anti-flag Ab. Fig. 3*B* shows that Rack-1 interacted with Tyk2 (266–733) and Tyk2 (600–1086) as well as full-length Tyk2. In addition, Rack-1 interacted weakly with Tyk2 (833–1187), which contains the kinase domain of Tyk2. As Rack-1 did not interact with Tyk2 (1–450), these data indicate that Rack-1 interacts weakly with the kinase domain of Tyk2 and interacts strongly with the pseudokinase domain of Tyk2. Furthermore, as there is no overlapping region in Tyk2 (266–733) and Tyk2 (833–1187), there is the possibility that multiple RACK-1 binding sites exist in Tyk2.

We next determined the region of Rack-1 required for the interaction with Tyk2. Fig. 3*C* shows the full-length and deletion mutants of Rack-1 used in this experiment. As shown in Fig. 3*D*, full-length Rack-1 (1–317), Rack-1 (125–317), and Rack-1 (1–204) bound to Tyk2. The C-terminal region of Rack-1 (aa 204–317) did not associate with Tyk2. Taken together with the fact that the two-hybrid candidate clone 4-86 encodes aa 137–317 of Rack-1, these data suggest that the middle portion of Rack-1, aa 137–203, may associate with Tyk2. We next examined the binding of Rack-1 Δ 138–203, which lacks aa 138–203, to Tyk2. Unexpectedly, Rack-1 Δ 138–203 also binds to Tyk2 (Fig. 3*D*). This indicates that two regions of Rack-1, the N terminus and the middle portion, might bind to Tyk2. This was confirmed by the fact that Rack-1 (1–137) binds to Tyk2 (Fig. 3*D*).

Rack-1 is phosphorylated on Tyr^{194}, a residue in the fifth WD repeat, by Jaks

As Tyk2 is a tyrosine kinase, we assessed whether Rack-1 could be phosphorylated by Tyk2. When Rack-1 was transiently cotransfected into 293T cells with Tyk2, Rack-1 was phosphorylated by Tyk2 (Fig. 4*A*). There are six tyrosine residues in Rack-1; to identify the specific tyrosine residue(s) that is phosphorylated by Tyk2 in vivo, we performed site-directed mutagenesis, substituting phenylalanine for tyrosine at individual and multiple sites in Rack-1 (Fig. 4*B*). The Rack-1 mutants were then transiently coexpressed with Tyk2 in 293T cells, and proteins were immunoprecipitated with anti-Rack-1 and subjected to immunoblot analysis with anti-phosphotyrosine (Fig. 4*C*). We found that the Rack-1 mutants containing phenylalanine at position 194 (the E, F, H, and I mutants) were not phosphorylated by Tyk2. Because the Rack-1 E mutant is a single substitution of tyrosine 194 to phenylalanine and this mutant is not phosphorylated, Tyk2 must phosphorylate Rack-1 on Tyr^{194}. In addition, the immunoprecipitates of all Rack-1 mutants contained phosphorylated Tyk2, indicating that the tyrosine phosphorylation of Rack-1 has no influence on the association of Rack-1 and Tyk2. This result was confirmed by the transient transfection of wild type or the E mutant of Rack-1 with or without Tyk2 in 293T cells. Proteins were immunoprecipitated with Tyk2 or Rack-1. The results shown in Fig. 5 demonstrate that Rack-1 was present in the Tyk2 immunoprecipitates at equivalent levels whether aa 194 of Rack-1 was tyrosine or phenylalanine. In addition, Tyk2 was present in the Rack-1 immunoprecipitates at equivalent amounts. Taken together, these results demonstrate that phosphorylation of tyrosine 194 of Rack-1 is not important for the interaction of Rack-1 and Tyk2.

We next examined whether other Jaks could phosphorylate Rack-1. In addition to Tyk2, Jak1, Jak2, and Jak3 were able to phosphorylate Rack-1 (Fig. 6). We also transfected kinase-dead Jak1 or Jak2 (KE mutant) with Rack-1 into 293T cells (Fig. 6, *lane 3* and *5*). In this case, Rack-1 was not phosphorylated, although Rack-1 associated with Jak1KE and Jak2KE. This indicates that Jaks directly phosphorylate Rack-1. Furthermore, none of the Jak family kinases phosphorylated the Rack-1 E mutant (Fig. 6, *right*), indicating that all Jaks phosphorylate Rack-1 on a single tyrosine, residue 194. Notably, immunoprecipitates containing the Rack-1 E

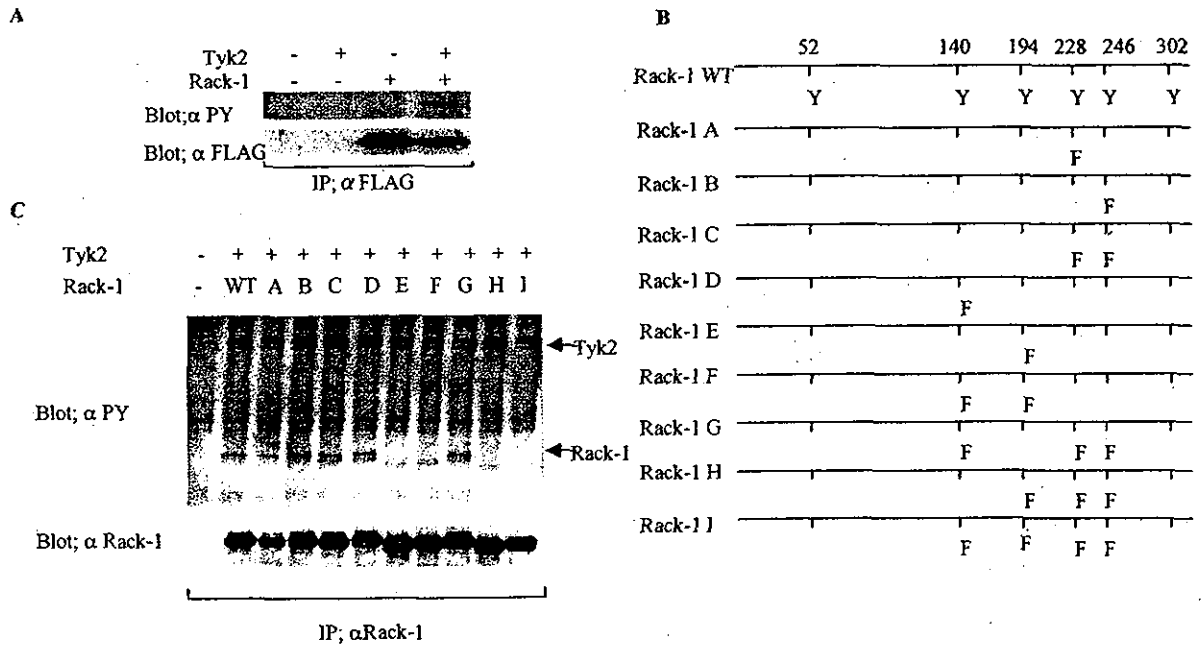

FIGURE 4. Tyk2 phosphorylates Rack-1 on Tyr^{194}, a residue in the fifth WD repeat. *A*, Rack-1 is phosphorylated by Tyk2. Tyk2 and/or Flag-tagged Rack-1 were expressed in 293T cells. Total cell lysates were immunoprecipitated with anti-Flag Ab and immunoblotted with anti-phospho tyrosine Ab (*upper panel*) or anti-Flag Ab (*lower panel*). *B*, Site-directed mutagenesis of Rack-1 was performed to substitute phenylalanine for tyrosine at individual and multiple sites. *C*, Tyk2 phosphorylates Rack-1 on Tyr^{194}, a residue in the fifth WD repeat. 293T cells were transfected with a series of wild-type or Rack-1 mutants. Forty-eight hours after transfection, cells were lysed, immunoprecipitated with anti-Rack-1 Ab, and immunoblotted with anti-phosphotyrosine Ab (*upper panel*) or anti-Rack-1 Ab (*lower panel*). The arrows indicate tyrosine phosphorylated Rack-1 and Tyk2 which coimmunoprecipitates with Rack-1.

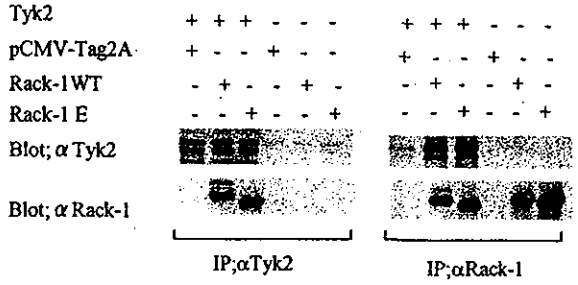

FIGURE 5. Tyrosine phosphorylation of Rack-1 has no influence on the association between Rack-1 and Tyk2. Empty vector (pCMV-Tag2A), wild-type Rack-1, or the Rack-1 E mutant (Y194F) were coexpressed with Tyk2 in 293T cells. Total cell lysates were immunoprecipitated with anti-Tyk2 Ab (*left panel*) or anti-Rack-1 Ab (*right panel*) and immunoblotted with anti-Tyk2 Ab (*upper panel*) or anti-Rack-1 Ab (*lower panel*).

mutant contained Jak1, Jak2, Jak3, and Tyk2. Therefore, Jaks associate with Rack-1 whether Rack-1 is tyrosine phosphorylated or not.

Perinuclear translocation of Rack-1 by Jaks

293T cells were used to localize the distribution of Rack-1 within cells with or without the activation of Jaks (Fig. 7*A*). When Rack-1 was transfected into 293T cells, it was detected throughout the cytoplasm. When both Rack-1 and Tyk2 were transfected into 293T cells, intracellular redistribution of Rack-1 toward the perinuclear area was observed. The transfection of Jak1 or Jak2 with Rack-1 in 293T cells also induced the perinuclear translocation of Rack-1. To determine whether the perinuclear translocation of Rack-1 was induced by Jaks, we transfected the Jak1 KE mutant or the Jak2 KE mutant with Rack-1 in 293T cells. Under these conditions, Rack-1 was detected throughout the cytoplasm, and was not translocated to the perinuclear region. Next, we examined whether perinuclear translocation of Rack-1 required the phosphorylation of Rack-1. When the Rack-1 E mutant (Y194F) was transfected with Tyk2 into 293T cells, perinuclear translocation of the Rack-1 E mutant was observed to the same extent as Rack-1 wild type (Fig. 7*B*).

