by the Institutional Ethics Committee of the Tokyo Medical and Dental University Hospital (#863 in 2010).

Results

Diagnosis and clinical characteristics of RA patients with PCP

We applied the above diagnostic criteria to the 17 RA patients in the PCP group. Of the 17 cases, three (patients 8, 14, and 17) met the criteria for definitive PCP, and 14 met the criteria for presumptive PCP. The clinical characteristics of each patient are summarized in Table 1. The median age of the 17 patients was 68 years (range 48–78 years), and 12 (71 %) were female. The median duration of RA was eight years. Fourteen patients were at Steinbrocker's stage III or IV. All patients received MTX and 13 (77 %) received corticosteroids from baseline to the onset of PCP. At the onset of PCP, the median dosages of prednisolone and MTX were 5.0 mg/day (range 2.5–9 mg/day) and 8.0 mg/week (range 4–15 mg/week), respectively. One patient was receiving another immunosuppressive drug, tacrolimus, at 3 mg/day. Eight patients had pulmonary comorbidities, including interstitial pneumonia ($n = 4$), chronic obstructive pulmonary disease ($n = 4$), and old pulmonary tuberculosis ($n = 2$). Four patients had diabetes mellitus. None of the patients received chemoprophylaxis for PCP at the time of PCP diagnosis. The median interval between the first injection of adalimumab and the onset of PCP was 12 weeks (range 4–38 weeks). Thirteen patients (76 %) developed PCP within 26 weeks after the first injection. Fever was the most common clinical symptom (it was observed in 15 patients; 88 %), followed by dyspnea on effort (82 %) and dry cough (41 %).

Laboratory and radiographic features of the PCP patients

Laboratory data at the onset of PCP are summarized in Table 2. Fourteen patients either had severe hypoxia (with $PaO_2 < 60$ mm Hg on room air) or required immediate oxygen therapy at the onset of PCP. Peripheral blood lymphocyte (PBL) counts at the onset of PCP were <500 cells/μl in three patients, 500–1,000 cells/μl in five patients, and $>1{,}000$ cells/μl in nine patients. *P. jirovecii* was microscopically identified in three patients. The polymerase chain reaction test for *P. jirovecii* DNA was positive in 13 patients, using either induced sputum (11 patients) or bronchoalveolar lavage fluid (four patients), but three patients were not examined. Serum levels of BDG, one of

Table 1 Characteristics of rheumatoid arthritis patients treated with adalimumab at the onset of PCP

Pt	Age/sex	Stage/class	Number of injections[a]	Treatment duration (days)[b]	MTX (mg/w)	PSL (mg/d)	Lung disease	DM	Clinical manifestations
1	48/F	III/I	7	105	8	2.5	–	–	Fever/DOE
2	69/M	IV/III	4	62	10	0	E	–	Cough/DOE
3	74/F	IV/II	9	131	8	5	IP E	–	DOE
4	52/M	III/II	5	59	4	8	IP	–	Fever/cough/DOE
5	61/F	IV/II	3	45	8	9	–	–	Fever
6	67/F	III/III	3	28	8	8	IP	–	Fever/cough/DOE
7	61/F	IV/II	4	59	6	0	Old TB	–	Fever/DOE
8	77/F	IV/II	6	129	6	5	–	+	Fever/DOE
9	52/F	III/I	3	55	8	5	–	–	Fever/DOE
10	78/M	III/III	6	86	8	0	IP	+	Fever/DOE
11	66/F	I/III	6	106	8	3	–	–	Fever/cough
12	70/F	II/II	2	23	8	5	Old TB	–	Fever/cough/DOE
13	68/M	I/II	3	28	8	0	E	+	Fever/DOE
14	71/F	III/II	15	214	8	7.5	–	–	Fever/DOE
15	73/M	III/II	18	268	15	3	–	+	Fever/cough/DOE
16	65/F	III/II	16	227	8	2	–	–	Fever/DOE
17	78/F	IV/II	16	252	4	4	–	–	Fever/cough

PCP Pneumocystis jirovecii pneumonia, *Pt* patient, *w* week, *d* day, *M* male, *F* female, *MTX* methotrexate, *PSL* prednisolone, *E* emphysema, *IP* interstitial pneumonia, *old TB* old tuberculosis, *DM* diabetes mellitus, *DOE* dyspnea on effort, *cough* dry cough

[a] Number of injections of ADA prior to the diagnosis of PCP

[b] Treatment duration with ADA before the onset of PCP

Table 2 Laboratory data of rheumatoid arthritis patients treated with adalimumab at the onset of PCP

Pt	WBC (/μl)	Lymphocytes (/μl)	SpO_2 or PaO_2 (Torr) [O_2, l/min][a]	Serum β-D-glucan (μg/ml) [normal range at the institute]	*Pneumocystis jirovecii* PCR
1	7,870	912	SpO_2 96 % [0]	289 [<11]	+
2	5,100	1,989	SpO_2 92 % [0]	30.5 [<11]	+
3	6,300	252	55.1 [0]	1041 [<11]	NA
4	6,200	874	68.0 [0]	25.76 [<11]	+
5	8,050	1,110	60.4 [0]	50.3 [<20]	NA
6	6,400	716	58.9 [0]	37.8 [<6]	+
7	5,660	1,041	71.8 [0]	22.1 [<11]	+
8	6,800	279	31.3 [0]	29 [<11]	+[b]
9	15,900	832	85.7 [3]	79.5 [<20]	+
10	7,500	1,350	65.4 [0]	22.3 [<20]	+
11	8,400	3,696	69.5 [0]	16.4 [<11]	+
12	11,700	1,029	26.1 [0]	21.06 [3.5]	+
13	7,950	1,761	SpO_2 85 % [2]	160 [<5]	+
14	9,580	34	56.7 [0]	13.0 [<11]	NA[b]
15	5,700	1,140	55.1 [0]	13.0 [<11]	–
16	7,000	1,330	56.1 [10]	21.38 [<11]	+
17	3,200	704	52.5 [0]	419 [<11]	+[b]
Median (IQR)	7,000 (5950–8225)	1,029 (710–1340)	Not applicable	Not applicable	Not applicable

PCP Pneumocystis jirovecii pneumonia, *Pt* patient, *WBC* white blood cell, *PCR* polymerase chain reaction, *NA* not assessed, *SpO2* oxygen saturation measured using a pulse oximeter, *IQR* interquartile range

[a] Oxygen therapy during the measurement of PaO_2

[b] *Pneumocystis jirovecii* microscopically detected in bronchoalveolar-lavage fluid

the major components of the cell walls of fungi and a serum maker for PCP [17, 18], were elevated in all patients. Results of sputum culture performed in 14 patients revealed no causative bacteria or fungi.

Chest radiographs and thoracic CT scans were analyzed for all 17 patients. The most common CT finding was ground-glass opacity (GGO) (in 17 patients), either with sharp demarcation by interlobular septa in one patient (type A GGO) (Fig. 1a) or without interlobular septal boundaries in 14 patients (type B GGO) (Fig. 1b). Two patients demonstrated mixed patterns (type C).

Treatment and clinical course of PCP in patients with RA receiving adalimumab

All patients were hospitalized on the same day that PCP was suspected. Fourteen patients (all except for patients 2, 5, and 11) received oxygen therapy on admission. MTX and adalimumab were immediately discontinued in all patients. All patients received therapeutic doses of trimethoprim/sulfamethoxazole (TMP/SMX). Because of adverse drug reactions that included skin eruptions, liver dysfunction, thrombocytopenia, and hyperpotassemia, TMP/SMX was reduced or stopped in eight patients. One patient was changed to pentamidine isethionate. Sixteen patients were concomitantly treated with high-dose corticosteroids within a few days after admission. Eleven patients were empirically treated with antibiotics and four with antifungal agents. Three patients (patients 1, 3, and 8) were intubated on the day of admission because of progressive respiratory failure; two of these patients responded to treatment and were successfully weaned from artificial ventilation. One patient (patient 17) died because of PCP with progressive respiratory failure. Two patients died because of multiple organ failure (patient 12) and gastrointestinal bleeding, cytomegalovirus infection, and multiple organ failure (patient 3) after improvement of PCP.

Case–control study

In order to characterize the PCP group more precisely, we compared demographic information, comorbidities, treatments, and laboratory data at baseline (i.e., at the initiation of treatment with adalimumab) between the PCP and non-PCP groups using a univariate analysis (Table 3). The PCP group was significantly older ($p = 0.003$) and had a more advanced radiographic stage (Steinbrocker's stage III or IV) ($p = 0.010$) than the non-PCP group. Although the rates of patients with preexisting pulmonary diseases and diabetes mellitus in the PCP group were numerically higher, these differences were not statistically significant. There were no differences in disease duration and the dosages of prednisolone and methotrexate between the two groups. None of the patients in the PCP group and fourteen patients in the non-PCP group received prophylaxis for

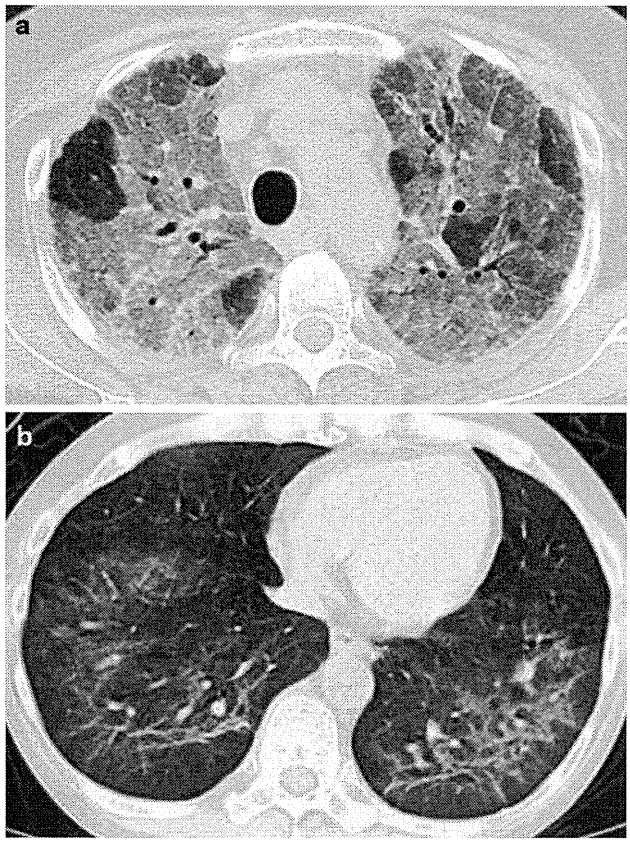

Fig. 1 Representative thoracic computed tomography findings of rheumatoid arthritis patients who developed *Pneumocystis jirovecii* pneumonia while receiving adalimumab. **a** Ground-glass opacity (GGO) with sharp demarcation by interlobular septa (type A) (patient 12). **b** Inhomogeneous GGO without obvious demarcation by interlobular septa (type B) (patient 1)

PCP for at least three months during the observation period. Twelve patients used TMP/SMX and two used aerosolized pentamidine.

