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Systemic Expression of Rat Soluble Retinal Antigen Induces Resistance to Experimental Autoimmune Uveoretinitis

Scott W. McPherson, Josh P. Roberts, and Dale S. Gregerson

To assess the role of sequestration in the maintenance of the immune privilege of the retina, retrovirally mediated gene transfer was used to express a defined, specific retinal autoantigen, rat soluble retinal Ag (S-Ag), in a systemic, nonsequestered manner. In this study we report the stable, long term transduction of rat retinal S-Ag into PBMC. Tolerance to S-Ag was assayed by challenging the S-Ag chimeric animals with S-Ag peptides in CFA and monitoring the time course and severity of experimental autoimmune uveoretinitis (EAU). The resulting data showed a correlation between the incidence of S-Ag chimerism and the loss of susceptibility to EAU. The development of resistance to EAU induction supports the hypothesis that Ag sequestration contributes to retinal immune privilege. The Journal of Immunology, 1999, 163: 4269–4276.

The rat soluble retinal Ag (S-Ag), also known as arrestin or 48-kDa protein, is a major soluble protein component of retinal rod photoreceptor cells and pinealocytes. The normal physiological role of S-Ag is to quench the visual transduction cascade (1). This is achieved by the binding of S-Ag to light-activated, phosphorylated rhodopsin, which then inhibits the binding of a photoreceptor cell-specific G protein (transducin) to rhodopsin (2–4). The uncoupling of rhodopsin and transducin ultimately prevents the propagation of signals from rod cells that are processed into visual images in the central nervous system. S-Ag is a member of the arrestin gene family, which includes β-arrestins (5–7) that regulate β-adrenergic receptors, various cone photoreceptor arrestins (8), and others. Studies have shown that members of the arrestin gene family are widespread and function in an analogous manner to S-Ag (9); they bind activated membrane-bound receptors of the rhodopsin family, preventing G protein binding and, in turn, downstream signaling events.

In addition to its normal physiological function, S-Ag is one of several ocular autoantigens that can induce experimental autoimmune uveoretinitis (EAU) upon immunization of susceptible hosts (10). EAU can also be induced by immunization with several different S-Ag peptides (11–13). EAU has been shown to be T cell mediated, because naive animals acquire the disease upon transfer of activated, S-Ag-specific, CD4+ T cell lines isolated from immunized animals (14, 15). Subsequent studies have described a number of specific S-Ag epitopes responsible for proliferative and/or pathogenic responses (13, 16–18). In addition to being a model of human uveitis/retinitis of autoimmune etiology, EAU is also a model for the study of mechanisms of immunological self-tolerance, particularly as they relate to the maintenance of the immune-privileged status of the eye (19). A long-standing question concerning mechanisms of ocular immune privilege is the role of sequestration. Sequestration refers to passive tolerance that is due to Ags being located behind anatomical or physiological barriers and has been proposed to account in part for the tolerance in normal animals to Ags located behind the blood-brain or blood-retinal barrier, such as S-Ag (20). Immune responses to sequestered Ags are thought to result from breakdowns of the barriers that might occur with immunization or injury. Recent studies on ocular immune privilege have elucidated several active mechanisms, including the anterior chamber-associated immune deviation phenomenon (21–23), Fas ligand expression (24), regulation of immune responses by corneal endothelial cells (25), and thymic expression of retinal Ags in certain animal strains (26). There is also evidence for lymphatic drainage of the eye (27), and dendritic cells have been found adjacent to the retinal pigment epithelium that forms part of the blood-retinal barrier (28). These processes and observations call the role and significance of sequestration into question. In any case, these mechanisms often fail to protect normal hosts against EAU induced by retinal autoantigens. The assessment of whether sequestration contributes to tolerance of retinal Ags in normal animals thus requires a comparison of the immune responses of animals that do and do not express a sequestered Ag in nonsequestered sites. We have recently shown that transgenic expression of β-galactosidase (β-gal) in the mouse retina creates a target for β-gal-mediated EAU, and those mice appeared ignorant of that β-gal unless Ag-specific, activated T cells were raised (29).