Discussion

The main substrates of Jaks are the Stats, and the phosphorylation of Stats is essential for the biological activities of cytokines (2). Analysis of Stat-deficient mice has demonstrated that Stats transduce almost all cytokine signaling (17). In the case of inhibition of IL-7-induced B cell growth by IFN-α, Stat1 activation was not required (12), although we found that Tyk2 activation was necessary (11). To find Tyk2-activated signaling molecules other than the Stats, we performed a yeast two-hybrid screen for proteins that associated with Tyk2. We found that Rack-1, which was originally described as a receptor for activated protein kinase C (PKC)-β (18), associated with Tyk2 (Fig. 1). Interestingly, it has recently been reported that Rack-1 associates with the β-chain of the type I IFNR and recruits Stat1 to the receptor complex. This raises the possibility that Rack-1 serves as a scaffolding protein during cytokine signal transduction. We have shown here that Tyk2 associates with Rack-1 in 293 T cells and BAF/3 cells (Fig. 2). This association was not altered by stimulation with IFN-α (Fig. 2*B*). Recently, Usacheva et al. (14) reported that Tyk2 and Jak1 associated with Rack-1 using GST-fusion proteins. Consistent with our result, they also reported that association of Jak1 and Rack-1 was not affected by IFN-β treatment. Additionally, we have determined the binding sites on Tyk2 and Rack-1. Tyk2 binds strongly to Rack-1 through the kinase domain, and binds weakly through the pseudo-

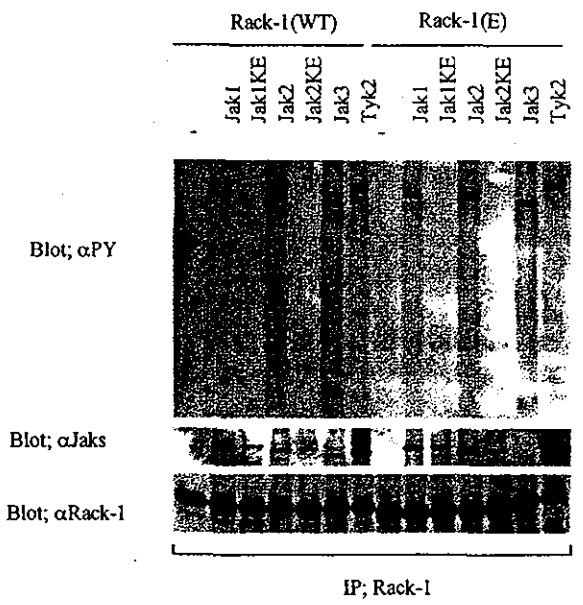

FIGURE 6. In addition to Tyk2, Jak1, Jak2, and Jak3 phosphorylate tyrosine 194 on Rack-1. Wild-type or the Rack-1 E mutant (Y194F) were coexpressed with either Jak1, the Jak1 KE mutant, Jak2, the Jak2 KE mutant, Jak3, or Tyk2 in 293T cells. Total cell lysates were immunoprecipitated with anti-Rack-1 Ab and immunoblotted with anti-phosphotyrosine Ab, anti-Rack-1 Ab, or anti-Jak Abs (mixture or individual anti-Jak1, anti-Jak2, and anti-Tyk2 Abs) as indicated.

kinase domain (Fig. 3*B*). Therefore, Tyk2 probably associates with Rack-1 through more than one binding site. Similarly, Rack-1 binds to Tyk2 through two regions, one in the N terminus and one in the middle portion of the protein (aa 137–203) (Fig. 3*D*).

As Tyk2 is a tyrosine kinase, we next analyzed whether Rack-1 could serve as a substrate of Tyk2. When Tyk2 and Rack-1 were transiently transfected into 293T cells, Rack-1 was phosphorylated by Tyk2 (Fig. 4*A*). In addition to Tyk2, other members of the Jak family (Jak1, Jak2, and Jak3) phosphorylated Rack-1 (Fig. 6). Kinase-dead Jaks (Jak1 KE or Jak2 KE mutants) were unable to phosphorylate Rack-1, suggesting that Jaks directly phosphorylate Rack-1.

When cells are stimulated by PMA, PKC is activated and Src phosphorylates Rack-1 on Tyr^{228} and/or Tyr^{246} (19). Therefore, we wanted to determine whether the site of Tyk2 phosphorylation was the same as the site of Src phosphorylation. We demonstrated that the A (Y-228-F), B (Y-246-F), and C (Y-228, 246-F) mutants of Rack-1 were phosphorylated by Tyk2 (Fig. 4*C*). Next, we substituted tyrosine 140 and/or 194 to phenylalanine. The substitution of tyrosine 194 to phenylalanine in Rack-1 (this mutation is present in the E, F, H, and I mutants) diminished the phosphorylation of Rack-1 by Tyk2 (Fig. 4*C*). In addition to Tyk2, other members of the Jak family (Jak1, Jak2, and Jak3) phosphorylated tyrosine 194 on Rack-1 (Fig. 6). Therefore, Jaks phosphorylate only tyrosine 194 of Rack-1. In addition, these results indicate that Jaks and Src kinase phosphorylate different tyrosine residues on Rack-1.

Although the binding of Rack-1 to Src required the phosphorylation of Rack-1 (19), Rack-1 mutants (E, F, H, and I) which were not phosphorylated by Tyk2 were still able to associate with Tyk2 (Fig. 4*C*). Indeed, wild-type Rack-1 and the Rack-1 E mutant associated with Tyk2 to the same degree (Fig. 5). These results indicate that the association of Rack-1 and Tyk2 occurred regardless of Rack-1 phosphorylation, and this association is not enhanced by tyrosine phosphorylation of Rack-1. In addition, catalytic activity

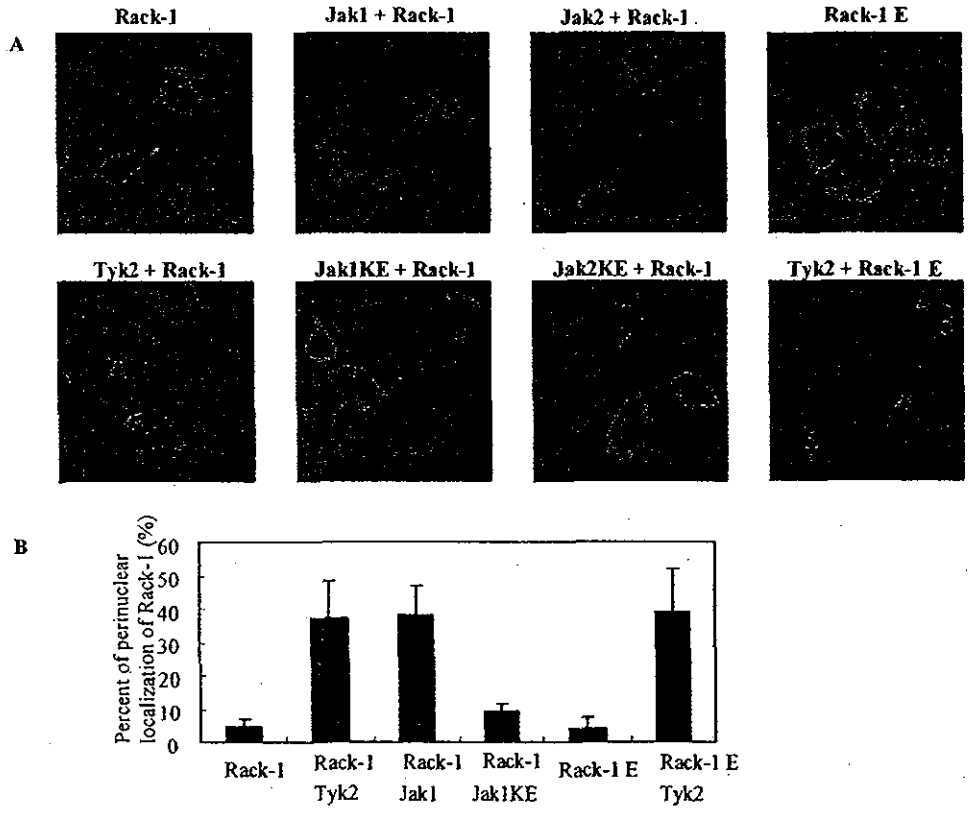

FIGURE 7. Localization of Rack-1. *A*, Flag-tagged Rack-1 (wild type or E mutant) was expressed either alone or in combination with one of Tyk2, Jak1, the Jak1 KE mutant, Jak2, or the Jak2 KE mutant in 293T cells. After 48 h, the localization of tagged proteins was visualized by confocal microscopy. *B*, The number of cells showing perinuclear localization of Rack-1 was calculated. Data represent the mean with SD of four different experiments.

of Jaks is not essential for binding to Rack-1, as the KE mutants of both Jak1 and Jak2 associated with Rack-1 (Fig. 6). This result is consistent with previous observations that Rack-1 associated with Tyk2 in BAF/3 cells in the presence or absence of IFN-α stimulation (Fig. 2*B*).