Based on the results of the univariate analysis, age, sex, pulmonary comorbidities and Steinbrocker's stage of RA were analyzed as candidate predictors for the development of PCP. The Cox proportional-hazards regression analysis revealed a significant association between advanced radiographic stage (stage III or IV) and development of PCP (hazard ratio (HR) 3.76, 95 % confidence interval (CI) 1.03–7.30, $p = 0.045$). While the hazard ratios of older age and preexisting pulmonary diseases tended to be higher, they did not reach statistical significance (Table 4).

Because 14 patients in the non-PCP group received prophylaxis for PCP, we performed the multivariate analysis after excluding these 14 patients, and found a significant association between older age and development of PCP (HR 3.31, 95 % CI 1.09–10.0, $p = 0.034$). The HR of the radiographic stage did not reach statistical significance (HR 2.82, 95 % CI 0.74–10.7) in this model.

Discussion

We accumulated the largest possible number of patients with RA who developed PCP during treatment with adalimumab, and described the clinical and radiologic characteristics of the 17 patients that we found.

Adalimumab is the third TNF antagonist to be approved in Japan. We have already reported the clinical characteristics and risk factors for PCP in RA patients treated with infliximab or etanercept [10–12]. The median interval (range) between the first dose of TNF antagonists and the onset of PCP was 12 weeks (range 4–38) for adalimumab, nine weeks (range 2–90) for infliximab [11], and 14 weeks (range 3–43) for etanercept [12]. PCP developed within six months in the majority of RA patients after the initiation of each TNF antagonist: 90 % for infliximab, 80 % for etanercept, and 76 % for adalimumab.

Previous studies have revealed that patients without HIV infection develop PCP abruptly and progress to fulminating pneumonia with acute respiratory failure [21, 22]. We also reported that RA patients treated with infliximab or etanercept developed PCP rapidly and progressed to severe respiratory failure [10–12]: 18 out of 21 PCP patients using infliximab, all 15 PCP patients using etanercept, and 14 of 17 PCP patients in this study showed severe hypoxemia and required oxygen therapy. The mortalities of the patients with PCP given infliximab (0 %) or etanercept (6.7 %) are numerically lower than the mortality of this study, in which three patients (17.6 %) died. Walzer etal. [23] identified older age, second or third episode of PCP, low hemoglobin level, low PaO_2 breathing room air at admission, pulmonary Kaposi sarcoma, and presence of medical comorbidity as early predictors of mortality of PCP in HIV-infected patients. Although such prognostic factors in non-HIV PCP patients are unknown, all three patients in our study who died were females over 70 years old, and their PaO_2 on admission was less than 60 Torr. Two of these patients had pulmonary comorbidities. One patient had a quite high serum level of BDG, and one was positive for both microscopic detection and the PCR test for the organism. These data would suggest severe pulmonary injury at presentation and a high burden from *P. jirovecii.*

In our study, all patients received therapeutic doses of TMP/SMX. However, eight patients (47.1 %) were obliged to reduce the dosage or stop using the drug due to adverse drug reactions, such as gastrointestinal symptoms and hematological abnormalities. Kameda et al. [24] also reported that more than one-third of the patients could not complete the standard protocol of the TMP/SMX treatment. These data indicate that the optimal dosage and treatment period of TMP/SMX for PCP should be investigated. The clinical benefit of adjunctive corticosteroid

Table 3 Baseline characteristics of patients with rheumatoid arthritis treated with adalimumab

Characteristic	PCP group ($n = 17$)	Non-PCP group ($n = 89$)	p value
Age (years)[a]	68 (48–78)	60 (24–79)	0.003
Female (%)	70.6	80.9	0.255
Disease duration (years)[a]	8.0 (0.7–36)	9.5 (3–40)	0.491
Chronic pulmonary disease (%)	47.1	22.5	0.107
Diabetes mellitus (%)	23.5	7.9	0.074
Steinbrocker's radiographic stage (III or IV) (%)	82.4	48.3	0.010
Steinbrocker's functional class (III or IV) (%)	17.6	19.1	0.596
MTX (%)	100	86.5	0.108
MTX (mg/week)[a]	8.0 (4–10)	8.0 (4–15)	0.119
MTX ≥ 8 mg/week (%)	11.8	28.1	0.228
PSL (%)	76.5	56.2	0.118
PSL (mg/day)[a]	5.0 (3–12)	5.0 (1–17)	0.529
PSL ≥ 5 mg/day (%)	52.9	33.7	0.131
WBC <4,000/μl (%)	0	2.2	0.731
Serum IgG (mg/dl)[a]	1421 (846–1954)	1316 (827–3165)	0.817

PCP Pneumocystis jirovecii pneumonia, *MTX* methotrexate, *PSL* prednisolone, *Chronic pulmonary disease* = interstitial pneumonia, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma, middle lobe syndrome, old pulmonary tuberculosis

p values were calculated using the Mann–Whitney test for continuous variables or χ^2 test for categorical variables

[a] Median (range)

Table 4 Cox regression analysis of risk factors for the development of PCP in rheumatoid arthritis patients treated with adalimumab

	Hazard ratio (95 % CI)	p value
Age (≥ vs. <65 years old)	2.38 (0.80–7.05)	0.119
Gender (female vs. male)	0.53 (0.18–1.58)	0.258
Chronic pulmonary disease (yes vs. no)	2.14 (0.79–5.76)	0.133
Steinbrocker's radiographic stage (III/IV vs. I/II)	3.76 (1.03–7.30)	0.045

PCP Pneumocystis jirovecii pneumonia, *CI* confidence interval

Chronic pulmonary disease = interstitial pneumonia, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma, middle lobe syndrome, old pulmonary tuberculosis

therapy for PCP patients without HIV infection has not been established [25]. All patients except for one in this study received adjunctive corticosteroid therapy with various treatment durations and dosages, including intravenous methylprednisolone pulse therapy. Nineteen out of 21 PCP patients who used infliximab and nine out of 15 PCP patients who used etanercept used adjunctive corticosteroid therapy as well [11, 12]. Pareja etal. [26] retrospectively analyzed the clinical courses of 30 cases of severe PCP without HIV infection, among which 16 cases who received high doses of adjunctive corticosteroid therapy presented a good clinical outcome. Considering the intense inflammatory response to the organism in non-HIV PCP patients [25] and the favorable effectiveness of adjunctive corticosteroid therapy in previous studies, it is necessary to consider treatment with corticosteroids for PCP patients with RA who show hypoxemia at presentation or during their clinical courses.

In the present study, using the Cox proportional-hazards analysis, Steinbrocker's radiographic stage III or IV was identified as a statistically significant risk factor for the development of PCP in patients receiving adalimumab. Although there was no significant difference in Steinbrocker's functional class, it is plausible that advanced radiographic stages associated with decreased physical function contributed to the development of PCP. Steinbrocker's functional class may be less sensitive to the detection of such differences in physical function. On the other hand, older age was a significant risk factor in another Cox proportional-hazards regression analysis after excluding those who received TMP/SMX or aerosolized pentamidine for prophylaxis at least three months from the non-PCP group. The different results from the Cox proportional-hazards regression analyses can be explained by the fact that nine out of 14 patients given prophylaxis were aged 65 or older. Pulmonary diseases were not significant risk factors for PCP in either Cox proportional-hazards analysis, perhaps because of the small number of PCP cases enrolled.

None of the 17 patients had received prophylaxis for PCP. Vananuvat etal. [27] conducted a retrospective cohort study for patients with connective tissue diseases (CTD) who were at risk for PCP in order to examine the effectiveness of primary prophylaxis with TMP/SMX and the incidence of adverse drug reactions (ADR) of TMP/SMX. Six patients without and none with prophylaxis developed PCP; the overall incidence rate was 4.3 % and the relative risk reduction was 100 %. Five patients (8.5 %) developed ADR: four had drug eruptions and one had mild hepatitis. These data indicate that TMP/SMX can be used effectively for primary prophylaxis against PCP.

There are definite limitations to our study. First, we included definite and presumptive cases of PCP in our analysis. It has been well documented that the microscopic detection of *P. jirovecii* is difficult in non-HIV PCP [28, 29], as confirmed in this and our previous studies. To increase the specificity of the diagnosis of PCP without detecting the organism microscopically, we utilized composite diagnostic criteria, including clinical symptoms, laboratory tests, radiological findings, and the clinical

course. Kameda etal. found no difference in clinical characteristics of PCP in RA patients between definite PCP (i.e., acute-onset diffuse interstitial lung disease and microscopic positivity for *P. jirovecii* or positivity in both PCR test and BDG) and probable PCP (acute-onset diffuse interstitial lung disease and positivity in either PCR test or BDG) [24]. Their data support the use of composite diagnostic criteria for PCP in patients with RA. Second, we had only 17 RA patients with PCP, which decreased the sensitivity of the Cox proportional-hazards analysis for detecting statistically significant risk factors. Third, a higher incidence of PCP in Japanese RA patients receiving TNF antagonists and their risk factors have gained widespead recognition in the past few years by Japanese rheumatologists who use TNF antagonists; this may have affected the characteristics of the patients who were treated with adalimumab. For example, we found a significant difference in the daily dose of PSL between the PCP and non-PCP groups in our previous two studies, but not in this study.

In summary, the results of this study show that PCP is a serious complication in patients with RA who receive treatment with adalimumab. The majority of the patients developed PCP early in the course of adalimumab treatment and progressed to respiratory failure. Treating physicians should therefore take prophylaxis with TMP/SMX or other agents into consideration in RA patients with a high risk for PCP. Careful monitoring of clinical manifestations and laboratory tests for early diagnosis and treatment of PCP are strongly recommended.

