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Suppression of experimental arthritis by gene transfer of interleukin 1 receptor antagonist cDNA

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ABSTRACT **Restoration of the impaired balance between pro- and antiinflammatory cytokines should provide effective treatment of rheumatoid arthritis. Gene therapy has been proposed as an approach for delivery of therapeutic proteins to arthritic joints. Here, we examined the efficacy of antiinflammatory gene therapy in bacterial cell wall-induced arthritis in rats. Human secreted interleukin 1 receptor antagonist (sIL-1ra) was expressed in joints of rats with recurrent bacterial cell wall-induced arthritis by using *ex vivo* gene transfer. To achieve this, primary synoviocytes were transduced in culture with a retroviral vector carrying the sIL-1ra cDNA. Transduced cells were engrafted in ankle joints of animals prior to reactivation of arthritis. Animals in control groups were engrafted with synoviocytes transduced with *lacZ* and *neo* marker genes. Cells continued to express transferred genes for at least 9 days after engraftment. We found that gene transfer of sIL-1ra significantly suppressed the severity of recurrence of arthritis, as assessed by measuring joint swelling and by the gross-observation score, and attenuated but did not abolish erosion of cartilage and bone. The effect of intraarticularly expressed sIL-1ra was essentially local, as there was no significant difference in severity of recurrence between unengrafted contralateral joints in control and experimental groups. We estimate that locally expressed sIL-1ra was about four orders of magnitude more therapeutically efficient than systemically administered recombinant sIL-1ra protein. These findings provide experimental evidence for the feasibility of antiinflammatory gene therapy for arthritis.**

Rheumatoid arthritis (RA) is an autoimmune disease in which almost every arm of the inflammatory response is activated (1). Local expression of antiinflammatory proteins in synovium by using gene transfer may provide efficient treatment for RA (2, 3). Recent publications have demonstrated the feasibility of using gene transfer to express reporter genes (2, 4, 5) and an antiinflammatory protein, the secreted interleukin 1 receptor antagonist (sIL-1ra) or IRAP (6–8), in normal joints. However, the therapeutic efficacy of antiinflammatory gene therapy in animal models of arthritis relevant to human disease remains obscure.

The present experiments were designed to evaluate efficacy of sIL-1ra gene transfer in recurrent bacterial cell wall (BCW)-induced arthritis in rats. The cytokine interleukin 1 (IL-1) plays an important role in pathogenesis of human RA (9). Its naturally occuring antagonist, sIL-1ra, is a glycoprotein which competetively binds IL-1 receptors without eliciting cell activation (10–13). Experimental evidence suggests that the balance between IL-1 and sIL-1ra is impaired in arthritic joints (14, 15). Previously, we have demonstrated that systemic administration of recombinant sIL-1ra protein significantly suppressed joint inflammation in BCW arthritis (16). However, as IL-1 mediates a broad array of local and systemic effects, including T- and B-cell activation, neutrophilia, induction of the acute phase, and neuroendocrine responses (17), it was unclear how the local expression of sIL-1ra protein would influence the outcome of disease. Thus, gene transfer of sIL-1ra cDNA was used to assess both the feasibility and the efficacy of antiinflammatory gene therapy for suppression of BCW arthritis. We demonstrate that retroviral *ex vivo* gene transfer of the sIL-1ra cDNA to synovium significantly suppressed recurrence of arthritis in rats. We estimated that intraarticularly (i.a.) expressed sIL-1ra protein was about four orders of magnitude more therapeutically efficient than systemically administrated recombinant human sIL-1ra protein. These findings suggest that local expression of antiinflammatory proteins in arthritic joints by using gene transfer may prove more beneficial in treatment of human disease than systemic delivery of recombinant proteins.