IFN-β stimulation has been reported to translocate Rack-1 toward the perinuclear region (13). Transient expression of Jaks in 293T cells led to the autophosphorylation of the Jaks. When Rack-1 alone was transfected into 293T cells, Rack-1 was present throughout the cytoplasm. In contrast, when both Jak (Jak1, Jak2 or Tyk2) and Rack-1 were transfected into 293T cells, Rack-1 was translocated to the perinuclear region (Fig. 7). This perinuclear translocation was not observed when Jak1 KE or Jak2 KE was cotransfected with Rack-1, indicating that the kinase activity of Jaks is required for the translocation of Rack-1. As Tyk2 phosphorylated Rack-1, and as the kinase activity was essential for the perinuclear translocation of Rack-1, it was possible that phosphorylated Rack-1 might translocate to the perinuclear region. To test this hypothesis, we transfected Tyk2 and the Rack-1 E mutant, which lacks tyrosine 194, the residue that is phosphorylated by Jaks. Surprisingly, the Rack-1 E mutant translocated to the perinuclear region in a similar manner as wild-type Rack-1 (Fig. 7). One possible explanation for this observation is that Tyk2 might phosphorylate another target, which forms a complex with Rack-1, and translocates Rack-1 to the perinuclear region. Another possible explanation is that Tyk2 might phosphorylate endogenous Rack-1 in 293T cells, this phosphorylated endogenous Rack-1 might form a complex with the transfected Rack-1 E mutant, and the two types of Rack-1 might then be translocated to the perinuclear region together. To determine the precise mechanism of Rack-1 translocation, similar analysis must be performed using Rack-1 null cells.

In summary, we have demonstrated that Rack-1 associates with Jaks. Specifically, Rack-1 interacts with the pseudokinase and kinase domains of Tyk2. Two regions of Rack-1, the N terminus and the middle portion of the protein (aa 138–203), contribute to binding Tyk2. In addition, we have shown that tyrosine 194 of Rack-1 is phosphorylated by Jaks. Neither the phosphorylation state of Rack-1 nor of the Jak has an influence on the association of the two proteins. Furthermore, Rack-1 is translocated to the perinuclear region by the activation of Jaks.

The function of Rack-1 in cytokine signaling is still unclear. Because Rack-1 is a WD repeat-containing protein with no enzymatic activity (20), and because Rack-1 binds to PKC, Src homology 2-containing proteins such as Src, phospholipase Cγ, and *ras*-GTPase-activating proteins (21, 22), it has been reported that Rack-1 functions as a scaffold protein that recruits specific signaling elements. Rack-1 also binds to the IFNR β-chain (13), Stat1 (15), and, as we have shown here, Jaks. It is possible that Rack-1 functions as a scaffold protein that aligns the signaling molecules in the cytokine-signaling cascade. Additionally, the fact that Rack-1 is phosphorylated by Jaks and is translocated to the perinuclear region by activation of Jaks raises the possibility that Rack-1 functions as a signaling molecule in the cytokine signaling cascade.

Acknowledgments

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Infections post transplant

A nationwide survey of deep fungal infections and fungal prophylaxis after hematopoietic stem cell transplantation in Japan

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Summary:

We conducted a nationwide survey to define incidence of deep fungal infections and fungal prophylaxis practices after HSCT. In all, 63 institutions responded. Total number of in-patient transplantations was 935: 367 autologous, 414 allogeneic myeloablative, and 154 allogeneic reduced-intensity (RIST) ($n=154$). Number of patients who were cared for in a clean room at transplant was 261 (71%) in autologous, 409 (99%) in conventional and 93 (66%) in RIST, respectively. All patients received prophylactic antifungal agents; 89% fluconazole. Number of patients who received the dosage recommended in the CDC guidelines (400 mg/day) was 135 (42%) in conventional transplant and 34 (30%) in RIST ($P=0.037$). Number of patients who received fluconazole until engraftment and beyond day 75 in conventional transplant *vs* RIST was, respectively, 324 (100%) *vs* 109 (97%), and 39 (12%) *vs* 18 (16%), with no significant difference between the two groups. A total of 37 patients (4.0%) were diagnosed with deep fungal infections; autologous transplantation (0.03%), conventional transplantation (6.0%) and RIST (7.1%). Wide variations in antifungal prophylaxis practice according to the type of transplant and the institutions, and deep fungal infection remain significant problems in RIST.
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Keywords: hematopoietic stem cell transplantation (HSCT); fungal infection; antifungal prophylaxis; reduced-intensity stem cell transplantation (RIST)

Fungal infection is a common complication after hematopoietic stem cell transplantation (HSCT), and the primary causative organisms are *Candida* and *Aspergillus*, with significant mortalities even if properly treated with antifungal agents.[1] Therefore, antifungal prophylaxis has been emphasized following HSCT.[2] In 2000, the Centers for Disease Control and Prevention (CDC) in the United States issued guidelines for the prevention of fungal infections in the setting of allogeneic HSCT and some cases of autologous transplantation,[3] and these are considered a gold standard in many countries throughout the world, including Japan. To prevent *Candida* infections, the CDC guidelines recommend the use of fluconazole (400 mg/day) until engraftment,[3] based on the results of two randomized controlled studies published in 1992 and 1995.[4,5] However, since then, the circumstances surrounding HSCT have been changing rapidly. The use of fluconazole has been questioned, since it is ineffective against *Aspergillus* species. Moreover, the rationale for selecting the recommended dose of 400 mg/day fluconazole and the optimal duration of prophylaxis need to be clarified.[6] A major concern regarding emerging fluconazole-resistant *Candida* species needs to be critically evaluated.[7,8]

Aspergillus is the most common pathogen in fungal infections in the course of transplantation or treatment for leukemia.[9] The importance of hospital environment control has been emphasized for effective prevention. The outbreak

of nosocomial infections due to the renovation of transplant buildings[10,11] and via the water system in transplant wards has been reported.[12] The CDC guidelines recommend the use of air conditioning systems/tools.[3] However, the guidelines may have become outdated due to changing circumstances. First, the timing of the development of aspergillosis has changed. Most *Aspergillus* infections occur late beyond 100 days after transplantation.[13] This trend may be enhanced by the wider application of reduced-intensity stem cell transplantation (RIST) on an outpatient basis.[14] Second, newly developed antifungal agents have been shown to be effective in the prevention of *Aspergillus* infection.[15,16] Moreover, a recent drastic change is that RIST has been extended to unrelated transplantation,[17] umbilical cord blood transplantation[18] and elderly patients.[19] RIST has the advantage of a shorter period of neutropenia, and it is likely that the incidence of *Candida* infection is reduced. On the other hand, the incidence of steroid-therapy requiring GVHD remains the same as in conventional transplantations, and the intensity of immune suppression is similar.[20] It is expected that late-occurring fungal infections by *Aspergillus*, etc may become a significant problem after RIST.[21–23]

Thus, a re-evaluation and update of the guidelines appears to be necessary, particularly in the area of RIST. There have been only a few studies on fungal infections after RIST.[21–23] The objective of this study was to survey antifungal prophylaxis in Japanese transplantation practice, and to compare the findings in RIST, conventional allogeneic transplantation (CST), and autologous transplantation.

Patients and methods

Data collection

Of the 418 Japanese medical institutions in which HSCT is performed, 122 institutions agreed to participate in this survey. Each of these institutions received a questionnaire that included the following items: the number of transplantations performed in 2001, the type of the patient's disease and the type of transplantation, use of a clean room or designated room for transplantation, practice regarding prophylactic use of antifungal agents, and occurrence of systemic fungal infections. For cases that developed deep fungal infections, age, sex, date of onset, diagnostic approaches, infected organs, pathogens, neutrophil count at onset, coexistence of GVHD, use of immunosuppressants, prophylactic use of antifungal agents, and patient outcome were also recorded.

We used the EORTC/NIH-MSG criteria for the diagnosis of deep fungal infections.[24] We defined both proven and probable infectious cases as deep fungal infection.

Antifungal prophylaxis measures

We defined the nine measures listed below as measures for preventing fungal infection. All of the methods were evaluated as A or B based on the evidence level in the CDC guidelines, as previously reported.[25] The guidelines use a combination of category (A–D) and the recommendation (I–III). We used two recommendation levels, A and B, which indicated strong or moderate evidence supported by well-established clinical trials (I or II) or respected authorities (III).

(a) Yeast infection following allogeneic HSCT

(a1) Medical staff in contact with HSCT recipients should follow appropriate hand-washing practices to safeguard patients from exposure (Evidence level A III).
(a2) Fluconazole (400 mg/day, orally or intravenously) should be administered from the day of transplant until engraftment (Evidence level A II).

(b) Mold infections following allo-SCT

(b3) HSCT recipients who remain immunocompromised should avoid hospital construction or renovation areas (Evidence level A III).
(b4) Use of high efficiency particulate air (HEPA) filtration (Evidence level B III).
(b5) Air should flow from patient rooms to corridors (Evidence level B III).
(b6) Correctly sealed rooms, including correctly sealed windows and electrical outlets (Evidence level B III).
(b7) High rates of room air exchange (ie, >12 air changes/h) (Evidence level B III).
(b8) Barriers between patient care areas and renovation or construction areas to prevent dust from entering patient care areas. The barriers must be impermeable to Aspergillus species.

(c) Autologous HSCT

(c9) Autologous HSCT recipients generally are at lower risk of invasive fungal infection than allogeneic HSCT recipients. Autologous HSCT recipients do not require routine intense anti-yeast prophylaxis. Nevertheless, some researchers recommend the use of an anti-yeast prophylaxis in patients who have underlying hematologic malignancies, and who have or will have prolonged neutropenia and mucosal damage from intense conditioning regimens or graft manipulation, or who have recently received purine analogues (Evidence level B III). Regarding mold infections, no guideline has been reported for autologous HSCT.

Prophylaxis for deep fungal infections in Japan

In Japan, the antifungal agents approved for the treatment of deep fungal infections in 2001 included amphotericin B, fluconazole, itraconazole, miconazole and terbinafine. With the exception of itraconazole, they can be administered either orally or intravenously. The only available formula of itraconazole was a capsule, and in this form, absorption through the gastrointestinal tract is inconsistent.[26] Itraconazole oral solution, voriconazole, liposomal amphotericin B and echinocandin have not yet been approved.

Governmental approval has not yet been granted for the prophylactic use of any antifungal agents, although this is widely applied as a practice in most medical institutions.[27] Fluconazole, up to 400 mg, is approved only for the treatment of fungal infection.

Use of a clean room within a transplant ward is reimbursed by insurance. We considered that prophylactic measures (b3)–(b8) were satisfied if HSCT was performed in a clean room. A clean room was defined as an isolated room equipped with HEPA filtration and air flow toward an exit of the room with sealed windows.