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CASE REPORT

Successful treatment of eosinophilic granulomatosis with polyangiitis (EGPA; formerly Churg–Strauss syndrome) with rituximab in a case refractory to glucocorticoids, cyclophosphamide, and IVIG

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Abstract A 44-year old woman with eosinophilic granulomatosis with polyangiitis (EGPA) developed sequential paralysis of different cranial nerves despite treatments including methylpredonisolone pulse therapy, intravenous immunoglobulins (IVIG), and cyclophosphamide. Infusions of rituximab ameliorated her neurological symptoms and serological inflammatory findings. Rituximab, a specific B cell-targeting therapy, might offer an alternative for refractory EGPA with possible advantages of cost and ease of use compared to IVIG, which also targets (at least in part) B lymphocytes and immunoglobulin production.

Keywords Eosinophilic granulomatosis with polyangiitis · IVIG · Rituximab

Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg–Strauss syndrome) is a systemic granulomatous vasculitis with eosinophilia in a patient with a history of allergic disease. The involvement of small to medium vessels is characteristic of EGPA, as is the presence of antineutrophil cytoplasmic antibody (ANCA) in the serum. Both of these features qualify EGPA for inclusion as an ANCA-associated vasculitis (AAV).

Although high-dose glucocorticoids (GC) with cyclophosphamide (CPA) has been the main treatment applied in severe cases of EGPA, about 10 % of these cases have been found to be treatment resistant [1]. Further therapeutic options have been sought for such cases, and successful induction of remission has been reported with the use of both intravenous immunoglobulins (IVIG) and rituximab (RTX) [2]. However, repeated administration and/or combination therapies are sometimes required to achieve maximum benefit [3].

Rituximab (RTX) is a chimeric anti-CD20 monoclonal antibody that has been approved for use in cases of lymphoid malignancy, rheumatoid arthritis, and, more recently, microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA). Although it has proven to be efficacious for AAV in two randomized controlled studies [4, 5], its efficacy in EGPA cases has remained unclear.

We hereby present a case of EGPA resistant to both CPA and IVIG, which was successfully treated with RTX.

Case report

A 44-year-old woman with a history of bronchial asthma was admitted to a local hospital in June 2011 for fever with numbness and weakness of her extremities. Laboratory data showed peripheral eosinophilia (6579/μl) and elevated CRP (34.5 mg/l), as well as the presence of MPO–ANCA (180 EU/l). Nerve conduction test revealed low amplitude in the right median, the left ulnar, and the right sural nerves, suggestive of mononeuritis multiplex. She was diagnosed with EGPA, and treatment was initiated with intravenous pulses of methylprednisolone.

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Peripheral blood eosinophil count and MPO–ANCA normalized within six weeks during the oral prednisolone (PSL) taper. However, she remained febrile, her neurological symptoms persisted, and her CRP remained elevated (Fig. 1). A trial of IVIG (400 mg/kg/day over five days) was ineffective, with a new left facial nerve paralysis developing during the second course. One intravenous dose of CPA (500 mg) was administered along with the initiation of trimethoprim-sulfamethoxazole for preventing pneumocystis pneumonia, during which time the facial nerve paralysis worsened. There was no evidence of meningitis or hypertrophic pachymeningitis by cerebrospinal fluid analysis and brain MRI.

At this point (in August), the patient was transferred to our hospital, and she still presented febrile. The physical examination revealed that the cranial nerves other than the left facial nerve were intact and that paresthesia and weakness of her extremities had persisted. Peripheral blood count showed an absence of eosinophils, with total white blood cells 17,200/μl, hemoglobin level 8.2 g/dl, and platelets 469,000/μl. Serum CRP (159 mg/l) and eosinophil cationic protein (ECP; 36 μg/dl) were elevated, while MPO–ANCA remained negative. Serum IgG concentration was 1517 mg/ml (normal range: 868–1780 mg/ml). Methylprednisolone pulses improved the facial nerve palsy and the patient became afebrile, although her CRP remained high. Two additional intravenous doses of CPA (750 mg, followed by 1000 mg) also failed to decrease the CRP. Moreover, the patient started to complain of difficulty swallowing, and paralysis of the right hypoglossal nerve

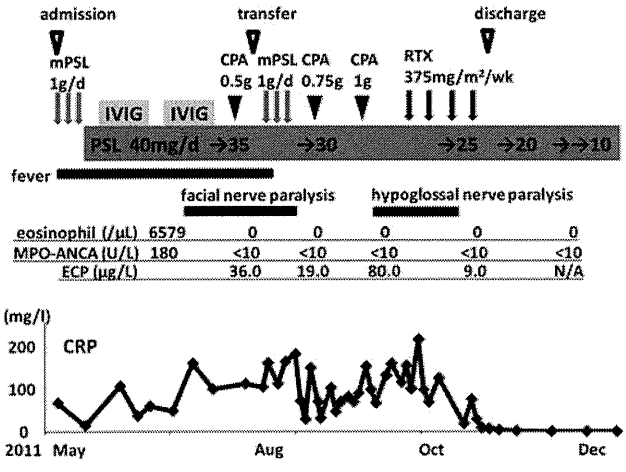

Fig. 1 Clinical course. This EGPA patient was resistant to corticosteroid, IVIG, and CPA. Treatment with RTX ameliorated cranial nerve involvement and reduced serum ECP and CRP levels. She remains in remission with B-cell depletion six months after RTX therapy. *mPSL* methylprednisolone, *PSL* prednisolone, *IVIG* intravenous immunoglobulin, *CPA* cyclophosphamide, *RTX* rituximab, *ECP* eosinophil cationic protein

was demonstrated clinically. Serum ECP rose to 80 μg/dl while serum IgG decreased to 801 mg/ml.

Subsequently, RTX was administered at a dose of 480 mg (375 mg/m^2) once a week for four weeks under written informed consent. Three weeks after the first dose, the hypoglossal nerve paralysis had disappeared completely, with decreased peripheral B-cell counts of 2 cells/μl as compared to 164 cells/μl before the RTX administration. Serum CRP and ECP diminished to within normal ranges by the time of the fourth dose, although the peripheral neuropathy at the extremities did not improve. There was no occurrence of adverse events despite the depletion of peripheral B cells and the low serum IgG concentration (760 mg/ml). At six months after completing treatment with RTX, the patient remained in remission on PSL 10 mg/day without peripheral B-cell recovery.

Discussion

Rituximab showed dramatic efficacy in our patient who had EGPA that was resistant to both conventional treatment and IVIG. The success obtained with RTX in the present case is consistent not only with the demonstrated efficacy of RTX in cases of MPA and GPA [4, 5], but also with the results achieved when it was used in eleven previously reported cases (Table 1) [6–11]. Among these 12 cases (including the case herein), three were resistant to previous IVIG, and at least five achieved remission without concomitant immunosuppressants or high-dose corticosteroids, suggesting a possible advantage of RTX in remission induction. As IVIG is presumed to exert its efficacy partly by providing a negative feedback signal on B cells mediated through FcγRIIB [12], the depletion of B cells using RTX may be a more potent and direct mechanism.

The efficacy of IVIG in EGPA has been previously demonstrated in two nonrandomized interventional studies. Tsurikisawa et al. [13] reported that IVIG showed significantly greater improvement of muscle weakness, dysesthesia, and cardiac output in 22 EGPA patients refractory to conventional treatment compared to 24 patients who did not receive IVIG. In a separate report, Danieli et al. [14] found that repeated IVIG combined with plasmapheresis in addition to conventional treatment achieved a significantly higher remission rate (100 %) than conventional treatment alone (44 %) in newly diagnosed EGPA. Based on these findings, IVIG can be recommended in particular for cases with persistent neurological deficits and/or cardiac dysfunction, as well as for difficult-to-treat cases. The efficacy of IVIG monotherapy for inducing remission remains unproven.

Although the potential clinical superiority of RTX needs to be demonstrated in larger studies, its economic and logistical advantages are clear. The administration of IVIG

Table 1 Cases of EGPA successfully treated with RTX

Patient no. [reference]	Age, sex	Involved organs	ANCA	Previous treatments (other than GC)	Concomitant treatments with RTX	Observation period after RTX administration/relapse	Additional RTX use/its indication
1 [6]	49, M	Kidney, skin	PR3	IVCY, AZA	PSL 30 mg/day	3 months/not relapsed	None
2 [7]	37, F	Myocarditis	(–)	IVCY, IVIG, MMF, alemtuzumab	PSL 15 mg/day	9 months/not relapsed	At 6 months/ prophylaxis
3 [7]	37, F	PNS, skin	(–)	CPA, AZA, MMF, alemtuzumab	PSL 10 mg/day	12 months/relapsed at 6 months	At 6 months/relapse
4 [8]	40, M	Lung	PR3	IVCY, PE	PSL[a] + CPA	9 months/not relapsed	None
5 [8]	66, M	PNS	MPO	IVIG, IVCY, PE	Low-dose PSL[a]	3 months/not relapsed	None
6 [9]	46, F	CNS	MPO	IVCY, MMF	PSL 5 mg/day + MMF	4 months/not relapsed	None
7 [10]	50, M	PNS, skin	(–)	MTX, CyA, AZA IFX, anakinra	PSL[a]	12 months/not relapsed	At 6 and 12 months/ prophylaxis
8 [10]	35, F	Lung	(–)	AZA	PSL[a]	6 months/not relapsed	At 6 months/ prophylaxis
9 [11]	54, F	Kidney	MPO	IVCY, MTX	PSL 1 mg/kg/day	12 months/relapsed at 6 months	At 6 months/relapse
10 [11]	54, F	Kidney, PNS	MPO	(–)	PSL 1 mg/kg/day	12 months/not relapsed	None
11 [11]	65, M	Kidney, PNS	MPO	(–)	PSL 1 mg/kg/day	12 months/not relapsed	None
12 (present case)	44, F	CNS, PNS	MPO	IVIG, IVCY	PSL 25 mg/day	6 months/not relapsed	None

PNS peripheral nervous system, *CNS* central nervous system, *IVCY* intravenous CPA pulse therapy, *IVIG* intravenous immunoglobulin, *AZA* azathioprine, *MMF* mycophenolate mofetil, *CPA* oral cyclophosphamide, *PE* plasma exchange, *MTX* methotrexate, *CyA* cyclosporine A, *IFX* infliximab

[a] The dosage of PSL was not available

requires hospitalization, which sometimes needs to be repeated. Furthermore, the clinical status of the patient may dictate the need for concomitant plasma exchange, significantly increasing the cost of treatment. Logistically, the availability of IVIG has been problematic globally, and the use of alternative treatments, when possible, has been recommended. Compared with IVIG, one course of RTX may provide sustained efficacy for approximately six months or longer, with at least some of the infusions possible in an outpatient setting.

