INTENSIFIED-DOSE (4 gm/m²) CYCLOPHOSPHAMIDE AND GRANULOCYTE COLONY-STIMULATING FACTOR ADMINISTRATION FOR HEMATOPOIETIC STEM CELL MOBILIZATION IN REFRACTORY RHEUMATOID ARTHRITIS

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Objective. To evaluate the feasibility, safety, and efficacy of intensified-dose cyclophosphamide (ID-CYC), followed by granulocyte colony-stimulating factor (G-CSF) administration for collection of peripheral blood hematopoietic stem cells (HSC), for patients with severe, refractory rheumatoid arthritis (RA).

Methods. Four patients with severe refractory RA were enrolled in this open study. They received a single infusion of CYC (4 gm/m²) at day 0 followed by G-CSF (5 µg/kg/day) from day 6 until the last day of leukapheresis (performed at the time of hematopoietic recovery) to harvest peripheral blood HSC. Patients were monitored for disease activity, adverse effects, and hematopoietic reconstitution following this procedure.

Results. For all patients, administration of ID-CYC induced an early, dramatic improvement of disease activity. Long-term followup indicates that partial disease relapse was observed for all patients. No adverse effect was directly attributable to the treatment procedure. For most patients, HSC collection was sufficient to provide a graft enriched in CD34+ cells by positive selection as well as an unselected rescue graft.

Conclusion. Patients with severe, refractory RA can benefit from ID-CYC. This procedure, followed by G-CSF administration, appears safe and technically suitable. In addition, it allows immediate improvement of RA activity that can occasionally persist beyond 6 months.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown origin affecting primarily the synovial tissue. The immune system, including T cells, probably plays a role in this disorder, based on findings from animal models, the predisposing role of specific HLA–DRB1 alleles, and the efficacy of immunosuppressive drugs such as cyclosporin A (CSA). The natural disease course of RA ranges from mild and limited with benign consequences to severe and progressive with polyarticular destruction and extraarticular manifestations that can lead to death. Although it is difficult to predict which patient will have the most severe disease course, highly active RA that is refractory to conventional therapy may justify early, aggressive therapies.

Recently, hematopoietic stem cell (HSC) transplantation has been proposed as a therapeutic option to be explored in severe autoimmune diseases, including RA (1). The rationale for such treatment is based on data from experimental animal studies and also on anecdotal reports of findings in humans. In adjuvant arthritis, considered to be a model for RA, disease was cured by bone marrow transplantation (2). Furthermore, several RA patients who underwent allogeneic bone marrow transplantation for aplastic anemia have remained free of disease for years (3), and long-term remission of RA has been reported after autologous stem cell transplantation (ASCT) for concomitant hematologic malignancy (4). The mechanism of efficacy of stem cell transplantation in RA is unknown. However, one can speculate that the intensive immunosuppressive conditioning used to prepare transplantation eliminates putative autoaggressive immune cells, or that the proce-
procedure as a whole contributes to resetting the immune system, allowing tolerance toward autoantigens to be restored (1).

ASCT has a procedure-related mortality rate of <5% and may be an appealing solution for the treatment of severe, refractory RA (1). However, there is limited information about its feasibility, safety, and efficacy for this indication. Harvesting of hematopoietic progenitor cells is required before ASCT. Mobilization of peripheral blood progenitor cells with granulocyte colony-stimulating factor (G-CSF) alone was shown to be both effective and safe in treatment-resistant, active RA (5,6). An alternative to this approach is to induce profound immunosuppression with a chemotherapeutic agent before HSC collection. In this study, we investigated the feasibility, safety, and efficacy of intensified-dose cyclophosphamide (ID-CYC), followed by G-CSF administration for HSC collection, in severe, refractory RA.

PATIENTS AND METHODS

Patients. Four patients with RA who fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria (7) were enrolled in this open study after being advised about expectations and risks of the procedure and providing informed consent. Their mean age was 48 years (range 32–62 years). The mean duration of RA was 7 years (range 1.5–16 years). All were seropositive for rheumatoid factor (RF), had erosive arthropathy, and were in Steinbrocker functional class III or IV. All patients had severe RA at the time of ID-CYC administration, with a Disease Activity Score (DAS) (8) of >3.7 (Figure 1). In addition, patient 1 had bilateral anterior and posterior nodular scleritis causing marked decrease in vision, and patient 2 had vasculitis manifested by cutaneous purpura, nailfold infarcts, glomerulonephritis (GN), and decreased C4 complement fraction serum level.