End points and statistical methods

The objective of this study was to conduct a survey of antifungal prophylaxis in HSCT. The levels of compliance with the established prophylactic measures in RIST and CST were compared. The second objective was to compare the CST, RIST and auto-SCT groups with respect to the incidence and characteristics of fungal infections.

We examined (a2) and (b3)–(b8) of the nine items listed above. Item (a1) was excluded from the survey because of inconsistency of proper evaluation in a retrospective survey. We considered that items (b3)–(b8) were satisfied when a clean room was used. We aimed to evaluate differences in fungal prophylaxis between conventional and reduced-intensity transplants. We did not collect detailed information on transplantation procedures such as stem cell sources and drugs used in the preparative regimens. A univariate analysis using Fisher's exact test and the Mann–Whitney U test was performed to compare the differences in prophylactic measures between RIST and CST. Values of $P<0.05$ were considered significant.

Results

Patients background

We received questionnaires from 63 medical institutions, representing 935 transplantations. In Japan, a total of 1964 transplantations were performed in 2001,[28] and approximately half of the patients were surveyed in this study. The median number of transplantations per institute/year was 10 (range, 1–108). The types of transplantation were autologous HSCT (367, 40%), CST (414, 45%), and RIST (154, 15%). All of the RIST recipients received purine-analog-based preparative regimens with or without low-dose TBI.

Patients' diseases included malignant lymphoma (191 cases, 21%), acute myelocytic leukemia (147, 16%), acute lymphocytic leukemia (107, 11%), multiple myeloma (80, 9%), chronic myelocytic leukemia (74, 8%), myelodysplastic syndrome (51, 5%), solid tumors (50, 5%), aplastic anemia (16, 2%), and others (219, 23%).

All patients were hospitalized during transplantation. The number of autologous HSCT, CST and RIST recipients who were in a clean room at the time of transplantation was 261/367 (71%), 409/414 (99%) and 93/154 (66%), respectively.

Prophylactic use of antifungal agents

All patients including autologous HSCT cases received an antifungal agent as a prophylaxis, and azole antifungal agents were administered in 742 patients (79%). The most commonly used azole antifungal agent was fluconazole (89%). Figure 1 shows the types of agents used in CST, RIST and autologous HSCT.

Fluconazole was administered to 324 (78%) CST and 112 (73%) RIST recipients. The numbers of patients who received fluconazole 400, 200 and 100 mg/day were 135 (42%), 169 (52%) and 20 (6%), respectively. Those patients who were given fluconazole 400, 200 and 100 mg/day were 34 (30%), 70 (63%) and 8 (7%), respectively. Significantly larger doses of fluconazole were used in CST than in RIST ($P=0.037$).

The numbers of patients who received fluconazole until engraftment and beyond day 75 in the CST and RIST groups were, respectively, 324 (100%) and 39 (12%), and 109 (97%) and 18 (16%). Duration of fluconazole use was not significantly different between CST and RIST.

Incidence and clinical characteristics of deep fungal infections

Of the 935 transplant cases, 37 (4.0%) were diagnosed with deep fungal infections (13 proven and 24 probable cases); 0.03% (1/367) of autologous HSCT, 6.0% (25/414) of CST, and 7.1% (11/154) of RIST. The causative organisms included *Candida* ($n=9$), *Aspergillus* ($n=16$), *Mucor* ($n=1$), *Fusarium* ($n=1$) and unknown ($n=10$). The median onset date from the time of transplant was 85 days (range, 1–392 days): 92 days in CST and 117 days in RIST, with nine cases developing within 30 days of transplant (one case in autologous HSCT, seven in CST and one in RIST). Three cases of 37 deep fungal infections had a previous history of fungal infection. All of them received fluconazole and AMPH-B intravenously as a prophylaxis.

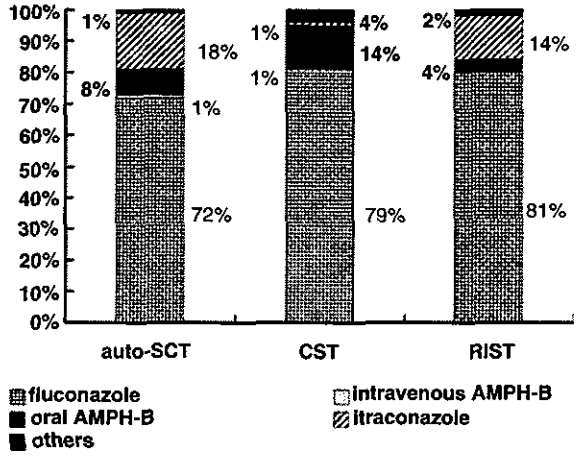

Figure 1 Types of prophylactic antifungal agents used in each type of HSCT Azole agents were the most-administered drugs for prophylactic use in HSCT patients.

The causative organisms for the nine cases of candidiasis included *C. albicans* ($n=4$), *C. glabrata* ($n=2$), *C. tropicalis* ($n=1$), *C. inconspicua* ($n=1$), and a combination of *C. lusitaniae* and *C. guilliermondii* ($n=1$). The infected organs included blood (fungemia, six cases), the respiratory system ($n=2$) and the liver and spleen ($n=1$). Among four patients who were infected with *C. albicans*, two had not received any antifungal agents including fluconazole as a prophylaxis, while the remaining two cases had been on fluconazole prophylaxis and were considered to be prophylaxis failure. Among these four patients, three started or continued to receive fluconazole following infection, and three patients subsequently died.

The causative organisms for the 16 cases of aspergillosis included *Aspergillus* spp. ($n=14$), *A. fumigatus* ($n=1$) and *A. terreus* ($n=1$). Six and 10 of these patients received RIST and CST, respectively. In all, 14 and two patients had received prophylactic fluconazole and itraconazole, respectively. GVHD requiring corticosteroid therapy was documented at the onset of the disease in 13 of the 16 cases. The initially infected organ was the respiratory system in all cases, with a median onset of invasive aspergillosis of 85 days (range, 9–392). Four of the 16 patients were neutropenic at the diagnosis of aspergillosis. Treatment for invasive aspergillosis included intravenous AMPH-B (13 cases), oral itraconazole (two cases), and fluconazole (one case). Eight patients subsequently died without resolution of invasive aspergillosis.

Comparison of CST and RIST recipients

Table 1 shows the characteristics of fungal infections developed in CST and RIST recipients. There was no significant difference between the two groups with respect to the incidence, date of onset or death rate.

Discussion

This study highlights the current problems in antifungal prophylaxis. In Japan, a total of 1964 transplantations were performed in 2001,[28] and about half of these were surveyed in this study. We believe that sufficient data were collected to characterize prophylaxis practice and fungal infection in this country, while it is limited by bias as a retrospective design, and we should be careful in interpreting the results of this study.

With improvements in prophylactic measures, the incidence of early mold infections has decreased,[14,29,30] and most infections occur late in association with GVHD.[13] The need for a HEPA filter or laminar air flow (LAF) may be questioned in RIST. All transplant wards in Japan, but not all nontransplant wards, are equipped with these systems. Although 99% of CST was performed in transplant wards, this percentage decreased to 71% in autografts and 66% in RIST. The potential economic benefits of RIST may be related to an increase in RIST performed in nontransplant wards without expensive air filtration systems.

In autologous HSCT, the prophylactic use of antifungal agents is recommended only for high-risk patients[3] based on several clinical studies.[4,31] However, this study demonstrates that all of the Japanese autologous HSCT recipients received some prophylactic antifungal agents. This is an overuse of antifungal agents because the overall risk of fungal infection in autologous transplants is lower than allogeneic transplant. There are several possible explanations for this overuse. Since the definition of 'low-risk patients' in the CDC guidelines is unclear, physicians take a more conservative way to reduce the risk of fungal infection. It has been suggested that long-term use of azole antifungal agents may cause resistance.[32] Additionally, azole antifungal agents are expensive, and long-term use is not economical.[33] On the other hand, for the prophylactic use of antifungal agents in allogeneic transplant, the CDC recommends prophylactic fluconazole 400 mg/day during the neutropenic period following transplantation. However, the rationale for the selection of fluconazole from among many other available antifungal agents, its dose setting and prophylaxis period remain unclear. Its usefulness in RIST recipients has not been established.

After the CDC issued its guidelines in 2000, various drugs, including voriconazole, itraconazole (oral solution and intravenous formulation), and echinocandin, have been developed and are now commercially available. Moreover, *Aspergillus* is not susceptible to fluconazole, and recent comparative studies suggest that itraconazole is more effective than fluconazole in the prevention of *Aspergillus* infection.[15,34] Hence, the recommendation of fluconazole over any other agent may no longer be defensible. Nevertheless, this study clearly documented that fluconazole was used in 75% of allogeneic transplant in Japan. This bias is likely due to the CDC recommendation and to the fact that an alternative antifungal agent is not commercially available in Japan. As of June 2003, the only available itraconazole formula in Japan is a capsule, which carries an obvious risk of inadequate

Table 1 Comparison on clinical features of deep fungal infection between CST and RIST

		CST ($n=414$)	*RIST ($n=154$)*
Number of deep fungal infection		26 (6.3%)	11 (7.1%)
Median onset (days after transplant) (range)		77 (1–392)	117 (28–182)
Presence of GVHD at the onset of fungal infection	Present/absent	12/14	9/2
Presence of neutropenia at the onset of fugal infection[a]	Present/absent	8/18	1/10
Use of corticosteroid at the onset of fungal infection	Yes/no	15/11	7/4
Causative organisms	Aspergillus/Candida/Others	17/7/2	9/1/1
Mortality		81%	82%

[a]Neutropenia was defined as neutrophils below 0.5×10^9/l.

absorption of the drug. Voriconazole and caspofungin have not yet been approved, and micafungin was approved only recently. Regarding *Aspergillus* infections, it is difficult to conclude that the CDC's recommendation for the prophylactic administration of fluconazole is useful. With the present availability of all of these alternative agents, a comparative study to identify a suitable procedure will be required.

