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Regression of adjuvant-induced arthritis in rats following bone marrow transplantation

(*Mycobacterium tuberculosis*)

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ABSTRACT **Total body irradiation followed by bone marrow transplantation was found to be an effective treatment for adjuvant arthritis induced in rats. This treatment is most effective when applied shortly after the clinical manifestation of arthritis—i.e., 4–7 weeks after administration of *Mycobacterium tuberculosis*. Transplantation of bone marrow at a later stage results in a limited recovery, in that the inflammatory reaction regresses but the newly formed excessive bone is not eliminated. Local irradiation of the affected joints had no effect on the disease. It could also be excluded that the recovery of arthritis following marrow transplantation is due to lack of available antigen. Transplantation of syngeneic bone marrow is as effective as that of allogeneic bone marrow from a rat strain that is not susceptible to induction of adjuvant arthritis. The beneficial effect of this treatment cannot be ascribed to the immunosuppressive effect of total body irradiation, since treatment with the highly immunosuppressive drug Cyclosporin A resulted in a regression of the joint swelling but relapse occurred shortly after discontinuation of the treatment.**

Chronic adjuvant-induced arthritis (AA) in rats is an autoimmune disease that can be elicited in susceptible rat strains by a single intracutaneous inoculation of *Mycobacterium tuberculosis* in incomplete Freund's adjuvant (IFA) (1). The disease is characterized by a subacute or chronic polyarthritis, chiefly involving the distal extremities, and has been proposed as a model of rheumatoid arthritis in human.

The susceptibility to arthritis is major histocompatibility complex-linked in humans and in rodents (2–5). In humans the disease is initiated by exposure to various (as yet unidentified) antigenic stimuli. It is also established that the pathogenesis of AA is fundamentally immunological (6, 7). The involvement of T lymphocytes in experimental AA has been well documented and is strongly supported by the observation that AA, like certain other experimental autoimmune diseases (e.g., experimental type I diabetes and experimental allergic encephalitis) can be transferred by T lymphocytes (8–13).

The question of whether susceptibility to the development of experimental autoimmune diseases is determined at the level of the hemopoietic stem cell or at a later stage of lymphoid differentiation was studied by various investigators in different animal models. Ikehara *et al.* (14) demonstrated that transplantation of bone marrow of nonsusceptible strains can prevent the onset of a systemic and an organ-specific autoimmune disorder—i.e., in MLR/lpr and type I diabetes in NOD mice, respectively—suggesting the determination of susceptibility at the level of the hemopoietic stem cell.

If the susceptibility to arthritis is, likewise, determined at the level of the hemopoietic stem cell, replacement of the hemopoietic system by that of a healthy/unsusceptible individual is expected to be of benefit, especially in the early stages of the disease—i.e., before irreversible damage to the joints has occurred. A single encouraging clinical case supporting this assumption has been reported. Jakobs *et al.* (15) described a patient suffering from disabling arthritis who responded very favorably to an allogeneic bone marrow transplantation that was initiated primarily to treat aplastic anemia that had developed in this patient.

The present study was undertaken to evaluate the effects of a replacement of the hemato-immunological system of arthritic animals by that of donor rats of a strain that is not susceptible to the induction of arthritis. When this treatment was found to be highly effective, a control experiment was performed with syngeneic bone marrow. To our surprise, syngeneic marrow was found to be just as effective as allogeneic marrow. Additional control experiments were carried out with an extra supply of adjuvant after the bone marrow transfer, and irradiation of affected paws only was studied to exclude the possibility that the observed curative effects were due to the local antiinflammatory effects of irradiation.

MATERIALS AND METHODS

Animals. In this study we used male and female rats of the inbred strains Buffalo ($RT1A^u$) and Wag/Rij ($RT1A^i$), 12–16 weeks of age. All rats were bred in our animal facilities in Rijswijk, The Netherlands, under specific pathogen-free conditions.

Induction and Evaluation of Arthritis. To induce arthritis, animals were injected intradermally with a suspension of 1.0 mg of *M. tuberculosis* (H37 Ra) (Difco) in IFA (Difco). A final volume of 0.1 ml was administered at the root of the tail, according to Pearson and Wood (1). This resulted in severe chronic arthritis in 70–80% of the Buffalo rats (male and female) within 3–4 weeks after immunization.

