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

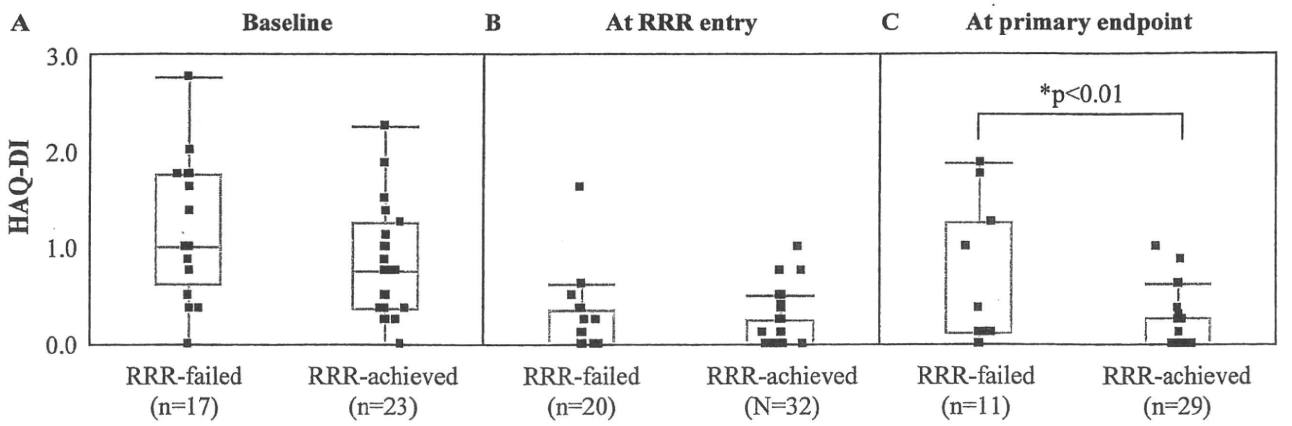

Figure 4 Health Assessment Questionnaire-Disability Index (HAQ-DI) in patients for whom remission induction by Remicade in rheumatoid arthritis failed (RRR-failed)' and in patients for whom 'RRR was achieved (RRR-achieved)' at (A) baseline, (B) RRR entry and (C) the primary end point. The line in the 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.

Acknowledgements We thank all medical staff in all institutions for providing the data.

Funding Supported in part by a research grant-in-aid for scientific research by the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and the University of Occupational and Environmental Health, Japan.

Competing interests YT has received consultant fees from Mitsubishi-Tanabe Pharma, Pfizer Inc; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, Chugai Pharma. TT has received consultant fees from Mitsubishi-Tanabe Pharma, Wyeth Japan, Abbott, Eisai Pharma, Janssen Pharma, Chugai Pharma, Bristol-Myers-Squibb, Novartis; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, Chugai Pharma. HK has received lecture fees from Mitsubishi-Tanabe Pharma, Centocor, Wyeth Japan, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, Chugai Pharma. NM has received consultant fees from Mitsubishi-Tanabe Pharma; Abbott, Eisai Pharma, Janssen Pharma, Chugai Pharma, Bristol-Myers-Squibb; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Wyeth Japan, Abbott, Eisai Pharma, Chugai Pharma. TK has received consultant fees from Bristol-Myers-Squibb, Abbott; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Wyeth Japan, Abbott, Eisai Pharma, Chugai Pharma.

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.

Provenance and peer review Not commissioned; externally peer reviewed.

RRR study investigators (other than the authors) Shunsuke Fukuyo (University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan), Hayato Nagasawa (Saitama Medical Centre, Saitama Medical University, Kawagoe, Japan), Yukitaka Ueki (Sasebo Central Hospital, Sasebo, Japan), Hideo Ohstubo (Japanese Red Cross Kagoshima Hospital, Kagoshima, Japan), Kosaku Murakami (Kyoto University, Kyoto, Japan), Hiroaki Dobashi (Kagawa University, Kagawa, Japan), Shigeru Honjo (Saiseikai Takaoka Hospital, Takaoka, Japan), Teruhisa Azuma (Tenri Hospital), Masato Yagita, Saori Hatachi and Kazuyasu Ushio (Kitano Hospital, Tazuke Kofukai Medical Resarch Institute and Ushio clinic, Osaka, Japan), Toshihide Mimura and Yuji Akiyama (Saitama Medical University, Saitama, Japan), Hiromitsu Takemori (Aomori Prefectural Hospital), Takao Takeuchi (Osaka Red Cross Hospital, Hayaishi Hospital, Osaka, Japan),Tsuyoshi Kasama (Showa University, Tokyo, Japan), Shunsuke Mori (Kumamoto Saishunsou National Hospital, Kumamoto, Japan), Shouhei Nagaoka (Yokohama Minami Kyousai Hospital, Yokohama, Japan), Masaaki Inaba and Hitoshi Goto (Osaka City University, Osaka, Japan), Toshihiko Hidaka (Zenjinkai Shimin-no-Mori Hospital, Miyazaki, Japan), Yasuaki Okuda (Dohgo Spa Hospital, Matsuyama, Japan), Yoshinari Takasaki and Naoto Tamura (Juntendo University, Tokyo, Japan), Kazuhide Tanimura (Tokeidai Memorial Hospital, Sapporo, Japan), Takayuki Sumida (University of Tsukuba, Tsukuba, Japan), Katsumi Eguchi (Nagasaki University, Nagasaki, Japan), Yho Ishiguro (Hirosaki University, Horosaki, Japan), Takeo Sakurai (Inoue Hospital, Gunma, Japan).

REFERENCES

1. **Klareskog L**, Catrina AI, Paget S. Rheumatoid arthritis. *Lancet* 2009;**373**:659–72.
2. **Emery P**, Breedveld FC, Hall S, *et al*. Comparison of methotrexate monotherapy with a combination of methotrexate and etanercept in active, early, moderate to severe rheumatoid arthritis (COMET): a randomised, double-blind, parallel treatment trial. *Lancet* 2008;**372**:375–82.
3. **Breedveld FC**, Weisman MH, Kavanaugh AF, *et al*. The PREMIER study: a multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum* 2006;**54**:26–37.
4. **Klareskog L**, van der Heijde D, de Jager JP, *et al*. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomised controlled trial. *Lancet* 2004;**363**:675–81.
5. **St Clair EW**, van der Heijde DM, Smolen JS, *et al*. For the Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset Study Group. Combination of infliximab and methotrexate therapy for early rheumatoid arthritis: a randomized, controlled trial. *Arthritis Rheum* 2004;**50**:3432–43.
6. **Lipsky PE**, van der Heijde DM, St Clair EW, *et al*. For the Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. *N Engl J Med* 2000;**343**:1594–602.
7. **Yamanaka H**, Tanaka Y, Sekiguchi N, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan (RECONFIRM). *Mod Rheumatol* 2007;**17**:28–32.
8. **Tanaka Y**, Takeuchi T, Inoue E, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: 1-year clinical and ragiographic outcomes (RECONFIRM-II). *Mod Rheumatol* 2008;**18**:146–52.
9. **Takeuchi T**, Yamanaka H, Inoue E, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: one-year outcome of joint destruction (RECONFIRM-2J). *Mod Rheumatol* 2008;**18**:447–54.
10. **Nawata M**, Saito K, Nakayamada S, *et al*. Discontinuation of infliximab in rheumatoid arthritis patients in clinical remission. *Mod Rheumatol* 2008;**18**:460–4.
11. **Quinn MA**, Conaghan PG, O'Connor PJ, *et al*. Very early treatment with infliximab in addition to methotrexate in early, poor-prognosis rheumatoid arthritis reduces magnetic resonance imaging evidence of synovitis and damage, with sustained benefit after infliximab withdrawal: results from a twelve-month randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 2005;**52**:27–35.
12. **Goekoop-Ruiterman YP**, Vries-Bouwstra JK, Allaart CF, *et al*. Comparison of treatment strategies in early rheumatoid arthritis: a randomized controlled trial. *Ann Intern Med* 2007;**146**:406–15.
13. **van der Kooij SM**, le Cessie S, Goekoop-Ruiterman YP, *et al*. Clinical and radiological efficacy of initial vs delayed treatment with infliximab plus methotrexate in patients with early rheumatoid arthritis. *Ann Rheum Dis* 2009;**68**:1153–8.
14. **Goekoop-Ruiterman YP**, de Vries-Bouwstra JK, Allaart CF, *et al*. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. *Arthritis Rheum* 2008;**58**:S126–35.
15. **van der Bijl AE**, Goekoop-Ruiterman YP, de Vries-Bouwstra JK, *et al*. Infliximab and methotrexate as induction therapy in patients with early rheumatoid arthritis. *Arthritis Rheum* 2007;**56**:2129–34.
16. **Goekoop-Ruiterman YP**, de Vries-Bouwstra JK, Kerstens PJ, *et al*. DAS-driven therapy versus routine care in patients with recent-onset active rheumatoid arthritis. *Ann Rheum Dis* 2010;**69**:65–9.
17. **Miyasaka N**, Takeuchi T, Eguchi K. Proposed (corrected) Japanese guidelines for the use of infliximab for rheumatoid arthritis. *Mod Rheumatol* 2005;**15**:4–8.
18. **Arnett FC**, Edworthy SM, Bloch DA, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
19. **van der Heijde DMPM**, van 't Hof MA, van Riel PLCM, *et al*. A comparison of validity of single variables and composed indices for measuring disease activity in rheumatoid arthritis. *Ann Rheum Dis* 1992;**51**:177–81.
20. **van der Heijde D**. Radiographic progression in rheumatoid arthritis: does it reflect outcome? Does it reflect treatment? *Ann Rheum Dis* 2001;**60**(Suppl 3):iii47–50.
21. **van der Heijde D**. How to read radiographs according to the Sharp/van der Heijde method. *J Rheumatol* 2000;**27**:261–3.
22. **Takeuchi T**, Tatsuki Y, Nogami Y, *et al*. Postmarketing surveillance of the safety profile of infliximab in 5000 Japanese patients with rheumatoid arthritis. *Ann Rheum Dis* 2008;**67**:189–94.

ARTHRITIS & RHEUMATISM
Vol. 62, No. 4, April 2010, pp 952–959
DOI 10.1002/art.27331

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

Norifumi Sawamukai,[1] Sonosuke Yukawa,[2] Kazuyoshi Saito,[2] Shingo Nakayamada,[2] Taku Kambayashi,[3] and Yoshiya Tanaka[2]

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

Supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan, by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the University of Occupational and Environmental Health, Japan.