The study of the immunological properties of S-Ag has been limited by the quantity and difficulty of its isolation from rodent retinas. Although bovine S-Ag has been used in most studies, the limitations of studying tolerance to self Ags by using heterologous Ags are obvious. To study immunological tolerance to rat S-Ag, several S-Ag-expressing eukaryotic cell lines were made using retroviral vectors. These lines produce virus that, through the transduction of bone marrow stem cells, provides a method in which the normally sequestered retinal S-Ag can be expressed in a systemic, nonsequestered manner in the cells of the immune system. Here we report the in vitro expression of S-Ag in eukaryotic cells, the long term transduction of rat bone marrow stem cells, and the induction of resistance to S-Ag-mediated EAU as a result of its systemic expression.
Materials and Methods

Cloning of rat S-Ag and construction of recombinant vectors

A cDNA clone containing the coding sequence of rat S-Ag with 5′- and 3′-flanking sequences was obtained from a rat retinal cDNA library made by us. The S-Ag sequence was amplified by PCR using primers (25 pmol each) with 5′ EcoRI sites just 5′ and 3′ of the start and stop codon (SWM-18 and SWM-19, respectively; Table I). The amplification was conducted in 50 μl of 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, and 50 mM KCl with 200 μM (each) dNTPs, and 2.5 U of Taq polymerase. The PCR was run under the following conditions: initial denaturation of 1 min at 94°C, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and 30 s, and a final extension of 7 min. The amplified DNA was purified in a C-400 spin column (Clontech, Palo Alto, CA), digested with EcoRI (Promega, Madison, WI), repurified in a C-400 spin column, and ligated with T4 DNA ligase (Promega) into EcoRI-digested retroviral vector pLXSN (30) (provided by Dr. S. McIvor, University of Minnesota, Minneapolis, MN). The recombinant plasmid was designated pS-Ag (Fig. 1A). All recombinant plasmids were transformed into MAX EFFICIENCY STBL2 competent cells (Life Technologies, Gaithersburg, MD). The orientation and sequence of S-Ag in the recombinant plasmid were confirmed by restriction enzyme digestion and/or nucleotide sequencing.

Preparation of nucleic acids

Plasmid DNA was prepared using standard methods (31). Genomic DNA from eukaryotic cells was prepared using a DNA extraction kit (Stratagene, La Jolla, CA), and mRNA was prepared using a Poly(A)Tract System (Promega) or an Oligotex Direct mRNA kit (Qiagen, Valencia, CA). After isolation, mRNA was routinely treated with DNase I (Life Technologies), reisolated with RNeasy spin columns (Qiagen), and eluted in 5 mM Tris (pH 7.5).

Cells lines and maintenance

The retroviral packaging cell line GP+E86 (32) and NIH-3T3 cells were provided by Dr. S. McIvor. KZO cells, a murine T cell hybridoma, were provided by Dr. N. Shastri (33). All cells were maintained in DMEM (Celox, Hopkins, MN) with 5500 mg/L glucose, 100 μg/ml sodium pyruvate, 784 μg/ml l-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 35 μg/ml gentamicin, and 1× MEM nonessential amino acids (Sigma, St. Louis, MO), supplemented with 10% FCS in an atmosphere of 6% CO₂ at 37°C.

Transfection and selection of cells

GP+E86 cells were transfected with 1–5 μg of pLXSN or the recombinant plasmid pS-Ag using a calcium phosphate transfection system (Life Technologies, Gaithersburg, MD). The transfections were performed according to the manufacturer’s protocol except that 5 × 10⁶ cells were initially plated per 60-mm tissue culture dishes, and reagents were used at half-volume. Transfected cells were selected in 400-2000 μg/ml geneticin (antibiotic G418, Life Technologies). Cells transfected with pLXSN and pS-Ag plasmids were designated LXSN and GP-SAg, respectively. KZO-SAg cells were made by coculturing KZO cells with GP-SAg for 24 h, followed by removal of the nonadherent KZO cells and selection of transformed cells in G418.