Evaluation of the progress of the disease was carried out by weekly measurements of the thickness of the ankles and wrists using an industrial caliper. The severity of arthritis was expressed as an arthritic score, calculated for individual animals as the sum of increased thickness (in mm) of the four paws compared to measures taken before inoculation [according to Nagler-Anderson *et al.* (16) with slight modifications]. In other words, the paw thicknesses measured prior to administration of adjuvant were substracted from the measures recorded afterward. The resulting values were added up and the score represents the arthritic score of an individual rat. Mean arthritic scores were derived by averaging the individual scores of rats within each experimental group. This

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Abbreviations: IFA, incomplete Freund's adjuvant; TBI, total body irradiation; CsA, Cyclosporin A; AA, adjuvant-induced arthritis; TLI, total lymphoid irradiation.

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scoring enabled us to follow closely the development and progression of the disease in individual rats. It was observed thereby that individual inbred rats respond differently to the same challenge with *M. tuberculosis*. Individual variations were recorded in latency, in severity, and in long-term development of the disease, making evaluation of the treatment more complex. Since the severity of the arthritis also varied among the experiments, we chose to include a control group in every experiment and related the response of treated animals to the controls of the same experiment.

In the experiment in which the effect of local irradiation of one paw was studied (results shown in Fig. 4) the arthritic score is the thickness of one paw minus the thickness of that paw measured before adjuvant administration. In this case, the mean arthritic score is the average of the values for one paw per rat of all rats of one group. This explains the much lower arthritic score in this particular experiment as compared to the other studies. For histopathological examination, animals were sacrificed, and joints of the hind legs were removed and fixed in 4% buffered formaldehyde. Microscopic sections of decalcified joints were stained with hematoxylin/phloxine/saffron.

Radiation Exposures. For total body irradiation (TBI), nine rats at a time were placed in a round perspex box and exposed to a radiation dose of 8.5 Gy, using a Phillips-Muller 300 machine (300-kV x-rays at a dose rate of 0.34 Gy·min^{-1}). For the irradiation of one hind leg, rats anesthetized with Ethrane (Abbott) were constrained in horizontally placed exposure polyvinyl chloride cylinders with an opening through which one hind leg could protrude. The whole body except the right hind leg was shielded with 5-mm lead, and rats were exposed to 8.5 Gy as above.

Bone Marrow Transplantation. Recipient Buffalo rats preexposed to TBI were injected intravenously with 5×10^7 nucleated viable bone marrow cells from syngeneic (Buffalo) or allogeneic (Wag/Rij) sex-matched donors. Bone marrow cells were obtained in suspension by flushing several times the cavities of femoral bones with Hanks' salt solution. For this purpose, a bent needle was inserted into the distal end of the bone and 2 ml of solution was forced through the bone shaft. The collected bone marrow was filtered on a nylon gauze. The number of nucleated cells present in the suspensions was counted in a Burker-type hemocytometer using Turk's solution (0.01% crystal violet/1% acetic acid in saline). The number of dead cells was determined by means of eosin (0.2%) uptake.

RESULTS

Effect of Allogeneic and Syngeneic Bone Marrow Transplantation on the Progression of Arthritis. In this study we used Buffalo (an arthritis-susceptible strain) males, 5 weeks after immunization with *M. tuberculosis*. Animals were divided into two groups matched for the severity of the manifested disease. Rats of one group were left untreated and served as controls. Those of the other group were irradiated (8.5 Gy) and transplanted with 5×10^7 bone marrow cells of Wag/Rij (an arthritis-unsusceptible strain) males. In a second experiment, similarly designed, the treated group received 4 weeks after immunization 5×10^7 syngeneic (Buffalo male) bone marrow cells. The progression of arthritis in the two experiments was recorded weekly. Results were expressed as the average arthritic score for each group (Fig. 1 *A* and *B*).