MATERIALS AND METHODS

Cloning of sIL-1ra cDNA. A human monocyte cDNA library in pcDNA I vector (Invitrogen) was generated essentially as described (18). With a probe containing a common sequence between intracellular IL-1ra and sIL-1ra (19), the partial clone of human sIL-1ra (hsIL-1ra) C15-6 (1.7 kb) was isolated. The C15-6 clone contained the sIL-1ra coding sequence and the 3′ untranslated region except for the first nucleotide of the initiation codon. To obtain the complete coding sequence of sIL-1ra, the partial-length cDNA was amplified by PCR with the primer **ACCATGGAAATCTGCAGAGG**, which contains the Kozak sequence and the missing nucleotide (in boldface type), and a downstream primer complementary to a noncoding region of sIL-1ra. The PCR fragment was inserted into pCRII (Invitrogen) vector and sequenced. The resulting construct was digested with *Bam*HI, and the 928-bp insert was inserted into *Bam*HI-digested pLXSN (20). The resulting pLXSN-SRAM3 plasmid contained the Kozak sequence, complete sIL-1ra protein coding sequence (11) and the 3′ untranslated region from bp 554 to 907.

Retroviral Vectors. Plasmids pLN (20) and pLNPOZ (21) were a gift from A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle). Retroviral vector LNPOZ carried *lacZ* and *neo* genes encoding bacterial proteins β-galactosidase (β-gal) and neomycin phosphotransferase, respectively, LN vector carried *neo* gene, and SRAM3 vector carried cDNA for sIL-1ra and *neo* gene. Clonal amphotropic packaging cells producing retroviral vectors were derived as described (20). The titers of vectors were approximately 2×10^5 to 3×10^6 colony forming units per ml.

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Abbreviations: RA, rheumatoid arthritis; IL-1, interleukin 1; sIL-1ra, secreted IL-1 receptor antagonist; hsIL-1ra, human sIL-1ra; BCW, bacterial cell wall; PG-APS, peptidoglycan–polysaccharide polymer complex; β-gal, β-galactosidase; i.a., intraarticular(ly).

Cell Culture. Primary synovial cells were obtained by enzymatic dispersal of tissue from the ankle joints of female Lewis rats with established BCW arthritis, as previously described (5). Cells were transduced at the first passage with retroviral vectors in presence of Polybrene (8 μg/ml) and selected with G418 (GIBCO/BRL) by using a concentration of 0.5 mg/ml.

Western Blot Analysis of SRAM3 Supernatants. Conditioned medium from SRAM3-transduced synoviocytes was concentrated by using Amicon-10 microconcentrators. To improve the protein recovery, microconcentrators were blocked with 10% (wt/vol) nonfat dry milk according to manufacturer's (Amicon) instructions. Concentrated samples were resolved on a 15% denaturating polyacrylamide gel and transferred to nitrocellulose. Serial dilutions of human recombinant sIL-1ra protein (R & D Systems) were used as a standard. Blots were incubated with primary rabbit polyclonal antibodies against hIL-1ra (19) and alkaline phosphatase-conjugated secondary antibodies. Concentrations of the hsIL-1ra protein were estimated by using a densitometer. Deglycosylation was carried out with *N*-glycanase (Genzyme) by following manufacturer's instructions.

Bioassay for Determination of Biological Activity of hsIL-1ra *in Vitro*. Conditioned medium from SRAM3-transduced cells was collected after a 24-h incubation in RPMI 1640 medium supplemented with 0.5% fetal bovine serum. The murine pre-B 70Z/3 cells (gift from T. Bender; University of Virginia) were resuspended in conditioned medium or in RPMI 1640 medium with 0.5% fetal bovine serum (with or without recombinant sIL-1ra) at 2×10^7 cells per ml. Cells were preincubated at 37°C for 10 min, the indicated concentrations of human recombinant IL-1β (R&D Systems) were added, and incubation was continued for another 30 min. Nuclear extracts from 70Z/3 cells were prepared as described (22). The DNA-binding NF-κB activity in nuclear extracts was determined by gel-shift assay (23). Binding reactions were performed by incubating 4 μg of nuclear extracts with a ^{32}P-labeled UV 21 double-stranded oligonucleotide probe containing κB-binding site from the enhancer of murine H-2K^b gene, as described (24). The oligonucleotide–NF-κB complex was separated from unbound oligonucleotide by nondenaturating 5% polyacrylamide gel electrophoresis and visualized with a PhosphorImager (Molecular Dynamics). The specificity of NF-κB binding was determined in a separate experiment by competition of binding with unlabeled probe (data not shown).