[1]Norifumi Sawamukai, MD, PhD: University of Occupational and Environmental Health, Kitakyushu, Japan, and University of Pennsylvania, Philadelphia; [2]Sonosuke Yukawa, MD, Kazuyoshi Saito, MD, PhD, Shingo Nakayamada, MD, PhD, Yoshiya Tanaka, MD, PhD: University of Occupational and Environmental Health, Kitakyushu, Japan; [3]Taku Kambayashi, MD, PhD: University of Pennsylvania, Philadelphia.

Dr. Tanaka has received consulting fees and honoraria from Astellas, Mitsubishi-Tanabe, Takeda, and Wyeth (more than $10,000 each).

Address correspondence and reprint requests to Yoshiya Tanaka, MD, PhD, First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Iseigaoka, Yahatanishi, Kitakyushu 807-8555, Japan. E-mail: tanaka@med.uoeh-u.ac.jp.

Submitted for publication May 8, 2009; accepted in revised form December 28, 2009.

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

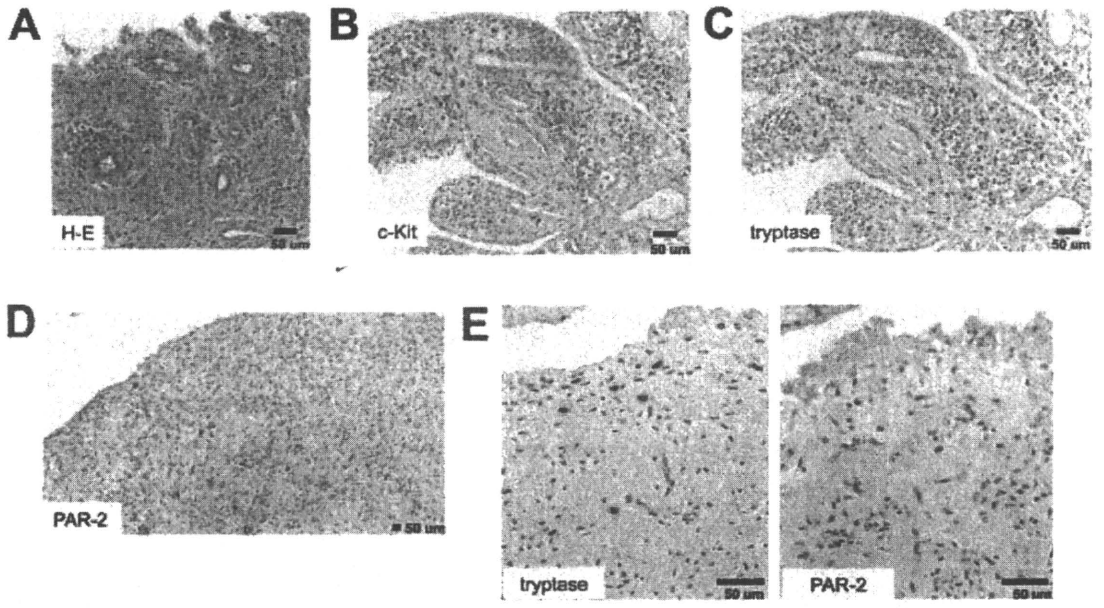

Figure 1. Detection of mast cells and protease-activated receptor 2 (PAR-2)–expressing synovial fibroblasts in rheumatoid arthritis (RA) synovial tissue. **A–D,** Synovial tissue specimens (surgically removed basal layer) from a patient with RA were stained with hematoxylin and eosin (H&E) **(A)** or 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)

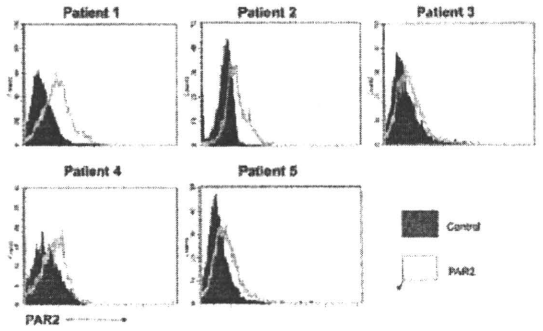

Figure 2. Expression of protease-activated receptor 2 (PAR-2) by isolated rheumatoid arthritis synovial fibroblasts (RASFs) from the synovial tissue of 5 patients with RA. RASFs were labeled with an antibody against PAR-2, in comparison with an isotype control, and the fluorescence intensity in 5 separate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

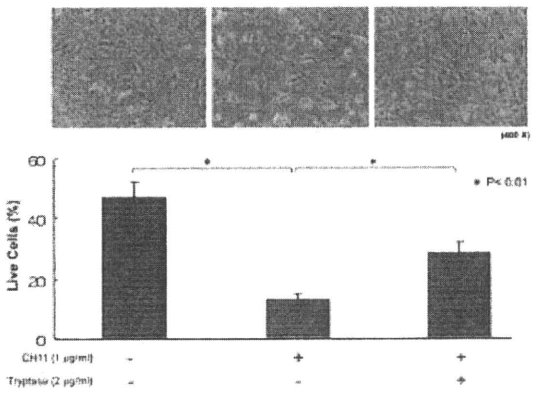

Figure 3. Inhibition of anti-Fas antibody–mediated apoptosis of rheumatoid arthritis synovial fibroblasts (RASFs) by tryptase. **Top,** RASFs were left untreated (**left**) or treated with anti-Fas antibody in the absence (**middle**) or presence (**right**) of tryptase. Cells were observed using an 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.

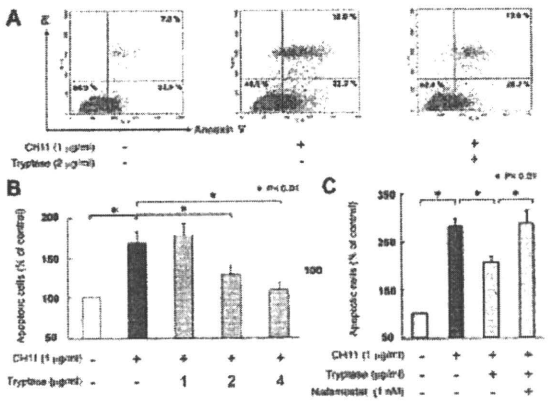

Figure 4. Inhibition of anti-Fas antibody–mediated early and late apoptosis of rheumatoid arthritis synovial fibroblasts (RASFs) by tryptase. **A,** RASFs were left untreated (**left**) or treated with anti-Fas antibody in the absence (**middle**) or presence (**right**) of tryptase. Twelve hours after 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.

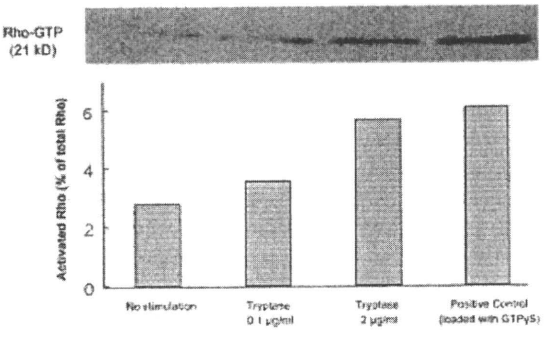

Figure 5. Activation of Rho kinase by tryptase in rheumatoid arthritis synovial fibroblasts (RASFs). RASFs were treated with tryptase (0.1 or 2 μg/ml) for 1 minute (or treated with GTPγS as a positive control). **Top,** After cell lysis, GTP-bound Rho was assessed using a pull-down assay involving the 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.

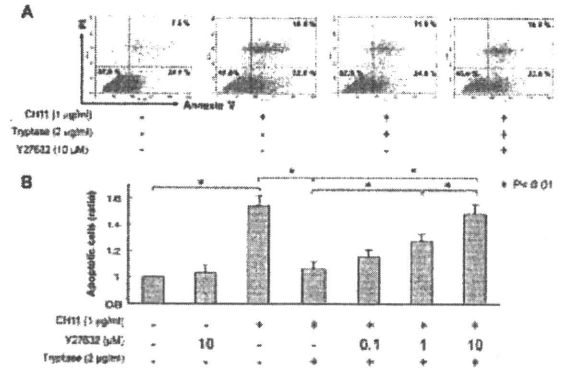

Figure 6. Rho kinase–dependent tryptase-mediated inhibition of apoptosis. **A,** Rheumatoid arthritis synovial fibroblasts (RASFs) were left untreated **(left)** or treated with anti-Fas antibody in the absence or presence of tryptase **(middle panels)** or treated with anti-Fas antibody in the presence 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.

ACKNOWLEDGMENT

The authors thank Ms T. Adachi for providing excellent technical assistance.

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.

Acquisition of data. Sawamukai, Yukawa.

Analysis and interpretation of data. Sawamukai, Saito, Nakayamada, Kambayashi.

REFERENCES

1. Gabriel SE. Why do people with rheumatoid arthritis still die prematurely? Ann Rheum Dis 2008;67 Suppl 3:iii30–4.
2. Nakayamada S, Saito K, Fujii K, Yasuda M, Tamura M, Tanaka Y. β1 integrin-mediated signaling induces intercellular adhesion molecule 1 and Fas on rheumatoid synovial cells and Fas-mediated apoptosis. Arthritis Rheum 2003;48:1239–48.
3. Fujii K, Fujii Y, Hubscher S, Tanaka Y. CD44 is the physiological trigger of Fas up-regulation on rheumatoid synovial cells. J Immunol 2001;167:1198–203.
4. Sawamukai N, Saito K, Yamaoka K, Nakayamada S, Ra C, Tanaka Y. Leflunomide inhibits PDK1/Akt pathway and induces apoptosis of human mast cells. J Immunol 2007;179:6479–84.
5. Nigrovic PA, Lee DM. Synovial mast cells: role in acute and chronic arthritis. Immunol Rev 2007;217:19–37.
6. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science 2002;297:1689–92.
7. Sandler C, Lindstedt KA, Joutsiniemi S, Lappalainen J, Juutilainen T, Kolah J, et al. Selective activation of mast cells in rheumatoid synovial tissue results in production of TNF-α, IL-1β and IL-1Ra. Inflamm Res 2007;56:230–9.
8. Woolley DE. The mast cell in inflammatory arthritis. N Engl J Med 2003;348:1709–11.
9. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
10. Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, et al. A proinflammatory role for IL-18 in rheumatoid arthritis. J Clin Invest 1999;104:1393–401.
11. Molino M, Raghunath PN, Kuo A, Ahuja M, Hoxie JA, Brass LF, et al. Differential expression of functional protease-activated receptor-2 (PAR-2) in human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 1998;18:825–32.
12. Tanaka Y, Wake A, Horgan KJ, Murakami S, Aso M, Saito K, et al. Distinct phenotype of leukemic T cells with various tissue tropisms. J Immunol 1997;158:3822–9.
13. Abe M, Tanaka Y, Saito K, Shirakawa F, Koyama Y, Goto S, et al. Regulation of interleukin (IL)-1β gene transcription induced by IL-1β in rheumatoid synovial fibroblast-like cells, E11, transformed with simian virus 40 large T antigen. J Rheumatol 1997; 24:420–9.
14. Van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW. Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ Res 2000;87:335–40.
15. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J 1999;18:578–85.
16. Aznar S, Lacal JC. Rho signals to cell growth and apoptosis. Cancer Lett 2001;165:1–10.
17. Vega FM, Ridley AJ. Rho GTPases in cancer cell biology. FEBS Lett 2008;582:2093–101.
18. Nakayamada S, Kurose H, Saito K, Mogami A, Tanaka Y. Small GTP-binding protein Rho-mediated signaling promotes proliferation of rheumatoid synovial fibroblasts. Arthritis Res Ther 2005; 7:R476–84.
19. Yagi Y, Otani H, Ando S, Oshiro A, Kawai K, Nishikawa H, et al. Involvement of Rho signaling in PAR2-mediated regulation of neutrophil adhesion to lung epithelial cells. Eur J Pharmacol 2006;536:19–27.
20. Wakisaka S, Suzuki N, Takeba Y, Shimoyama Y, Nagafuchi H, Takeno M, et al. Modulation by proinflammatory cytokines of Fas/Fas ligand-mediated apoptotic cell death of synovial cells in patients with rheumatoid arthritis (RA). Clin Exp Immunol 1998; 114:119–28.
21. Dery O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases [review]. Am J Physiol 1998;274:C1429–52.
22. Meyer MC, Creer MH, McHowat J. Potential role for mast cell tryptase in recruitment of inflammatory cells to endothelium. Am J Physiol Cell Physiol 2005;289:C1485–91.
23. Zvaifler NJ, Firestein GS. Pannus and pannocytes: alternative models of joint destruction in rheumatoid arthritis. Arthritis Rheum 1994;37:783–9.