While all of the patients received prophylactic fluconazole at least until engraftment, only 44% received the recommended dose of 400 mg/day. Moreover, only 20% of both the CST and RIST recipients received fluconazole beyond 75 days following transplantation, as recommended in a previous study.[6] It has been reported that *C. albicans* can be controlled at a lower dose of 200 mg/day.[35,36] Many physicians believe that 400 mg of fluconazole is not required for prophylactic use, and optimal duration of fluconazole prophylaxis remains to be established. Since fluconazole is expensive and costs about 100 000 yen ($850) when used at 400 mg/day to cover from the commencement of pre-transplant treatment and engraftment, validation of the adequate dose and the duration for prophylactic use is important.

With an increasing number of patients undergoing transplant, establishment of fungal management is important in RIST. The practice for the prevention and treatment of fungal infection varies among institutions. Mortality of invasive aspergillosis was 50% in this survey, which were far lower than reported previously.[1] The differences might be attributable to diagnostic approaches. In Japan, diagnostic measures using computed tomography and blood tests such as beta-D-glucan assay or an enzyme-linked immunosorbent test detecting galactomannan antigen are widely used.[37–39] These tests might have contributed to make an early diagnosis of aspergillosis, improving its prognosis. These situations are similar to antifungal prophylaxis. The guidelines for antifungal prophylaxis, which were prepared based on previous clinical studies, should be updated, since the circumstances surrounding transplantation have been changing. However, there are little data to make new recommendations for guidelines of antifungal prophylaxis, and more information is needed regarding fungal infections following RIST. Further investigation is needed to determine what measures are effective to accommodate the changes in transplant practices.

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Appendix

This study was conducted at the following institutions under the auspices of the following investigators in Japan:

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□ CASE REPORT □

Rapidly Progressed Secondary Amyloidosis in a Patient with Mixed Connective Tissue Disease

Hirotaka KIMURA, Atsushi KOMATSUDA*, Ken-ichi SAWADA*, Akio MIMORI**, Satoshi BABA*** and Seiji MINOTA****

Abstract

A 39-year-old woman with mixed connective tissue disease suddenly developed repeated watery diarrhea two years after the onset of the disease. A colonic biopsy specimen revealed amyloid A protein deposition and the diagnosis of secondary amyloidosis was established. The amyloid deposition disappeared after the 8-month course of the treatment with prednisolone and azathioprine. Molecular genetic analysis showed the presence of the γ-allele in her serum amyloid A protein 1 gene. This might be associated with the early onset and progression of secondary amyloidosis in our case, just like cases reported in rheumatoid arthritis.
(Internal Medicine 43: 878–882, 2004)

Key words: MCTD, secondary amyloidosis, serum amyloid A gene, γ-allele

Introduction

Mixed connective tissue disease (MCTD) was first described by Sharp and colleagues in 1972. It is an overlapping disease, including symptoms and signs of systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and polymyositis, with a high titer of autoantibodies against a ribonuclease-sensitive extractable nuclear antigen (now called anti-U1-RNP antibody) (1). The inflammation which can cause secondary amyloidosis (SA) in this disease such as arthritis, lymphadenitis and myositis usually tends to occur in the early stage of the disease, and to decline gradually in cases with long disease duration (2). However, to our knowledge, there are few reports of patients with SA due to MCTD.

Several reports show that the presence of the γ-allele in the serum amyloid A protein (SAA) 1 gene is a risk factor for SA in patients with rheumatoid arthritis (RA) (3–5). Here, we report a case with MCTD who developed SA in a very short time after the onset of the disease with the γ-allele in the SAA1 gene. We also discuss the association between the early onset of SA and allelic variants of the SAA 1 gene in our patient.

Case Report

A 39-year-old woman presented with intermittent fever, symmetrical polyarthralgia lasting all day, and Raynaud's phenomenon accompanied by swelling of her digits and hand in April 2000. Each of her digits was swollen as a whole like a sausage but there were no pulsions located in her wrists, metacarpophalangeal, or proximal interphalangeal joints. The dorsal aspects of both hands were swollen as well. Her knees had mild soft tissue swelling though predominantly in the right hand side. The hand and wrist radiographs showed no bony erosions or remarked decalcification adjacent to the joints. Although she was suspected of having rheumatic disease of some sort, the classification criteria for RA (6) was not fulfilled. She was treated with nonsteroidal anti-inflammatory drugs (NSAIDs) in a local clinic; a favorable response was not achieved. Thus, she was referred to our hospital for further evaluation. Nothing particular was found in her past history.

In spite of the treatment with a varying combination of NSAIDs, bucillamine, and low dose prednisolone, poly-

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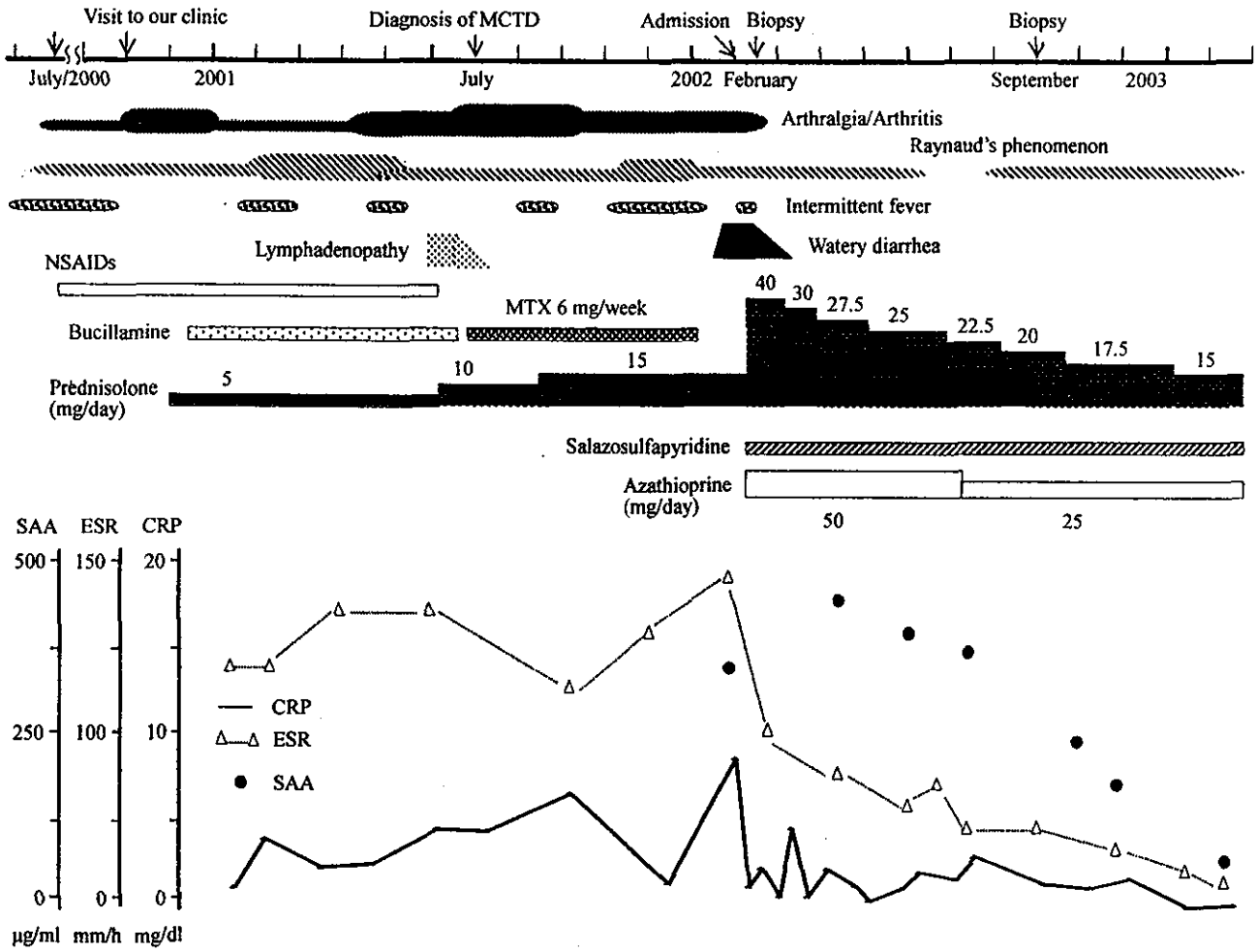

Figure 1. Clinical course of the present case. NSAIDs: nonsteroidal anti-inflammatory drugs, MTX: methotrexate.

arthralgia persisted with an increased level of serum C-reactive protein (CRP). In July 2001, she noticed swelling of the neck lymph nodes with mild tenderness and simultaneously complained of persistent finger-stiffness without atrophy of finger pad. Sense of stiffness lasted all day long without relief. Sclerosis of the skin was limited to her fingers and the internal organ was completely spared, excluding the diagnosis of SSc. Laboratory data showed the following: slightly increased rheumatoid factor; positive rheumatoid factor to degalactosylated IgG; positive anti-nuclear antibody (ANA) with a titer of 1 : 2,048; and positive anti-U1-RNP antibody with a titer higher than 1 : 128. The following symptoms and signs fulfilled the Japanese criteria for MCTD (7): Raynaud's phenomenon, swollen digits and dorsal aspects of hands, the high titer of anti-U1-RNP antibody, polyarthritis, lymphadenopathy and sclerodactyly as the partial findings of SLE and SSc, respectively. Thus, she was diagnosed with MCTD. She received oral prednisolone at a daily dose of 10 to 15 mg as well as a low dose of methotrexate (8 mg a week) from July 2001 (Fig. 1).