Model of Recurrent BCW Arthritis. The model of synchronized recurrence of BCW arthritis was reproduced as previously described (16). Female Lewis rats (Charles River Breeding Laboratories) weighing ≈150 g were injected i.a. into both tibiotalar (ankle) joints with 5 μg of a rhamnose equivalent of peptidoglycan–polysaccharide polymer complex (PG-APS) isolated from BCW of group A streptococci. PG-APS (fraction 100 p) was injected in 10 μl of pyrogen-free saline. Four weeks later, animals were randomly distributed into groups, and on day 0 each rat was injected i.a. into one ankle joint with approximately 1×10^5 transduced cells suspended in 10 μl of Hanks' balanced salt solution (HBSS). The number and viability of engrafted cells after injection were estimated by mock-injecting cells into HBSS. The viability of mock-injected cells was routinely 75–95%, as assessed by exclusion of trypan blue and by plating efficiency. Reactivation of arthritis was induced on day 1 by i.v. injection of a dose of 225 μg of a rhamnose equivalent PG-APS. The lateral diameters of ankle joints were measured in quadruplicate by using a digital caliper, as previously described (16). To reduce bias, all animals were coded and measurements were done randomly. The blind gross-observation score was based on assessment of erythema, swelling, and joint displacement on a scale of 0 (normal) to 4 (maximum inflammation), as described (25). For the histological study, joints were fixed in formalin, decalcified, embedded in paraffin, sectioned sagitally, and stained with hematoxylin/eosin.

Detection of Viable Transplanted Cells in Joints. LNPOZ-transduced cells were detected in joint explants by enzymatic staining for β-gal activity. Cell cultures were established from joint explants as described (5). After 9 days in culture, fibroblast-like adherent cells were fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (GIBCO/BRL). The PCR analysis of genomic DNA was performed in whole-cell lysates as described (26). Control genomic DNA or cell lysates prepared from 10^4 recovered cells were subjected to 35 cycles of PCR amplification with primers GM398 and GM344 specific for the hsIL-1ra cDNA (19). The PCR products were resolved by agarose gel electrophoresis, transferred to a nylon membrane, hybridized with a ^{32}P-labeled hsIL-1ra cDNA, and visualized with a PhosphorImager (Molecular Dynamics).

Statistical Analysis. Significance of differences between groups in the course of arthritis based on joint diameter measurements were analyzed by using analysis of variance (ANOVA) statistical program. Significance of differences based on gross-observation score was calculated by Student's unpaired two-tailed *t* test.

RESULTS

***Ex Vivo* Reporter Gene Transfer to Inflamed Synovium.** Initial experiments were designed to determine the feasibility of transplanting genetically modified cells into inflamed synovium. Although several experiments on gene transfer to normal synovium have been reported to date (2, 4, 6), it was not clear how the inflammatory milieu would influence expression of the transferred gene and the viability of transduced cells.

Chronic, remitting, erosive polyarthritis in the rat can be induced by a single i.p. injection of PG-APS. Inflammation of peripheral joints is characterized by repeated episodes of remission and recurrence, progressing over a period of several months to joint destruction (27). In a modified model, which was used in the studies reported here, i.a. injection with a small dose of PG-APS produces monoarticular arthritis which recedes to a low-level chronic inflammation. Several weeks later, an i.v. injection with PG-APS provokes a recurrence of chronic inflammation in previously injured joints (28). The distinct advantage of this model is that it provides predictable, synchronized recurrence that reaches a peak in 2–4 days and continues for several weeks after i.v. injection.