24. Sandberg WJ, Halvorsen B, Yndestad A, Smith C, Otterdal K, Brosstad FR, et al. Inflammatory interaction between LIGHT and proteinase-activated receptor-2 in endothelial cells: potential role in atherogenesis. Circ Res 2009;104:60–8.
25. Shpacovitch VM, Seeliger S, Huber-Lang M, Balkow S, Feld M, Hollenberg MD, et al. Agonists of proteinase-activated receptor-2 affect transendothelial migration and apoptosis of human neutrophils. Exp Dermatol 2007;16:799–806.
26. Shin K, Nigrovic PA, Crish J, Boilard E, McNeil HP, Larabee KS, et al. Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. J Immunol 2009; 182:647–56.
27. Palmer HS, Kelso EB, Lockhart JC, Sommerhoff CP, Plevin R, Goh FG, et al. Protease-activated receptor 2 mediates the proinflammatory effects of synovial mast cells. Arthritis Rheum 2007; 56:3532–40.

Overcoming drug resistance induced by P-glycoprotein on lymphocytes in patients with refractory rheumatoid arthritis

S Tsujimura, K Saito, M Nawata, S Nakayamada, Y Tanaka

The First Department of Internal Medicine, University of Occupational & Environmental Health, School of Medicine, Kitakyushu, Japan

Correspondence to: Professor Y Tanaka, The First Department of Internal Medicine, University of Occupational & Environmental Health, School of Medicine, 1–1 Iseigaoka, Yahata-nishi, Kitakyushu 807–8555 Japan; tanaka@med.uoeh-u.ac.jp

Accepted 14 July 2007
Published Online First 27 July 2007

ABSTRACT

Objective: P-glycoprotein (P-gp), a member of the ATP-binding cassette transporter family, causes drug resistance by exclusion of intracellular drugs. Here, we elucidate the clinical relevance of P-gp expression on lymphocytes to drug resistance in patients with rheumatoid arthritis (RA).

Methods: P-gp expression on lymphocytes from 20 normal volunteers and 100 RA patients was analysed by flow cytometry. Drug exclusion analysis of lymphocytes was conducted by radioisotope-labelled dexamethasone.

Results: P-gp was overexpressed on RA lymphocytes compared with normal lymphocytes. P-gp expression levels were higher in partial responders with a Disease Activity Score (DAS) 28-3 of >5.1 despite taking at least two disease-modifying antirheumatic drugs (DMARDs) or one DMARD and corticosteroids for at least 2 years. P-gp expression levels correlated with DAS28-3. Intracellular dexamethasone levels (IDLs) in RA lymphocytes decreased according to P-gp expression. Tacrolimus, a P-gp inhibitor, restored IDLs in RA lymphocytes. P-gp overexpression in patients with highly active RA was suppressed by methotrexate but enhanced by corticosteroids. Furthermore, infliximab (3 mg/kg) resulted in improvement of RA disease activity, reduction of P-gp and recovery of IDLs.

Conclusions: P-gp overexpression on lymphocytes might cause efflux of corticosteroids and DMARDs, P-gp substrates, from lymphocytes, resulting in drug resistance in patients with highly active RA. P-gp inhibition/reduction could overcome such drug resistance. Measurement of P-gp expression on lymphocytes could be a potentially useful marker for assessing drug resistance in RA, and may be suitable for selecting infliximab or DMARDs including tacrolimus for RA treatment.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by progressive polyarthritis with occasional extra-articular involvement.[1] Progression of RA results in severe disability and loss of function with severe pain.[2] The ultimate goals of effective treatment are the control of abnormal immune system, erosive synovitis and prevention of joint destruction by administration of disease-modifying antirheumatic drugs (DMARDs) without delay. The main target of these DMARDs is the lymphocyte, but we often encounter refractory RA patients. Therefore, resistance to antirheumatic drugs in lymphocytes is one of the important issues to be overcome with regard to the treatment of RA.

Among the multiple mechanisms of multidrug resistance, overexpression of P-glycoprotein (P-gp), which is a 170-kDa product of the multidrug resistance (MDR)-1 gene, has emerged as the major molecule involved in multidrug resistance and prognosis during chemotherapy for various malignancies.[3–7] P-gp is a member of ATP-binding cassette transporter superfamily and functions as an energy-dependent transmembrane efflux pump. Overexpression of P-gp results in reduction of intracellular concentrations of substrates involving xenobiotics and various drugs such as vinca alkaloids, anthracyclines, verapamil, some DMARDs (eg, hydroxychloroquine, D-penicillamine, colchicines), and corticosteroids.[7–11] Thus, P-gp appears to be a double-edged sword, involved in protecting cells from these drugs and in the development of resistance to them.

In a previous study, we found that the transcription of MDR-1 gene in lymphocytes is induced by various lymphocyte-activation stimuli, eg, cytokines such as interleukin (IL)-2[12] and tumour necrosis factor (TNFα), and adhesion to extracellular matrix such as hyaluronan.[13] In this regard, immune stimuli, that play a critical role in the pathogenesis of RA,[14–20] might induce the expression of P-gp on RA lymphocytes. However, the expression of P-gp on lymphocytes, the regulatory mechanisms of P-gp expression, and clinical relevance of P-gp to drug resistance are not clear in RA.

The present study was designed to elucidate the relation between P-gp expression on lymphocytes in refractory RA patients and clinical features including drug resistance. We also determined the significance of evaluation of P-gp on peripheral blood lymphocytes on clinical decision-making regarding treatment strategies.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs from 20 normal volunteers and 100 RA patients who fulfilled the American College of Rheumatology (ACR) revised criteria for RA were isolated by density gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden) as described previously.[21 22] We confirmed by immunostaining that purified PBMCs contained more than 90% lymphocytes (CD4-, CD8- or CD19-positive cells) and less than 10% CD14-positive monocytes. The clinical activity of RA was assessed by the Disease Activity Score (DAS) 28-3 that was calculated by using tender joint counts, swollen joint counts, and erythrocyte sedimentation rate.[23 24] Table 1 summarises the demographic characteristics and clinical features of the RA patients.

Ann Rheum Dis 2008;**67**:380–388. doi:10.1136/ard.2007.070821

The study was approved by the institution human subjective research committee and informed consent was obtained from all the donors who were enrolled in the study.

Flow cytometry

Staining and flow cytometric analysis of PBMCs were conducted by standard procedures as described previously using a FACScan (Becton Dickinson, Mountain View, California, USA).[21 22] Briefly, PBMCs were initially plated onto a 96-well culture dish (2×10^5 cells/well) and incubated with polyclonal γ-globulin (10 μg/ml, Mitsubishi Welpharma Co., Osaka, Japan) to block Fc-receptors. These cells were then incubated with MRK-16 (100 μg/ml, Kyowa medex, Tokyo, Japan), a specific monoclonal antibody (mAb) against P-gp,[25] followed by the addition of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG Ab (5 μg/ml, Fujisawa, Osaka, Japan) in FACS medium consisting of phosphate-buffered saline (PBS), 0.5% human serum albumin (HSA; Mitsubishi Welpharma), and 0.2% NaN_3 (Sigma Aldrich, Tokyo, Japan) for 30 min at 4°C. For the two-colour analysis, we incubated PBMCs with phycoerythrin (PE)-conjugated CD4mAb or CD19mAb (1.25 μg/ml, Fujisawa, Osaka, Japan) after blocking of free anti-mouse IgG-binding sites with irrelevant antibodies. The two-colour-stained cells were detected by electronic gating based on their CD4 or CD19 expression using a FACScan. Quantification of the cell surface antigens on one cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as described previously.[26] The data were used to construct a calibration curve of the mean fluorescence intensity vs anti-body-binding capacity. The cell specimen was analysed on the FACScan and the antibody-binding capacity calculated by interpolation on the calibration curve. When the green fluorescence laser detection was set at 500 nm in the FACScan, the antibody-binding capacity was equal to (202.98×exp (0.0092×mean fluorescence intensity), ($R^2 = 0.9995$)). Subsequently, the specific antibody-binding capacity was obtained after correcting for the background, and apparent antibody-binding capacity of the negative control anti-mouse IgG Ab. The specific antibody-binding capacity is the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

Dexamethasone accumulation

^{14}C-Labelled *n*-butanol (1.61 mCi/mmol; Toho Biochemical, Tokyo, Japan) was diluted with unlabelled butanol (Sigma Aldrich Japan) at a concentration of 0.5 MBq/ml. ^{3}H-Labelled dexamethasone (40.0 Ci/mmol; Perkin, Boston, Massachusetts, USA) was dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Tokyo, Japan) and then diluted in PBS (final concentration of DMSO 0.1%). PBMCs were suspended in PBS with 7 mM of dextrose for ATP supply, which is dispensable in this assay,[27] at a cell density of 5×10^6 cells/ml. The PBMCs were then incubated with 5.0×10^{-5} M of ^{14}C-labelled *n*-butanol and 3.0×10^{-8} M of ^{3}H-labelled dexamethasone for 20 min time range at 37°C.