All patients had previously been treated unsuccessfully with each of the following disease-modifying antirheumatic drugs (DMARDs): hydroxychloroquine (HCQ), intramuscular gold salts, methotrexate, sulfasalazine, D-penicillamine or tiopronin, and CSA. Each had received multiple courses of corticosteroids that were insufficiently effective. In addition, the first 2 patients had each received 4 monthly pulses of 600 mg/m² of CYC, the third patient had received a combination of azathioprine (AZA) (2 mg/kg) and HCQ, and the fourth patient had received anti-CD4 monoclonal antibody (mAb) therapy, all without clinical effect. Only patient 3 was receiving DMARD therapy at the time of inclusion (i.e., AZA and HCQ). AZA was interrupted for this patient before ID-CYC administration, but the patient continued taking HCQ throughout the study. Other treatments for RA were limited to 10 mg/day of oral prednisone, local corticosteroid injections, nonsteroidal antiinflammatory drugs, and analgesic drugs for up to 6 months after ID-CYC administration.

Peripheral blood HSC mobilization. Patients were admitted to the intensive care unit (ICU) of the Cochin Hospital hematology department and were closely monitored in a sterile room until the end of aplasia. Evaluation of cardiac, pulmonary, and renal functions and an extensive search for occult bacterial or viral infection were performed before treatment. A single infusion of CYC (4.0 gm/m²) was administered on day 0 through a central venous catheter. Mesna (total dose equal to the dose of CYC) was administered during and after ID-CYC treatment for a total of 12 hours. Recombinant human G-CSF (Lenograstim: Rhône Poulenc Rorer, Montrouge, France) (5 µg/kg/day) was administered subcutaneously from day 6 until the last day of leukapheresis. Prophylactic antibiotics were used during aplasia, and trimethoprim/sulfamethoxazole was administered up to 6 months after discharge.

The number of peripheral blood CD34+ cells (hematopoietic stem and progenitor cells) was monitored by flow cytometry, as previously described (9). Peripheral blood HSC were harvested by leukapheresis using a Cobe Spectra Cell Separator (Cobe, Denver, CO) as soon as the white blood cell count reached 1.0 × 10⁹/liter and the count of CD34+ peripheral blood cells was >10 × 10⁹/liter. Leukapheresis was performed daily for 1–3 days until the number of harvested
progenitor cells reached $7.0 \times 10^6 \text{CD34}^+ \text{cells/kg body weight}$, to yield a minimum of $2.5 \times 10^6 \text{CD34}^+ \text{cells/kg after positive selection}$. For potential future ASCT, HSC were stored after positive selection for CD34$^+$ cells with an Isolex-300 Cell Separator (Baxter Healthcare, Deerfield, IL). In addition, an unselected graft that contained a minimum of $2.5 \times 10^6 \text{CD34}^+ \text{cells/kg was spared for rescue}$. The actual proportion of CD34$^+$ cells in the graft was determined by flow cytometry. The number of granulocyte–macrophage colony-forming units (GM-CFU)/kg contained in the graft was assessed by clonogenic progenitor assay as follows: $10^6$ cells from CD34$^+$-selected graft or $10^5$ cells from unselected graft were seeded in 1 ml of ready-to-use methylcellulose medium HC 4431 (StemCell Technologies, Vancouver, British Columbia, Canada) in triplicate; GM-CFU colonies were numbered at day 14 using an inverted microscope.

Clinical monitoring. Disease activity was repeatedly assessed by the same trained research nurse, who collected clinical and laboratory data including the Ritchie Articular Index (10), the number of swollen joints (44-joint count), a French equivalent of the Stanford Health Assessment Questionnaire (11), patient’s assessment of pain by 100-mm visual analog scale (VAS), patient’s and assessor’s global health assessment (each by 100-mm VAS), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) level.

Flow cytometric detection of cell surface antigen. Single-, double-, or triple-staining was performed using the following mouse anti-human mAb: phycoerythrin (PE)–labeled anti-CD34 (Becton Dickinson, Le Pont de Claix, France), PE–Texas Red-x–labeled anti-CD3 (Coulter, Marseille, France), PE–labeled anti-CD4 (Coulter), PE–labeled anti-CD8 (Coulter), fluorescein isothiocyanate (FITC)–labeled anti-CD7 (Becton Dickinson), FITC–labeled anti-CD5 (Immunotech, Marseille, France), PE–labeled anti-CD34R (Immunotech), and FITC–labeled anti-CD45RA (Immunotech). Cells were analyzed using an EPICS XL flow cytometer (Coulter, Miami, FL).