A clear-cut complete regression of the arthritis, expressed as a gradual decrease of paw thickness, was observed in the marrow-treated groups. After 14 weeks of observation of the rats in group A and 9 weeks in group B the experiment was discontinued. The course of the disease in the control animals, though not identical in the two experiments, runs

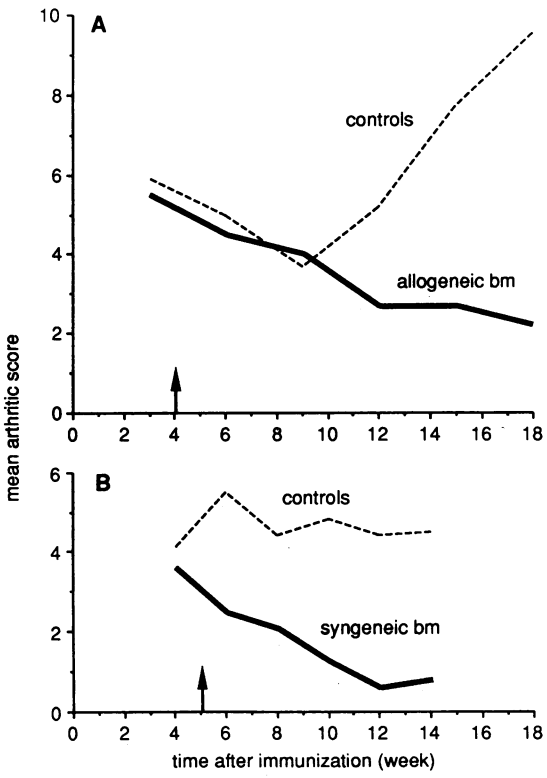

FIG. 1. Effect of bone marrow transplantation on the progression of arthritis. In two experiments (*A* and *B*) Buffalo male rats were inoculated with *M. tuberculosis*. In each experiment 20 arthritic positive rats were divided in two equal groups: (*i*) animals with no further treatment, controls; (*ii*) animals irradiated (8.5 Gy TBI) and transplanted with 5×10^7 bone marrow (bm) cells (syngeneic, *A*; allogeneic, *B*). Arrows indicate the time of bone marrow transplantation. Results are expressed as the arithmetic mean of the arthritic score. The SEM for each group is ±10%.

significantly different from that of the marrow-treated animals.

Histological examination of sections through the hind legs revealed virtually complete absence of inflammatory reaction in the bone marrow-grafted rats at 4 weeks after the treatment with normal interarticular surfaces and synovia. In the control rats, studied at a similar time after immunization, the classical severe inflammation and destruction process was apparent (see Fig. 2 *a* and *b*).

Effect of Marrow Transplantation on Late-Stage Arthritis. To answer the question of whether marrow transplantation is beneficial at all stages of the disease, the following study was initiated. Male and female Buffalo rats suffering from arthritis 4–11 months after immunization were irradiated and transplanted with 5×10^7 sex-matched syngeneic bone marrow cells. Thereafter measurements of the thickness of the paws were continued. For evaluation of effectiveness of treatment the animals were divided into two subgroups according to the course of the disease before transplantation. One subgroup included animals with progressive arthritis, as estimated by a continuing increase in paw thickness (5 or 31 weeks after immunization) (Fig. 3*A*). The second subgroup included animals in which the disease had come to a steady state, as manifested by a stable paw thickness for a period of >10 weeks (Fig. 3*B*).

The curves, which represent the arthritic score of individual animals, show no effect of the bone marrow transplantation treatment for animals represented in Fig. 3*B*. The treatment of animals in Fig. 3*A* results in an arrest of the progression of the disease either immediately after transplan-