Primary synoviocytes obtained from syngeneic rats with BCW arthritis were transduced in culture with the retroviral vector LNPOZ (21) carrying reporter genes *lacZ* and *neo*. The frequency of transduction *in vitro* was routinely 5–40%. After drug selection, cells were engrafted into ankle joints of animals with BCW arthritis at the peak of inflammation following reactivation. Nine days after engraftment, cell cultures were established from joint explants and stained for β-gal enzymatic activity (Fig. 1). It was found that expression of transferred genes in engrafted cells continued in inflamed joints for at least 9 days. These results suggested that the expression of a transferred antiinflammatory gene would last long enough to provide suppression of recurrence of arthritis.

Primary Synoviocytes Transduced in Culture Secrete Biologically Active hsIL-1ra Protein. To express the sIL-1ra protein in synoviocytes, we constructed the retroviral vector SRAM3 carrying the hsIL-1ra cDNA and the *neo* gene. Primary synovial cells transduced *in vitro* with the SRAM3 vector and selected with G418 secreted about 200 ng of hsIL-1ra per 10^6 cells per day, as estimated by Western blotting of conditioned medium (Fig. 2*A*). An apparent molecular mass of secreted protein (22–25 kDa) was consistent with the reported value (10). After deglycosylation, the secreted pro-

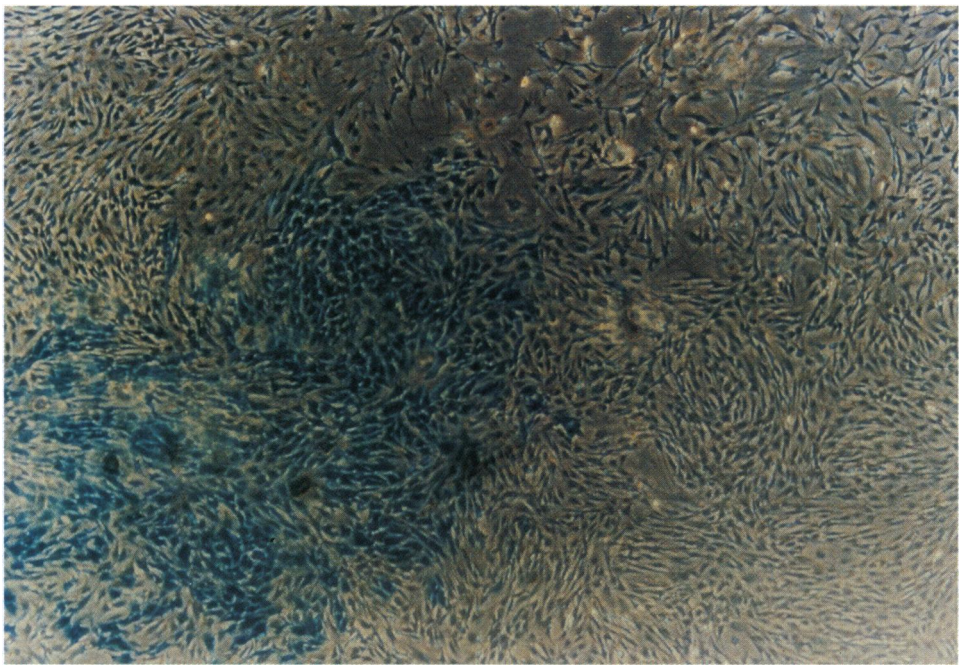

FIG. 1. Retroviral-mediated *ex vivo* reporter gene transfer to inflamed synovium. Primary synovial cells transduced with recombinant amphotropic retroviral vector LNPOZ carrying both *lacZ* and *neo* genes were engrafted into one inflamed ankle joint in Lewis rats at a peak of recurrence of BCW arthritis. Nine days later, cell cultures were established from joint explants and stained for the presence of β-gal activity. No staining was observed in cells from contralateral joints (data not shown). (×100.)

