clinical remission, structural remission and functional remission.[1–5] The next goal should be remission without the use of biological agents and subsequent drug-free remission. Although global evidence of the efficacy and safety of TNF inhibitors such as infliximab has accumulated, including the ATTRACT study, ASPIRE study, our RECONFIRM studies and many others,[5–10] there is no well-established firm evidence for remission free from the use of biological agents.

The initial report of the potential for remission without the use of biological agents in patients with RA was reported by a British group (TNF20 study).[11] The combination of infliximab and MTX in patients with early RA who had fewer than 12 months of symptoms provided tight control of the disease activity and a significant reduction in MRI evidence of synovitis and erosions at 1 year. At 2 years, functional and quality of life benefits were sustained, despite withdrawal of infliximab treatment. On the other hand, the Behandelstrategieën (BeSt) study was conducted to observe clinical and radiological outcomes of patients with early RA treated with initial infliximab and MTX who discontinued infliximab after achieving a sustained Disease Activity Score (DAS) ≤2.4. Five years after receiving infliximab and MTX as initial treatment for RA, 58% of 120 patients discontinued infliximab because of a continuous DAS ≤2.4 and 19% of patients have stopped all antirheumatic drugs and remain in clinical remission, with minimal joint damage progression. These findings indicate that treatment using infliximab and MTX, guided by DAS, is an effective and tight control to maintain low disease activity (LDA) and may alter the course of early RA.[12–16]

Discontinuation of TNF inhibitors after acquisition of LDA is important for reasons of safety and economy. For instance, the problem of the incidence of haematological malignancy owing to the long-term use of TNF inhibitors remains unresolved. In Japan a large majority of patients have to pay 30% of their medical costs and all wish to know for how long biological agents must be continued, but we have no answer. We successfully discontinued infliximab after attaining DAS-guided remission for >24 weeks,[10] but evidence based on multicentre studies is needed. Reports published to date on this topic are confined to those from the BeSt study and TNF20 study involving only patients at an early stage of RA.[11–16]

Thus, this multicentre study was undertaken to seek the possibility of discontinuing infliximab after attaining DAS-guided remission and maintaining LDA without infliximab, in patients with RA, including patients with long-established disease, and to evaluate progression of articular destruction and functional disabilities during the discontinuation.

PATIENTS AND METHODS

Patients

Data and information on patients with RA fulfilling the diagnostic criteria of the American College of Rheumatology were collected from 26 centres of remission induction by Remicade in RA (RRR) investigator groups in Japan.[17] Disease activity of individual patients was assessed by Disease Activity Score, including a 28-joint count (DAS28)-erythrocyte sedimentation rate (ESR) or DAS28-C reactive protein (CRP) that was calculated according to the authorised formula (http://www.das-score.nl/, accessed 15 February 2010).[18] Since none of the patients, except for one, achieved LDA measured by DAS28 despite MTX or a combination of MTX and other disease-modifying antirheumatic drugs for at least 3 months, infliximab treatment (3 mg kg, every 8 weeks) was administered in the investigators' institutions, according to the treatment guideline proposed by the Japan College of Rheumatology.[17] Joint damage was assessed by the van der Heijde-modified total Sharp score (mTSS)[19] and for 102 patients, x-ray images of the hands and feet at baseline, RRR-study entry and 1 year after the study were available; these were evaluable for 49 patients owing to loss of the radiographs and/or low quality of the x-ray images.[20 21] Two blinded expert readers independently scored articular damage and progression according to the mTSS scoring method. The difference between the two readers' scores for each patient's radiographs was <1% of the maximum mTSS score—that is, 448.[9 20 21] To confirm that the x-ray results of the 49 patients represented the outcomes of the whole group, we compared multiple background characteristics and changes of each characteristic from baseline to RRR-study entry between 49 patients with evaluable x-ray images and 53 patients without them and no significant difference was seen between the two groups.

After patients had achieved DAS28 (ESR)<3.2 (LDA) for >24 weeks, informed consent to discontinue infliximab was obtained from 126 patients. Other criteria were that patients were controlled with <5 mg/day of oral prednisolone (PSL) and were >18 years old. Concomitant use of MTX was started in all patients, and the dose of MTX was determined by each attending doctor. Twelve patients dropped out at the screening period, and 114 patients were enrolled in the study and discontinued infliximab (figure 1). The demographic indicators and baseline disease characteristics of the 114 patients enrolled are summarised in table 1.

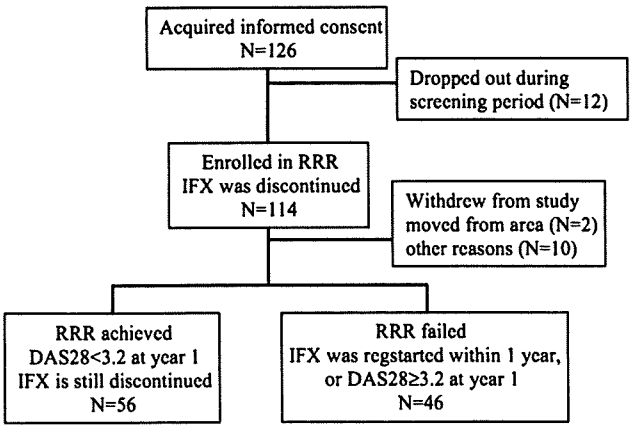

Figure 1 Study design and profile. DAS28, Disease Activity Score, including a 28-joint count; IFX, infliximab; RRR, remission induction by Remicade in rheumatoid arthritis.