Tacrolimus, which is effective for refractory RA patients,[28 29] had been reported to inhibit P-gp competitively.[29 30] The clinical safety dose of tacrolimus for RA patients is less than 3 mg/day and the mean (SD) blood concentration of tacrolimus (about 12 h after 3 mg dose of tacrolimus) is 5.3 (2.0) ng/ml (SD).[31] In preliminary studies, we prepared PBMCs that overexpressed P-gp by stimulation with IL-2. Tacrolimus inhibited the secretion of dexamethasone from these cells in a concentration-dependent manner, from 1–50 ng/ml, through a significant inhibition of P-gp by 10 ng/ml of tacrolimus (data not shown). The latter concentration was used in the following experiments. For competitive studies with tacrolimus, PBMCs were incubated with 10 ng/ml of tacrolimus (Fujisawa) for 15 min and then incubated with ^{14}C-labelled *n*-butanol and ^{3}H-labelled dexamethasone. Tacrolimus was dissolved in DMSO before diluting with PBS (final concentration of DMSO 0.03%). After incubation, 100 l of aliquots were layered on 80 l of a mixture of lauryl bromide and silicone oil (2:1 ratio; Nacalai Tesque) in an Eppendorf tube (Assist, Tokyo, Japan). After centrifugation at 10 000 rpm for 2 min, the aliquots were rapidly frozen in liquid nitrogen, the frozen tube was cut between medium–mixture bound areas, thereby obtaining the upper layer as the medium fraction and the lower layer as the cell fraction.

The cell fractions were melted with soluene-350 and 10 ml of Hionic-Fluor (Packard, Meriden, Connecticut, USA) was added. The medium fractions were mixed with 10ml of mixtures of toluene (Wako, Osaka, Japan), methanol (Wako), ethylene glycol monoethyl ether (Nacalai Tesque) and Permafluor (200:50:50:12 ratio; Packard). The radioactivity of each fraction was counted with a scintillation counter. The cell to medium ratio (C/M ratio), which is an index of intracellular and extracellular dexamethasone concentration ratio, was computed using the following formula: C/M ratio = ((^{3}H in cell fraction/^{14}C in cell fraction)/(^{3}H in medium fraction/^{14}C in medium fraction)).

Table 1 Characteristics of the study subjects

	RA patients (n = 100)
Age (years)	57 (24–85)*
Sex (females/males)	91/9
Disease duration (years)	8.8 (0.4–32)*
DAS28-3	5.5 (2.3–7.6)*
Steinbrocker stage (I/II/III/IV)	6/35/19/40
ACR functional class (1/2/3/4)	5/81/11/3

*Data are median (range).
ACR, American College of Rheumatology; DAS28-3, Disease Activity Score 28-3; RA, rheumatoid arthritis.

Infliximab infusion

Eleven patients with refractory RA and P-gp-overexpressing lymphocytes were treated with infliximab. After an induction regimen of 3 mg/kg at weeks 0, 2, and 6, all patients received maintenance therapy every 8 weeks. PBMCs from these 11 RA patients were isolated before infliximab infusion and at weeks 0 and 2. During the first 2 weeks, corticosteroids, methotrexate (MTX) and other DMARDs dosages were kept unchanged.

Statistical analysis

Results are expressed as mean (SD). Student t test was used to compare data of two groups. One way ANOVA followed by Fisher PLSD for multicomparison was used to compare data among three or more groups. Correlations between two variables were examined using Pearson correlation analysis. In the figures, the linear regression line is shown together with the Pearson correlation coefficient (r) and the respective correlation p value. p Values less than 0.05 were considered statistically significant.

RESULTS

Expression of cell surface P-glycoprotein on peripheral lymphocytes of RA patients

We examined the expression of P-gp using mAb against the MRK-16 epitope of P-gp on peripheral blood lymphocytes from

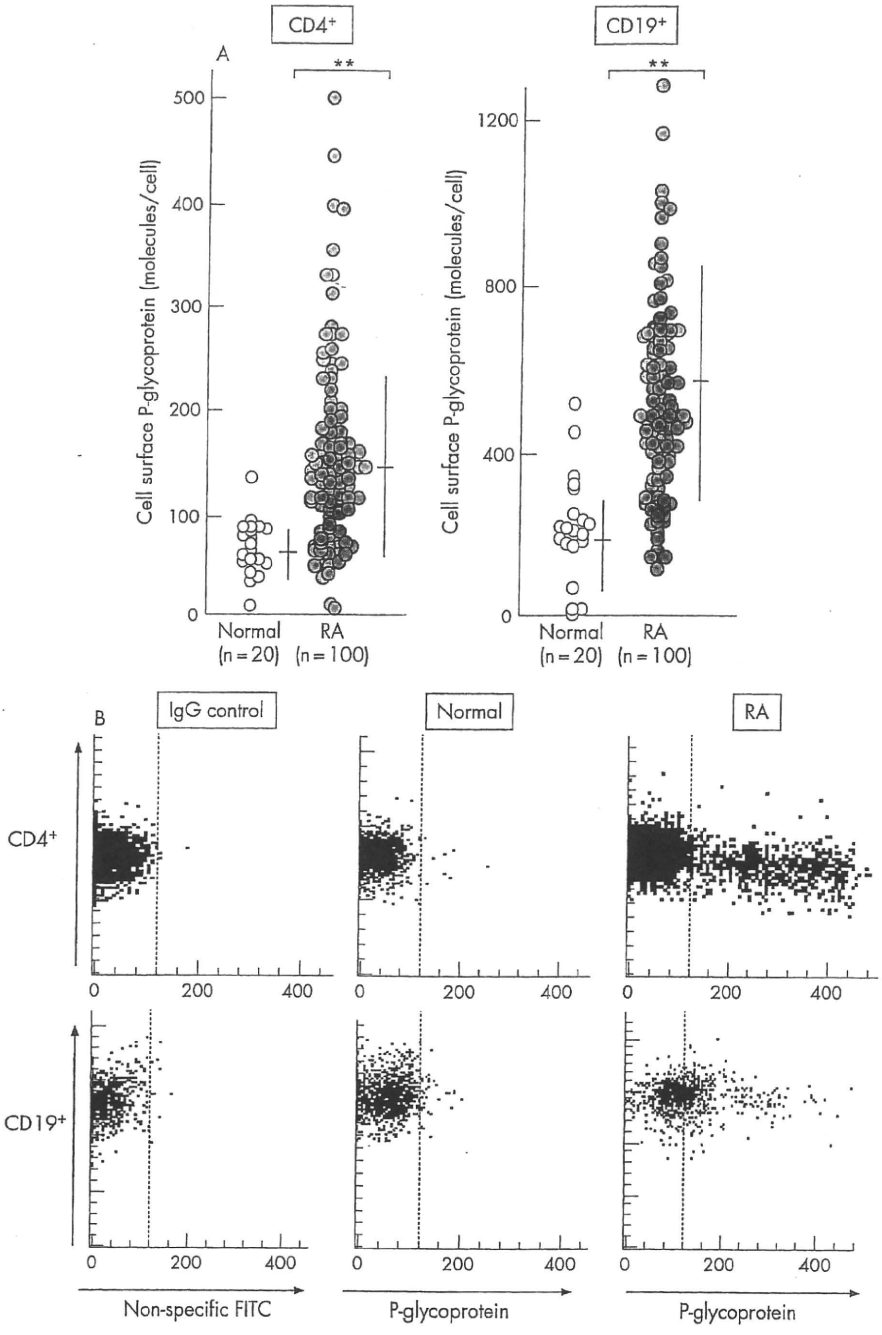

Figure 1 Expression of P-glycoprotein (P-gp) on lymphocytes from patients with rheumatoid arthritis (RA), as determined by flow cytometry. A. P-gp expression on peripheral CD4+ and CD19+ peripheral blood lymphocytes from 20 normal volunteers (open circles) and 100 RA patients (dotted circles). Results were calculated with the use of standard QIFIKIT beads. Values are the mean and SD of independent experiments. **$p<0.01$, by unpaired t test. B. Typical P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from a normal volunteer and an RA patient with high P-gp expression. The dotted line represents the gate set to discriminate negative from positive stained cells as determined by control FITC-conjugated anti-mouse IgG Ab.

100 RA patients and 20 normal volunteers. The expression of P-gp was significantly high on most of peripheral CD4+ and CD19+ lymphocytes of RA patients compared with normal lymphocytes. The level of P-gp expression on RA lymphocytes ranged from moderate to high, whereas the expression on normal lymphocytes ranged from marginal to moderate, with most expressing marginal levels (fig 1A). Figure 1B shows typical pattern of P-gp expression on lymphocytes of RA patients and normal volunteers. In RA patients, CD4+ lymphocytes were divided into P-gp high or low expressing subgroups, and most CD19+ lymphocytes expressed P-gp at moderate or high levels.

Relationship between drug resistance and expression of P-gp

Clinically, we sometimes encounter RA patients with high disease activity who do not respond to treatment with DMARDs or oral corticosteroids, although the mechanism of this drug resistance is not clear. To analyse the relationship between clinical responsiveness to treatment and the level of expression of P-gp on lymphocytes, we defined partial responders as those: (1) with DAS28-3 of >5.1, (2) previously treated with at least two DMARDs or one DMARDs and corticosteroids, and (3) treated for at least 2 years. The P-gp expression levels on CD4+ and CD19+ lymphocytes were significantly increased in the partial responders (table 2).

Ann Rheum Dis 2008;**67**:380–388. doi:10.1136/ard.2007.070821

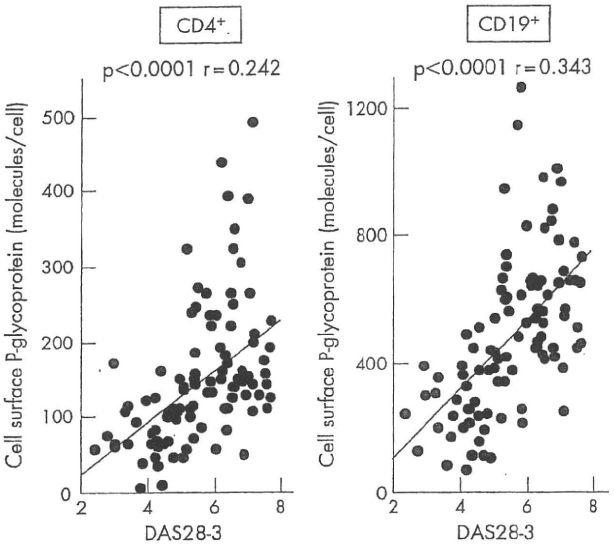

Figure 2 Correlation between P-glycoprotein (P-gp) expression on lymphocytes and scores on the Disease Activity Score (DAS) 28-3 in 100 patients with rheumatoid arthritis (RA). Levels of P-gp expression on RA lymphocytes correlated closely with disease activity of each patient, as estimated by the DAS28-3 score. Vertical axis: numbers of P-gp molecules per cell were calculated with the use of standard QIFIKIT beads. Horizontal axis: the disease activity of each RA patient, as estimated by DAS28-3. Pearson correlation analysis was used to determine statistical significance. n = 100.