tein comigrated with the recombinant hsIL-1ra (17 kDa) (Fig. 2*B*). The biological activity of sIL-1ra was confirmed in a bioassay based on IL-1-induced activation of NF-κB in murine pre-B lymphoid 70Z/3 cells (29). In resting cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB. Stimulation of cells with IL-1 causes degradation of IκB followed by nuclear translocation of NF-κB (30). The nuclear

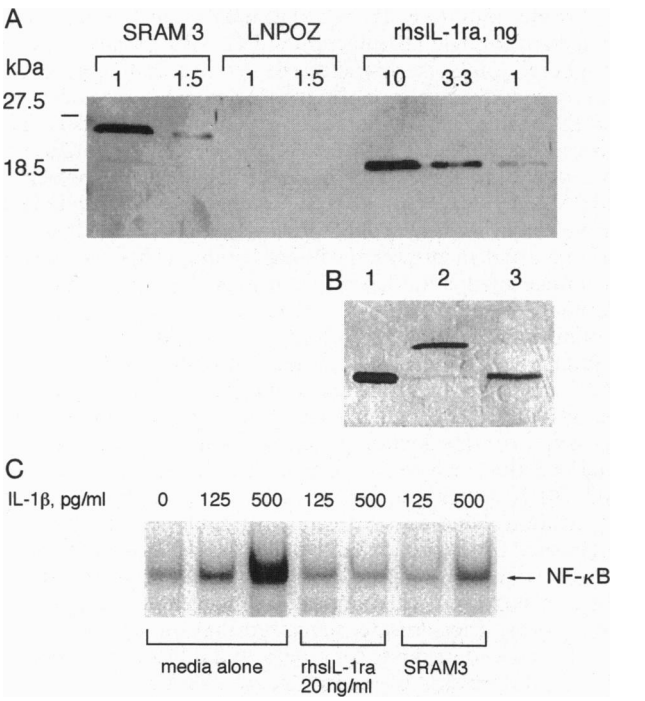

FIG. 2. Secretion of the sIL-1ra protein in culture by SRAM3-transduced synoviocytes. (*A*) Western blotting of secreted protein in supernatants of primary rat synoviocytes transduced with the retroviral vector SRAM3 carrying cDNA of hsIL-1ra and *neo* gene or with the LNPOZ vector (see legend of Fig. 1). (*B*) Western blotting of sIL-1ra after deglycosylation. Lane 1, recombinant hsIL-1ra; lane 2, supernatant incubated for 12 h with reaction buffer alone; and lane 3, supernatant reacted with *N*-glycanase for 12 h. (*C*) Biological activity of sIL-1ra *in vitro* assessed by the competitive inhibition of the IL-1-induced NF-κB DNA-binding activity in 70Z/3 cells.

concentration of NF-κB in IL-1β-treated 70Z/3 cells increased in a dose-dependent manner (Fig. 2*C*). Upon coincubation of cells with IL-1β and recombinant sIL-1ra or with conditioned medium from SRAM3-transduced synoviocytes, nuclear translocation of the NF-κB was inhibited. Similarly, in human endometrial stromal cells, IL-1β-stimulated degradation of IκB was prevented by coincubation of cells with conditioned medium from SRAM3-transduced synoviocytes (data not shown).

Suppression of Recurrence of BCW Arthritis by the hsIL-1ra *ex Vivo* Gene Transfer. To investigate the effect of the *ex vivo* gene transfer on the course of recurrence of arthritis, ≈1 × 10^5 SRAM3-transduced synoviocytes were engrafted into one of the ankle joints of animals in the experimental group previously injected in both joints with PG-APS (Fig. 3*A*). To account for nonspecific effects unrelated to sIL-1ra expression, an equal number of cells transduced with the LNPOZ vector were engrafted in a similar fashion in joints of animals in the control group. The severity of arthritis was evaluated by measuring the ankle joint swelling (31).