Table 1 Demographic indicators and baseline disease characteristics

	Enrolled patients (N=114)	RRR-achieved (N=56)	RRR-failed (N=46)	p (probability $>\chi^2$)
Women	87 (76%)	42 (75%)	38 (83%)	0.4691
Age (years)	51.4 (20.0–73.0)	49.5±12.6	56.1±12.2	*0.0053*
Disease duration (years)	5.9 (0.1–38.0)	4.8±5.9	7.8±7.7	*0.0238*
Tender joint count (0–28)	8.2±6.7	8.6±7.0	7.5±5.8	0.5798
Swollen joint count (0–28)	9.0±7.2	10.1±7.7	7.6±5.8	0.1674
PaGA (0–100 mm, VAS)	50.0±23.0	50.0±24.2	49.3±23.1	0.9520
CRP (mg/dl)	2.5±3.0	2.6±2.6	2.7±3.7	0.5531
ESR (mm/h)	46.2±26.9	43.1±24.2	54.1±30.1	0.1555
DAS28 (ESR) score	5.5±1.2	5.5±1.4	5.6±1.1	0.9112
DAS28 (CRP) score	4.9±1.2	5.1±1.3	4.8±1.3	0.5486
HAQ-DI	1.0±0.7	0.9±0.6	1.2±0.7	0.1112
mTSS*	63.3 (1.0–314.0)	46.9±46.5	97.2±86.9	*0.0207*
RF (U/ml)	201.9±496.5 (68.5%)	225.7±583.3	197.9±427.8	0.5190
MTX (mg/week)	7.7±2.3	7.9±1.9	7.8±2.8	0.3232
PSL (mg/day)	2.5±3.4 (45.6%)	2.4±3.5	2.8±3.5	0.5223

Data are number of patients (%) for categorical data and the means for continuous data. Statistical difference was assessed by non-parametric Wilcoxon t test and p (probability $>\chi^2$) values are shown. Values in italic indicate a significant difference ($p<0.05$).
*Data supplied for 33 patients who achieved RRR and 16 patients for whom RRR failed.
CRP, C-reactive protein; DAS28, Disease Activity Score, including a 28-joint count; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; mTSS, modified total Sharp score; MTX, methotrexate; PaGA, patient global assessment of disease activity; PSL, prednisolone; RF, rheumatoid factor; RRR, remission induction by Remicade in rheumatoid arthritis; VAS, visual analogue scale.

Procedures

Study protocol was a simple observation after discontinuation of infliximab. The follow-up observation was monitored by symptoms, signs and DAS28 (ESR) every 4–13 weeks for 2 years. The dose of concomitant MTX was basically consistent, but tapering of non-steroidal anti-inflammatory drugs and glucocorticoid was allowed during the study period. The primary end points were that after discontinuing infliximab, DAS28 remains <3.2 (LDA) for 1 year and (B) yearly progression of mTSS remains <0.5 (structural remission) for 1 year. Secondary end points were DAS28 remains <2.6 (clinical remission) for 1 year, DAS28 remains <3.2 for 2 years, yearly progression of mTSS remains <0.5 for 2 years and no rescue with infliximab for 1 or 2 years is needed, after discontinuing infliximab. When a flare-up occurred in patients after the discontinuation, restart of infliximab was allowed and patients were categorised into the 'RRR-failed' group. For the restart of infliximab, the same dose (3 mg/kg) and the same premedication as used before the study entry were used.

Statistical analysis

Baseline characteristics of patients are summarised in table 1 using the mean values for continuous variables. All multivariate analyses were conducted using the variables gender, age, duration of disease, DAS28 (ESR) score, DAS28 (CRP) score, tender joint count (0–28), swollen joint count (0–28), patient global assessment of disease activity (PaGA, 0–100 mm, visual analogue scale), ESR, CRP, Health Assessment Questionnaire-Disability Index (HAQ-DI), rheumatoid factor (RF), MTX dose and PSL dose at baseline. Spearman correlation analyses were performed to evaluate the

association between multivariables at RRR-study entry and DAS28 at the primary end point (last observation carried forward) of 102 patients. Logistic regression analysis was carried out to estimate DAS28 at the primary end point as dependent variables (probability) by DAS28 at RRR entry as independent variables. A receiver operating characteristic (ROC) curve was developed based on the logistic analysis and the significant cut-off point was determined from the curve. For categorical response parameters, group comparisons were made using a non-parametric Wilcoxon t test. Statistical analyses were performed using JMP software version 7 (SAS Institute, Cary, North Carolina, USA). All reported p values are two sided and p values <0.05 were considered significant.

RESULTS

Study end points

The demographic indicators and baseline characteristics of the 114 patients enrolled were as follows: mean age 51.4 years, mean disease duration 5.9 years and mean mTSS 63.3, indicating that the population included patients with long-established disease, and the mean DAS28 (ESR) score was 5.5, implying that most patients had highly active disease (table 1). Figure 1 shows the study profile. After maintaining DAS28<3.2 (LDA) for >24 weeks by infliximab treatment, infliximab was discontinued in 114 patients. Twelve patients withdrew because they moved from area (n=2) and for other reasons (n=10), and thus DAS28 could be evaluated in 102 patients at year 1.