As pharmacotherapeutic decisions need to be made by weighing up the balance of efficacy and safety, the risk of progressive multifocal leukoencephalopathy (PML) needs to be considered specifically for RTX. Fortunately, these cases are rare; most of them are associated with either previous or concomitant exposure to other immunosuppressive agents. Still, when compared to the safety of IVIG, the risk of developing serious infections should be an important consideration with RTX.

RTX could be an alternative for remission induction in EGPA cases. However, it remains unclear whether the scheduled RTX treatment or treatments with other oral immunosuppressants should be followed as maintenance therapy. In the present case, azathioprine was used successfully, in accordance with the EULAR recommendation for maintenance therapy after the conventional remission induction treatments [2]. An ongoing randomized controlled trial of RTX versus azathioprine as maintenance therapy (MAINRITSAN) will provide us with important information.

In general, peripheral eosinophil counts as well as serum ECP levels serve as biomarkers of the disease activity of EGPA [15, 16]. In the present case, the ECP level reflected the disease activity better than the eosinophil count. Together with another report that described a discrepancy between the levels of these two biomarkers [17], our observation suggests that serum ECP might be the more sensitive biomarker.

In conclusion, RTX is a potent alternative therapy for refractory EGPA. Further clinical investigations, especially with larger numbers of patients, are needed to confirm its efficacy and safety, as well as its most appropriate position in the therapeutic armamentarium.

Conflict of interest PYS is currently employed by UCB Japan, Co., Ltd. All other authors have declared no conflict of interest.

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CASE REPORT

Recurrent mitral valve regurgitation with neutrophil infiltration in a patient with multiple aseptic abscesses

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Abstract Aseptic abscess (AA) is characterized by accumulation of neutrophils without evidence of infection, no response to antibiotics, and rapid response to corticosteroids. We report a case of multiple abscesses in the subcutaneous tissues and joints, and severe mitral valve regurgitation. Although AA did not respond to antibiotic therapy, it improved dramatically with corticosteroid treatment. However, repeated valvuloplasty was required for the mitral valve regurgitation. The mitral valve tissue showed neutrophil infiltration without any bacterial invasion. This is the first case of AA to show involvement of cardiac valves, indicating the importance of systematic examination for patients with AA and cardiac valve involvement.

Keywords Aseptic abscesses · Mitral valve regurgitation · Neutrophil

Introduction

Aseptic abscess (AA) was first described in 1995 [1] and is characterized by accumulation of neutrophils without evidence of infection, no response to antibiotics, and rapid response to corticosteroids. It was reported that 31 of 49 patients with AA had inflammatory bowel diseases, whereas only three patients had no underlying disease [2]. We describe a case of AA with multiple abscesses in the subcutaneous tissues and joints in a patient with severe mitral valve regurgitation with neutrophil infiltration in the valve.

Case report

A 57-year-old woman without any previous medical history presented with a 3-month history of fever, and pain in the ankle and hip joints. Physical examination identified systolic murmur at the apex and tenderness and swelling of the right shoulder and pubic region. Laboratory tests showed increased leukocyte count [13,100/μl (neutrophil 81.8 %)] and C-reactive protein (CRP 22.72 mg/dl). Autoantibodies, such as antinuclear antibodies, rheumatoid factor and antineutrophil cytoplasmic antibodies, were negative. Human leukocyte antigen (HLA) typing was A24, A26, B54, and B62. Echocardiography showed mild mitral valve regurgitation without vegetation. Although bacterial endocarditis was initially suspected, repeated blood cultures were negative. Magnetic resonance imaging revealed multiple cystic lesions in the pubic joint and subcutaneous tissue of the coccygeal region (Fig. 1a, b). Contrast-enhanced computed tomography showed similar lesions around the right shoulder (Fig. 1c). Increased fluorine-18-deoxyglucose (FDG) uptake was found in both shoulders and pubic joint by positron emission tomography (FDG-PET) (Fig. 1d). Arthrocentesis from the pubic joint showed increased inflammatory cells with a cell count of 33,333/μl (polymorphonuclear cells 90 %), but cultures for bacteria and acid-fast bacilli were negative.

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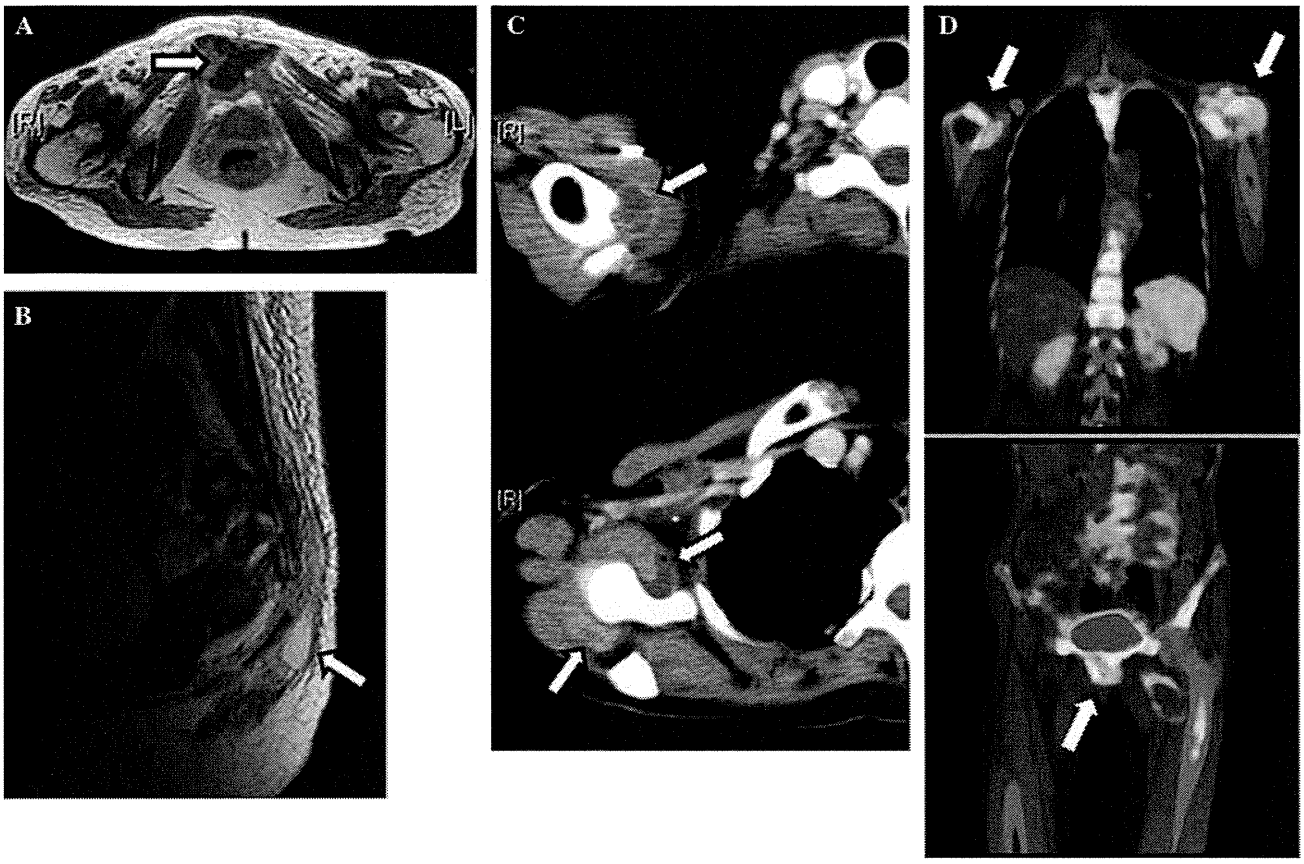

Fig. 1 Findings of magnetic resonance (MR) and computed tomography (CT) imagings. **a** Gadolinium-enhanced T1-weighted MR imaging showed low-intensity areas with enhanced margin in the pubic joint. **b** T2-weighted imaging showed high-intensity mass in the subcutaneous tissues of the coccygeal region. **c** Contrast-enhanced CT showed multiple cystic lesions with enhanced margins around the right shoulder. **d** Fluorine-18-deoxyglucose positron emission tomography (FDG-PET) showed increased FDG uptake in both shoulders and pubic joint. *Arrows* indicate affected lesions

The patient was initially treated with several kinds of antibacterial, antituberculosis, and antifungal agents for more than 2 months, but showed no clinical response. Mitral valve regurgitation had been worsening and resulted in heart failure, which was treated with furosemide. As antibiotic therapy was not at all effective for the abscesses and the mitral valve regurgitation, in addition to negative cultures from the abscesses and blood, we finally diagnosed her as AA. Accordingly, she was treated with 30 mg/day prednisolone (PSL), which resulted immediately in resolution of the fever, marked improvement of joint pain, and reduction in CRP to an undetectable level (Fig. 2). Repeated imaging showed cystic lesions became smaller in all areas. Subsequently, PSL was gradually tapered to 15 mg/day, however; this resulted in a rise in CRP to around 1 mg/dl, necessitating the addition of methotrexate (MTX) at 8 mg/week. As severe mitral valve regurgitation persisted even with corticosteroid therapy, following the tapering of PSL to 5 mg/day, mitral valvuloplasty was performed. During surgery, perforations at the posterior leaflet and posterolateral commissure were revealed, which were sutured. She was discharged 1 month after the surgery.

While our patient was maintained by 5 mg/day PSL and 8 mg/week MTX in the outpatient clinic, any symptom such as high-grade fever or arthralgia did not develop. However, serum CRP level was around 3 mg/dl, suggesting that the disease activity was not fully controlled. Two months after discharge, she was hospitalized again because of syncope and severe anemia. Physical examination identified systolic murmur at the apex, and laboratory tests showed decreased hemoglobin (8.8 g/dl) and increased lactase dehydrogenase (2940 IU/l) and CRP (6.3 mg/dl). Severe mitral valve regurgitation was revealed again by echocardiogram. Therefore, we diagnosed her condition as mechanical hemolytic anemia due to relapsed valve regurgitation.