Detection of nucleic acids

The presence and transcription of the S-Ag gene in chimeric rats (described below) were detected by PCR and RT-PCR on DNA and mRNA from PBMCs purified by density gradient centrifugation (Lympholyte-Rat, Accurate Chemicals, Westbury, NY). The PCR reactions were conducted using 500 ng of genomic DNA and 12.5 pmol of primers SWM-30 and SWM-31 in 50 μl of PCR reaction buffer (10 mM Tris (pH 8.8), 1.5 mM MgCl₂, and 75 mM KCl) containing 200 μM (each) dNTPs and 2.5 U Taq polymerase under the following conditions: initial denaturation at 92°C for 1 min, then 40 cycles of denaturation at 92°C for 30 s, annealing at 45°C for 1 min, extension at 72°C for 1 min, followed by a final extension of 7 min. A control PCR reaction specific for the neo gene of the retroviral vector (primers SWM-25A and SWM-26A) was performed using similar conditions (10 mM Tris (pH 9.2) and 15 pmol primers, 58°C annealing). The RT-PCR amplifications contained the mRNA, 20 U of RNase inhibitor (RNasin, Promega), 12.5 pmol of the 3′-downstream primer (SWM-23), and 20 pmol of the 5′ upstream primer (SWM-24). The RT portion of the reaction was conducted in 20 μl of 10 mM DTT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂ (RT buffer), 1 mM (each) dNTPs, and 200 U of reverse transcriptase (SuperScript II, Life Technologies) and incubated at 37°C for 1 h. The RT reaction was then inactivated at 95°C for 10 min and added to a solution containing the upstream primer, 5 μl of PCR reaction buffer (100 mM Tris (pH 8.3), 15 mM MgCl₂, and 500 mM KCl), and 2.5 U Taq polymerase to a final volume of 50 μl. The PCR portion of the amplification was run under the following conditions: initial denaturation of 92°C for 30 s, then 40 cycles of denaturation at 92°C for 20 s, annealing at 52°C for 30 s, extension at 72°C for 1 min and 30 s, followed by a final extension of 7 min. The products of all PCR and RT-PCR reactions were analyzed by Gel Star (FMC, Rockland, ME)-stained agarose gels.

Table I. Sequence of oligonucleotide primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>SWM-18</td>
<td>5′-GGAGAATTCCATCCCAAGGGACAGAAC</td>
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<tr>
<td>SWM-19</td>
<td>5′-GGAGATCCACGCCATGCGCTAAGTC</td>
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<td>SWM-23</td>
<td>5′-GAAGATCTTGGTCAATGGCTGTTG</td>
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<td>SWM-24</td>
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<td>SWM-25A</td>
<td>5′-GGCAACAAGACAACTTGCGCTC</td>
</tr>
<tr>
<td>SWM-26A</td>
<td>5′-TCTTGCAGATCATCCCTGATCG</td>
</tr>
<tr>
<td>SWM-30</td>
<td>5′-CTGCTCGTGTCCCTGACTACCTA</td>
</tr>
<tr>
<td>SWM-31</td>
<td>5′-CTTGGACGCTAAATATATCTCGA</td>
</tr>
</tbody>
</table>

FIGURE 1. Construction of recombinant vector and rat S-Ag expression. A, Construction of pS-Ag. A cDNA clone of rat S-Ag was PCR amplified and cloned into pLXSN as described in Materials and Methods. B, Expression of S-Ag. Hypotonic cell lysates from the indicated cell lines were Western blotted and probed with a mixture of mAbs (A9C6, 5C6.47, H11A2, and M9F10) to bovine S-Ag. +, PMA/ionomycin-activated cell lines.
Activation and detection of S-Ag proteins

Expression of the S-Ag protein in cell lines and rat PBMCs was assayed by Western blotting. Cell lines were activated by the addition of PMA (10 ng/ml) and/or ionomycin (400 ng/ml) for 6 h before harvest. The cells were then trypsinated, counted, washed twice in PBS, resuspended in hypotonic cell lysis buffer (34) to a concentration of 1 × 10^8 cells/μl, chilled on ice for 10 min, and briefly spun in a microcentrifuge. Rat PBMCs were purified from whole blood using a density gradient, resuspended in PBS, counted, and then subjected to hypotonic lysis as described above. The supernatants were then subjected to SDS-PAGE (35) and Western blotted using a mixture of mAbs to S-Ag including A9C6 (36), H11A2 (37), and M9F10 (a gift from Dr. J.-P. Faure).

Transduction of bone marrow (BM)

The transduction of BM was based on protocols modified from Kang (39, 40) and Fletcher (41). Briefly, donor BM was prepared from Lewis (LEW) rat or (LEW × Buffalo rat)F1 animals. The animals were treated with 5’-fluorouracil (150 mg/kg) and sacrificed after 2 days. BM was isolated by femoral perfusion with PBS followed by centrifugation and lysis of RBC using 0.17 M NH₄Cl. The cells were recentrifuged, and the remaining cells were placed in culture medium (RPMI 1640 medium (Cambrex) supplemented with 100 μg/ml sodium pyruvate, 784 μg/ml L-glutamine, 50 μM 2-ME, 1× MEM nonessential amino acids, 2.5 mg/ml glucose, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml gentamicin, 10% FCS, 10% U-937 conditioned medium, and 15% WEHI-conditioned medium).