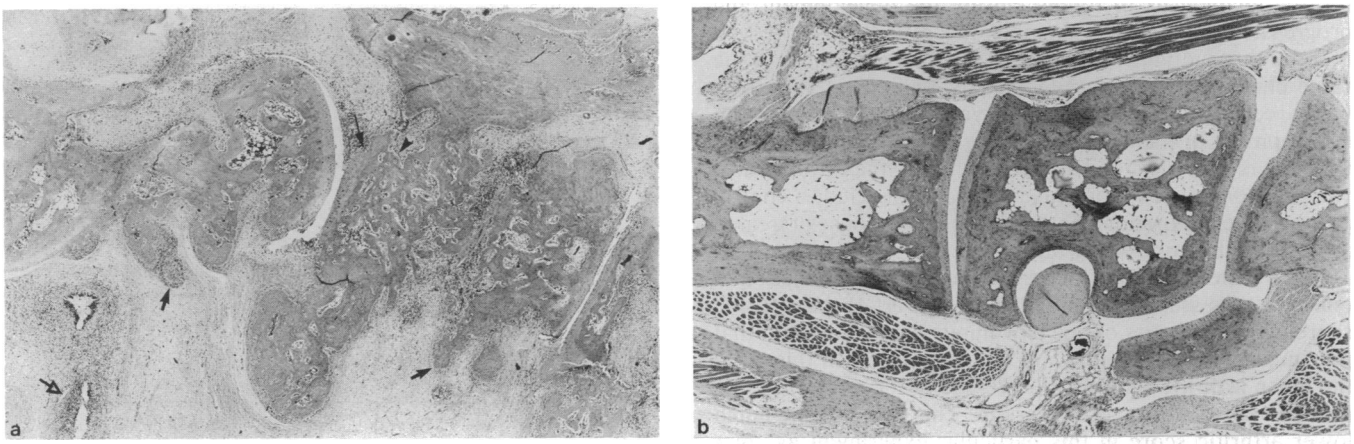

FIG. 2. Histopathology of the tarsal bones of arthritic rats (*a*) and of arthritic rats treated with bone marrow transplantation (*b*). (*a*) Area of tarsal bones of Buffalo rats, 8 weeks after immunization with *M. tuberculosis*, showing chronic proliferative synovitis with pannus formation (⟶), destruction of subchondral bone (▶), reactive bone formation (➔), vasculitis (➪), and pericapsular fibrosis. (*b*) Comparable area of tarsal bones without any lesions of a Buffalo rat 8 weeks after immunization with *M. tuberculosis* and 4 weeks after syngeneic bone marrow transplantation. (Hematoxylin/phloxine/saffron, ×16.)

tation (animals 1 and 2) or after a short lag time (animals 3, 4, and 5).

Effect of Local Irradiation of the Paw on the Development of Arthritis. The possibility that the beneficial effect of bone marrow transplantation is due to a direct effect of 8.5 Gy of x-rays on the affected joints was investigated in male Buffalo rats expressing a similar degree of arthritis in both hind legs. Four weeks after immunization, the right hind leg of each animal was irradiated (8.5 Gy) while the rest of the body was shielded. The progression of arthritis in the two hind legs after irradiation was compared. Results presented in Fig. 4 clearly demonstrate that irradiation of an arthritic joint only does not affect further development of the disease in that joint.

Effect of Preceding Syngeneic Bone Marrow Transplantation on the Susceptibility to Induction of Arthritis. To investigate whether the curative effect of bone marrow transplantation on arthritis is simply due to an inability of the reconstituted immune system to mount an autoimmune response, male Buffalo rats that had been treated with TBI plus syngeneic bone marrow transplantation 10 weeks previously were immunized with adjuvant. The development of arthritis in these animals was compared to that of naive immunized Buffalo rats. The data presented in Fig. 5 show no significant difference in the severity or the incidence of arthritis in the two groups, indicating that prior syngeneic marrow transplantation does not affect the susceptibility to arthritis.

Effect of a Second Immunization after Bone Marrow Transplantation. A possible explanation for the regression of arthritis observed after allogeneic or syngeneic marrow transplantation might be the unavailability of the arthritogen after transplantation. To ensure sufficient available arthritogen in the reconstituted arthritic rats the animals were reimmunized with *M. tuberculosis* after marrow transplantation. Groups of Buffalo rats were irradiated and transplanted with syngeneic marrow at 1 week after the onset of arthritis. Thereafter, they were reimmunized with *M. tuberculosis* (1.0 mg) 24 hr after bone marrow transplantation. Development of arthritis was measured at various time points after treatment and compared to that of control groups receiving bone marrow