Other investigators have reported that transcription of MDR-1 is induced in response to various drugs, including corticosteroids[52] in various malignancies.We then analysed the relationship between prior received drugs (DMARDs and corticosteroids) and the level of expression of P-gp on lymphocytes. However, P-gp expression on RA lymphocytes was not correlated with either the number of previously received DMARDs or prior corticosteroid therapy (table 2).

Relationship between disease activity and expression of P-gp

We investigated the relationship between DAS28-3 scores and P-gp expression on peripheral blood CD4+ and CD19+ lymphocytes from patients with RA. The levels of P-gp expression on RA lymphocytes correlated closely with the disease activity in each patient, as estimated by the DAS28-3 score (fig 2). Several RA patients with high disease activity (DAS28-3>5.1) had extremely high expression of P-gp on lymphocytes.

The level of P-gp on CD4+ and CD19+ lymphocytes was markedly increased in RA patients with high disease activity (DAS28-3 >5.1) than mild and moderate active RA patients (DAS28-3<5.1) (fig 3A). In the next step, we investigated the association between P-gp expression and drug exclusion through P-gp. For this purpose, dexamethasone accumulation analysis of PBMCs was conducted using radioisotope labelled dexamethasone. Intracellular dexamethasone levels in PBMCs of these highly active RA patients (DAS28-3>5.1) with high expression of P-gp on lymphocytes was significantly decreased compared with those in mild to moderate active RA patients (DAS28-3<5.1) and normal volunteers (fig 3B). To confirm the functional involvement of P-gp in the low levels of intracellular dexamethasone, we added tacrolimus, a competitive inhibitor of P-gp, to PBMCs from RA patients with high disease activity. Tacrolimus inhibited the secretion of dexamethasone from PBMCs of highly active RA patients (fig 3C).

Effects of MTX and corticosteroids on P-gp expression in highly active RA patients

The level of P-gp on lymphocytes in highly active RA patients (DAS28-3>5.1) varied extensively among individuals despite almost similar disease activity (figs 2 and 3A). MTX is the preferred first anchor drug in the treatment of RA. Recent evidence indicates that MTX efflux via P-gp is uncommon.[53] We found that immunosuppressants, not substrates of P-gp, induced reduction of P-gp expression on lymphocytes.[11] Therefore, in highly active RA patients, we analysed the relationship between P-gp expression level on lymphocytes and prior treatment with non-P-gp substrate drugs (eg, MTX), or corticosteroids (which are inducers and substrates of P-gp).[32] The disease activity was not significantly different between MTX users and MTX non-users or between corticosteroid users and corticosteroid non-users. However, the expression level of P-gp on CD19+ lymphocytes was significantly higher in corticosteroids users or MTX non-users compared with MTX users (table 3). We also analysed interaction between MTX and corticosteroids at the level of P-gp expression on lymphocytes.

Table 2 Expression of P-glycoprotein (P-gp) on lymphocytes of rheumatoid arthritis (RA) patients with resistance to treatment and those previously treated with disease-modifying antirheumatic drugs (DMARDs)

	P-glycoprotein, molecules/cell	
	CD4+ lymphocytes	CD19+ lymphocytes
Responders (n = 54)	123.5 (94.4)	365.0 (210.3)
Partial responders (n = 46)	167.1 (85.0)*	526.8 (209.0)*
Number of DMARDs used previously for treatment:		
0–1 (n = 20)	161.2 (111.0)	449.7 (219.8)
2 (n = 24)	116.5 (83.6)	358.6 (214.9)
3 (n = 24)	150.1 (73.1)	447.8 (243.6)
4 (n = 14)	141.5 (120.8)	433.2 (176.9)
5 (n = 18)	153.0 (80.8)	529.5 (230.1)
Corticosteroids		
User (n = 63)	145.5 (95.7)	469.5 (231.0)
Non-user (n = 37)	140.3 (87.7)	388.2 (204.0)

Values are mean (SD). *p<0.01, compared with responders. There were no differences in P-gp expression on each CD type based on the number of DMARDs or the use of corticosteroids.

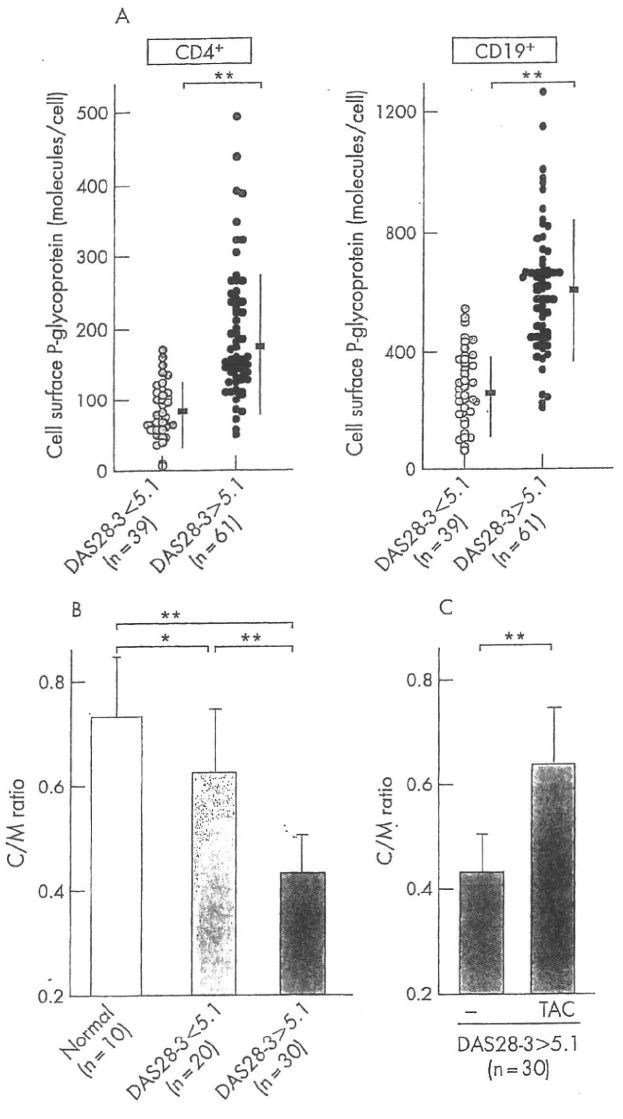

Figure 3 Decrease in intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients based on levels of P-glycoprotein (P-gp) expression on lymphocytes and inhibition of dexamethasone excretion through P-gp by tacrolimus. A. Flow cytometric analysis showed P-gp expression on peripheral CD4+ and CD19+ lymphocytes in 39 RA patients whose DAS28 were less than 5.1 points (hatched circles) and 61 highly active RA patients whose DAS28 were more than 5.1 points (solid circles). Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data are mean (SD). **p<0.01, by non-paired t test. B. Intracellular dexamethasone was evaluated by the C/M ratio of PBMCs in 10 normal volunteers (open bar), 20 RA patients whose DAS28 were less than 5.1 points (hatched bar) and 30 highly active RA patients whose DAS28 were more than 5.1 points (solid bar). Data are mean (SD). Statistical analysis was performed using the non-paired t test. *p<0.05, **p<0.01. C. C/M ratio of PBMCs in 30 highly active RA patients whose DAS28 were more than 5.1 points was evaluated in the absence or presence of 10 ng/ml of tacrolimus. Values are mean and SD of 30 independent experiments. Statistical analysis was performed using the paired t test. **p<0.01.

The expression of P-gp on CD19+ lymphocytes was significantly high in patients treated with corticosteroids without MTX, compared with those treated with MTX without corticosteroids, despite the same disease activity level (table 4, fig 4). These results indicate that the expression of P-gp on lymphocytes in highly active RA is enhanced with increased disease activity, in association with lymphocyte activation, and is modified by medication.

Effects of infliximab on P-gp expression

The expression of P-gp is induced by extracellular stimuli such as lymphocyte-activating cytokines, including IL-2, and adhesion to extracellular matrix proteins such as hyaluronan.[12 13] In fact, cytokines and other immune-stimuli activated lymphocytes in RA patients with high disease activity apparently acquired P-gp overexpression. Based on the above experiments, we provided infliximab as an intravenous infusion (3 mg/kg) in 11 patients with refractory RA whose lymphocytes expressed high levels of P-gp despite being treated with MTX (table 5). Then, we evaluated P-glycoprotein expression on lymphocytes and the levels of intracellular dexamethasone before and 2 weeks after infusion.

Surprisingly, a single infusion of infliximab significantly reduced P-gp on CD4+ and CD19+ lymphocytes (fig 5A). Figure 5B demonstrates down-regulation of P-gp expression on lymphocytes from four representative cases. Two weeks after infliximab infusion, the P-gp high-expressing subgroups of CD4+ lymphocytes were eliminated, and most CD19+ lymphocytes expressed P-gp at only low levels. Reduction of P-gp resulted in recovery of intracellular dexamethasone levels in PBMCs of these RA patients (fig 5C), and then acquired improvement of disease activity (fig 5D).

DISCUSSION

In pathogenesis of RA, lymphocytes are the main orchestrators of immune responses. Intercellular adhesion such as antigen presentation, cytokines, and adhesion to the extracellular matrix (ECM) transduce signals via functional molecules on lymphocytes, resulting in activation of lymphocytes in RA.[14] These activated lymphocytes enhance inflammatory and destructive arthropathy.