Reactivation of arthritis was induced by the i.v. injection of PG-APS 24 h after cell engraftment. In 2 days, the recurrence reached a peak. In the control group, ankle joints with engrafted LNPOZ-transduced cells developed a strong inflammation. The inflammation was substantially inhibited in joints with engrafted SRAM3-transduced cells (Fig. 3*B*). The difference between control and experimental groups was significant over the period of observation ($P < 0.04$). There was no significant difference in severity of recurrence between unengrafted contralateral ankle joints in experimental and control groups ($P > 0.3$) (Fig. 3*C*).

To substantiate the assessment based on measurement of joint diameter, in an additional experiment, the severity of arthritis was evaluated by using blind assessment based on gross-observation score (25) on day 8. In this study, recurrence of arthritis was compared between animals with engrafted SRAM3-transduced cells and animals which received synoviocytes transduced with retroviral vector LN carrying only the *neo* gene. Consistent with the previous experiments, the scores of joints with engrafted cells in the experimental (mean group score 0.9 ± 0.6) and control (mean score 2.5 ± 1.0) groups were significantly different ($P < 0.007$) (Fig. 4). The four most severely affected animals in each group were selected on day 8 for histological determi-

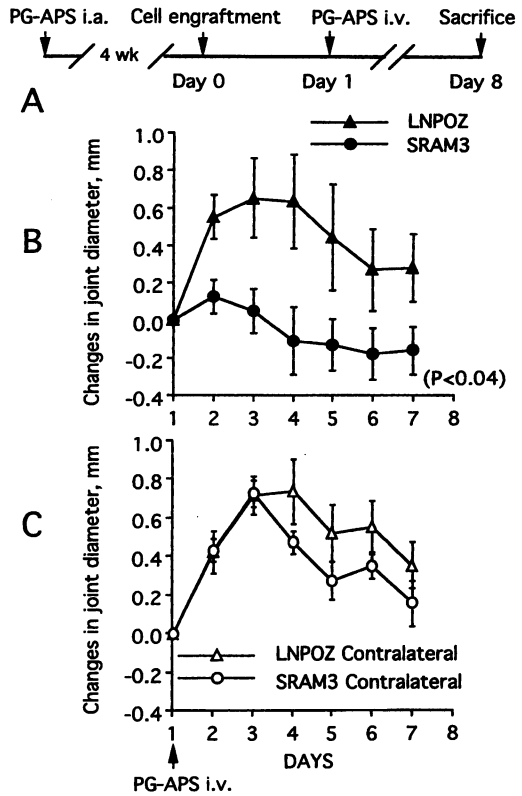

FIG. 3. Effect of antiinflammatory gene transfer on the course of recurrence of arthritis. (*A*) Schedule of injections. Rats were injected i.a. in both ankle joints with PG-APS. Four weeks later, on day 0, animals were divided into groups of 10, and each rat was injected in one ankle joint with primary synoviocytes transduced with the SRAM3 (experimental group) or LNPOZ (control group) retroviral vectors. On day 1, animals in both groups were injected i.v. with PG-APS. Joint measurements were made from day 0 through day 7. (*B*) Time course of recurrence in joints engrafted with LNPOZ- (▲) or SRAM3- (●) transduced cells. Changes in diameters were calculated as $d_n = D_n - D_1$, where D_1 and D_n were joint diameters at day 1 and day n, respectively. Each point was the mean value in the group. The increase in joint diameter from day 0 to day 1 was 0.50 ± 0.17 mm in experimental and 0.57 ± 0.27 mm in control groups. By using day 1 measurements as a baseline, the data presented adjusts for this initial increase. (*C*) Time course of recurrence in contralateral unengrafted ankle joints, in control (△) and experimental (○) groups. The experiment was carried out twice with a similar outcome. *P* values of comparison were calculated by using two-factor repeated measurement analysis of variance. Error bars represent standard deviation.

nation of arthritis. The histological observation confirmed that the histopathology was very similar to that illustrated previously (16, 28). Synovitis and pannus formation were present in the ankle joints from both groups. Consistent with our studies of recombinant sIL-1ra in the recurrent model of BCW arthritis (16), erosion of cartilage and subchondrial bone was less advanced in the experimental group but not abolished (data not shown).