RESULTS

Tolerance of ID-CYC and G-CSF administration. Patients were hospitalized in the ICU for 13–20 days. The whole treatment procedure was uneventful for 3 of 4 patients, and, notably, no flare of arthritis was observed during or after administration of G-CSF. Patient 2 had cutaneous and renal vasculitis at the time of ID-CYC. A sudden loss of hematocrit was noticed 3 days after ID-CYC for this patient, and this was associated with hemoptysis, shortness of breath, hypoxemia, and bilateral lung infiltrates seen on chest radiograph. Bronchoscopic examination was performed with alveolar lavage, which revealed a diffuse, hemorrhagic bronchus but no intraalveolar hemorrhage. Thoracic microbial investigations yielded negative findings. All symptoms rapidly regressed within a few days. This episode was most likely related to a flare of the lung vasculitis this patient had initially presented with in association with GN and RA.

Subsequent followup ranged from 43 weeks (patients 1 and 3) to 2 years (patient 2). No adverse effects directly related to the treatment were noticed during this period. Patient 1 had a femoral neck fracture 13 weeks after ID-CYC. This was complicated by a sacral scab and required several surgical procedures. The patient died 11 months after ID-CYC from a staphylococcal septicemia secondary to local infection of a great toe.

Effect of ID-CYC and G-CSF administration on disease course. Figure 1 summarizes the time course of the synovitis (joint) count and the DAS for all patients following ID-CYC administration. Administration of ID-CYC and G-CSF was quickly followed by a dramatic clinical improvement of RA activity. Within the first month, 1 patient had an ACR 70% response (12), 2 others had ACR 50% responses, and the last patient had an ACR 20% response. This beneficial effect was noticeable for arthritis (Figure 1) and extraarticular manifestations. Indeed, scleritis and vision both improved for patient 1. The scleritis remained stable and, apparently, inactive throughout followup. For patient 2 (who had skin, renal, and, presumably, lung vasculitis flares at the time of ID-CYC), skin lesions scarred, hematuria disappeared, complement level normalized within 2 months after ID-CYC, and there was no vasculitis relapse within the next 2 years of followup.

After initial improvement, a clear relapse of arthritis was observed for all patients, reaching a peak 4–6 months after ID-CYC. Thereafter, patient 1's disease progressively remitted without additional treatment. For the 3 other patients, RA activity persisted and tended to increase with time but never reached pretreatment intensity levels, even 2 years after the procedure (patient 2) (data not shown).

The course of systemic inflammation assessed by ESR and serum CRP level was quite variable, neither mirroring the clinical pattern precisely nor showing consistent improvement at any time point (not shown). A consistent, but transient, decrease in RF level was noted during the first month after ID-CYC (not shown).

Efficiency of HSC collection. Leukapheresis was started 10–14 days after ID-CYC. Results of HSC collection are summarized in Table 1. In 3 of 4 patients, sufficient numbers of CD34$^+$ cells were obtained with 2 cycles of leukapheresis to provide both a CD34$^+$-selected and an unselected graft. After positive selection, the proportion of CD34$^+$ cells averaged 97% for those patients (Table 1), corresponding to a 3-log depletion of T cells (Marolleau J-P: unpublished results). For
For all patients, ID-CYC induced short remission, and only an unselected graft could be stored. The yield of CD34+ cells from this patient was presumably related to prior AZA treatment that had been maintained until the time ID-CYC was administered.

### Hematologic and lymphoid reconstitution following ID-CYC

For all patients, ID-CYC induced short aplasia, defined by a peripheral blood neutrophil count of \(<0.5 \times 10^9/\text{liter}\) lasting 4–6 days. The platelet count was reduced to a minimum of 32–195 \(\times 10^9/\text{liter}\). Only patient 2 required transfusion because of the hemorrhagic anemia described above. Thereafter, the peripheral blood white cell count, hemoglobin level, and platelet count normalized and remained stable throughout followup (not shown).

Flow cytometry was used for patients 2–4 to study variations in the total number of circulating T lymphocytes and in specific T cell subpopulations after ID-CYC (Figure 2). For patient 2, we observed increased levels of all T lymphocyte subsets after ID-CYC, including CD45RO+ memory and CD45RA+ naive subsets. For patients 3 and 4, levels of CD4+ T cells dropped or remained stable after ID-CYC. A particularly marked drop was noted for the CD4+,CD45RA+ subset, and this persisted up to 7 months after ID-CYC. Interestingly, variations in the CD4+,CD7− T cell subset paralleled the clinical evolution of disease for all patients tested. Levels of this subset decreased for patient 3 during disease remission, while they increased for patients 2 and 4 during clinical disease relapse (Figure 2). Total levels of CD8+ T cells increased for patient 3 and remained stable for patient 4 (e.g., the CD8+,CD45RO+ T cell subset), while levels of the CD8+,CD45RA+ subset dropped at first and thereafter increased slowly for the same 2 patients (Figure 2). No concordance was noted for CD8+,CD57+ T cell variations and clinical evolution of disease (Figure 2).