The valvuloplasty was thus repeated. In the open heart surgery, another perforation was found next to the sutured one at the posterior leaflet. Histological findings of mitral valve tissue showed neutrophil infiltration (Fig. 3). No bacterial invasion was detected by Gram staining. Disease

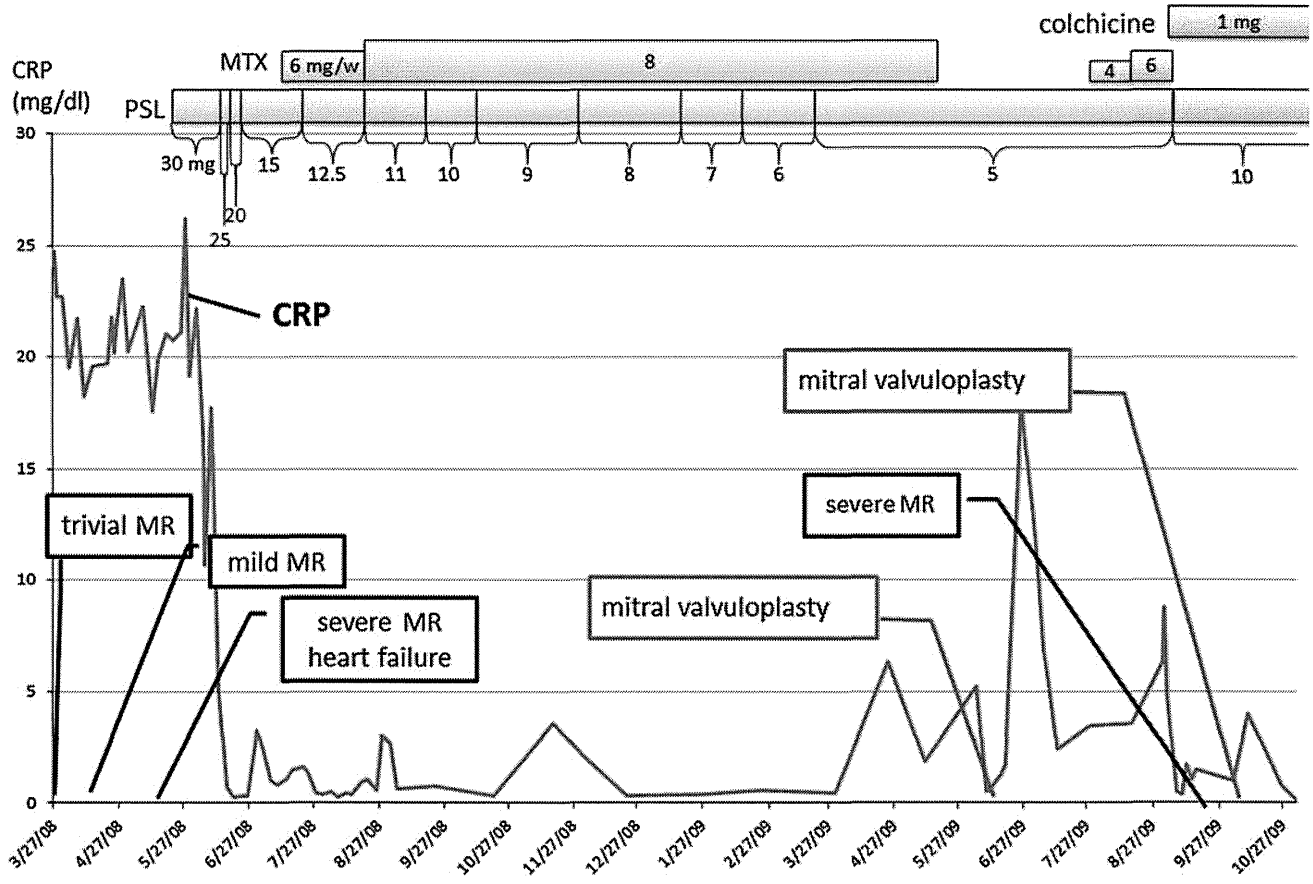

Fig. 2 Clinical course

activity was subsequently well controlled with maintenance therapy of 10 mg/day PSL and 1 mg/day colchicine.

Discussion

Aseptic abscess is diagnosed based on criteria by André et al. [2]: (1) deep abscess detected by radiologic examination associated with neutrophilia; (2) negative cultures of blood and pus from abscess; (3) lack of response to antibiotic therapy; (4) rapid clinical improvement following initiation of corticosteroids. Previous case series reported AA appeared mostly in viscera, such as spleen (70 %), abdominal lymph nodes (53 %), liver (40 %), lung (17 %), pancreas (10 %), and brain (10 %). In addition, involvement of soft tissue in AA patients was also reported [2–4]. All patients were treated with corticosteroids; however, some patients required additional drugs, such as azathioprine (30 %), colchicine (27 %), cyclophosphamide (17 %), antitumor necrosis factor agent (10 %), and MTX (3 %) [2]. We treated our patient with 30 mg/day (0.6 mg/kg/day) of PSL with MTX. Furthermore, colchicine was added with the expectation of suppressing augmented neutrophil activity.

In this case, mitral valve regurgitation without vegetation occurred with multiple abscesses. Antibiotics were not effective, and cultures from the abscesses and blood were negative. The mitral valve regurgitation relapsed during maintenance therapy, with CRP elevation, suggesting disease activity was not well controlled. Moreover, histological findings of the mitral valve revealed neutrophil infiltration without bacterial invasion. Taken together, we considered that this mitral valve regurgitation was derived from AA. To our knowledge, this is the first case in the literature to show involvement of the cardiac valves in AA.

Although autoinflammatory diseases such as familial Mediterranean fever, hyperimmunoglobulinemia D with periodic fever syndrome, pyogenic arthritis, pyoderma gangrenosa and acne syndrome, tumor-necrosis-factor-receptor-associated periodic syndrome, and cryopyrin-associated periodic syndromes were suspected in our patient, she did not have any disease-related nucleotide polymorphisms [5]. Genetic abnormalities of AA have not been reported.

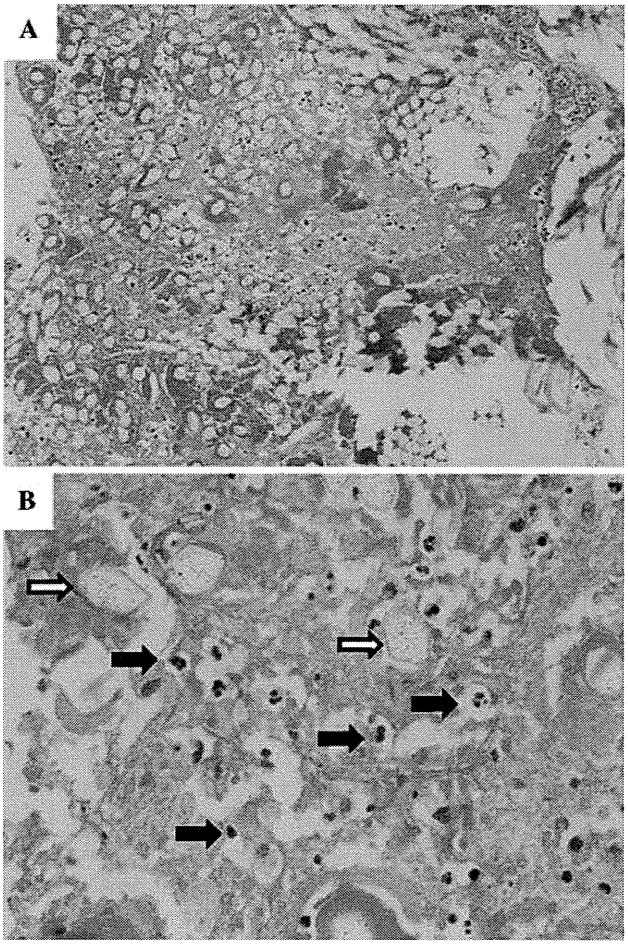

Fig. 3 Histological examination of the mitral valve. Note infiltrated polymorphonuclear neutrophils in the mitral valve (*black arrows*). *White arrows* indicate filaments of suture. Hematoxylin and eosin staining: **a** ×100, **b** ×400

It is reported that 7–46 % of patients with Behçet's disease develop cardiovascular complications—including valve diseases, which frequently relapse [6, 7]—and some require valvuloplasty [8]. However, this patient showed no typical manifestations of Behçet's disease, such as oral ulcerations, urogenital lesions, cutaneous lesions, or ocular disease.

In conclusion, ours is the first case of AA affecting the mitral valve reported in the literature. Patients with AA should be systemically and carefully examined, including cardiac valve involvement. Furthermore, once AA is diagnosed, disease activity should be tightly controlled.

Conflict of interest None.