We found that lymphocyte activating cytokines, such as IL-2[12] and TNF, up-regulated the expression of P-gp on lymphocytes via activation of transcriptional factor YB-1 and that such up-regulation markedly reduced intracellular corticosteroid concentration in vitro.[12] The serum and synovial concentrations of these cytokines are high in active RA patients.[15–17] We also found that fragmented hyaluronan, detected in certain pathological conditions such as inflammation and tumours,[19 34] induced YB-1 activation followed by P-gp expression in accordance with activation of CD4+ T cells. Fragmented hyaluronan is increased in the RA synovium and synovial fluid.[19 20] Although the production of native hyaluronan by RA synovial cells is promoted according to the aggravation of inflammation by inflammatory cytokines including IL-1 and TNF,[35] the activity of hyaluronidase produced by CD14+ monocytes is so high that native hyaluronan is digested to fragmented hyaluronan immediately at the loci of inflammation.[36] Furthermore, the production of fragmented hyaluronan is accelerated due to oxygen-derived free radicals in the inflammation locus, as in synovitis.[19 20]

Therefore, lymphocytes in RA patients with high disease activity are activated by cytokines and ECM adhesion, resulting in acquiring MDR-1-mediated multidrug resistance. Thus, the results of our studies showed overexpression of P-gp on CD4+

Ann Rheum Dis 2008;**67**:380–388. doi:10.1136/ard.2007.070821

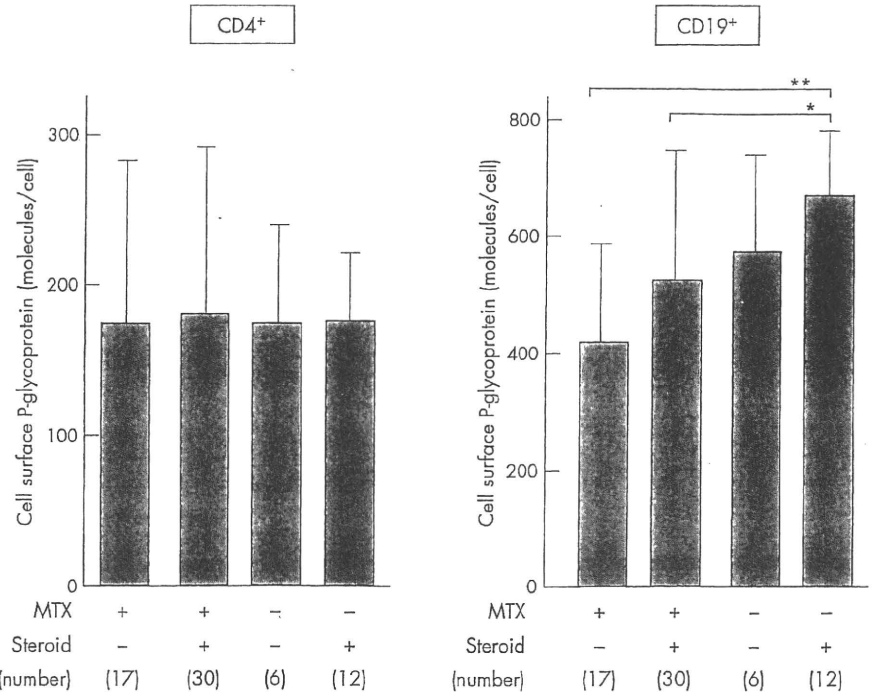

Figure 4 Effects of methotrexate (MTX) and corticosteroids on P-glycoprotein (P-gp) expression in patients with highly active rheumatoid arthritis (RA). Flow cytometric analysis for P-gp expression on peripheral CD4+ and CD19+ lymphocytes in 61 patients with highly active RA and DAS28 of more than 5.1 points treated with or without MTX or corticosteroids. Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data are mean (SD). *p<0.05, **p<0.01, by one-way ANOVA.

Table 3 Expression of P-glycoprotein on lymphocytes in rheumatoid arthritis (RA) patients with high disease activity

	DAS28-3	P-glycoprotein, molecules/cell CD4+ lymphocytes	CD19+ lymphocytes
Corticosteroids:			
Users (n = 42)	6.35 (0.72)	175.8 (94.3)	565.3 (204.1)
Non-users (n = 23)	6.22 (0.73)	169.7 (96.0)	459.6 (200.3)
p Value	NS	NS	<0.05
Methotrexate:			
Users (n = 47)	6.38 (0.73)	174.6 (106.9)	487.3 (216.6)
Non-users (n = 18)	6.21 (0.71)	171.3 (49.6)	633.9 (136.6)
p Value	NS	NS	<0.01

p Values are for comparison between groups with different characteristics in patients with high RA disease activity. Values are mean (SD).
DAS28-3, Disease Activity Score 28-3; NS, not significant.

and CD19+ lymphocytes of RA patients with high disease activity.

Other investigators and our group have reported that low cytoplasmic corticosteroid concentrations (caused by increased P-gp-mediated efflux of corticosteroid from lymphocytes) is one of the mechanisms of corticosteroid resistance in inflammatory bowel disease, asthma and systemic lupus erythaematosus.[11 37–39] Some DMARDs, eg, chloroquine, hydroxychloroquine, D-penicillamine and colchicines, are substrates of P-gp as well as corticosteroids.[8–10] Yudoh *et al* reported that P-gp expression on lymphocytes in RA patients might be also involved in resistance to bucillamine and sulfasalazine.[40] We noted that P-gp acts as a "hydrophobic vacuum cleaner", that is, P-gp captures those drugs that are substrates of P-gp when they pass through the cell membrane and then releases them outside the cell. Thus, when the number of P-gp molecules expressed on the lymphocyte cell surface increases, corticosteroids and some DMARDs that are substrates of P-gp, cannot reach the cytoplasm, resulting in low response to treatment with these drugs. We demonstrated that the levels of intracellular dexamethasone in PBMCs from RA patients correlated closely with P-gp expression on lymphocytes. Our results imply that the high expression of P-gp on lymphocytes, which correlated

Table 4 Expression of P-glycoprotein on lymphocytes in rheumatoid arthritis patients with high disease activity, according to treatment

Methotrexate	Corticosteroids	n	DAS28-3
+	−	17	6.14 (0.67)
+	+	30	6.48 (0.72)
−	−	6	6.39 (0.85)
−	+	12	6.14 (0.69)

Values are mean (SD). There were no differences in DAS28-3 based on treatment regimen.
DAS28-3, Disease Activity Score 28–3.

Table 5 Infliximab infusion in 11 patients with refractory rheumatoid arthritis

Case	Age (years)	Disease duration (years)	Steinbrocker stage	Previously used DMARDs and PSL	DAS28-3
1	44	1	II	MTX	6.3
2	57	7	III	MTX, D-pen, SSZ	6.2
3	53	10	II	MTX, SSZ, PSL	5.2
4	40	14	III	MTX, PSL	5.8
5	76	10	II	MTX, SSZ	5.2
6	48	3	II	MTX, LFF, SSZ	6.0
7	60	15	IV	MTX, LFN	7.0
8	66	15	IV	MTX, Gold, PSL	7.6
9	51	17	IV	MTX, Gold, SSZ, PSL	7.4
10	55	9	IV	MTX, SSZ	7.6
11	60	7	IV	MTX, Gold, PSL	6.2

DAS28-3, Disease Activity Score 28-3; DMARD, disease-modifying antirheumatic drugs; D-pen, D-penicillamine; Gold, parenteral gold; LFN, leflunomide; MTX, methotrexate; PSL, prednisolone (or equivalent); SSZ, sulphasalazine.

with disease activity, might lead to active efflux of corticosteroids and P-gp substrate DMARDs to the cell exterior, resulting in the development of drug resistance and failure to control disease activity in RA patients with highly active disease.

Therefore, when one of the targets of overcoming treatment resistance is P-gp on lymphocytes, one of the good countermeasures could be administration of competitive inhibitors. Administration of substrates that have high affinity to P-gp, and inhibit P-gp mediated exclusion of other substrates, could overcome the multidrug resistance induced by P-gp expression. Tacrolimus is such a competitive inhibitor.[29 30] We demonstrated that levels of intracellular dexamethasone in PBMCs of highly active RA patients actually increased with tacrolimus treatment. Therefore, we suggest that tacrolimus could be used not only to inhibit NF-AT-dependent transcription of cytokines in lymphocytes, but also as a competitive inhibitor of P-gp, and propose that combination treatment with tacrolimus as a competitor of P-gp is a useful treatment for highly active refractory RA patients. Indeed, the efficacy of tacrolimus has been already reported in refractory RA patients.[28 41 42]

By contrast, MTX could also suppress P-gp expression on activated lymphocytes. Although MTX has been reported to efflux to the cell exterior through the ABCC subfamily of proteins, which are members of ATP-binding cassette transporters superfamily,[33 43] the mechanisms that regulate the ABCC subfamily on lymphocytes, relevance of the ABCC subfamily to RA clinical features and clinical relevance of the ABCC subfamily to drug resistance in RA, are not clear at present. Our results suggest that MTX might prolong the effects of combined DMARDs by preventing acquisition of drug resistance with restriction of P-gp expression in patients with highly active RA. Conversely, corticosteroid might enhance P-gp expression on activated lymphocytes, resulting in enhancement of multidrug resistance. In such cases, the dose of corticosteroids should be kept as low as possible.

We propose that overcoming treatment resistance might require reduction of P-gp levels on activated lymphocytes in patients with highly active RA. TNF plays a critical role in mediation of the pathogenic actions of inflammation and bone erosion in RA resulting in enhancement of RA disease activity, and thus is an important molecular target for directed biologic intervention.[14 17 18 44] It is noteworthy that infliximab successfully improved disease activity and reduced P-gp expression on lymphocytes in 11 patients with refractory RA who had high levels of P-gp expression. In these patients, lymphocyte activation could not be suppressed when they were treated with MTX. Furthermore, we demonstrated the preferential expression of P-gp on activated subgroups (had high levels of CD69 expression) among CD4+ T cells.[13] In this report, infliximab infusion resulted in elimination of the P-gp high-expressing CD4+ lymphocytes. Therefore, we propose that infliximab inhibits activated lymphocytes resulting in reduction of P-gp expression. This is the first report that demonstrates the effect of infliximab on the reduction of P-gp and addressed the possible beneficial actions of infliximab. Recovery drug concentrations in lymphocytes associated with marked reduction of P-pg on lymphocytes by treatment with infliximab might result in overcoming treatment resistance in refractory RA. Translated clinically, these findings indicate that when a patient with RA fails to develop clinical remission with DMARDs and corticosteroids, overexpression of P-gp might be involved in treatment resistance. In such a case, a better option is administration of DMARDs and immunosuppressive agents that are not actively exteriorised from lymphocytes by P-gp, and, more properly, biological agents such as anti-TNF compounds, which are not affected by P-gp, should be initiated.

In conclusion, we have demonstrated in the present study that lymphocytes activated by various stimuli in RA patients with highly active disease apparently acquire MDR-1-mediated multidrug resistance against corticosteroids and probably some DMARDs, which are substrates of P-gp. Our results suggest that in patients with highly active RA who develop P-gp-mediated multidrug resistance, treatment with MTX is necessary first, and that it is not advised to supplement treatment with P-gp substrate DMARDs without P-gp inhibitors or increase the dose of corticosteroid only. Furthermore, our results suggest that the inhibition of P-gp by competitive inhibitors and the reduction of P-gp by biological agents could overcome drug resistance induced by P-pg expression on lymphocytes in refractory RA. Accordingly, we propose that measurement of P-gp expression level on peripheral blood lymphocytes is useful for the assessment of drug resistance and lymphocyte activation, and is a good tool for selection of DMARDs including P-gp competitors such as tacrolimus, and for application of biological agents in RA patients with highly active disease.