She was admitted to our hospital because of frequent watery diarrhea without abdominal pain, lasting more than a month, on January 31, 2002. Her body temperature was 37.8 °C; respiratory rate, 16 breaths/min; blood pressure, 112/68 mmHg; pulse rate, 84 beats/min. A physical examination showed hand edema and sausage-like edema in her whole digits. Raynaud's phenomenon and sclerodactyly were also present. There were no swollen joints, especially on her digits or hands at this time at all, except for her bilateral knees. Elastic soft lymph nodes were palpated on her neck. Examinations of the lungs and heart were normal. The bowel sound was moderately increased without tenderness, and hepatosplenomegaly was absent. Results of muscle and neurological examinations were normal. Her laboratory data were as follows: erythrocyte sedimentation rate, 68 mm/h; CRP, 12.0 mg/dl; leukocyte count, 7,200/μl (neutrophils 65%, eosinophils 2%, monocytes 9%, lymphocytes 24%); erythrocyte count, 452×10^4/μl; hemoglobin, 10.4 g/dl; hematocrit 33.4%; and platelet count 56.4×10^4/μl. The serum total protein was 6.2 g/dl, with serum albumin 3.4 g/dl.

Figure 2. Light microscopic examination of biopsied colonic mucosa at the onset of diarrhea. A: Homogeneous deposits are seen in the parenchyma and walls of vessels (HE stain, ×100). B: Congo-red positive homogeneous deposits are present in the parenchyma and walls of vessels (arrows) (Congo-red stain, ×100). C: Application of potassium permanganate to a Congo-red stained section abolished the staining, indicating that the deposited material is amyloid A protein (Congo-red stain, ×100).

Blood urea nitrogen was 9.9 mg/dl, and serum creatinine was 0.4 mg/dl. Transaminases were normal, with lactate dehydrogenase 144 IU/*l*, creatine kinase 10 IU/*l*, ferritin 118 ng/ml, and serum amyloid A protein (SAA) 255 μg/ml (normal range, less than 8 μg/ml). Although urinalysis showed no abnormal findings, stool occult blood was positive. Serological studies revealed that the titer of ANA was higher than 1 : 2,048, with a speckled pattern under immunofluorescence. Anti-U1-RNP antibody was positive and its titer was higher than 1 : 128. Anti-double-stranded DNA, anti-Scl-70, anti-SS-A (Ro), anti-SS-B (La) antibodies and rheumatoid factor were all negative. Myeloperoxidase-antineutrophil cytoplasmic antibody was also negative. Serum complement and circulating immune complexes were not detected. Ultrasonic cardiography showed no findings suggestive of pulmonary hypertension or myocardial amyloidosis. Repeated cultures of the stool ruled out an infection due to bacteria or fungi. Cytomegalovirus antigenemia was also negative. Colonic endoscopy showed erosive mucosa and small ulcers were distributed from the transverse to the sigmoid colon. Although the lesions involved a part of the descending colon diffusely, resembling ulcerative colitis, the rest of the colon had similar lesions irregularly scattered. Endoscopically biopsied specimens revealed Congo-red positive homogeneous deposition and mild mononuclear cell infiltration in the parenchyma without cryptic abscess and goblet cell depletion (Fig. 2B). Application of potassium permanganate to Congo-red stained sections abolished the staining (Fig. 2C), indicating that the stained material was amyloid A protein. Immunohistochemistry showed positive staining for amyloid A protein (Fig. 3A). Therefore, she was diagnosed with SA due to MCTD.

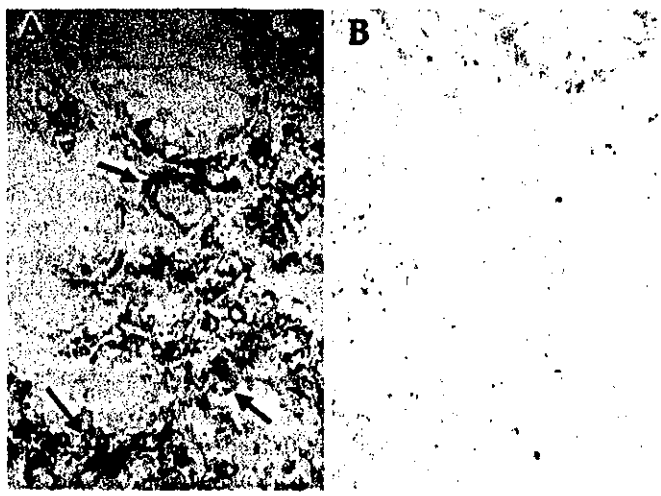

Figure 3. A: Immunohistological study using an anti-amyloid A protein antibody shows that the deposits are positive for amyloid A protein (arrows). B: Normal control.

After treatment with prednisolone (40 mg/day), azathioprine (50 mg/day) and salazosulfapyridine (3 g/day) were initiated orally, intractable watery diarrhea waned within two weeks under the concomitant use of antidiarrheal drugs although she infrequently experienced sporadic diarrhea for a few months. Serum levels of CRP and SAA decreased gradually and were maintained within normal ranges by these treatments. Three weeks later, prednisolone was re-

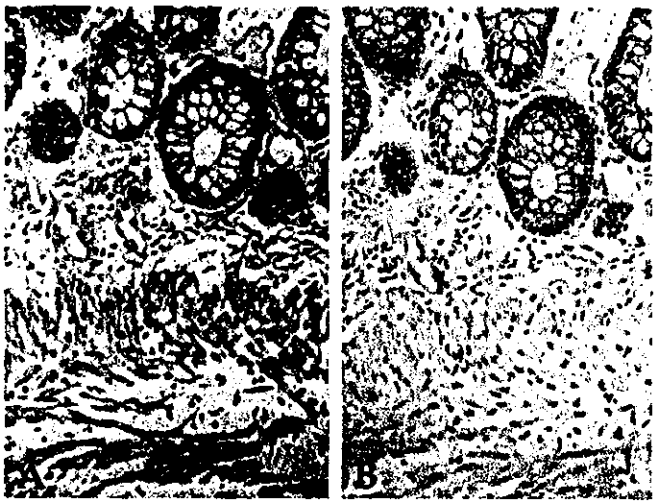

Figure 4. Light microscopic examination of repeated biopsied colonic mucosa after the 8-month treatment. A: Homogeneous deposits are scarcely identified (HE stain, ×100). B: Light microscopy shows that amyloid deposits are markedly diminished (Congo-red stain, ×100).

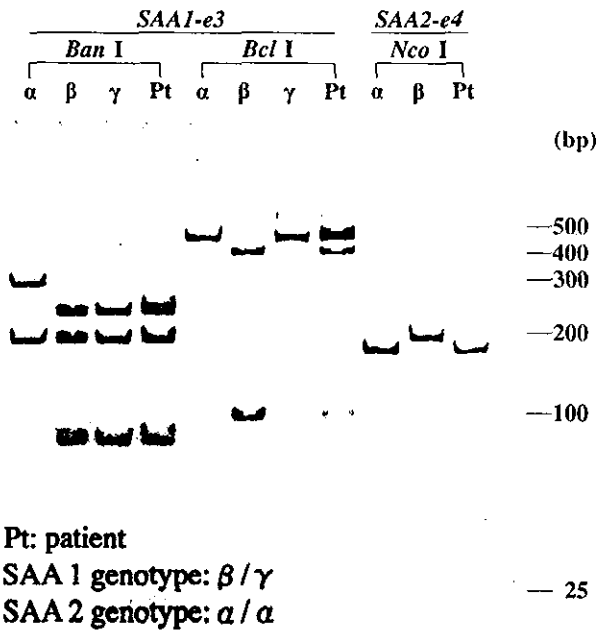

Figure 5. Genotypes of the SAA 1 and SAA 2 gene. Polymerase chain reaction amplified restriction fragment length polymorphism (PCR-RFLP) applied to amplified exon 3 for her SAA 1 gene and exon 4 for her SAA 2 gene reveals that this case has the β/γ heterozygote in the SAA1 gene and the α/α homozygote in the SAA 2 gene.

duced to 30 mg/day, and subsequently it was tapered by 5 mg at two- to four-week intervals. Azathioprine was continued at the same dose and was then reduced to 25 mg/day five months after the start of the therapy. Because repeated diarrhea did not relapse since her admission despite the tapering of the prednisolone dosage, she was discharged in March 19, 2001, and followed in our outpatient clinic. Symptoms and signs of organ dysfunction were not observed and had not developed in her stomach, heart, thyroid gland and kidneys. In November 2001, a repeated biopsy of colonic mucosa revealed a marked diminution of parenchymal amyloid deposition (Fig. 4A and B).

Molecular genetic analysis of allelic variant of SAA 1 and SAA 2 genes was performed. Their genotypes were determined by polymerase chain reaction amplified restriction fragment length polymorphism method (PCR-RFLP) using genomic DNA derived from peripheral leukocytes. The analysis revealed that her SAA 1 gene had β and γ-forms (β/γ) heterozygotes and SAA 2 gene had α/α homozygotes (Fig. 5).

Discussion

The present case developed SA within 2 years after the onset of MCTD. SA due to MCTD is quite rare and actually we could not find such a case in the literature.

Recentry, RA is the main disease causing SA (8) although chronic infectious diseases such as tuberculosis once stayed one of the top causes of SA in Japan (9). SA is a grave complication in cases with RA with a poor life prognosis (10, 11).

SAA is an acute phase protein and behaves just like CRP. It is reported that SAA is a precursor of amyloid A fibrils and persistently increased levels of SAA cause and promote SA (12, 13). However, not all cases with SA have higher levels of SAA than those without it. Furthermore, not all cases with persistent increased levels of SAA develop SA (13). Therefore, unknown factors other than the levels of SAA might contribute to the development of SA.

The SAA family consists of three members including SAA 1, SAA 2 and SAA4. SAA 1 has three forms of allelic variants; α-, β- and γ-forms. SAA 2 also has α- and β-forms. The levels of both SAA 1 and SAA 2 increase in response to inflammatory stimulation and they exhibit amyloidogenesis, except for SAA4 (3). In 1995, Baba and colleagues reported that the γ-allele of the SAA1 gene is a risk factor for SA in RA (3). A subsequent study carried out by Okuda and colleagues confirmed the higher frequency of the γ-allele of the SAA 1 gene in RA patients with SA (4). Moriguchi et al also confirmed the frequency of the γ-allele of the SAA 1 gene mainly in Japanese patients with RA (5). Molecular analysis was carried out in the present case to determine the allelic variant of the SAA 1 gene. It showed that the genotype of her SAA1 was a β/γ heterozygote and that of SAA 2 was an α/α homozygote. Thus, it is possible that γ-allele of the SAA 1 gene might have facilitated the development of SA and caused the rapid progression of SA in the present case.