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Am80, a Retinoic Acid Receptor Agonist, Ameliorates Murine Vasculitis Through the Suppression of Neutrophil Migration and Activation

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Objective. **Vasculitis is characterized by leukocyte infiltration in the vessel walls, with destructive damage to mural structures. Retinoids are compounds that bind to retinoic acid receptors and exert biologic activities similar to those of vitamin A, including modulatory effects on cell proliferation and differentiation. This study was undertaken to examine the therapeutic effects of a synthetic retinoid, Am80, in a murine model of vasculitis induced by *Candida albicans* water-soluble fraction (CAWS).**

Methods. **Vasculitis was induced in BALB/c mice by intraperitoneal injection of CAWS. Neutrophils were depleted by injection of antineutrophil antibody–positive serum. Am80 was administered orally once daily. Vasculitis was evaluated histologically. Migration of labeled adoptively transferred cells was quantified. Chemotaxis was assessed by cell mobility analysis. Production of reactive oxygen species (ROS) and phosphorylation of MAPKs were measured by flow cytometry. Concentrations of elastase were measured by enzyme-linked immunosorbent assay.**

Results. **Administration of CAWS induced vasculitis in the coronary arteries and aortic root, with abundant neutrophil infiltration. Depletion of neutrophils reduced CAWS-induced vasculitis. Treatment with Am80 led to a significant attenuation of the vasculitis score and inhibition of the migration of transferred neutrophils into the site of vasculitis. In vitro, Am80 suppressed fMLP-induced chemotaxis of human peripheral blood neutrophils. ROS production and elastase release by stimulated neutrophils were reduced by AM80 treatment, and Am80 also inhibited phosphorylation of ERK-1/2 and p38 in neutrophils stimulated with fMLP plus lipopolysaccharide.**

Conclusion. **Am80 significantly suppressed CAWS-induced vasculitis. This effect was presumably exerted via inhibition of neutrophil migration and activation.**

Vasculitis is defined by the presence of inflammatory leukocytes in vessel walls, with destructive damage to mural structures. Antineutrophil cytoplasmic antibodies are often detected in certain types of vasculitis, such as microscopic polyangiitis. The affected vessels vary in size, type, and location according to the type of vasculitic disease (1). Although the exact mechanisms

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underlying these disorders are unclear, activated polymorphonuclear cells (neutrophils) in the vascular endothelium are thought to play an important role in the pathogenesis of the vasculitides (2). Oral corticosteroids and immunosuppressive agents are commonly used for the treatment of vasculitis. However, in some cases the disease is refractory to these treatments, and immunosuppression often leads to significant clinical complications. Therefore, there is a need for a new low-cost therapy for vasculitis that is more effective and safer than currently used treatments.

An experimental mouse model of vasculitis has been developed in which the disease is induced by administration of *Candida albicans* water-soluble fraction (CAWS) (3–7). The experimental mice exhibit severe coronary arteritis accompanied by neutrophil activation and production of proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-6 (8). CAWS-induced coronary arteritis is considered to be an appropriate model for use in studies aimed at understanding the pathogenesis of arteritis as well as developing novel treatments.

Retinoid, a derivative of vitamin A, is a general term for compounds that bind to and activate retinoic acid receptors (RARs [RARα, RARβ, and RARγ]) and/or retinoid X receptors (RXRs [RXRα, RXRβ, and RXRγ]), members of the nuclear receptor superfamily (9). RARs are transcriptional regulators that bind to specific retinoic acid response elements present in the promoters of their target genes. Retinoids have important roles in cell proliferation, differentiation, and morphogenesis (10). They have also been reported to promote differentiation of Th2 and Treg cells and to suppress Th1 and Th17 differentiation (11). In addition, retinoids inhibit tumor necrosis factor α and nitric oxide production by murine peritoneal macrophages and human keratinocytes (12,13). Furthermore, previous studies have indicated that retinoids inhibit neutrophil activation, including superoxide anion and protease release (14–17). Clinically, retinoids are used for the treatment of cutaneous inflammatory disorders such as psoriasis and acne (18,19). Therefore, retinoids may have a beneficial effect in neutrophil-dominant diseases. All-*trans*-retinoic acid, the most notable endogenous retinoid, is a ligand for RARα, RARβ, and RARγ.

Am80 is a specific ligand for RARα and RARβ but not for RARγ (20), and is characterized by higher stability, fewer side effects, and superior bioavailability, compared with all-*trans*-retinoic acid (20,21). All-*trans*-retinoic acid and Am80 are used for the treatment of acute promyelocytic leukemia (21,22). The present study was designed to determine the effects of Am80 on CAWS-induced vasculitis and on neutrophil migration and activation.

MATERIALS AND METHODS

Induction of CAWS-induced vasculitis, depletion of neutrophils, and treatment with Am80. CAWS was prepared from *C albicans* strain IFO1385, using a previously described method (23). Six-week-old male BALB/c mice were purchased from Oriental Yeast Company. To induce vasculitis, CAWS (1 mg) was injected intraperitoneally into the mice in a volume of 0.2 ml once daily for 5 days (day 1 to day 5).

Neutrophils were depleted using a modification of a previously described method (24). Briefly, 0.2 ml of saline-diluted (1:10) rabbit anti–mouse neutrophil antibody–positive serum (Accurate) was injected intraperitoneally once daily from day 1 to day 5. Subsequently, an additional dose was injected once every other day to maintain neutropenia. The control group received normal rabbit serum (Accurate) in the same volume. As a therapeutic administration after vasculitis had developed, rabbit anti–mouse neutrophil antibody–positive serum was injected from day 8 to day 12, with an additional dose injected every other day.

Am80 was suspended in carboxymethylcellulose. Carboxymethylcellulose alone as vehicle or Am80 (1.0 mg/kg or 4.0 mg/kg) in carboxymethylcellulose was administered orally once daily from day 1 to day 35 (prophylactic administration) or from day 8 to day 35 (therapeutic administration). On day 36 the mice were killed and the hearts were harvested and examined histologically. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Immunohistochemistry. Immunohistochemical analysis was conducted on OCT-embedded sections of frozen heart tissue. Briefly, 6-μm–thick cryostat sections were fixed in 4% paraformaldehyde for 10 minutes, and then the samples were rehydrated in 0.1% Triton X-100 in phosphate buffered saline (PBS) for 3 minutes. Endogenous peroxidase activity was blocked by incubation in 1.5% H_2O_2 in PBS for 15 minutes, followed by rinsing with PBS. Nonspecific binding was blocked by addition of 10% normal goat serum in PBS for 40 minutes. The sections were incubated overnight at 4°C with 10 μg/ml rat anti-mouse Ly-6G monoclonal antibody (mAb) (RB6-8C5; eBioscience), 5 μg/ml rat anti-mouse F4/80 mAb (C1:A3-1; Serotec), 5 μg/ml rat anti-mouse CD4 mAb (GK1.5; Cymbus Biotechnology), or normal rat IgG in antibody diluent (BD PharMingen). The samples were washed 3 times in PBS and then incubated for 30 minutes with 2.5 μg/ml biotin-conjugated rabbit anti-rat IgG (DakoCytomation) pretreated with 5% normal mouse serum to reduce nonspecific binding. After washing in PBS, the sections were incubated for 30 minutes with streptavidin–horseradish peroxidase. After washing in PBS, diaminobenzidine (DakoCytomation) was used for visualization. The sections were counterstained with hematoxylin for 10 seconds and washed in tap water for 5 minutes. F4/80-positive and CD4-positive cells were counted in 4 randomly selected fields examined at 200× magnification under light microscopy.

Histologic evaluation. The fixed hearts were embedded in paraffin and sectioned. In order to observe the histologic changes in the coronary arteries and the aorta in detail, we

prepared step sections 20 μm apart in a horizontal direction. The sections were stained with hematoxylin and eosin. For quantitative evaluation of vascular inflammation, each of 5 areas (3 aortic root areas and both coronary arteries) was scored as 0 (no inflammatory cell migration) or 1 (presence of panvasculitis). The severity of the arteritis in each mouse was defined as the sum of the scores of the 5 segments (maximum possible score of 5).

Migration of Ly-6G–positive splenocytes into the aortic wall. Ly-6G–positive splenocytes from normal BALB/c mice were purified using MACS Microbead–coupled mAb and magnetic cell separation columns (Miltenyi Biotec). The cells were shown to be >95% pure. Most of the purified cells were polymorphonuclear, indicating that they were neutrophils. The purified Ly-6G–positive cells were labeled with CellTracker Orange 5-(and-6)-([4-chloromethyl] benzoyl amino) tetramethylrhodamine (CMTMR) according to the protocol supplied by the manufacturer (Molecular Probes). The CMTMR-labeled cells (1.6×10^7) were injected intravenously into the tail vein of mice with CAWS-induced vasculitis on day 15. The recipient mice were administered vehicle or Am80 (4 mg/kg) orally, 24 hours and 2 hours before and 22 hours after the transfer. Twenty-four hours after the transfer, the mice were killed, hearts were harvested and embedded in OCT compound, and horizontal 5-μm–thick sections were prepared. The total number of CMTMR-labeled cells that had migrated into the aortic wall was counted under fluorescent microscopy (Biozero). Two slides from each heart were evaluated independently by 2 observers, and the mean number of cells was recorded.

Expression of RARs in human peripheral blood neutrophils. Neutrophils from peripheral blood of healthy human controls were obtained using Mono-Poly resolving medium (Dainippon), followed by positive removal of all contaminating cells with mAb against CD3, CD56, CD19, CD36, CD49d, and glycophorin A using a custom-made EasySep kit (Stemcell Technologies) as previously described (25). Total RNA was prepared from human peripheral blood neutrophils and synovial fibroblasts obtained from a patient with rheumatoid arthritis (26), and first-strand complementary DNA (cDNA) was synthesized using 2 μg total RNA. Polymerase chain reaction (PCR) was performed in a total volume of 50 μl containing 1 μl cDNA, 0.2 m*M* dNTP, 0.02 μ*M* of each primer, 1× PCR buffer (Roche Molecular Systems), and FastStart *Taq* DNA polymerase (Roche Molecular Systems), with a thermal cycler (PTC-200; MJ Research). After the initial denaturing step (94°C for 5 minutes), amplification was performed for 35 cycles at 94°C for 60 seconds, 66°C for 30 seconds, and 72°C for 60 seconds. The final cycle was followed by an extension step of 5 minutes at 72°C. The sequences of the primers were as follows: RARα 5′-TGG-GTG-GAC-TCT-CCC-CGC-CA-3′ (sense), 5′-CCC-ACC-TCC-GGC-GTC-AGC-GTG-3′ (antisense) (product size 438 bp); RARβ 5′-CAC-TGG-CTT-GAC-CAT-CGC-AGA-CC-3′ (sense), 5′-GAG-AGG-TGG-CAT-TGA-TCC-AGG-3′ (anti-sense) (product size 500 bp); RARγ 5′-GGC-CTG-GGC-CAG-CCT-GAC-CTC-3′ (sense), 5′-CAG-CCC-CAG-ATC-CAG-CTG-CAC-G-3′ (antisense) (product size 537 bp); β-actin 5′-GTC-CTC-TCC-CAA-GTC-CAC-ACA-3′ (sense), 5′-CTG-GTC-TCA-AGT-CAG-TGT-ACA-GGT-AA-3′ (antisense) (product size 239 bp). PCR products were resolved by electrophoresis on 1.5% agarose gels (Takara Bio) containing ethidium bromide.