To assess the viability of engrafted cells, animals were sacrificed on the eighth day after engraftment, and cell cultures were established from joint explants. The presence of hsIL-1ra cDNA in genomic DNA was detected in cells recovered from joints with engrafted hsIL-1ra$^+$ cells but not from contralateral joints (Fig. 5). As in the above described experiments (see Fig. 1), both recovered LNPOZ- and SRAM3-transduced cells maintained expression of transferred genes, as indicated by their growth in the presence of G418 (data not shown).

DISCUSSION

The basis of the concept of antiinflammatory gene therapy for arthritis is the hypothesis that the local expression of therapeutic proteins by transduced synovial cells would circumvent the problems associated with inefficient systemic delivery to joints (2, 3). The feasibility of using gene transfer for delivery of antiinflammatory proteins to synovium was tested in recent studies (6–8). Both *ex vivo* (6, 7) and *in vivo* (8) approaches were used to express sIL-1ra in normal rabbit joints. The locally produced sIL-1ra was functionally active, as evidenced by its ability to block the inflammatory response to a subsequent i.a. injection of recombinant IL-1. However, in human RA, IL-1 is just one cytokine that acts in cooperation with other cytokines and growth factors. Accordingly, in the absence of other stimuli, the i.a. injected IL-1 elicits only a transient, reversible inflammatory reaction (31). Thus, in previous studies, the prevention of inflammatory effects of exogenous IL-1 by the sIL-1ra gene transfer demonstrated the biological activity of expressed sIL-1ra *in vivo* rather than the antiarthritic properties of antiinflammatory gene transfer.

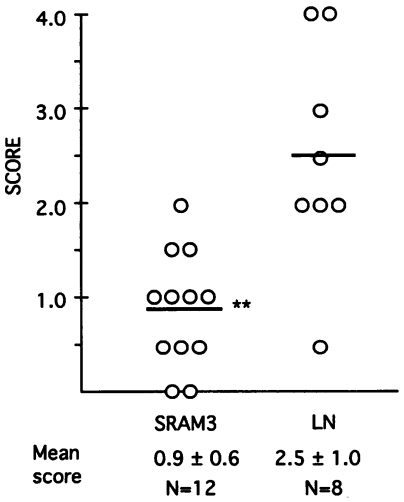

FIG. 4. Inhibition of chronic inflammatory reaction by hsIL-1 gene transfer: gross-observation scores. Gross-observation score of arthritis in joints with LN- or SRAM3-transduced cells 8 days after reactivation by the i.v. injection of PG-APS. The ankle joints were graded on a scale of 0 (normal) to 4 (maximum erythema, swelling, and joint displacement). The significance of difference between groups was calculated by using unpaired two-tailed Student's *t* test. **, $P < 0.007$.