GP-SAg cells were grown to near confluence in DMEM with G418 in T150 flasks. This medium was replaced with the supplemented RPMI 1640 medium, and after 24 h BM cells were added (BM cells from one donor animal per flask) and were cocultured for 24–72 h with the GP-SAg cells in medium containing 0.5 μg/ml polyclrene.

BM chimeric animals and EAU induction

After the GP-SAg/BM coculture, nonadherent cells were collected by straining the suspension through a 70-μm pore size nylon cell strainer, washed, resuspended in saline, and injected i.p. into irradiated (1100 rad) LEW recipients. Nonadherent cells (20–40 × 10^6) were used to reconstitute each recipient. At a minimum of 12 wk postreconstitution, the animals were injected s.c. with 75 μg of rat S-Ag peptide (aa 343–362, TSSEVATEVPFRMLHQPPEQ) or rat S-Ag peptide (aa 273–289, TLTKTLVLV-PPLANNRE) in CFA along with an i.p. injection of 5 × 10^5 preserved Bordetella pertussis organisms (Michigan Department of Public Health, Lansing, MI). Peptides 273–289 and 343–362 were previously shown to be uveitogenic in LEW rats (13, 16). The time course and severity (based on a 0–4 scale) of EAU induced was followed clinically and was verified by histopathology after sacrifice (42).

Results

Presence, transcription, and expression of S-Ag in fibroblasts

Following transfection of GP+E86 cells with pLXSN or pS-Ag, the resulting G418-selected cells (LXSN and GP-SAg, respectively) were assayed for the presence and expression of the S-Ag gene. PCR and RT-PCR analysis of the cell lines confirmed the presence and transcription of the gene in GP-SAg cells (data not shown). To determine whether GP-SAg cells express S-Ag protein, whole cell lysates were examined by Western blot analysis with mAbs to bovine S-Ag. S-Ag was found in GP-SAg cells, but not in LXSN cells (Fig. 1B). Densitometric analysis of the blot indicated that protein expression levels increased 2- to 9-fold upon treatment of the cells with the nonspecific activators PMA and ionomycin.

Titration of virus and viral transduction

To evaluate the ability of the GP-SAg cell line to produce significant levels of infectious, S-Ag-transducing virions, the virus was titrated on NIH-3T3 cells. Viral titration showed that the GP-SAg cell line produced ~7.5 × 10^9 infectious particles/ml based on counting foci of G418-resistant NIH-3T3 cells. To show that cells of BM lineage could be transduced by the virus and express S-Ag, KZO cells were cocultured with GP-SAg cells and then selected in G418 (KZO-SAg cells). Western blot analysis of whole cell lysates (Fig. 1B) shows the presence of S-Ag protein in the G418-resistant
KZO-SAg cells as well as NIH-SAg cells (from a G418-resistant foci of NIH-3T3 cells isolated during viral titration; not shown). Taken together, these data show that GP-SAg cells produce a high level of virus carrying the S-Ag gene, and that the virus is capable of infecting cells of BM origin. As with GP-SAg cells, S-Ag protein levels increased in KZO-SAg and NIH-SAg cells upon PMA/ionomycin activation (Fig. 1).