In vitro chemotaxis assay. The purified human peripheral blood neutrophils were incubated for 2 hours at 37°C in RPMI 1640 medium (Sigma-Aldrich) with 10% fetal calf serum (FCS), without Am80 or in the presence of Am80 at 10^{-7}, 10^{-6}, or 10^{-5} moles/liter. Chemotaxis of the neutrophils was examined using an EZ-TAXIScan (ECI, Inc.) for quantitative measurement of cellular chemotaxis, as previously described (27). Neutrophils were aligned to one edge of a TAXIScan holder, and 10 n*M* fMLP was applied to the opposite compartment. Cell migration at 25°C was recorded every 30 seconds for 45 minutes. After the assay, the digital images were converted into videos for analysis, and the number of cells moving into the assay field was analyzed using TAXIScan Analyzer software (ECI, Inc.). Cell viability was measured using a Cell Counting Kit-8 according to the instructions of the manufacturer (Dojindo). The viable cell number of neutrophils treated with 10^{-5} moles/liter Am80 for up to 5 hours was >90% compared with that of vehicle-treated neutrophils, suggesting that culture with Am80 did not affect neutrophil viability. Concentrations of Am80 in the in vitro experiments were determined, according to the plasma concentration of Am80 administered to the mice (28).

Measurement of generated reactive oxygen species (ROS). Human peripheral blood neutrophils were incubated for 5 hours at 37°C, without Am80 or in the presence of Am80 at 10^{-8}, 10^{-7}, or 10^{-6} moles/liter in RPMI 1640 with 10% FCS. Cells were stimulated with 1 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 10 minutes at 37°C, 1 μg/ml palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4; Calbiochem) for 90 minutes at 37°C, or 5 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 90 minutes at 37°C. The Pam_3CSK_4- and LPS-treated cells were then stimulated with 100 n*M* fMLP (Sigma-Aldrich) for 5 minutes. The neutrophils were subsequently incubated with 1 μ*M* dihydrorhodamine 123 (DHR-123; Marker Gene Technologies) for 5 minutes and then washed with cold PBS. To estimate intracellular peroxide production, the fluorescence intensity of rhodamine 123, which is a product of cellular oxidation of DHR 123, in 10,000 cells was recorded using an Accuri C6 flow cytometer. Three independent experiments were performed in triplicate.

Measurement of elastase release. Human neutrophils were incubated for 5 hours at 37°C, without Am80 or in the presence of Am80 at 10^{-8}, 10^{-7}, or 10^{-6} moles/liter in RPMI 1640 with 10% FCS. The cells were then incubated for 30 minutes with 10 μg/ml cytochalasin B (Sigma-Aldrich) and 1 μ*M* fMLP. The concentration of elastase in the culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer (Hycult).

Phosphospecific flow cytometry. The neutrophils were stimulated with fMLP (1 μ*M*) and LPS (1 μg/ml) at 37°C. At various time intervals, 1×10^6 cells were fixed in 3% formaldehyde, washed in PBS, and resuspended in ice-cold 90% methanol at 4°C for 30 minutes. The cells were then washed twice in PBS with 2% FCS before incubation with rabbit anti–phosphorylated ERK-1/2 antibody (9101; Cell Signaling Technology) or rabbit anti–phosphorylated p38 antibody (9211; Cell Signaling Technology) for 20 minutes at room temperature. Phosphospecific antibodies were detected by subsequent incubation with phycoerythrin-conjugated goat anti-rabbit antibody (2.5 μg/ml; Beckman Coulter). Cells were then analyzed by flow cytometry using an Accuri C6 flow cytometer.

To evaluate the effect of Am80 on the phosphorylation of ERK-1/2 or p38, the cells were incubated with 10^{-6} moles/liter Am80 or control medium for 3 hours at 37°C before stimulation with fMLP and LPS. Unstimulated samples showed no increase in phosphorylation of ERK-1/2 or p38 throughout the experiment.

Statistical analysis. All data are expressed as the mean ± SEM. The statistical significance of differences between groups was assessed by Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Effects of neutrophil depletion on CAWS-induced vasculitis. We induced vasculitis in BALB/c mice by administration of CAWS once daily for 5 days (days 1–5). On day 36, the mice were killed and the hearts were evaluated histologically. Although normal mice exhibited no inflammatory changes (Figure 1A), CAWS-injected mice showed inflammatory cell infiltration into the aortic root and coronary arteries (Figure 1B). Immunohistochemical analysis revealed massive invasion of Ly-6G–positive neutrophils in the vascular wall (Figure 1C). Moderate infiltration of F4/80-positive macrophages and CD4-positive T cells was also observed (Figures 1D and E).

Next we analyzed the effect of neutrophil depletion on CAWS-induced vasculitis. Rabbit anti–mouse neutrophil antibody–positive serum was administered from day 1 to day 5, and an additional dose was subsequently injected every other day to maintain neutropenia. The number of peripheral blood neutrophils was decreased to 43% of baseline by day 17. The neutrophil depletion reduced inflammatory cell infiltration into the aortic root and coronary arteries compared to that observed in mice treated with control serum (Figures 1G and H). The vasculitis score was significantly lower in neutrophil-depleted mice (Figure 1I). Neutrophil depletion also reduced the number of F4/80-positive macrophages and CD4-positive T cells infiltrating into the vessel wall (mean ± SEM 43.0 ± 4.2 F4/80-positive macrophages per field and 29.0 ± 2.7 per field in control serum–treated and antineutrophil antibody–positive serum–treated animals, respectively, and 20.0 ± 1.8 CD4-positive T cells per field and 13.25 ± 1.5 per field, respectively; $P < 0.05$ for both). Neutrophil depletion

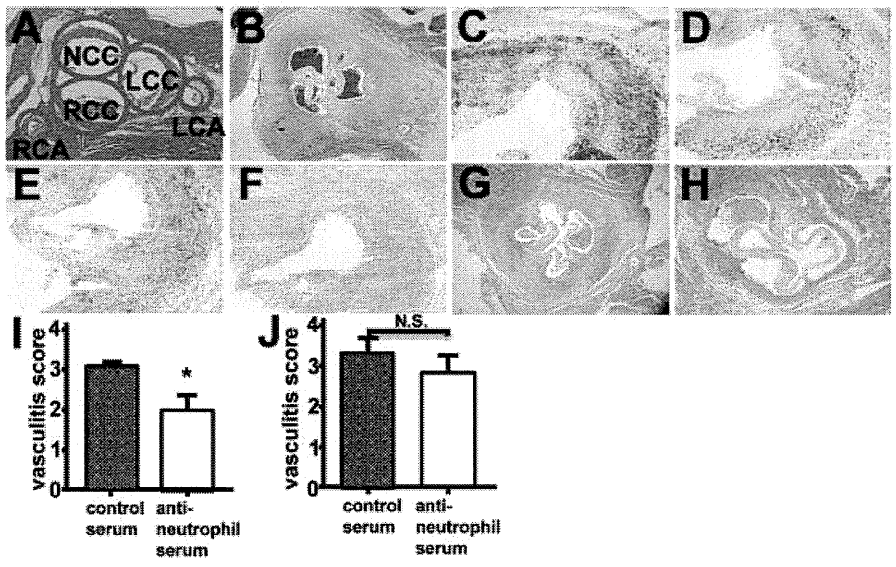

Figure 1. Histologic findings in BALB/c mice with *Candida albicans* water-soluble fraction (CAWS)–induced vasculitis. **A** and **B,** Hematoxylin and eosin (H&E)–stained heart specimens obtained on day 36 from a normal mouse (**A**) and a mouse that was injected with CAWS once daily from day 1 to day 5 (**B**). **NCC** = noncoronary cusp; **LCC** = left coronary cusp; **RCC** = right coronary cusp; **LCA** = left coronary artery; **RCA** = right coronary artery. **C–F,** Identification of infiltrated cells in the aortic wall. The hearts of CAWS-injected mice were stained with Ly-6G (**C**), F4/80 (**D**), CD4 (**E**), or isotype control (**F**). All sections were counterstained with hematoxylin. **G** and **H,** H&E-stained heart specimens obtained on day 17 from a CAWS-injected mouse administered control serum (**G**) and a CAWS-injected mouse administered antineutrophil antibody–positive serum (**H**) from day 1 to day 5 and subsequently every other day. **I,** Mean ± SEM vasculitis scores in mice treated as described in **G** and **H** (n = 8 in the control serum–treated group; n = 11 in the antineutrophil antibody–positive serum–treated group). * = $P < 0.05$ versus control serum–treated group. **J,** Mean ± SEM vasculitis scores in mice treated with control serum or antineutrophil antibody–positive serum beginning on day 8 (n = 12 in each group). NS = not significant. Specimens shown in **A–H** are representative of the respective treatment groups. Original magnification × 40 in **A, B, G,** and **H**; × 100 in **C–F**.

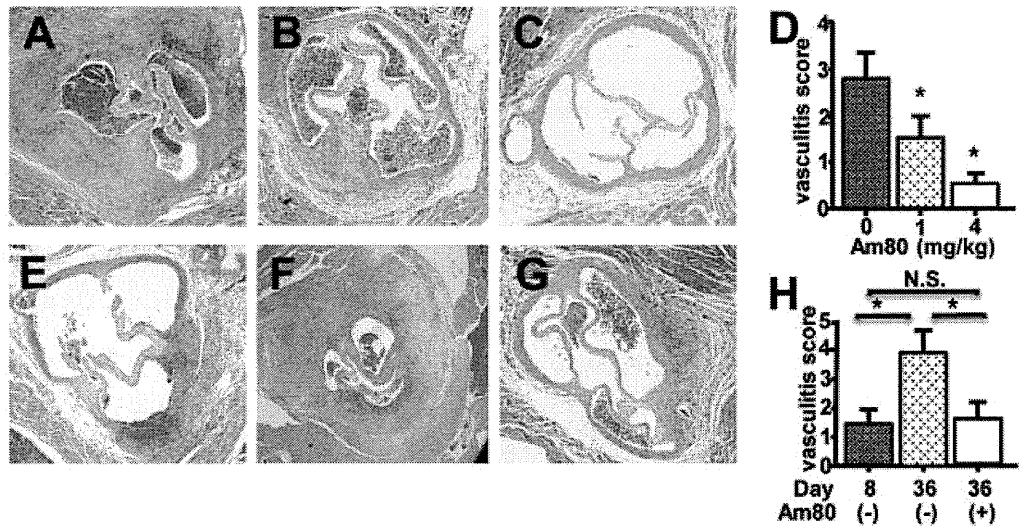

Figure 2. Effect of Am80 on *Candida albicans* water-soluble fraction (CAWS)–induced vasculitis. **A–C,** Hematoxylin and eosin (H&E)–stained heart specimens obtained on day 36 from CAWS-treated BALB/c mice administered vehicle (**A**) or Am80 at 1 mg/kg (**B**) or 4 mg/kg (**C**) (n = 10 per group) once daily for 5 weeks, as prophylaxis. **D,** Mean ± SEM vasculitis scores in mice treated as described in **A–C**. * = $P < 0.05$ versus vehicle-treated group. **E–G,** H&E-stained heart specimens from CAWS-treated BALB/c mice administered vehicle (n = 24) (**E** and **F**) or Am80 at 4 mg/kg (n = 25) (**G**) for 5 weeks beginning on day 8, as therapy for existing vasculitis. Specimens were obtained on day 8 (**E**) or day 36 (**F** and **G**). **H,** Mean ± SEM vasculitis scores in mice treated as described in **E–G.** * = $P < 0.05$. NS = not significant. Specimens shown in **A–C** and **E–G** are representative of the respective treatment groups. Original magnification × 40.