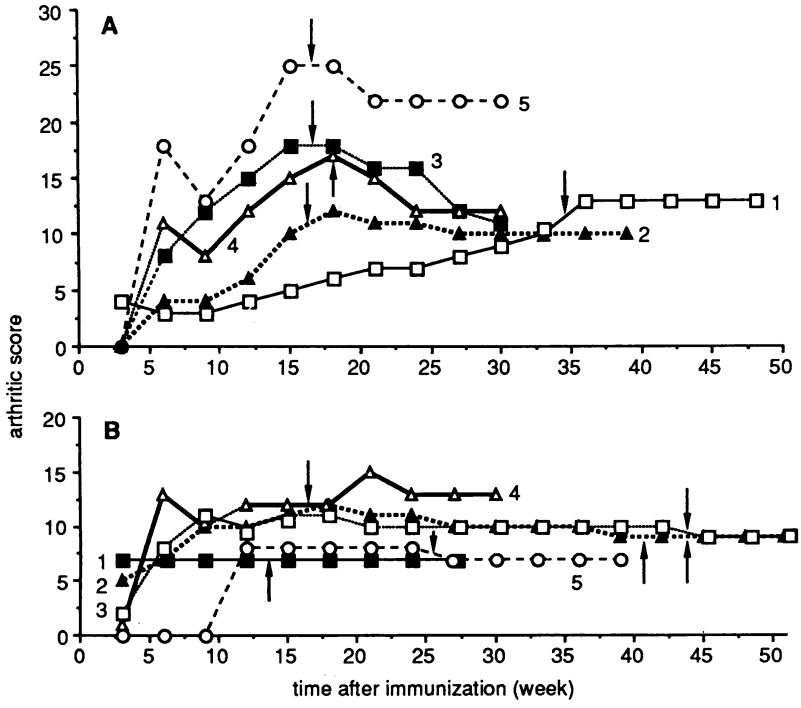

FIG. 3. Effect of bone marrow transplantation on late-stage arthritis. Buffalo male and female rats at various late stages of established arthritis were irradiated with a dose of 8.5 Gy TBI and transplanted with syngeneic marrow. (*A*) Selected animals in the progressive chronic stage. (*B*) Selected animals in the stabilized chronic stage. The arthritic score of individual animals is presented. Arrows indicate the time point of bone marrow transplantation. The end of each line is the time of sacrifice of the animal.

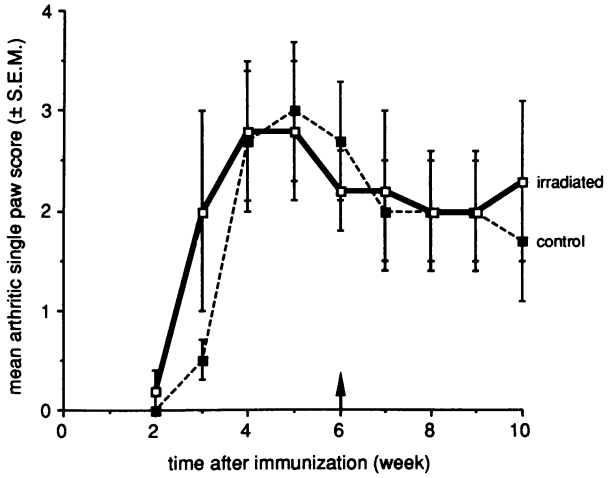

FIG. 4. Effect of paw irradiation on the development of arthritis. Ten Buffalo male rats with a similar arthritic score for both hind legs were used. Six weeks after immunization (arrow) the right hind leg was irradiated; the left leg served as control. Results are expressed as the arithmetic mean of the single paw swelling ± SEM. The experiment was discontinued at week 10.

transplantation only and arthritic rats that were not treated at all. The results (Fig. 6) show that reimmunization does not influence the remission induced by TBI and bone marrow grafting. This experiment was repeated twice with the following modifications: in the second experiment, rats were reimmunized at 28 days after the bone marrow transplantation and in the third experiment two reimmunizations were given, the first at 24 hr and the second at 28 days after bone marrow transplantation. In both experiments, the reimmunizations did not affect the regression of the arthritis (results not shown).

Effect of Cyclosporin A (CsA) on the Development of Arthritis. Buffalo male rats, 7 weeks after immunization with *M. tuberculosis*, were treated with CsA. Rats received daily subcutaneous injections of 5 mg of CsA per kg of body weight dissolved in ethanol, 5 days per week, for a period of 5 weeks.