**Transduction of rat BM**

To express S-Ag as a systemic, extraocular (nonsequestered) Ag, BM cells were transduced with the S-Ag-carrying retrovirus produced by the GP-SAg cells. The transduced marrow was then used to reconstitute lethally irradiated LEW rats. Twenty-five S-Ag chimeric rats (S-1 through S-25) and 12 vector only chimeric rats (L-1 through L-12) were made. Stable transduction of the S-Ag gene was confirmed by PCR analysis on genomic DNA harvested from PBMCs at time points ranging from 4–21 wk post-BM reconstitution. Animals S-1 through S-11 were analyzed at 12–21 wk post-BM transduction, but before S-Ag peptide immunization. Animals S-12 through S-25 were analyzed at 4 and 8 wk after BM reconstitution and at 5 wk posttransduction (minimum of 17 wk post-BM reconstitution). PCR analysis (Fig. 2A) of PBMC genomic DNA indicated the presence of the S-Ag gene in 17 of the 25 chimeric rats at a minimum of one time point. The chimeric rats were simply classified as being either S-Ag positive (a positive PCR at any time point) or negative (S-9, S-10, S-14, and S-15 shown, S-22 to S-25 not shown) because of the highly variable efficiency of retroviral transduction of stem cells and the difficulties of establishing truly quantitative PCR. The extensive genomic intron/exon structure of arrestin gene family members makes it unlikely that the 417-bp PCR product is the result of nonspecific amplification of other arrestins. This was confirmed by subjecting positive PCR reactions to digestion with the *Sphl* restriction enzyme that cuts at a site unique to S-Ag located between primers SWM-30 and SWM-31 (data not shown). Additional PCR reactions performed on selected animals with another set of rat S-Ag specific primers yielded the same result as those with primers SWM-30 and SWM-31 (data not shown). Nine of the 12 vector chimeric rats analyzed at 8 wk post-BM reconstitution harbored the transduced retroviral sequences (Fig. 2B) as evidenced by the presence of the neo' gene. All vector-only control rats were negative for S-Ag (data not shown).

mRNA isolated from S-Ag chimeric rat PBMCs was then analyzed for S-Ag gene transcription by RT-PCR. To verify the integrity of the mRNA, all samples were assayed and were positive for transcription of the ubiquitously expressed gene G3PDH (data not shown). Although our assay could detect S-Ag mRNA at a level of less than one cell equivalent per aliquot tested (Fig. 2C, SC-5 when overexposed; mRNA from 10 GP-SAg cells in 10^7 rat PBMCs in a final volume of 100 μl, 2 μl assayed), S-Ag transcription was detectable in only one chimeric rat (S-5, Fig. 2C). The use of an upstream primer located in the retroviral sequences 5' of S-Ag in pS-Ag allowed us to determine that the transcript was specific to the transduced S-Ag gene.

**EAU analysis of chimeric rats**

After at least 12 wk post-BM reconstitution, the chimeric and control rats were immunized with either the rat S-Ag_{343–362} peptide or the rat S-Ag_{273–280} peptide, and the progression of EAU was monitored by clinical examination. Twenty-three S-Ag chimeric animals survived to be fully analyzed for EAU. As shown in Fig. 3, Fig. 4, and Table II, the severity of EAU decreased significantly in animals harboring the rat S-Ag gene regardless of the immunizing peptide. Normal animals that were immunized with the 343–362 peptide developed very severe EAU, with an average clinical score of 3.0 on days 15 and 17. Similar clinical EAU scores were observed in both the vector only rats (Fig. 3C) and the S-Ag-negative rats (Fig. 3B), indicating that the transduction protocol and the retroviral vector, by itself, did not affect EAU susceptibility. However, compared with either S-Ag-negative rats or vector control.
raths, S-Ag-positive rats exhibited reduced severity of EAU (Fig. 3A and Table II). Similar results were obtained in animals immunized with the rat 273–289 peptide (Fig. 4 and Table II). Normal and vector control animals exhibited comparable levels of EAU, while S-Ag chimeric animals displayed reduced severity of EAU.

Once signs of clinical disease had abated, or after 28–35 days postimmunization, the animals were sacrificed, and their eyes were examined histologically for EAU. The histological score from each eye (Table II) correlated well with the maximum score seen by clinical examination. Normal, vector controls, and S-Ag-negative animals had similar histological scores, while S-Ag-positive animals had significantly lower scores, indicating a reduction in the severity of EAU as a result of the systemic presence of S-Ag.

Detection of S-Ag protein in chimeric rats

To determine the actual level of S-Ag protein expressed in PBMCs, we subjected lysed cells to Western blotting against various mAbs to bovine S-Ag. Although our assays could detect as little as 0.2 ng of bovine S-Ag or rat S-Ag expressed in as few as \(1 \times 10^6\) GP-SAg cells, we could not specifically detect S-Ag in chimeric rat PBMCs (Fig. 5). These results indicate that even low level and/or transient systemic expression of a previously sequestered Ag can induce a degree of tolerance manifested as resistance to EAU induction. Western blots probed with only the H11A2 Ab resulted in the detection of several nonspecific interactions, which were likely the result of the high amount of Ab used on those blots. Because the polypeptides were the appropriate size of arrestin family members, these interactions probably represent epitope sequence homology between S-Ag and other arrestin-like molecules.