Of the 102 patients, 56 patients achieved the primary end point having a DAS28<3.2 and remaining without infliximab for 1 year after the discontinuation (figure 2A). Thus, 55% of the enrolled patients met the primary end point that LDA was maintained for 1 year after discontinuing infliximab. Furthermore, 44 patients (43%) reached DAS<2.6 after the discontinuation. On the other hand, 29 patients flared within 1 year (mean duration was 6.4 months) after the discontinuation and in 17 patients DAS28 was >3.2 at year 1 and thus RRR failed for 46 patients (45%) at year 1. Re-treatment with infliximab in 32 patients was effective and the majority of patients reached DAS28<3.2 within 24 weeks (figure 2B). Minimal adverse reactions at infusion of the agent were seen in five patients only at the first or second infusion.

To clarify the background factors related to the RRR-achievement, multiple clinical parameters at baseline were compared between patients for whom RRR was achieved and those for whom it failed. Patients for whom RRR was achieved were younger (49.5 vs 56.1), their disease duration was shorter (4.8 vs 7.8) and mTSS was lower (46.9 vs 97.2) than for those for whom RRR failed. Among 56 patients who achieved RRR, 10 patients had early RA (disease duration <1.0 year) and eight long-established disease (>10 years). Of 46 patients for whom RRR failed, eight had early RA and 12 established disease. These results imply that infliximab can be discontinued in patients with long-established RA. In contrast, no significant difference was seen in gender, DAS including DAS28, tender or swollen joint count, ESR and CRP, HAQ-DI, RF and the dose of MTX and PSL. Since these factors interact with one another, we analysed the relationship between RRR-achievement and a series of clinical parameters at baseline using multivariate analysis after adjusting for confounding variables. No significant relations

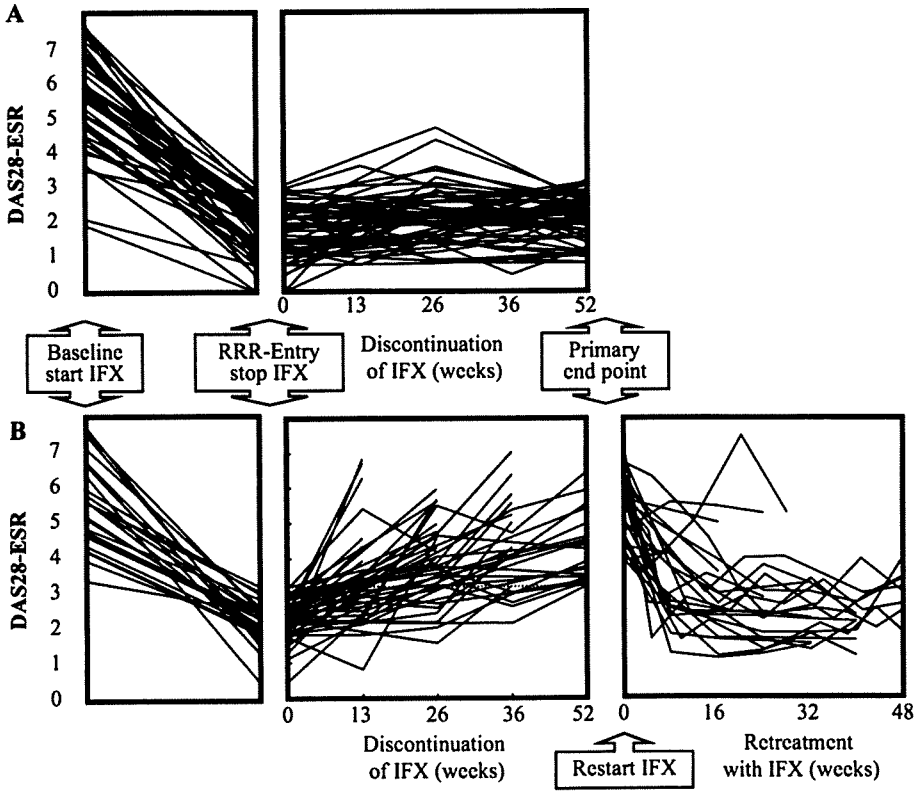

Figure 2 Changes of Disease Activity Score, including a 28-joint count (DAS28) in patients with remission induction by Remicade in rheumatoid arthritis-achieved (RRR-achieved) and patients for whom RRR failed (RRR-failed). (A) Changes of Disease Activity Score, including a 28-joint count (DAS28) at baseline when infliximab (IFX) was administered, at RRR-study entry when infliximab was discontinued and at the primary end point at week 52 after discontinuing IFX in 56 patients who were still satisfied with DAS28 (erythrocyte sedimentation rate (ESR)) <3.2 at week 52, RRR-achieved'. (B) Changes of DAS28 at baseline, at RRR entry and the end point in 46 patients whose disease activity flared after the discontinuation of IFX or DAS28 >3.2 at week 52, 'RRR-failed'. The lower right panel shows changes of DAS28 after the restarting IFX in 32 patients for whom RRR failed.

between RRR-achievement and age, gender, DAS28 (ESR) score, PaGA and CRP were found, whereas a significant correlation was found with disease duration (p=0.0019) and serum levels of RF (p=0.0128) in RRR-achievement.