In this regard, the major result of our experiments is the demonstration that local suppression of endogenous IL-1 by gene transfer of sIL-1ra can inhibit the recurrence of chronic arthritis in the BCW model, in which the pathogenesis is relevant to human disease. In contrast to systemically administered recombinant sIL-1ra protein (16), the effect of i.a. expressed sIL-1ra was essentially local, as there was no significant difference in severity of recurrence between unengrafted contralateral joints in control (*lacZ* or *neo*) and experimental (sIL-1ra) groups. Therefore, it demonstrates the predominant role of local rather than systemic effects of IL-1 in pathogenesis

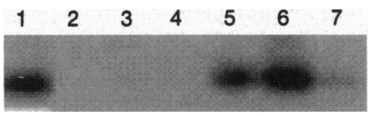

FIG. 5. Detection of viable engrafted SRAM3-transduced cells in joints. Cell cultures were established from joint explants obtained from animals 8 days after engraftment (see Fig. 3*A*). The presence of sIL-1ra cDNA in genomic DNA of recovered cells was detected by using PCR analysis. Lane 1, positive control (genomic DNA isolated from SRAM3-transduced primary rat synoviocytes); lane 2, negative control (genomic DNA from non-transduced cells); lanes 3 and 4, cells from contralateral joints (no cell engrafted) of animals from the experimental group. Lanes 5–7, cells from joints with engrafted SRAM3-transduced cells.

of BCW arthritis. The sIL-1ra protein expressed by gene transfer was extremely effective when compared with systemically delivered recombinant sIL-1ra. In previously reported studies on the antiarthritic properties of recombinant sIL-1ra protein in the reactivation model of BCW arthritis in rats (16), we estimated that the 50% effective dose of systemically delivered recombinant sIL-1ra protein was 0.5 mg/kg when injected i.p. immediately prior to reactivation and s.c. each 6 h thereafter. Here, using gene transfer, the upper limit of the sIL-1ra production by 1×10^5 engrafted cells, based on secretion in culture, was estimated as 20 ng per 24 h, which corresponds to a dose of 100 ng/kg. Comparison of these values suggests that the protein expressed in synovium after gene transfer was at least four orders of magnitude more effective than systemically delivered recombinant IL-1ra. Several factors could account for this striking difference. First, delivery of systemic recombinant sIL-1ra to synovium is hindered by the short half-life of the recombinant protein [$t_{1/2}$ for recombinant sIL-1ra in humans is 21 min (32)]. Second, it is possible that systemically administrated recombinant sIL-1ra protein and the sIL-1ra expressed by engrafted synoviocytes are localized differently in joints. Systemically administered protein would be expected to localize primarily in synovial fluid and perivascular subsynovium, whereas protein secreted by transduced synoviocytes is released directly into synovial tissue. Third, the glycosylation of i.a. expressed sIL-1ra protein also might contribute to its high efficacy. Although both glycosylated and recombinant sIL-1ra were shown to be equally active *in vitro* (10), the glycosylated protein may be less vulnerable to degradation *in vivo*. The impact of these factors are currently under investigation. Our observations are consistent with reported studies (7), where local expression of sIL-1ra was found to be more biologically effective in antagonizing exogenous recombinant IL-1 than i.a. injected recombinant sIL-1ra protein.

The achieved therapeutic effect of the specific inhibitor of a single proinflammatory mediator, IL-1, emphasizes the prominent role of IL-1 in pathogenesis of BCW arthritis. However, consistent with the study of recombinant sIL-1ra protein in the model of BCW arthritis (16), suppression of inflammation was significant but not complete, and destructive changes in bone and cartilage were attenuated but not abolished. The complexity of the cytokine networks in RA suggests that the simultaneous suppression of alternative inflammatory pathways will be needed for an adequate treatment of the chronic inflammatory process. One of those cytokines, tumor necrosis factor α (TNF-α), shares many proinflammatory properties and acts in a synergism with IL-1; yet each of these cytokines may exhibit distinct effects in RA (9). Studies in the BCW model of arthritis suggest that simultaneous inhibition of TNF-α and IL-1 may be more therapeutically efficacious than blockade of either agent alone (33).

In conclusion, the assessment of therapeutic activity of sIL-1ra gene transfer in the experimental model of recurrent BCW arthritis demonstrates the feasibility of antiinflammatory gene therapy for RA and the high biological efficacy of local delivery of therapeutic proteins to arthritic joints by gene transfer.

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