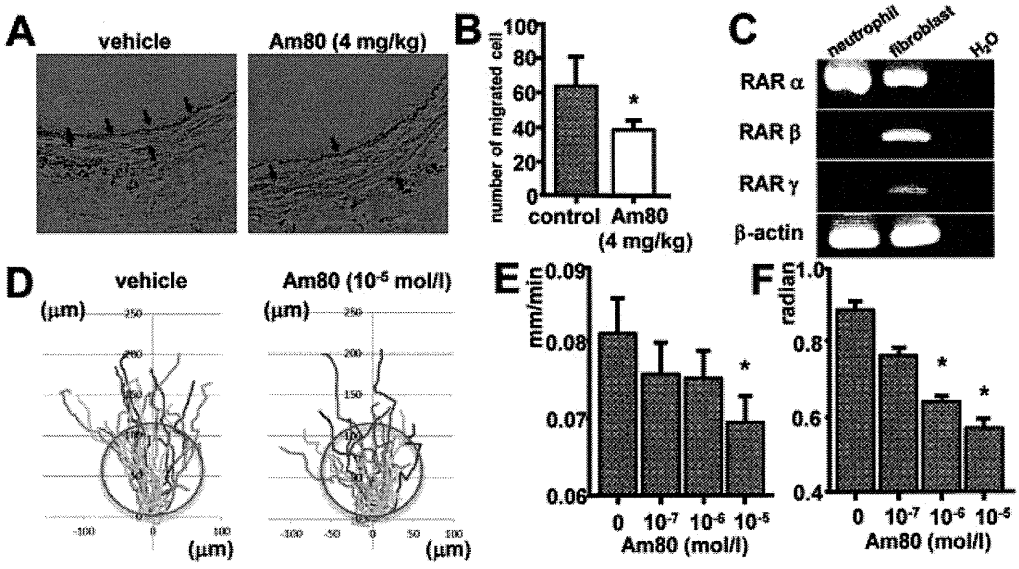

Figure 3. Inhibition of neutrophil migration by Am80. **A,** Labeled neutrophils were adoptively transferred into BALB/c mice with *Candida albicans* water-soluble fraction (CAWS)–induced vasculitis. Vehicle or Am80 at 4 mg/kg was administered orally to recipient mice (n = 6 per group) 24 hours and 2 hours before and 22 hours after the adoptive transfer. Twenty-four hours after the transfer, the animals were killed, and hearts were harvested and examined by fluorescent microscopy for migrated cells (**arrows**). Specimens shown are representative of the respective treatment groups. Original magnification × 80. **B,** Labeled cells in the aortic wall of mice treated as described in **A** were counted. Values are the mean ± SEM. * = $P <$ 0.05 versus vehicle-treated group. **C,** Total RNA was extracted from purified human peripheral blood neutrophils and synovial fibroblasts from a patient with rheumatoid arthritis, as a positive control. Expression of the retinoic acid receptors (RARs) RARα, RARβ, and RARγ was analyzed by reverse transcription–polymerase chain reaction. **D,** The purified neutrophils were incubated with vehicle or Am80 (10^{-7}, 10^{-6}, or 10^{-5} moles/liter), and then exposed to fMLP in a chemotactic chamber within an EZ-TAXIScan device. Single motile cells were tracked every 30 seconds for 45 minutes. The cell paths of the neutrophils treated with vehicle or Am80 at 10^{-5} moles/liter are shown. Results are representative of 3 independent experiments. **E** and **F,** Parameters of average migration velocity (**E**) and directionality (**F**) were measured. Values are the mean ± SEM from 3 video sequences of 22 cells in each treatment group. * = $P < 0.05$ versus no Am80 treatment.

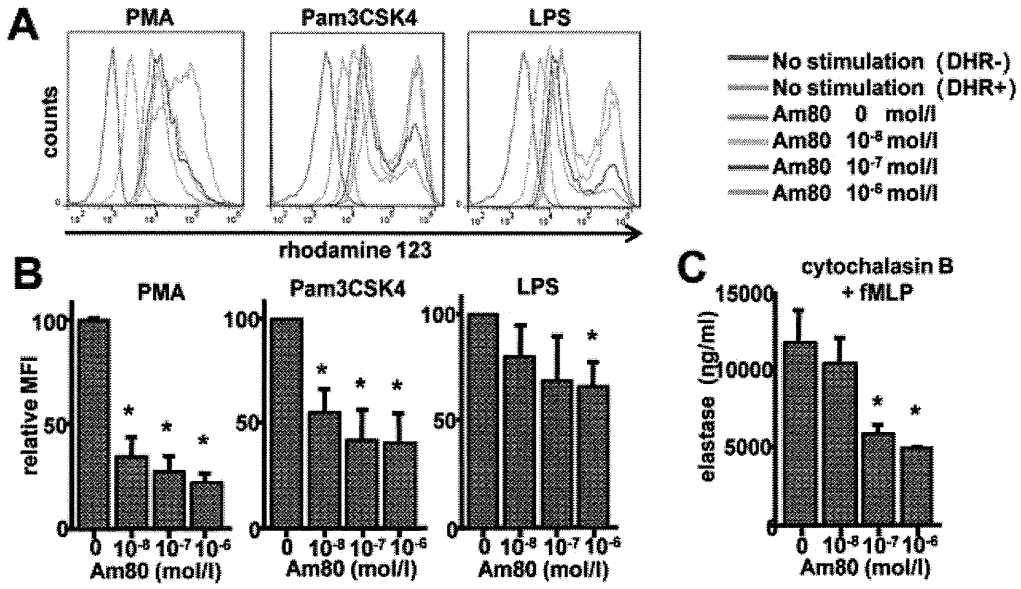

Figure 4. Effect of Am80 on reactive oxygen species (ROS) production and elastase release from neutrophils. **A,** Purified human peripheral blood neutrophils were incubated without Am80 or in the presence of Am80 at 10^{-8}, 10^{-7}, or 10^{-6} moles/liter and stimulated with phorbol 12-myristate 13-acetate (PMA) (1 ng/ml), palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) (1 μg/ml), or lipopolysaccharide (LPS) (5 μg/ml). ROS levels in the neutrophils were determined based on the fluorescence intensity of dihydrorhodamine 123 (DHR). Representative histograms are shown. **B,** Neutrophils were treated as described in **A,** and the relative mean fluorescence intensity (MFI) ratio was calculated ([MFI of cells treated with Am80/MFI of cells without Am80 treatment] $\times$ 100). **C,** The purified neutrophils were incubated without Am80 or in the presence of Am80 at 10^{-8}, 10^{-7}, or 10^{-6} moles/liter and stimulated with cytochalasin B (10 μg/ml) and fMLP (1 μM). Elastase concentrations in the culture supernatant were measured by enzyme-linked immunosorbent assay. Values in **B** and **C** are the mean $\pm$ SEM of triplicate wells from at least 3 independent experiments. $* = P < 0.05$ versus no Am80 treatment.

beginning on day 8 (after the onset of vasculitis) did not significantly reduce the vasculitis score (Figure 1J).

Effects of Am80 on CAWS-induced vasculitis. Next we examined the effect of Am80 on CAWS-induced vasculitis, since previous studies have suggested that retinoids inhibit the activation of neutrophils (14–17). Am80 (1 mg/kg or 4 mg/kg) was administered orally once daily from days 1 to 35. Prophylactic Am80 administration reduced inflammatory cell accumulation in the aortic root and coronary arteries by day 36 (Figures 2A–C) and significantly reduced the vasculitis score in a dose-dependent manner (Figure 2D). We also analyzed the therapeutic effects of Am80 (4 mg/kg) administered from day 8 to day 35. Vasculitis was already present on day 8 (Figure 2E). On day 36, the above treatment schedule also significantly suppressed the histologic features of vasculitis compared with vehicle (Figures 2F–H); however, the vasculitis score on day 36 was not decreased compared with that on day 8 (Figure 2G).

Effects of Am80 on neutrophil migration. We also analyzed the effect of Am80 on neutrophil migration into the inflamed aortic wall. CMTMR-labeled neutrophils were adoptively transferred into mice with CAWS-induced vasculitis. The recipient mice were treated with 4 mg/kg Am80 24 hours and 2 hours before and 22 hours after the transfer. This short-term administration of Am80 did not reduce the vasculitis score (data not shown). Twenty-four hours after the transfer, the number of cells that had migrated into the aortic wall was counted. In normal mice, no labeled neutrophils migrated into the aortic wall. In contrast, significant numbers of labeled neutrophils migrated into the aortic wall in the vasculitic animals. However, treatment with Am80 reduced this migration of neutrophils to the vasculitic site (Figures 3A and B).

The effect of Am80 on chemotaxis of human peripheral blood neutrophils was also analyzed in vitro; synovial fibroblasts from a patient with rheumatoid arthritis were used as a positive control for RAR expression in this experiment (29). The purified human neutrophils expressed RARα, but not RARβ or RARγ (Figure 3C). After culture with Am80 for 3 hours, fMLP-induced cell movement was video-recorded (see Supplementary videos 1 [control] and 2 [Am80-treated], available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-