To determine the correlation of DAS28 at the primary end point with clinical parameters at RRR-study entry, univariate analysis of multiple variables was carried out. No significant correlations between DAS28 (ESR) at the primary end point and a series of clinical parameters were found, whereas DAS28 (ESR) and DAS28 (CRP) at RRR-entry were significantly correlated with DAS28 (ESR) at the primary end point. Subsequently, logistic regression analysis to estimate the probability of DAS28<3.2 at the primary end point as dependent variable by DAS28 at RRR-entry as independent variable was assessed. A significant logistic regression curve was drawn between the dependent and independent variables (p=0.0005) (figure 3A). Thus, DAS28 at RRR-study entry had the most marked correlation with the maintenance of LDA for 1 year after the discontinuation. By reciprocal statistics, DAS28 at RRR-study entry was estimated as 2.22, to attain DAS28<3.2 at the end point in 50% of the 102 patients (figure 3A). Furthermore, 71.4% of patients whose DAS28 at study entry was <2.225, a cut-off point calculated from the ROC curve, continued to have DAS28<3.2 for 1 year, whereas only 32.6% of patients whose DAS28 at RRR-entry was 2.225–3.2 continued to have DAS28<3.2 (figure 3B), indicating that 'deep remission' was required to maintain lower disease activity for 1 year after discontinuation of infliximab.

Structural and functional changes

From the 102 patients enrolled in the study, 49 patients were selected in whom both hand and feet x-ray data were available and evaluable; experts examined the structural damage before and after the infliximab treatment. When the baseline characteristics of the 49 patients in the study were compared with the rest of the patients in the study with insufficient x-ray data (n=53), no significant difference was seen. Next, the baseline characteristics of the 33 patients who achieved RRR and 16 patients for whom RRR failed were compared. As described in table 1, disease duration was shorter and mTSS was lower in patients who achieved RRR than in patients for whom RRR failed, but yearly progression of mTSS (ΔmTSS) was comparable between two groups (table 2). ΔmTSS at RRR entry was also comparable between two groups. However, means (0.3 vs 1.6) and medians (0.0 vs 1.5) of ΔmTSS were lower in the RRR-achieved group than in the RRR-failed group and more patients in the RRR-achieved group (67%) achieved ΔmTSS<0.5, radiographic remission, than patients in the RRR-failed group (44%). Thus, another primary end point for structural remission was achieved for 1 year after the discontinuation. Furthermore, HAQ-DI at baseline and RRR entry was comparable between patients for whom RRR was achieved and those for whom it failed, whereas HAQ-DI at the primary end point in patients who achieved RRR was significantly lower than that in patients for whom RRR failed (0.174 vs 0.614) (figure 4).

DISCUSSION

This multicentre study was undertaken to determine the possibility of discontinuing infliximab treatment in patients with RA after acquiring DAS-guided LDA, including those with long-established disease. Among 102 patients who could be evaluated at year 1, 56 patients (55%) satisfied the primary end point by maintaining DAS28<3.2 (LDA) and 44 patients (43%) reached DAS<2.6 (remission), remaining without infliximab at year 1 after the discontinuation. Of the 102 patients, 83 (81.4%) were in clinical remission at study entry and after discontinuing infliximab, 39/83 patients (47%) remained in remission and 10/83 patients (12%) progressed to LDA at the primary end point.

These data are similar to those of the BeSt study. However, the greatest difference between the patient populations enrolled in the two studies is mean disease duration—0.4 years in the BeSt study versus 5.9 years in our RRR study.[12–16] Joint destruction also differed between the two studies—mean mTSS 7.0 in the BeSt study versus 63.3 in our RRR study—suggesting that discontinuation of infliximab after reaching LDA is possible in patients with early RA and also in patients with long-established disease.[13–15] On the other hand, among multiple clinical parameters at baseline, disease duration was statistically related to RRR-achievement by multivariate analysis and disease duration was shorter (4.8 vs 7.8) and mTSS was lower (46.9 vs 97.2) in patients who achieved

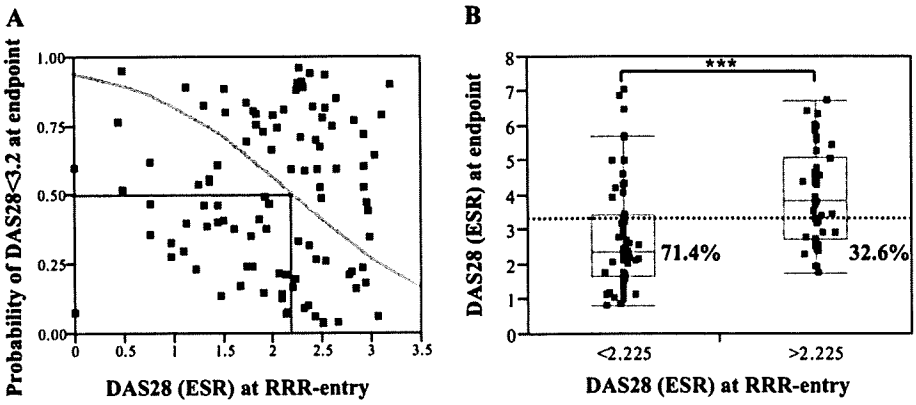

Figure 3 Logistic analysis of probability of Disease Activity Score, including a 28-joint count (DAS28) was <3.2 at primary end point by DAS28 at remission induction by Remicade in rheumatoid arthritis entry (RRR entry). (A) Logistic regression analysis to estimate DAS28 at primary end point as dependent variables by DAS28 at RRR entry as independent variables. The y-axis shows the probability of DAS28 <3.2 at the primary end point after the 52 weeks discontinuation of infliximab and a scatter diagram of an individual patient and logistic regression curve (solid line) are shown. To attain DAS28 <3.2 at the end point in 50% of the 102 patients, DAS28 at RRR study entry was estimated by reciprocal statistics. (B) From the receiver operating characteristic curve based on the logistic regression analysis above, the cut-off point of DAS28 at RRR-study entry was 2.225. Subsequently, one-way analysis of DAS28 at the primary end point by DAS28 at study entry, <2.225 versus between 2.225 and 3.2, was performed and the statistical difference of the two groups was sought by non-parametric Wilcoxon t test (***p<0.001). ESR, erythrocyte sedimentation rate.