Kaneko and colleagues reported a case of Still's disease

with SA which developed very rapidly (14). In that case, SA developed in 2 years after the onset of Still's disease. The patient also had the γ-allele in his SAA 1 gene. At the moment, this is the only case, to our knowledge, who was examined for the allelic variants of the SAA 1 gene other than RA. Although several reports described cases with rapidly progressive SA in the short periods of time in rheumatic diseases such as SLE (15, 16), vasculitis syndrome (17, 18) and Behçet's disease (19, 20), allelic variants of the SAA 1 gene were not pursued. In general, it takes long time, often more than 15 years, to develop SA in RA patients (11, 21, 22). On the contrary, RA patients with the γ/γ homozygotes in the SAA 1 gene have shorter durations for the development of SA (5). However, there have been very few reports describing the relationship between SA and SAA 1 alleles except for RA. Thus, it seems necessary to analyze the allelic variants of the SAA 1 gene in rheumatic diseases other than RA to confirm this relationship.

We speculate that inadequate control of inflammation of MCTD might have caused SA in our case. It is known that the presence of intractable diarrhea or chronic renal failure caused by SA usually leads to a poor prognosis in RA patients (23). However, we previously reported that treatment with steroids sufficient to suppress the inflammation successfully improved SA in two cases with RA and systemic vasculitis syndrome (24, 25). Because diarrhea was quickly ameliorated by the combination therapy with steroids and an immunosuppressant in this case, inflammation accompanying amyloid A deposition might have been contributing to diarrhea to a large extent.

It is important for physicians to prevent the development of SA in cases with rheumatic disease, even if it is a rare complication. The presence of γ-allele in SAA 1 gene might be a prediction for the development of SA, although further studies are definitely needed to confirm this.

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Case report

Rectal cancer associated with chronic lymphocytic leukemia

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It has been reported that chronic lymphocytic leukemia (CLL) often occurs concomitantly with other malignant neoplasms. However, because CLL is rare in Japan, there are only a limited number of reports of the occurrence of malignant neoplasia in Japanese patients with CLL. We report here the simultaneous occurrence of rectal cancer and CLL in a 57-year-old man. Because the clinical stage of CLL was Rai system I, we decided, in accordance with the National Cancer Institute–Sponsored Working Group guidelines, to monitor him without therapy for CLL until evidence of disease progression, and we performed abdominoperineal resection of the rectum for the cancer. The small rectal tumor was associated with aggressive lymphangiosis carcinomatosa, and multiple nodal metastases were observed in the pool of CLL cells. He died of rectal cancer 7 months after the operation, and autopsy revealed extensive metastases of the cancer. Cellular and humoral immunity is often impaired in patients with CLL, and the defective immunity in this patient may have had an etiological role in the development and rapid progression of the cancer. In the follow-up of CLL patients, we must always be aware of the possible existence of a second malignant disease. Particular attention should be paid to those with defective immunity, and screening should be performed, especially for pulmonary and gastrointestinal malignancies.

Key words: rectal cancer, chronic lymphocytic leukemia, synchronous cancer

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Introduction

Chronic lymphocytic leukemia (CLL) is a rare form of leukemia in Japan, though it is the most common form in Western countries.[1–5] Several studies from Western countries have indicated a significantly increased risk of subsequent neoplasms in patients with CLL.[6,7] Though the precise etiology remains unclear, it has been suggested that concurrent defective immunity may play a central role in the development of subsequent neoplasms.[6,7] Because of the rarity of CLL in Japan, there are only a limited number of reports of the occurrence of additional malignancy in Japanese patients with CLL. To supplement existing information on the simultaneous occurrence of CLL and other malignant neoplasms, we report here a case of rectal cancer associated with CLL.

Case report

A 57-year-old man noticed a right cervical mass in 1999. It gradually enlarged, and he noticed another mass, on the left side of his neck, in 2001. He consulted a doctor in September 2001, and physical examination revealed bilateral enlargement of the superficial cervical lymph nodes. Malignant lymphoma was suspected on biopsy, and colonoscopic examination performed for screening revealed a synchronous rectal cancer. In October he was referred to the Department of Hematology of Gunma Prefectural Cancer Center for detailed investigation.

He had undergone surgery for a herniated disk at the age of 42 years, and there was nothing of note in his family history. On presentation to our hospital, multiple enlarged nodes were palpable on both sides of his neck, in the right axilla, and in the bilateral inguinal regions. Digital examination revealed a hard tumor on the anterior wall of the rectum, 7 cm from the anal verge.

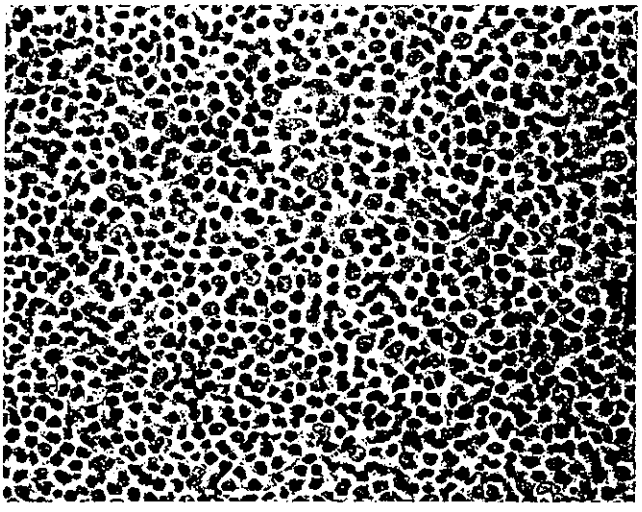

Fig. 1. Biopsy specimen, showing diffuse infiltration of mature lymphocytes. H&E, ×400

Re-examination of the biopsy specimen revealed a picture consistent with CLL (Fig. 1). Laboratory findings showed a hemoglobin concentration of 13.9 g/dl, white cell count of 15.8 × 10⁹/l with 79.0% lymphocytes; platelet count of 154 × 10⁹/l; IgG, 582.5 mg/dl (normal range [NR], 800.0–1800.0 mg/dl); IgA, 73.5 mg/dl (NR, 90.0–450.0 mg/dl); and IgM, 12.9 mg/dl (NR, 60.0–250.0 mg/dl). A bone marrow aspirate smear showed that 76.4% of all nucleated cells were lymphocytes. The predominant population of lymphocytes shared B-cell markers (CD19 and CD20) with the CD5 antigen, and the B cells were monoclonal with regard to the expression of IgM, κ. These data met the diagnostic criteria proposed by the National Cancer Institute–Sponsored Working Group (NCI-WG).[8] Barium enema showed a rectal tumor on the anterior wall of the middle of the rectum, with thickened folds and large nodules around it (Fig. 2). Colonoscopic examination revealed an ulcerated rectal tumor 7 cm from the anal verge (which was adenocarcinoma histologically) and also revealed large nodules with a smooth surface around the tumor (Fig. 3). Computed tomography (CT) scans of the neck, chest, and abdomen revealed enlarged nodes in the perirectal and right obturator spaces, around the aorta, behind the portal vein, in the mediastinum, and in the superficial and deep cervical regions. There was no sign suggestive of distant metastasis of cancer cells. These findings indicated that the clinical stage of CLL was Rai system I and Binet system B. We decided, in accordance with the NCI-WG guidelines,[8] to monitor him without therapy for CLL until evidence of disease progression, and we performed abdominoperineal resection of the rectum for the rectal cancer in November. The surgical specimen showed an ulcerated rectal tumor with enlarged folds and nodules caused by lymphangiosis

Fig. 2. Barium enema (lateral view), showing a middle rectal tumor (*arrow*) on the anterior wall, with thickened folds and large nodules around it

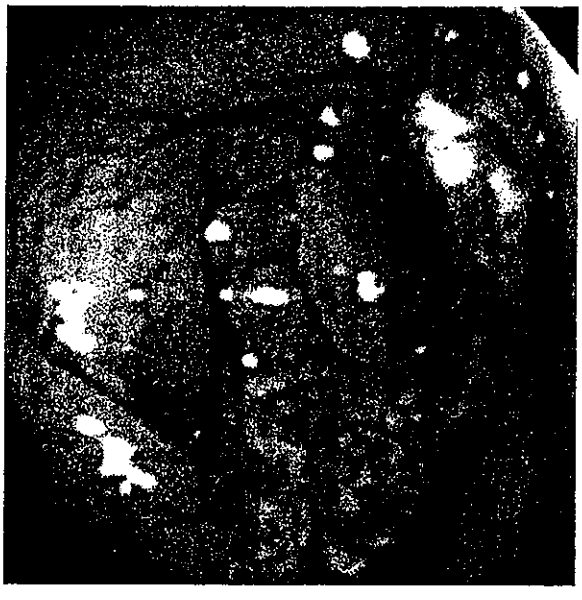

Fig. 3. Colonoscopic examination, showing an ulcerated rectal tumor and nodular change of the mucosa with smooth surface

carcinomatosa (Fig. 4). A total of 27 nodes were detected in the specimen, and diffuse invasion of CLL cells was observed in all of them. Metastatic carcinoma nests were observed in 19 nodes, in the pool of CLL cells. Two enlarged obturator nodes also included carcinoma