Discussion

If retinal-restricted expression of specific Ags, such as S-Ag, contributes to ocular immune privilege by a mechanism involving sequestration, we reasoned that expression of S-Ag in a systemic, nonsequestered manner should lead to tolerance to S-Ag that would result in resistance to S-Ag-induced EAU. Through the use of retrovirally mediated gene transfer into BM cells, S-Ag was transduced into LEW rat BM-derived cells, allowing the extracellular expression of S-Ag. Chimeric rats were then challenged with two different EAU-inducing S-Ag peptides. The resulting data showed a correlation between the incidence of S-Ag chimerism and reduced susceptibility to EAU. The development of immune tolerance to systemic S-Ag supports the hypothesis that Ag sequestration has a role in maintaining the immune privilege of the retina. It is well established that self Ag expression in the thymus leads to T cell tolerance to that Ag by either clonal deletion or clonal inactivation (43). We did not attempt to identify the mechanism(s) responsible for S-Ag tolerance, although both deletion and inactivation of T cells have been reported in response to proteins expressed as neo-self Ags by retroviral transduction (39, 44).

Endogenous S-Ag is expressed in the retinal photoreceptor cells, which are class I and class II MHC negative. In S-Ag-specific
EAU, it is thought that S-Ag from the photoreceptor cells is gathered, by unknown processes, into local APC, which then present peptides of S-Ag on class II MHC to CD4+, pathogenic T cells. Because S-Ag is an intracellular protein, virally transduced BM-derived cells will contain endogenously synthesized S-Ag, which would be expected to load mostly into class I MHC (45). Consequently, it is not obvious that expression of S-Ag peptides in class I MHC would lead to tolerance in CD4+, class II-restricted T cells. To directly alter the pathogenicity of CD4+, S-Ag-specific T cells would require that S-Ag peptides occupy class II on APC to delete these cells in the thymus or render them unresponsive as mature cells in the periphery. Alternatively, regulatory T cells might be raised by the systemic, virally transduced S-Ag by a variety of mechanisms, but we have no evidence for these.

Several observations bear on this question. There are clear examples of exogenous Ags being presented in class I MHC (46–48). Dendritic cells are reported to be particularly able to process exogenous Ag for presentation in class I MHC (49). Endogenous, intracellular Ags have found direct expression in class II via an endogenous presentation pathway (50, 51). In the thymus, with its rapid cellular turnover and apoptosis, there is further potential for intracellular Ags to be gathered and presented on class II MHC by cross-priming. As a result, the virally transduced S-Ag is likely to appear on class II MHC in the thymus. There is also opportunity for the transduced S-Ag to be presented on class II MHC elsewhere.

The level of S-Ag expression resulting from the viral transduction is of interest. Transduced S-Ag expression was, in all but one case, undetectable by reasonably sensitive RT-PCR and Western immunoblotting protocols. This low level was still able to reduce EAU susceptibility, indicating that very little systemic exposure is needed to be immunologically significant. On the other hand, endogenous retinal S-Ag expression is very high, reportedly 45 μg/retina (52), or ~4–5 mg/ml in the retina. Apparently, very little of this S-Ag leaves the retina, whether by leaking through the barriers or being conducted by dendritic cells, because it does not induce the same level of tolerance, if any, that was found to result from the very low expression levels obtained in BM-derived cells following viral transduction.

It has been observed that while retrovirally transduced cells stably harbor the vector sequences in vivo, protein expression levels may significantly decrease within periods as short as 1 mo (53). We could only detect transcription in one chimeric rat and were unable to detect definitive S-Ag protein expression in any of the rats that were assayed. Because we did not assay for S-Ag transcription for at least 4 wk postreconstitution or assay for S-Ag protein for at least 12 wk reconstitution, it is possible that an early burst of S-Ag expression resulted in the deletion of S-Ag autoreactive T cells by central or peripheral mechanisms. This could account for the resistance to EAU despite the lack of detectable protein expression at the time of assay. Because we assayed S-Ag transcription and expression in PBMCs, which are generally quiescent, it is possible that the S-Ag gene expression was up-regulated in more activated areas of the immune system, such as in thymic, BM-derived APC, which could affect selection of S-Ag-reactive precursors and contribute to the inactivation or deletion of peripheral T cells ordinarily activated by S-Ag peptide immunization (54).