Ann Rheum Dis 2008;**67**:380–388. doi:10.1136/ard.2007.070821

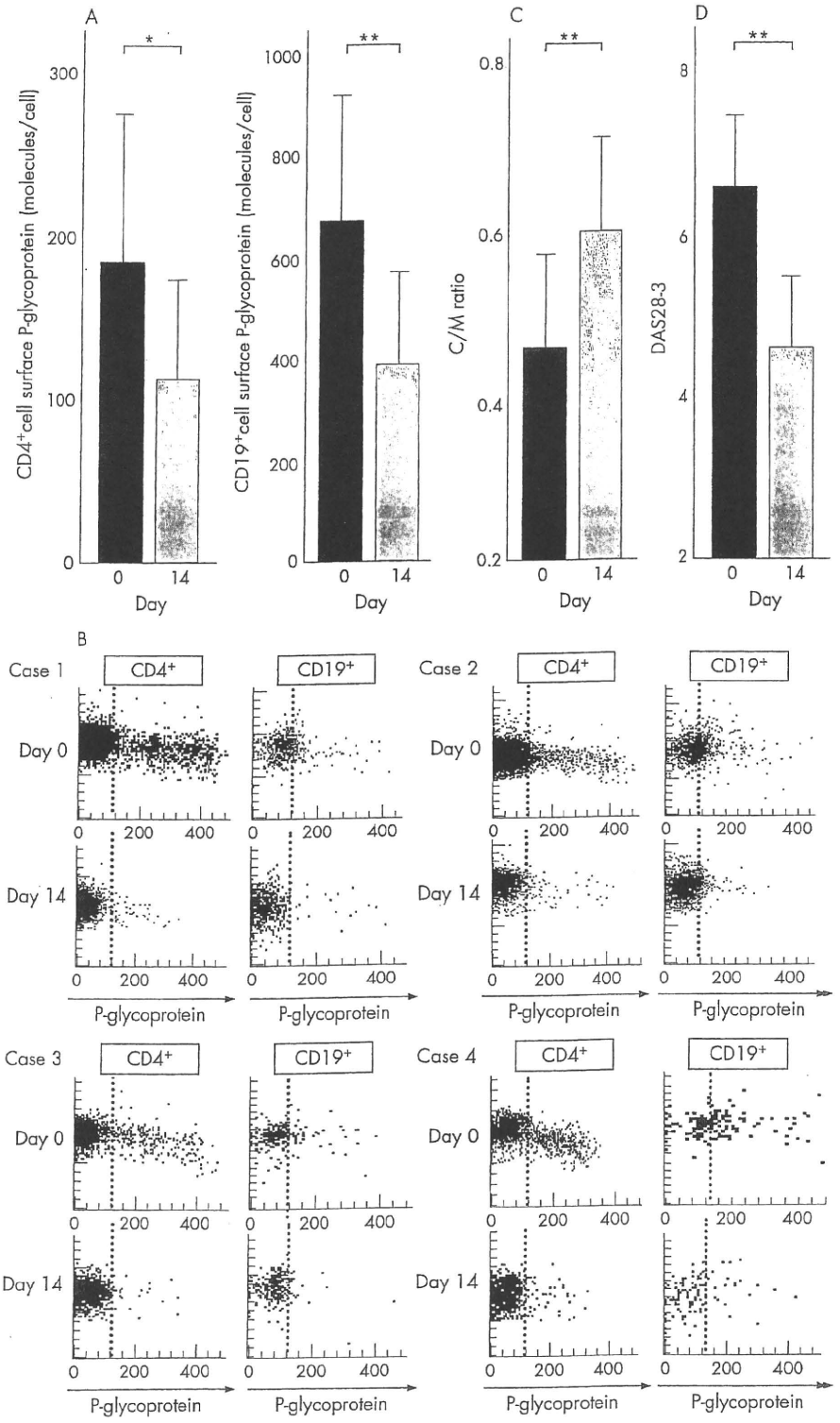

Figure 5 Effects of infliximab infusion on P-glycoprotein (P-gp) expression and intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients with high disease activity. A. P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from 11 patients with highly active RA, whose Disease Activity Score (DAS)28 were more than 5.1 points, before (closed bars) and 14 days after (hatched bars) infliximab infusion. Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Values are mean and SD of 11 independent experiments. *$p<0.05$, **$p<0.01$ by paired t test. B. Typical P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from four patients with active RA (Cases 1, 2, 3 and 4 in table 5) before and 14 days after infliximab infusion. The dotted line represents the gate set to discriminate negative from positive stained cells as determined by control FITC-conjugated anti-mouse IgG Ab. C. Intracellular dexamethasone levels were evaluated by determining the cell to medium (C/M) ratio in PBMCs from the same patients. The C/M ratio was evaluated before (closed bars) and 14 days after (dotted circles) infliximab infusion. Values are mean and SD of 11 independent experiments. **$p<0.01$ by paired t test. D. Patients showed clinical improvement following the aforementioned infliximab infusion therapy. The disease activity of 11 RA patients before (closed bars) and 14 days after (hatched bars) infliximab infusion was estimated by DAS28-3. Values are mean and SD of 11 independent experiments. **$p<0.01$ by paired t test.

Acknowledgements: The authors thank Ms T Adachi for the excellent technical assistance.

Funding: This work was supported in part by a Research Grant-In-Aid for Scientific Research by the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and University of Occupational and Environmental Health, Japan.

Competing interests: None declared.

Tacrolimus was kindly donated by Fujisawa. Co., Osaka, Japan.

REFERENCES

1. **Harris ED Jr.** Rheumatoid arthritis: pathophysiology and implications for therapy. *N Engl J Med* 1990;**322**:1277–89.
2. **Hochberg MC.** Adult and juvenile rheumatoid arthritis: current epidemiologic concepts. *Epidemiol Rev* 1981;**3**:27–44.
3. **Beck WT,** Grogan TM, Willman CL, Cordon-Cardo C, Parham DM, Kuttesch JF, *et al.* Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. *Cancer Res* 1996;**56**:3010–20.
4. **Advani R,** Visani G, Milligan D, Saba H, Tallman M, Rowe JM, *et al.* Treatment of poor prognosis AML patients using PSC833 (valspodar) plus mitoxantrone, etoposide, and cytarabine (PSC-MEC). *Adv Exp Med Biol* 1999;**457**:47–56.
5. **List AF,** Kopecky KJ, Willman CL, Head DR, Slovak ML, Douer D, *et al.* Cyclosporine inhibition of P-glycoprotein in chronic myeloid leukemia blast phase. *Blood* 2002;**100**:1910–2.
6. **Linn SC,** Honkoop AH, Hoekman K, van der Valk P, Pinedo HM, Giaccone G. p53 and P-glycoprotein are often co-expressed and are associated with poor prognosis in breast cancer. *Br J Cancer* 1996;**74**:63–8.
7. **Tsuruo T.** Reversal of acquired resistance to vinca alkaloids and anthracyclines antibiotics. *Cancer Treat Rep* 1983;**67**:889–94.
8. **Ueda K,** Cardarelli C, Gottesman MM, Pastan I. Expression of a full length cDNA for the human "MDR-1" gene confers resistance to colchicines, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* 1987;**84**:3004–8.
9. **Salmon SE,** Dalton WS. Relevance of multidrug resistance to rheumatoid arthritis: development of a new therapeutic hypothesis. *J Rheumatol* 1996;**44**(Suppl):97–101.
10. **Chen C,** Pollack GM. Enhanced antinociception of the model opioid peptide [D-penicillamine] enkephalin by P-glycoprotein modulation. *Pharm Res* 1999;**16**:296–301.
11. **Tsujimura S,** Saito K, Nakayamada S, Nakano K, Tanaka Y. Clinical relevance of expression of p-glycoprotein on peripheral lymphocytes to steroid-resistance in systemic lupus erythematosus patients. *Arthritis Rheum* 2005;**52**:1676–83.
12. **Tsujimura S,** Saito K, Nakayamada S, Nakano K, Tsukada J, Kohno K, *et al.* Transcriptional regulation of multidrug resistance-1 gene by interleukin-2 in lymphocytes. *Genes Cells* 2004;**9**:1265–73.
13. **Tsujimura S,** Saito K, Kohno K, Tanaka Y. Fragmented hyaluronan induces transcriptional up-regulation of the multidrug resistance-1 gene in CD4+ T cells. *J Biol Chem* 2006;**281**:38089–97.
14. **Choy EH,** Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med* 2001;**344**:907–16.
15. **Kuroda T,** Tanabe N, Sakatsume M, Nozawa S, Mitsuka T, Ishikawa H, *et al.* Interleukin-2 levels are elevated in the bone marrow serum of patients with mutilans-type rheumatoid arthritis. *Clin Rheumatol* 2002;**21**:23–7.
16. **Camilleri JP,** Amos N, Williams BD, Emery P, Williams LA, Jessop JD. Serum soluble interleukin 2 receptor levels and radiological progression in early rheumatoid arthritis. *J Rheumatol* 2001;**28**:2576–78.
17. **Saxne T,** Palladino MA Jr, Heinegard D, Talal N, Wollheim FA. Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum. *Arthritis Rheum* 1988;**31**:1041–5.
18. **Keffer J,** Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, *et al.* Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J* 1991;**10**:4025–31.
19. **Balazs EA,** Watson D, Duff IF, Roseman S. Hyaluronic acid in synovial fluid. I. Molecular parameters of hyaluronic acid in normal and arthritis human fluids. *Arthritis Rheum* 1967;**10**:357–76.
20. **Dahl LB,** Dahl IM, Engstrom-Laurent A, Granath K. Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. *Ann Rheum Dis* 1985;**44**:817–22.
21. **Tanaka Y,** Minami Y, Mine S, Hirano H, Hu CD, Fujimoto H, *et al.* H-Ras signals to cytoskeletal machinery in induction of integrin-mediated adhesion of T cells. *J Immunol* 1999;**163**:6209–16.
22. **Tanaka Y,** Wake A, Horgan KJ, Murakami S, Aso M, Saito K, *et al.* Distinct phenotype of leukemic T cells with various tissue tropisms. *J Immunol* 1997;**158**:3822–9.
23. **Balsa A,** Carmona L, Gonzalez-Alvaro I, Belmonte MA, Tena X, Sanmarti R. Value of Disease Activity Score 28 (DAS28) and DAS28-3 compared to American College of Rheumatology-defined remission in rheumatoid arthritis. *J Rheumatol* 2004;**31**:40–6.
24. **Prevoo ML,** van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995;**38**:44–8.
25. **Hamada H,** Tsuruo T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci USA* 1986;**83**:7785–9.
26. **Tanaka Y,** Kimata K, Wake A, Mine S, Morimoto I, Yamakawa N, *et al.* Heparan sulfate proteoglycan on leukemic cells is primarily involved in integrin triggering and its mediated adhesion to endothelial cells. *J Exp Med* 1996;**184**:1987–97.
27. **Richard C,** John RR. Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. *J Biol Chem* 1993;**268**:16059–64.
28. **Furst DE,** Saag K, Fleischmann MR, Sherrer Y, Block JA, Schnitzer T, *et al.* Efficacy of tacrolimus in rheumatoid arthritis patients who have been treated unsuccessfully with methotrexate: a six-month, double-blind, randomized, dose-ranging study. *Arthritis Rheum* 2002;**46**:2020–8.
29. **Parasrampuria DA,** Lantz MV, Benet LZ. A human lymphocyte based ex vivo assay to study the effect of drugs on P-glycoprotein (P-gp) function. *Pharm Res* 2001;**18**:39–44.
30. **Saeki T,** Ueda K, Tanigawara Y. Human P-glycoprotein transports cyclosporin A and FK506. *J Biol Chem* 1993;**268**:6077–80.
31. **Kawai S,** Yamamoto K. Safety of tacrolimus, an immunosuppressive agent, in the treatment of rheumatoid arthritis in elderly patients. *Rheumatology (Oxford)* 2006;**45**:441–4.
32. **Chaudhary PM,** Roninson IB. Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J Natl Cancer Inst* 1993;**85**:632–9.
33. **Szakacs G,** Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006;**5**:219–34.
34. **Liu D,** Pearlman E, Diaconu E, Guo K, Mori H, Haqqi T, *et al.* Expression of hyaluronidase by tumor cells induces angiogenesis in vivo. *Proc Natl Acad Sci USA* 1996;**93**:7832–7.
35. **Wells AF,** Klareskog L, Lindblad S, Laurent TC. Correlation between increased hyaluronan localized in arthritic synovium and the presence of proliferating cells. A role for macrophage-derived factors. *Arthritis Rheum* 1992;**35**:391–6.
36. **Nagaya H,** Ymagata T, Ymagata S, Iyoda K, Ito H, Hasegawa Y, *et al.* Examination of synovial fluid and serum hyaluronidase activity as a joint marker in rheumatoid arthritis and osteoarthritis patients (by zymography). *Ann Rheum Dis* 1999;**58**:186–8.
37. **Farrell RJ,** Kelleher D. Glucocorticoid resistance in inflammatory bowel disease. *J Endocrinol* 2003;**178**:339–46.
38. **Montano E,** Schmitz M, Blaser K, Simon HU. P-glycoprotein expression in circulating blood leukocytes of patients with steroid-resistant asthma. *J Investig Allergol Clin Immunol* 1996;**6**:14–21.
39. **Tsujimura S,** Saito K, Tokunaga M, Nakatsuka K, Nakayamada S, Nakano K, *et al.* Overcoming treatment unresponsiveness mediated by P-glycoprotein overexpression on lymphocytes in refractory active systemic lupus erythematosus. *Mod Rheumatol* 2005;**15**:28–32.
40. **Yudoh K,** Matsuno H, Nakazawa F, Yonezawa T, Kimura T. Increased expression of multidrug resistance of P-glycoprotein on Th1 cells correlates with drug resistance in rheumatoid arthritis. *Arthritis Rheum* 1999;**42**:2014–15.
41. **Yocum DE,** Furst DE, Kaine JL, Baldassare AR, Stevenson JT, Borton MA, *et al.* Efficacy and safety of tacrolimus in patients with rheumatoid arthritis: a double-blind trial. *Arthritis Rheum* 2003;**48**:3328–37.
42. **Kawai S,** Hashimoto H, Kondo H, Murayama T, Kiuchi T, Abe T. Comparison of tacrolimus and mizoribine in a randomized, double-blind controlled study in patients with rheumatoid arthritis. *J Rheumatol* 2006;**33**:2153–61.
43. **van der Heijden JW,** Dijkmans BA, Scheper RJ, Jansen G. Drug insight: resistance to methotrexate and other disease-modifying antirheumatic drugs – from bench to bedside. *Nat Clin Pract Rheumatol* 2007;**3**:26–34.
44. **Maini R,** St Clair EW, Breedvel d F, Furst D, Kalden J, Weisman M, *et al.* Infliximab (chimaeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomized phase III trial. ATTRACT Study Group. *Lancet* 1999;**354**:1932–9.