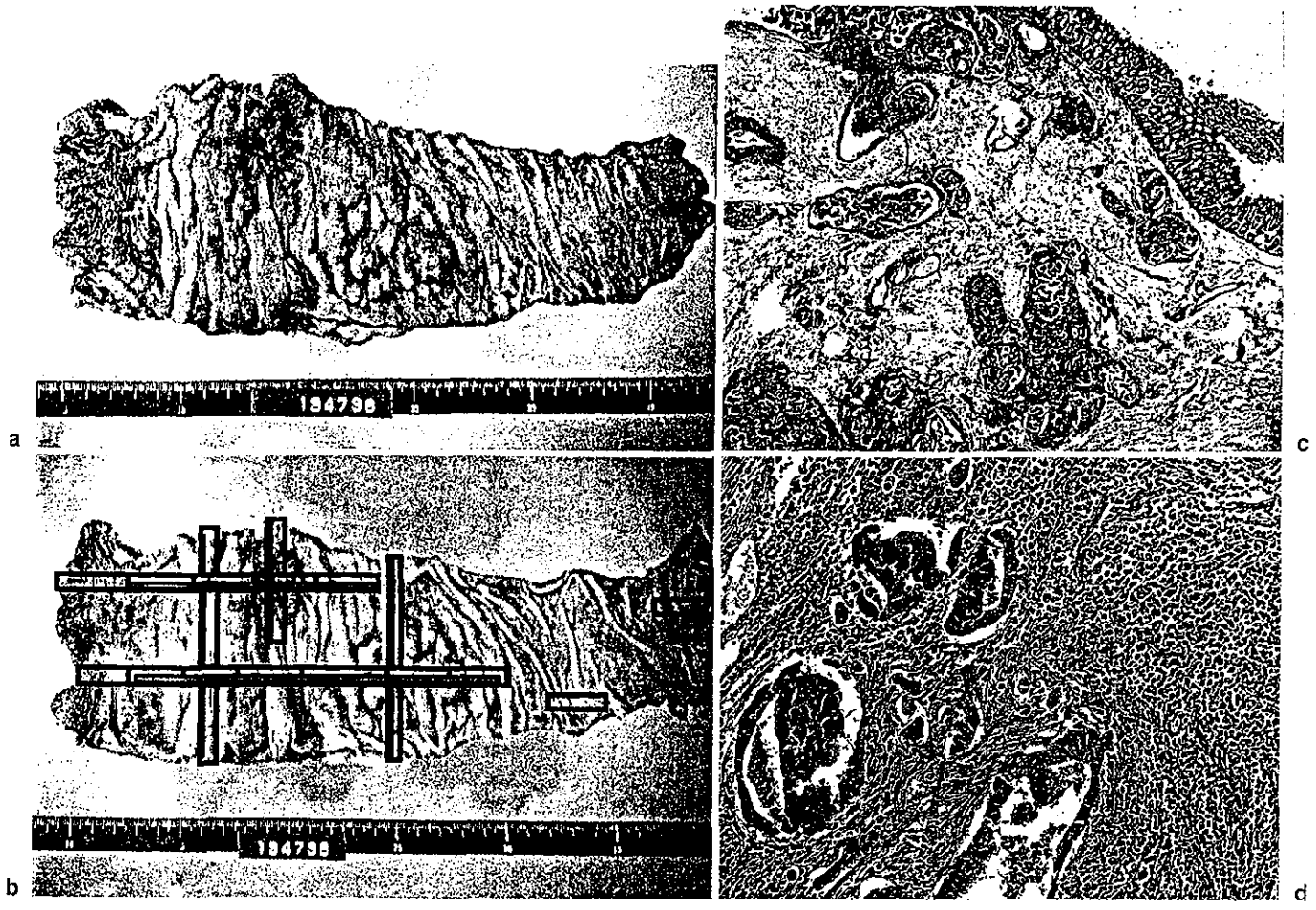

Fig. 4. **a** Macroscopic view of specimen removed at surgery. *Arrow* indicates an ulcerated rectal tumor. **b** Scheme showing infiltration of cancer cells and leukemia cells. The range of infiltration of both cell types is indicated by *gray bars*. **c** Microscopic view of submucosal layer of a thickened fold. Cancer cells have infiltrated laterally and vertically, by way of lymphatics. They appear to float in the expanded lymph vessels or block them. The submucosal layer is markedly edematous because of congestion with lymph. **d** Chronic lymphocytic leukemia (CLL) cells appear to gather around cancer cells in the subserosal layer. **c** H&E, ×12.5; **d** H&E, ×100

nests in the large pool of CLL cells. Adjuvant chemotherapy was administered, using fluorouracil and leucovorin.

It was interesting that all of the enlarged superficial nodes were markedly decreased in size after the operation. However, the patient developed difficulty in breathing because of right pleural effusion, and cytological examination of the effusion revealed numerous CLL cells. Low-dose cyclophosphamide (50 mg per day) and prednisolone (5 mg per day) were administered, and he had no complaints until May 2002, when he was readmitted with shortness of breath. Chest X-ray revealed bilateral pleural effusions and reticular shadows on the whole lung fields. Cytological examination of the effusion revealed numerous cancer cells, and he died 1 month later. Autopsy revealed bilateral pleuritis carcinomatosa with cancer metastases to the bilateral lungs, thyroid, liver, and right adrenal gland. Diffuse infiltration of cancer cells was observed in the cervical, mediastinal, and abdominal lymph nodes, and small numbers of CLL cells were also found in these nodes peripherally.

Discussion

CLL is a rare form of leukemia in Japan, though it is the most common form in Western countries.[1–5] It accounted for 3% of all leukemias according to a Japanese nationwide survey in 1978.[1] A recent study reported that is comprised 6%, of all leukemias, suggesting an increase, partly because of the detection of asymptomatic patients by nationwide regular health check-ups.[2] The annual incidence of CLL was estimated to be 0.27–0.48 per 100000 in Japan,[2,3] one-tenth to one-fifth of that in Western countries, where CLL accounts for 30% of

all leukemias, and the incidence was estimated to be 2.7 per 100000.[4,5]

CLL occasionally occurs concomitantly with other malignant neoplasms, and previous reports have indicated a modest but significant increase in the risk of subsequent neoplasms in CLL patients.[6,7] In a large study including 4869 patients with CLL diagnosed from 1935 through 1971, subsequent neoplasms were observed in 4.8% of them, and the relative risk (RR) was estimated to be 1.1, with a 95% confidence interval (CI) of 1.0–1.3.[6] In a recent study including 16367 CLL patients registered between 1973 and 1996, subsequent neoplasms were observed in 11.1% of them (RR, 1.20; 95% CI, 1.15–1.26).[7] Though significant excesses were found in both studies, particularly for sarcomas, including Kaposi sarcoma (RR, 5.3 and 5.09, respectively) and malignant melanoma (RR, 6.7 and 3.18, respectively), the most common neoplasms with an elevated RR were cancers of the lung (RR, 1.5 and 1.66, respectively), colon (RR, 1.0 and 1.13, respectively), and rectum (RR, 1.6 and 1.09, respectively).[6,7] In Japan, cancers were observed in 5.2% of CLL patients, and the most common neoplasm was gastric cancer, followed by cancers of the lung and large bowel.[9,10] Only 30 cases of malignant neoplasms associated with CLL have been reported in Japan, including 4 cases of colorectal cancer; 3 of sigmoid colon cancer, and 1 of rectal cancer.[11,12] This is the second reported case of rectal cancer associated with CLL.

Though the precise etiology of the development of additional malignancy is not clear, it has been suggested that defective immunity in CLL patients may have an etiological role,[6,7] from the observation that a similar array of tumors, particularly lung cancer and sarcomas, develop following therapeutic immunosuppression in transplant recipients.[13] Though CLL is a B-cell proliferation in most cases,[2,14] patients with CLL often display defective cellular immunity.[15–19] The CD5-positive neoplastic B cells often show decreased expression, not only of immunoglobulins but also of important glycoproteins on the cell surface. Specifically, qualitative or quantitative decreases in major histocompatibility complex molecules or critical activation ligands such as CD80 on the neoplastic B cells directly impairs their ability to function as antigen-presenting cells, resulting in their inability to provide the essential costimulatory signals required for T-cell activation.[15,16] In addition, several cytoskeletal abnormalities in neoplastic B cells not only cause reduced antigen presentation but also prevent effective B-cell-T-cell conjugate formation.[17] Reduced antigen presentation by neoplastic B cells, as well as defective B-cell-T cell interaction, results in T-cell anergy. Furthermore, because natural killer (NK) cells mediate cell killing by antibody-dependent cell-mediated cytotoxicity (ADCC), the opportunity for ADCC to occur in vivo is greatly reduced in CLL patients with hypogammaglobulinemia, which can alter the potential benefits of NK cells in the host's control of tumor growth.[18,19] Thus, B-cell CLL is often accompanied by a multitude of immune abnormalities. Though we did not examine cellular immunity, concurrent hypogammaglobulinemia in our patient may have caused dysfunction of T cells and NK cells, and this, in turn, could have been associated with the development of the cancer. It has been demonstrated that CLL patients with an additional malignancy more often show low immunoglobulin levels than those patients without it,[20] and that T-cell abnormality is more prominent in CLL patients with hypogammaglobulinemia.[21]

As the present patient showed an aggressive course of rectal cancer, the defective immunity may also have been associated with the rapid progression of the second malignancy. It has been shown, in mice, that highly immunogenic tumors exhibit more aggressive behavior in the presence of immunosuppression.[22] While Greene et al.[6] reported clustering of lung cancer and sarcoma among CLL patients who died early in the course of their disease,[6] case reports show an unusually aggressive course of additional malignancies other than lung cancer and sarcoma. For example, a sigmoid colon cancer infiltrating the muscular layer in a CLL patient with a low level of IgM and depressed lymphocyte response to phytohemagglutinin showed extensive metastases to the lungs and liver.[11] Skin cancers in CLL patients are also known to be unusually aggressive.[23,24]

The reason that the superficial nodes in our patient decreased in size after operation is not clear. One possible explanation is that cytokines such as granulocyte colony-stimulating factor might have been produced in the tumor as a paraneoplastic syndrome.[25]

Because CLL patients have an increased risk of additional malignancy, physicians who deal with them must always be aware of the possible existence of a second malignant disease. Particular attention should be paid to those with defective immunity, and screening should be performed, especially for pulmonary and gastrointestinal malignancies.

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