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Table 2 Radiographic indicators and baseline disease characteristics

	RRR-achieved (N=33)	RRR-failed (N=16)	p (probability $>\chi^2$)
Baseline			
Disease duration (years)	4.7 (0.5–14.0)	8.6 (0.5–25.0)	*0.0280*
DAS28 (ESR) score	5.5 (1.9–7.6)	5.7 (4.2–6.8)	0.6976
HAQ-DI	1.0 (0.0–2.3)	1.1 (0.0–1.8)	0.6271
mTSS	46.9 (1.0–216.5)	97.2 (6.0–314.0)	*0.0207*
Bone erosion score	23.7 (0.0–127.5)	55.5 (1.5–192.5)	*0.0119*
Joint space narrowing score	23.2 (1.0–89.0)	41.6 (4.5–121.5)	*0.0621*
Yearly progression of mTSS	13.1 (0.8–51.3)	15.0 (1.0–47.8)	0.5794
RRR-entry			
Yearly progression of mTSS	1.0 (−2.9 to 10.5)	0.7 (−2.0 to 6.7)	0.5788
Primary end point			
Yearly progression of mTSS	0.3 (−3.6 to 8.5)	1.6 (−3.6 to 7.0)	0.1087
Median of yearly progression of mTSS	0.0	1.5	–
Yearly progression of mTSS <0.5 (%)	67	44	0.2161

Data are number of patients (%) for categorical data and the means for continuous data. Statistical difference was assessed by non-parametric Wilcoxon t test and p (probability $>\chi^2$) values were shown. Values in italic indicate a significant difference ($p<0.05$). DAS28, Disease Activity Score based on assessments of 28 tender and 28 swollen joints; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; mTSS, modified total Sharp score; RRR, remission induction by Remicade in rheumatoid arthritis.

RRR than in those for whom RRR failed. These results imply that remission free from biological agents can be more easily obtained in patients with shorter disease duration than in those with more established disease, but discontinuation of infliximab is still possible even in patients with long-established RA, because eight patients whose disease duration was >10 years successfully remained without infliximab for 1 year.

Among 102 evaluated patients, disease in 29 patients flared within 1 year, 17 patients had DAS28≥3.2 at year 1 after discontinuing infliximab and 32 patients had already been re-treated with infliximab. One of the major concerns of restarting infliximab is the possibility of an infusion reaction after the long-term discontinuation, partly owing to human anti-chimeric antibodies.[22] However, minimal adverse reactions at infusion of the agent were seen only in five patients at the first or second infusion. Another concern is the progress of joint damage after discontinuation of infliximab. However, although the yearly progression of mTSS at RRR-study entry was also comparable between two groups, means (0.3 vs 1.6) and medians (0.0 vs 1.5) of ΔmTSS were shorter in the RRR-achieved group than in the RRR-failed group. Furthermore, at year 1 after the discontinuation of infliximab, more patients in the RRR-achieved group (67%) tended to satisfy ΔmTSS<0.5—that is, structural remission, than those in the RRR-failed group (44%) and HAQ-DI in patients who achieved RRR was significantly lower than that in patients for whom RRR failed (0.174 vs 0.614). These results indicate that both structural remission and functional remission were maintained for 1 year in patients with LDA even after discontinuing infliximab.

This study also shows the significance of DAS-guided tight control of RA in order to maintain remission free from biological agents. There was a significant correlation between DAS28 (ESR) or DAS28 (CRP) at RRR entry and DAS28 (ESR) at year 1 after the discontinuation of infliximab by univariate analysis of multiple variables and a logistic regression analysis. Thus, DAS28 at RRR-study entry had the greatest correlation with maintenance of LDA for 1 year after discontinuation. Also, DAS28 at study entry was mainly influenced by PaGA and ESR among the composite measures. By reciprocal statistics, the estimated DAS28 (ESR) at RRR-study entry was 2.22 (1.85–2.70), to attain DAS28<3.2 at the primary end point in 50% of the 102 patients studied. Also, 71.4% of patients whose DAS28 at study entry was <2.225, a cut-off point calculated from ROC curve, remained DAS28<3.2 for 1 year, whereas only 32.6% of patients whose DAS28 at RRR-entry was 2.225–3.2 remained DAS28<3.2. These results indicate that 'deep remission' appears to be required to maintain lower disease activity for 1 year after discontinuation of infliximab.

About 55% of the 102 patients, who were in an LDA state for >24 weeks with infliximab and MTX treatment, could discontinue infliximab for >1 year without progression of radiological articular destruction or functional disturbance. These data may have significant implications for the optimal use of expensive biological treatments: (*a*) re-treatment with infliximab is efficient and tolerable in the patients for whom RRR failed; (*b*) DAS-guided monitoring is valuable to keep tight control; (*c*) 'deep