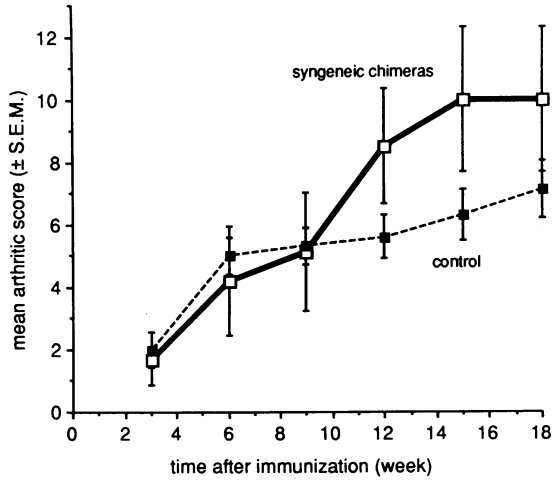

FIG. 5. Induction of arthritis in syngeneic radiation chimeras. Ten Buffalo male rats exposed 10 weeks previously to TBI and syngeneic bone marrow transplantation (solid line) and 20 normal untreated Buffalo males (broken line) were immunized with *M. tuberculosis*. The incidence of arthritis was 63% (15/20) in the control group and 70% (7/10) in the marrow-transplanted group. Results are expressed as the mean ± SEM of the arthritic score of the positive rats in each group. The experiment was discontinued at week 18.

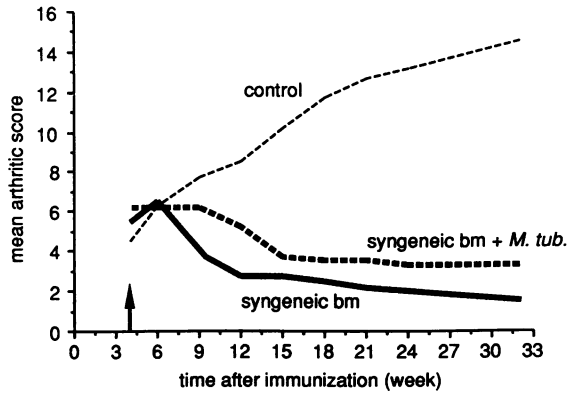

FIG. 6. Effect of reimmunization with *M. tuberculosis* after bone marrow transplantation on the development of arthritis. Buffalo male rats immunized with *M. tuberculosis* either were not further treated (8 rats) and served as controls or were transplanted with syngeneic bone marrow (bm) at 4 weeks (arrow) after immunization (16 rats). Of the latter group, 8 rats received a second inoculation of *M. tuberculosis* 24 hr after the marrow treatment. Results are expressed as the mean arthritic score per group. The SEM for each group is <10%. The experiment was discontinued at week 16.

Arthritic scores in the treated animals were compared with those of a control group injected with ethanol (the solvent) only. Results presented in Fig. 7 show a response to CsA. However, the remission of arthritis sustained only during the treatment period. Following withdrawal of CsA, the arthritis relapsed within a few weeks.

DISCUSSION

Our findings indicate that treatment with high-dose TBI and bone marrow transplantation induces a long-lasting remission of AA in rats. Marrow transplantation applied shortly (2–3 weeks) after the clinical manifestation of the disease results in a complete and lasting elimination of joint thickness. The same treatment given later, during the progressive phase of the disease, arrests the arthritic response. However, if treatment is applied after the arthritic process has reached a steady state (expressed as a long-lasting constant paw thickness), no effect of the transplantation treatment is observed.

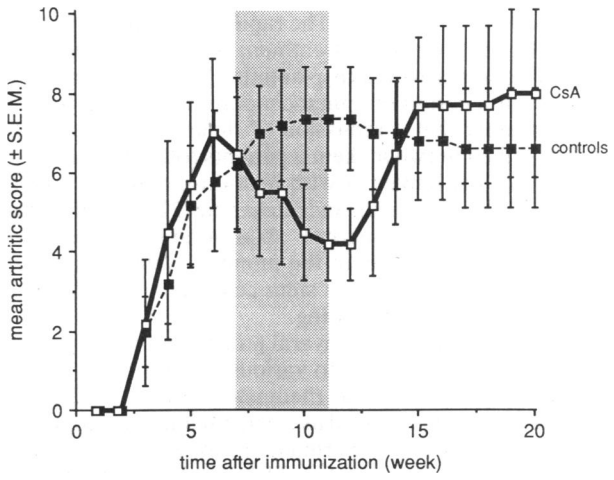

FIG. 7. Effect of CsA on the development of arthritis. Buffalo male rats, 7 weeks after immunization with *M. tuberculosis*, were treated with CsA (broken line) or ethanol (solid line). CsA was administered daily subcutaneously (5 mg/kg), 5 days/week, for 5 weeks (shaded area). Each group includes six animals. Results are expressed as the mean arthritic score per group ± SEM. The experiment was discontinued at week 20.