Postmarketing surveillance of the safety profile of infliximab in 5000 Japanese patients with rheumatoid arthritis

T Takeuchi,[1,14] Y Tatsuki,[2] Y Nogami,[3] N Ishiguro,[1,14] Y Tanaka,[5,14] H Yamanaka,[6,14] N Kamatani,[6,14] M Harigai,[7,14] J Ryu,[8,14] K Inoue,[9,14] H Kondo,[10,14] S Inokuma,[11,14] T Ochi,[12,14] T Koike[13,14]

[1] Division of Rheumatology and Clinical Immunology, Saitama Medical Center, Saitama Medical University, Saitama, Japan; [2] Pharmaceuticals Sales & Marketing Headquarters, Tanabe Seiyaku Co., Ltd, Osaka, Japan; [3] Compliance & Assurance Headquarters, Tanabe Seiyaku Co., Ltd, Osaka, Japan; [4] Department of Orthopaedic Surgery, Nagoya University School of Medicine, Aichi, Japan; [5] The First Department of Internal Medicine, University of Occupational & Environmental Health, School of Medicine, Fukuoka, Japan; [6] Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan; [7] Department of Pharmacovigilance and Department of Medicine and Rheumatology, Tokyo Medical and Dental University, Tokyo, Japan; [8] Department of Orthopaedic Surgery, Nihon University School of Medicine, Tokyo, Japan; [9] Department of Orthopaedic Surgery, Tokyo Women's Medical University, Medical Center East, Tokyo, Japan; [10] Kitasato Institute Medical Center Hospital, Saitama, Japan; [11] Department of Allergy and Immunological Diseases, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan; [12] National Hospital Organization Sagamihara National Hospital, Kanagawa, Japan; [13] Department of Medicine II, Hokkaido University Graduate School of Medicine, Hokkaido, Japan; [14] Member of the Committee on Post-Marketing Surveillance of Japan College of Rheumatology

Correspondence to: Tsutomu Takeuchi, Division of Rheumatology and Clinical Immunology, Saitama Medical Center, Saitama Medical University, 1981 Kamoda, Kawagoe, Saitama 350-8550, Japan; tsutake@saitama-med.ac.jp

Accepted 14 July 2007
Published Online First 2 August 2007

ABSTRACT

Objectives: A large-scale postmarketing surveillance (PMS) study was carried out to determine the safety profile of infliximab in Japanese patients with rheumatoid arthritis (RA).

Methods: The PMS study was performed for all patients with RA who were treated with infliximab. They were consecutively registered in the PMS study at the initiation of infliximab treatment and were prospectively monitored with all adverse events noted for a period of 6 months. All case reports, which include safety-related events, were collected monthly.

Results: Adverse drug reactions (ADRs) were assessed for 6 months in 5000 patients who were consecutively enrolled in the PMS study. The incidence rates of total and serious ADRs were 28.0% and 6.2%, respectively. "Infections" or "respiratory disorders" were most commonly observed among serious ADRs. Bacterial pneumonia developed in 2.2%, tuberculosis in 0.3%, suspected *Pneumocystis jiroveci* pneumonia (PCP) in 0.4% and interstitial pneumonitis in 0.5%. Bacterial pneumonia (for which individuals of male gender, of older age and those with advanced rheumatoid arthritis and comorbid respiratory disease were most at risk) began to develop immediately after the start of treatment, while tuberculosis, PCP and interstitial pneumonitis developed about 1 month later. Serious infusion reactions were observed in 0.5% and were more likely to occur in patients who had participated in previous clinical trials of infliximab.

Conclusion: This postmarketing surveillance study of patients treated with infliximab showed that infliximab in combination with low-dose MTX was well tolerated in Japanese patients with active RA.

Infliximab, an antihuman TNF-α chimeric monoclonal antibody, has been proven to be efficacious in the treatment of a number of inflammatory diseases (ATTRACT, ASPIRE, ASSERT, IMPACT ACCENT, ACT 1,2, SPIRIT and BeSt).[1–9] Clinical trials of RA treatments have demonstrated that infliximab not only improves clinical signs and symptoms, but also inhibits joint destruction. Given that TNF is necessary for host defense against infections, one can speculate that the incidence of infection may increase during treatment with TNF inhibitors. Although the incidence of serious infections in patients treated with infliximab plus methotrexate (MTX) has been shown to be comparable with those treated with MTX alone in a number of clinical trials, the incidence of *Mycobacterium tuberculosis* is reported to be higher in RA patients treated with infliximab in endemic regions, suggesting that its appearance is due to reactivation of latent tuberculosis.[10] The incidence of tuberculosis in Japan is 24.8/100 000 per year (2003 statistics), which is 5 times greater than that of the US. Thus, the potential risk of tuberculosis is a major concern, considering that TNF inhibitors would be widely used after their approval for the treatment of RA in Japan. The Ministry of Health, Labour and Welfare (MHLW) required that a large-scale postmarketing surveillance (PMS) study be carried out as a condition for the approval of infliximab. This PMS study was developed to fulfil that requirement to monitor all adverse drug reactions (ADRs) in every RA patient treated with infliximab and to add to the literature on the risks and benefits of biologic therapy. This report presents the ADRs observed in the 5000 patients enrolled in the study. Bacterial pneumonia, *P jiroveci* pneumonia (PCP), interstitial pneumonitis and infusion reactions are also discussed in this report.

METHODS

The specifics of the infliximab PMS study included a prospective collection of all adverse events (AEs) that all patients beginning infliximab therapy might encounter for a period of 6 months. To ensure complete registration and quality data, the registration and reporting were all centrally performed to maximise completeness in capturing information. Only institutions willing to comply with the protocol had access to infliximab during this investigation. The Committee on PMS under the auspices of the Japan College of Rheumatology (JCR) evaluated data in collaboration with a pharmaceutical company (Tanabe Seiyaku Co. Ltd). The MHLW approved the protocol of the PMS study and instructed the investigators to perform the PMS study according to Good PostMarketing Surveillance Practice, which is the authorised standard for PMS studies of approved drugs in clinical practice; therefore, no formal ethics committee approval was necessary.

Patients

All patients treated with infliximab between July 2003 and December 2004 were enrolled in the study, and each individual patient was followed up for 6 months. The study ended in June 2005. According to the Japanese guidelines for the use of