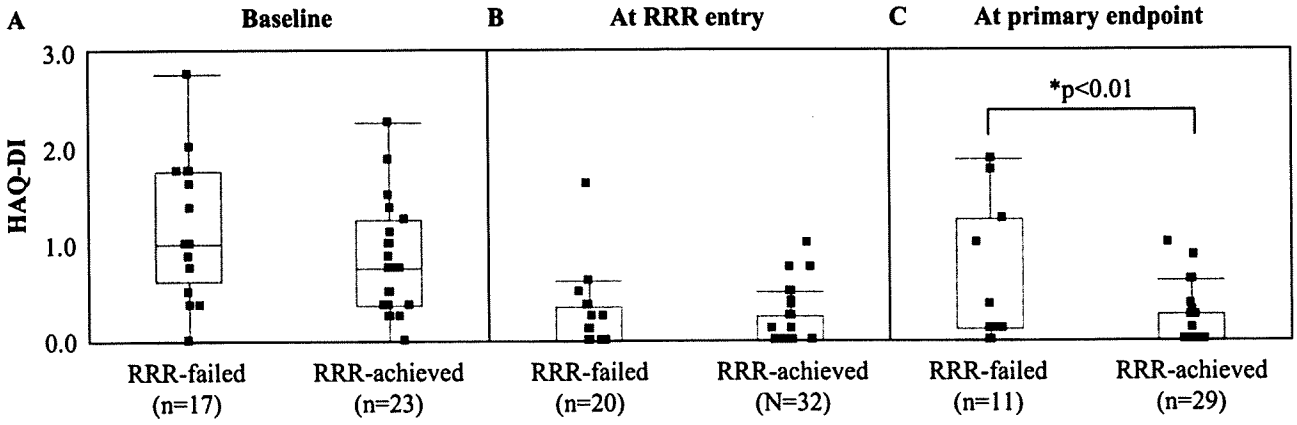

Figure 4 Health Assessment Questionnaire-Disability Index (HAQ-DI) in patients for whom remission induction by Remicade in rheumatoid arthritis failed (RRR-failed)' and in patients for whom 'RRR was achieved (RRR-achieved)' at (A) baseline, (B) RRR entry and (C) the primary end point. The line in the box represents the median value and the upper and lower ends of the box indicate the 25th and 75th centiles of the population. Statistical difference was assessed by non-parametric Wilcoxon t test.

remission' by tight control is required to maintain discontinuation of infliximab; (*d*) remission free from biological agents may be easier to attain in patients with early RA, but is possible for patients with long-established disease; (*e*) treatment aimed at reaching a target of LDA is pivotal to the approach to remission free from biological agents. Finally, TNFα is not a cause of RA, but if appropriate treatment with infliximab can lead to drug-free remission, TNF inhibitors may shut down pathological processes and may change or modify the disease course in RA. Thus, a clinical and basic research approach to the 'process-driven disease' of RA is warranted.

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Patient consent Obtained.

Ethics approval This study is an observational study and is registered with the University Hospital Medical Information Network-Clinical trials Registry, number R000002571. Also, ethics committees of the participating centres approved the study protocol.

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The Effects of Phosphatidylserine-Dependent Antiprothrombin Antibody on Thrombin Generation

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Objective. **Antibodies to prothrombin (APTs) and to β_2-glycoprotein I are the major autoantibodies responsible for lupus anticoagulant (LAC) activity. APTs comprise antibodies against prothrombin alone as well as antibodies against phosphatidylserine/prothrombin complex (anti-PS/PT), the latter being highly associated with the antiphospholipid syndrome (APS). The effect of anti-PS/PT on thrombin generation has not been elucidated, and the paradoxical effect of LAC (an anticoagulant in vitro, but a procoagulant in vivo) remains an enigma. The purpose of this study was to investigate the effects of anti-PS/PT on thrombin generation and to examine the LAC paradox.**

Methods. **We evaluated 36 anti-PS/PT–positive APS patients and 127 healthy subjects. Markers of in vivo thrombin/fibrin generation, including prothrombin fragment F_{1+2}, thrombin–antithrombin III complex, soluble fibrin monomer, D-dimer, and fibrin degradation products, were measured. Mouse monoclonal anti-PS/PT antibody 231D was established, and its effects on in vitro thrombin generation were investigated by chromogenic assay.**

Results. **Significantly elevated levels of markers of thrombin/fibrin generation were observed in anti-PS/PT–positive patients, regardless of the presence or absence of anticardiolipin antibodies, as compared with healthy subjects. In the presence of low concentrations of human activated factor V (FVa), monoclonal antibody 231D increased thrombin generation in a dose-dependent manner. In contrast, when high concentrations of FVa were added, monoclonal antibody 231D decreased thrombin generation. Under a constant concentration of FVa, a high concentration of human FXa enhanced the effect of 231D.**

Conclusion. **The presence of anti-PS/PT greatly correlated with increased thrombin generation in APS patients. The in vitro effects of monoclonal antibody 231D on thrombin generation are "biaxial" according to the FVa/FXa balance. These data may serve as a clue to understanding the LAC paradox and the thrombogenic properties of anti-PS/PT.**

Antiphospholipid antibodies (aPL) are immunoglobulins that are related to diverse clinical phenomena, such as arterial and venous thrombosis, complications of pregnancy, livedo reticularis, valvular disease, neurologic disorders, and thrombocytopenia. The term antiphospholipid syndrome (APS) is used to link thrombosis or pregnancy morbidity to the persistence of aPL as one of the most common causes of acquired thrombophilia (1).

It has been shown that despite their name, aPL are not directed against anionic phospholipids, as was previously thought, but are part of a large family of autoantibodies against phospholipid-binding plasma proteins or phospholipid–protein complexes (2). The most common and best characterized antigenic target of these antibodies is β_2-glycoprotein I (β_2GPI) (3–5), a phospholipid binding protein that has been extensively studied and has been shown to play a prominent role in the binding of aPL to phospholipid. Anticardiolipin

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antibodies (aCL), which are associated with APS, are not directed against cardiolipin alone, but require β_2GPI as a cofactor for the binding of cardiolipin in enzyme-linked immunosorbent assay (ELISA) plates. Beta$_2$-glycoprotein I bears the epitopes for aCL binding that are exposed when β_2GPI binds to negatively charged phospholipids (6,7).