This study does not include extensive histopathological examinations in the various groups of rats. However, the histopathology of AA in rats has been described in detail by others (1, 17). It was established that during the early period after immunization—first 4 weeks—an intense cellular reaction in the subsynovial tissue occurs, including monocyte, macrophage, and lymphatic activity, leading to pannus formation and subchondral erosion and destruction of the cartilage. After 2 months there is usually evidence of a decrease of the inflammatory and connective tissue reactions and an increasing active bone formation replacing the scar tissue. Still, in about 25% of the animals, foci with active lymphocytes and plasma cells can be found up to 12 months after immunization, indicating a progression of the pathological process. When comparing histopathological findings, radiographs of the arthritic joints at varying times after immunization, and clinical changes after marrow treatment, it can be concluded that bone marrow transplantation affects only the inflammatory/autoimmune activity in the joints and has little or no effect on existing excess bone deposits.

Osteoclasts have been shown *in vitro* to originate from the hemopoietic stem cells (18). The restored bone resorption in osteopetrosis patients and mice observed after allogeneic bone marrow transplantation (19, 20) implies that osteoclasts may differentiate *in vivo* from grafted hemopoietic stem cells and function normally in bone tissue. It may therefore be suggested that in the AA rat model the failure to absorb the anomalous osseous tissue should be attributed to an abnormality of the osseous deposits rather than to a dysfunction of osteoclasts.

Our results show a clear-cut curative effect of bone marrow transplantation when the animals are treated during the progressive stage of the disease. The histology of the joints showed complete disappearance of synovial inflammation in the treated animals. However, the similar effects of allogeneic and syngeneic marrow grafts suggest that suppression of the arthritic response cannot be attributed to the genetic makeup of the transplanted marrow.

In rats exposed 10 weeks previously to a lethal dose of irradiation and syngeneic bone marrow transplantation, full-fledged AA could be induced by immunization with antigen. This suggests that the curative effect of TBI and bone marrow transplantation in arthritic rats has to be ascribed to mechanisms associated with reconstitution of the immune system in the presence of antigens. The rapid relapse of arthritis that occurred after 5 weeks of treatment with CsA suggests that TBI and bone marrow transplantation do not act merely by induction of a period of immunosuppression. In this context it is of interest that a remission of rheumatoid arthritis has been described in two leukemia patients following successful treatment of the leukemia with high-dose cytosine arabinonucleoside, daunorubicin, and amsacrine (21). This treatment involves a temporary marrow ablation and may lead to a comparable regeneration of the immune system from a limited number of hemopoietic stem cells, as occurs following TBI and bone marrow grafting.

It has been reported by several groups that total lymphoid irradiation (TLI) alleviates to various degrees the symptoms in patients with intractable rheumatoid arthritis (22–24). In these patients TLI is applied as a general immunosuppressive treatment. Studies in experimental models reveal that the immune-suppressed state after TLI may be due to the presence of large numbers of natural suppressor cells (25, 26). Cells with similar suppressive capacity have also been demonstrated during ontogeny, in the neonatal spleen and thymus (27–29), and in spleens of mice regenerating after TBI and marrow transplantation (30, 31). It is therefore of interest to investigate whether the beneficial effects of TBI and bone marrow transplantation can be explained by mechanisms involving the generation of suppressor cells.

It is obvious that treatment with supralethal TBI and bone marrow grafting still carries too many risks to be considered as a therapy for severe cases of rheumatoid arthritis. However, the prospects for development of less toxic conditioning regimens are at present favorable. In addition, our results seem to provide opportunities to analyze the immune modulations underlying the observed curative responses associated with bone marrow transplantation.

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