### DISCUSSION

In this study, 4 patients with severe RA refractory to conventional therapy received a single infusion of 4 \(\text{gm/m}^2\) of CYC followed by administration of G-CSF. The immunosuppressive treatment strikingly reduced disease activity. In addition, the procedure appeared safe, and appropriate mobilization of HSC was achieved with a view toward potential ASCT.

Daily administration of low-dose immunosuppressive agents such as CYC or AZA is currently used to treat patients with severe RA. However, this therapeutic option is questionable because its efficacy is inconsistent and it bears a high risk of side effects, including secondary malignancy. As a treatment alternative, monthly pulses of CYC (600 mg/m²) have reduced toxicity and may improve extraarticular manifestations of RA, but do not seem effective for arthritis. Indeed, 3 of our patients had received AZA or monthly pulses of CYC without any effect on their articular or extraarticular RA manifestations.

Increasing the one-time dosage of immunosuppressive agent to induce prolonged remission at the price of fewer long-term complications is currently proposed as an approach to treating severe, refractory RA (13,14). The level of immunosuppression needed may require ASCT, and harvesting HSC after their mobilization would be a necessary first step for the whole treatment procedure. Mobilization with G-CSF treatment alone has been validated for RA as a suitable technique for this purpose (5,6). However, adding ID-CYC before G-CSF administration may offer several advantages. It adds a first round of immunosuppressive therapy that may limit the risk of disease flare following G-CSF administration (6). It may increase the yield of HSC and thereby facilitate graft manipulation (6,13–15). It may reduce the number of mature and potentially pathogenic lymphoid cells reinfused to the patient.
Finally, it stimulates self renewal of immunocompetent cells and may contribute to resetting the immune system without any requirement for HSC reinfusion.

Our study adds significant information relevant to this discussion. We show that clinical parameters of RA activity are profoundly improved by increasing immunosuppression to a level that does not require reinfusion of autologous HSC. Indeed, scleritis and vasculitis were completely and persistently cleared for the 2 patients with these extraarticular manifestations, and all patients had an immediate, dramatic reduction of synovitis. Long-term followup was also of interest, since, after an early disease relapse, one of the patients (who also had the shortest duration of RA prior to receiving ID-CYC) had a spontaneous disease remission. A possible (although speculative) interpretation for this finding is that pathogenic cells underwent apoptosis after an early period of expansion following ID-CYC (16).

Figure 2. Evolution of T cell subsets in peripheral blood of patients 2–4 following intensified-dose cyclophosphamide (ID-CYC) (at week 0) and granulocyte colony-stimulating factor (at week 1). Peripheral blood mononuclear cells were isolated by density gradient centrifugation on a Ficoll separation medium, and numbers of circulating T cells were calculated from the lymphocyte count and the percentage of each subset, determined by flow cytometry using appropriate monoclonal antibodies.
Indeed, it is likely that after inducing an initial drop in the number of immunocompetent cells, ID-CYC stimulates their self renewal. Because T cells are suspected in the pathogenesis of RA, we examined more precisely the effects of ID-CYC on specific T cell subpopulations. The most consistent effect was a prolonged reduction in the number of circulating CD45RA+ naive T cells, while numbers of CD45RO+ memory T cells were more stable. These findings are consistent with those of postchemotherapy CD4+ T cell reconstitution studies in adult populations, and probably reflect the limited ability of the adult thymus to reconstitute naive CD45RA+ T cells, as opposed to limited peripheral expansion of primed CD45RO+ T cells (16). Interestingly, an increase in levels of the circulating CD4+,CD7− T cell subset appeared to be associated with disease relapse. This finding is remarkable, since expansion of this latter T cell subset is suspected of playing a role in RA pathogenesis (17).

No side effect was attributed to the mobilization procedure itself, indicating that it is reasonably safe and may be considered as a future outpatient procedure. In particular, hematologic reconstitution was not delayed, even though all patients had previously received multiple DMARDs. For a majority of our patients, the yield of HSC was greater than that reported after mobilization with G-CSF alone (6), which suggests that the present protocol is preferred if graft manipulation is required.

At the dose used in this study, ID-CYC was insufficient to induce long-term disease remission for most patients. We therefore anticipate that more intense immunosuppressive therapy will be required for the majority of RA patients, for whom such treatments will be proposed. Based on findings of this pilot study, we suggest that ID-CYC with G-CSF should be used as a first step, both to treat severe RA refractory to conventional treatment and to harvest HSC. This strategy may offer a means of selecting patients with disease that is responsive to chemotherapy, and may also increase the efficacy of subsequent ASCT. However, the possibility must be considered that a first round of ID-CYC may result in subsequent spontaneous remission. Further studies are necessary in order to determine the optimal period of monitoring needed before deciding upon more intense immunosuppressive therapy requiring ASCT rescue.

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References