Prothrombin, another main phospholipid binding protein, was first reported by Loeliger in 1959 (8) to be a probable cofactor for the lupus anticoagulant (LAC). Fleck et al (9) subsequently confirmed that antiprothrombin antibodies (APTs) are responsible for the LAC activity, and in 1991, Bevers et al (10) emphasized the importance of prothrombin in generating LAC activity. Some years later, the inhibitory effect of LAC on endothelial cell–mediated prothrombinase activity was reported, and it was also demonstrated that the IgG fraction containing LAC activity bound to the phospholipid–prothrombin complex (11). Therefore, prothrombin was recognized as another target for autoantibodies with LAC activity. Accordingly, it is widely accepted that APTs and anti-β_2GPI antibodies are the 2 major autoantibodies responsible for LAC activity: APTs for prothrombin-dependent LAC and anti-β_2GPI antibodies for β_2GPI-dependent LAC.

An ELISA for the detection of APTs using prothrombin alone as the antigen coated onto irradiated plates (APT-alone assay) was described in 1995 (12). Since then, a number of studies have investigated the clinical relevance of testing APT alone; nevertheless, the association between APT alone and clinical manifestation of APS is still a subject of controversy (13). In 1996, antibodies against the phosphatidylserine/prothrombin complex (anti-PS/PT; or phosphatidylserine-dependent APTs) were described in LAC-positive patients (14). Moreover, the ELISA using phosphatidylserine-bound prothrombin as antigen was reported to be more sensitive for detecting the presence of APTs than the ELISA using prothrombin alone as antigen (15). Our group assessed the anti-PS/PT ELISA in a large population of patients with autoimmune diseases and found that IgG anti-PS/PT were highly prevalent in patients with APS as compared with patients with other diseases (16). We also showed that the detection of anti-PS/PT strongly correlated with the clinical manifestations of APS and with the presence of LAC.

In APS patients, the LAC paradox, that is, the behavior of LAC as an anticoagulant in vitro but a procoagulant in vivo, remains unresolved. In addition, the effects of anti-PS/PT on thrombin generation, whether in vitro or in vivo, have not been clarified. In order to investigate the effects of anti-PS/PT on thrombin generation, we evaluated markers of thrombin generation and fibrinolytic turnover in plasma samples from APS patients with anti-PS/PT antibodies. Furthermore, we established a mouse monoclonal anti-PS/PT antibody (231D) and used this monoclonal antibody to analyze thrombin generation in vitro.

PATIENTS AND METHODS

Patients. Plasma and serum samples were obtained from 36 APS patients with IgG and/or IgM anti-PS/PT antibodies (32 women and 4 men with a mean age of 46 years [range 22–74 years]) who fulfilled the revised Sapporo criteria for APS (1). Fifteen patients were diagnosed as having primary APS, and 21 patients had APS in association with other connective tissue diseases. Twenty-six patients (72%) had experienced arterial thrombotic events, such as stroke, myocardial infarction, and iliac artery occlusion, as confirmed by computed tomography scanning, magnetic resonance imaging, or conventional angiography. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis (12 of 36 patients [33%]) and were confirmed by angiography or scintigraphy. Thirteen women (36%) had pregnancy morbidity as defined by the APS criteria. Anti-PS/PT antibodies of IgG, IgM, and both isotypes were detected in 47%, 22%, and 31% of patients, respectively.

None of the patients had thrombotic events or pregnancy complications within 3 months before blood collection. Signs of acute thrombosis were not detected in any patient at the time blood was drawn. The time since the latest manifestation of APS varied from 4 months to 6 years. Therefore, our data correspond to the baseline of thrombin generation in anti-PS/PT–positive patients. When blood was drawn for this study, no patients were receiving heparin; some were taking warfarin, but there had been no modification of any medications within the 3 previous months. None of the patients had a tendency toward bleeding.

Blood samples were also collected from 127 apparently healthy subjects who consented to join the study. There were a total of 51 women and 76 men with a mean age of 34 years (range 18–65 years).

The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the Local Ethics Committee, and informed consent was obtained from each study subject before enrollment.

Plasma samples. Venous blood was collected into tubes containing a one-tenth volume of 0.105*M* sodium citrate and was centrifuged immediately at 4°C. Plasma samples were depleted of platelets by filtration then stored at −80°C until they were used in the experiments.

ELISA for the detection of anti-PS/PT. Anti-PS/PT antibodies were detected by ELISA, as previously described (16). Briefly, nonirradiated microtiter plates (Sumilon Type S; Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of a 50 μg/ml preparation of phosphatidylserine (Sigma, St. Louis, MO) and dried overnight at 4°C. To avoid nonspecific binding of proteins, the wells were blocked with 150 μl of Tris buffered