Our data suggest that even a very low level of S-Ag systemically expressed in BM-derived cells is capable of inducing tolerance. Retrovirally transduced expression of phage λ cI repressor in activated and resting B cells as well as lymphoid-deficient bone marrow cells from SCID mice has been demonstrated to be efficient at inducing tolerance to a potent peptide epitope of this Ag (55, 56). Thus, it is likely that our expression of S-Ag in BM-derived cells induces tolerance by similar mechanisms. Our data are also consistent with findings obtained in a study concerning the development of quantitative tolerance to the retrovirally transduced superantigen gene of mouse mammary tumor virus (39), demonstrating that low levels of superantigen gene expression were sufficient to induce partial deletion of Ag-reactive T cells. The fact that the S-Ag gene could be detected by PCR in PBMCs following immunization with the S-Ag peptides and the ability to culture G418-resistant cells from chimeric rats at that time (data not shown) suggest that cells capable of expressing S-Ag persisted in the periphery and were not a target for potentially S-Ag-reactive cells induced by peptide immunization. This is consistent with the evidence that persistence of Ag is important in maintaining tolerance (57). Because low or even undetectable levels of systemic S-Ag resulting from the retroviral gene transfer was able to induce resistance to EAU in our model, it would seem likely that in the absence of rigorous sequestration of the large amount of S-Ag normally found in photoreceptor cells, normal LEW rats would be spontaneously resistant to S-Ag-induced EAU. Such is not the case.

Critical to the idea that S-Ag sequestration contributes to the immune-privileged state of the retina is the absence of S-Ag expression in the thymus of normal LEW rats. Because many autoimmune diseases affect immune-privileged tissues, it has been suggested that autoimmunity against these tissues is the result of the specific autoreactive Ag(s) not being available for tolerogenic immune processes. This idea is supported by experiments demonstrating tolerance and loss of susceptibility to autoimmune disease in diabetic animal models (58, 59) and in myelin basic protein (MBP)-induced experimental autoimmune encephalomyelitis (60) when the tissue-specific Ag was introduced into the thymus. However, other studies have found that tissue-specific Ags such as insulin (61) and MBP (62, 63), are expressed in the thymus. Even viral transgenes controlled by tissue-specific regulatory sequences have been found to be expressed in the thymus (61) of mice. Despite concerns about MBP isoforms and a lack of defined autoantigen in the case of diabetes, these studies suggest that thymic expression could contribute to the immune tolerance of otherwise tissue-specific Ags (64).

We have previously assayed the LEW rat thymus for S-Ag expression (65) to examine the possibility that thymic expression of S-Ag contributes to retinal immune privilege. Transcripts from multiple members of the arrestin gene family, including β-arrestin-1 and β-arrestin-2, but not specifically S-Ag, were detected when using mixed oligonucleotide primers to sequences conserved among all known arrestins, including S-Ag. Further, we could not detect S-Ag expression in fetal, neonatal, or adult thymus when using primers whose sequences were predicted to be unique to retinal rod photoreceptor S-Ag. Our data were supported in a recent study correlating thymic expression of ocular autoantigens and the resistance to autoimmune disease (26) that, again, failed to detect retinal S-Ag expression in the LEW rat thymus, although expression was detected in mice. These results suggest that the lack of retinal S-Ag expression in the thymus could account for the presence of S-Ag-reactive T cells in normal LEW rats and could contribute to the ease of EAU induction in these rats.

Based on previous studies showing retroviral-mediated gene transfer leading to the induction of tolerance to viral and λ phage Ags expressed as systemic neo-self Ags (39, 44, 55, 56) and our present report of the use of an autoantigen-expressing retroviral vector to induce resistance to autoimmune disease, the potential for treating autoimmune disease with retrovirally mediated gene therapy is clear. In addition to the expression of wild type S-Ag, the
retroviral gene transfer system will permit the systemic expression of mutant S-Ags as self Ags, allowing us to further characterize the immune response to specific epitopes of rat retinal S-Ag. Our expression system allows significant levels of protein to be made in vitro, which can facilitate biochemical studies of S-Ag.

Acknowledgments
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References