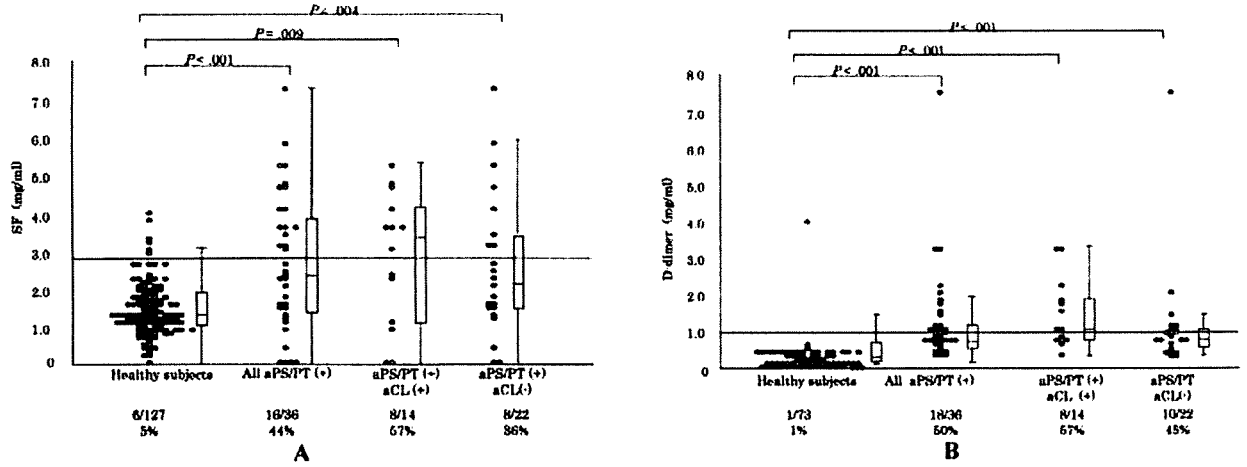

Figure 1. Distribution of plasma levels of soluble fibrin antigen (SF) and D-dimer in patients with antiphospholipid syndrome (APS) and in healthy individuals. Plasma levels of **A**, soluble fibrin antigen and **B**, D-dimer were measured in healthy controls, in all APS patients with anti-phosphatidylserine/prothrombin complex (anti-PS/PT) antibody, and in anti-PS/PT antibody–positive APS patients with or without anticardiolipin antibody (aCL). Horizontal line shows the cutoff level of positivity, which was defined as the mean ± 2SD of the level in control subjects. Data are shown as individual results as well as box plots, where each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Values across the bottom are the number of subjects positive/total number tested, as well as the percentages.

saline (TBS) containing 1% fatty acid–free bovine serum albumin (BSA) (catalog no. A6003; Sigma) and 5 m*M* $CaCl_2$ (BSA–$CaCl_2$). After 3 washes in TBS containing 0.05% Tween 20 (Sigma) and 5 m*M* $CaCl_2$ (TBS–Tween–$CaCl_2$), 50 μl of a 10 μg/ml preparation of human prothrombin (Diagnostica Stago, Asnières-sur-Seine, France) in BSA–$CaCl_2$ was added to half of the wells in the plates, and the same volume of BSA–$CaCl_2$ alone (as sample blank) was added to the other half.

After 1 hour of incubation at 37°C, the plates were washed, and 50 μl of serum diluted 1:100 in BSA–$CaCl_2$ was added to duplicate wells. Plates were incubated for 1 hour at room temperature, and alkaline phosphatase–conjugated goat anti-human IgG or IgM and substrate were added. The optical density of wells coated with phosphatidylserine alone was subtracted from that of wells containing phosphatidylserine/prothrombin. The anti-PS/PT antibody titer of each sample was derived from the standard curve according to dilutions of the positive control.

Determination of aCL and LAC. IgG and IgM aCL were measured according to a standard aCL ELISA, as described elsewhere (17).

Two clotting tests were performed for LAC determination, using a semiautomated hemostasis analyzer (STart 4; Diagnostica Stago) according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (18). For measurement of the activated partial thromboplastin time (APTT), a sensitive reagent with a low phospholipid concentration (test PTT-LAC; Diagnostica Stago) was used for screening, and the results were confirmed with the use of a StaClot LAC kit (Diagnostica Stago). The dilute Russell's viper venom time (dRVVT) was screened for and confirmed by use of a Gradipore LAC test (Sydney, New South Wales, Australia). LAC was considered positive when at least 1 of these tests confirmed its presence.

Assessment of markers of thrombin and plasmin generation in vivo. Plasma levels of soluble fibrin antigen (Mitsubishi Kagaku Iatron, Tokyo, Japan), prothrombin fragment F_{1+2} (Enzygnost F1+2 Micro; Dade-Behring, Marburg, Germany) and thrombin–antithrombin III complex (TAT test Kokusai F; International Reagent Corporation, Kobe, Japan) were assessed as markers of thrombin generation. Among them, F_{1+2} was not measured in patients receiving warfarin. We also evaluated D-dimer (D-dimer test-F; International Reagent Corporation) and fibrin/fibrinogen degradation products (Nonapia p-FDP; Daiichi Kagaku, Tokyo, Japan) as markers of fibrinolytic turnover.

Establishment of a mouse monoclonal anti-PS/PT antibody using prothrombin as antigen. Eight-week-old female BALB/c mice were immunized intraperitoneally and were given 2 booster injections with 50 μg of human prothrombin (Enzyme Research Laboratories, Swansea, UK) emulsified with Freund's complete adjuvant and with Freund's incomplete adjuvant (Difco, Detroit, MI), respectively. The spleens were excised from the mice, and spleen cells were fused with P3U1 mouse myeloma cells (19). Cells producing antibodies against the phosphoserine/prothrombin complex were screened by anti-PS/PT ELISA. Antibody-producing hybridomas were cloned by serial limiting dilution and injected intraperitoneally into pristane-pretreated BALB/c nude mice to obtain ascitic fluid. Monoclonal antibody 231D was sequentially purified by protein G–Sepharose affinity chromatogra-