Immunomodulation of Experimental Autoimmune Uveoretinitis by Intravenous Injection of Uveitogenic Peptides

Yoichi Sasamoro,* Yoh Ichi Kawano,* Randy Bouligny,*† Barbara Wiggert,‡ Gerald J. Chader,‡ and Igal Gery*

Intravenous (IV) injection of antigenic proteins induces specific unresponsiveness, as shown by the diminished response to a challenge with these proteins in complete Freund's adjuvant. This study examined the effect of IV treatment with uveitogenic peptides on the development of experimental autoimmune uveoretinitis (EAU). The peptides used were derived from the sequence of bovine interphotoreceptor retinoid-binding protein (IRBP) and included R16 (sequence, 1177–1191), which is immunodominant and highly uveitogenic, and R4 (sequence, 1158–1180), which is nondominant and weakly uveitogenic. The efficacy of this treatment was found to depend on both the dose used for the IV injection and that used for the challenge. Thus, EAU induced by R16 at a dose of 0.2 nmol/rat was inhibited completely in all rats treated with the peptide at doses of 400 or 133 nmol and partially by the low dose of 5 nmol/rat. However, the EAU induced by a R16 challenge of 40 nmol/rat was inhibited only partially by the high treatment dose of 400 nmol/rat. The IV treatment was found to be effective in inhibiting the EAU induced by peptide R4. A large dose of R4 was needed to induce EAU (40 nmol/rat), and the disease was inhibited completely in all rats treated IV with this peptide at doses of 800, 400, or 133 nmol. In most animals injected with the 44-nmol dose, also, inhibition was complete. These data show that there is a correlation between the doses needed for achieving inhibition and those used for the challenge. The ratios between these doses in all experiments were found within the range 1–20. The IV treatment of rats with the dominant peptide R16 effectively inhibited the development of EAU induced by whole IRBP. By contrast, treatment with peptide R4 had no effect on the disease induced by the native protein. These data therefore show the effectiveness of IV treatment with uveitogenic peptides as a procedure to inhibit EAU. This study also established a new relationship between the immunodominance of peptide determinants and their capacity to induce immune unresponsiveness.


Experimental autoimmune uveoretinitis (EAU) is an intraocular inflammatory disease that can be induced in various animals by immunization with retinal-specific antigens. Two retinal antigens are particularly uveitogenic: S-antigen and the interphotoreceptor retinoid-binding protein (IRBP). Both antigens have been sequenced, and consequently, several peptide determinants with uveitogenic capacity were identified for each protein. The uveitogenic peptides were found to have different levels of immunologic and immunopathogenic activities. Of interest to us were the findings with peptides of bovine IRBP. One peptide, which occupies sequence 1177–1191 (designated R16) was highly uveitogenic, inducing EAU at doses as low as 0.01 nmol/rat. Other uveitogenic peptides of IRBP induce disease only at much higher doses, eg, 40 nmol/rat for peptide sequence 1158–1180 (R4) or 100 nmol/rat for peptide sequence 1091–1115 (R23).

In general, EAU is considered a model for certain uveitic conditions in humans in which autoimmune processes are assumed to play a pathogenic role (in particular, sympathetic ophthalmia, Vogt-Koyanagi-Harada syndrome, Behcet’s disease, and birdshot retinochoroidopathy). This animal disease has been used, therefore, to test new treatments, some of which have been applied successfully clinically. In addition to various immunosuppressive or antiinflamatory treatments, EAU can be inhibited by induction of immune unresponsiveness that is specific for the antigens used for its production. The approaches used for induction of unresponsiveness mainly include the oral feeding of the antigen, injection of the...
antigen into the anterior chamber,16 or systemic administration of the uveitogenic antigens in large amounts.17,18
The classic procedure to induce unresponsiveness, or tolerance, is to inject antigens into newborn animals.19-20 In addition to inducing tolerance effectively against cells and proteins, this procedure also was useful for inducing unresponsiveness toward short peptides.21-24 Unresponsiveness also may be induced in adult animals by injecting antigens in soluble form, usually by the intravenous (IV) route. This procedure can be an effective mode for inducing unresponsiveness toward various antigens,25-28 including tissue-specific proteins.27,28 Little is known, however, about the usefulness of this procedure for inducing unresponsiveness toward peptides.

We tested the possibility of inhibiting EAU by IV administration of uveitogenic peptides. The tested peptides were two determinants of bovine IRBP, R4 and R16, which are weakly and highly uveitogenic, respectively. Both peptides were able to induce unresponsiveness, but profound differences were found between their activities.

**Materials and Methods**

**Animals**

Male inbred Lewis rats, 8–12 weeks old, were supplied by Charles River Laboratories (Raleigh, NC). The animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

**Antigens**

Bovine IRBP was purified as described in detail elsewhere.29 The IRBP-derived peptides we tested were synthesized and purified by Applied Biosystems (Foster City, CA) with a model 430A peptide synthesizer, using tertbutyloxycarbonyl chemistry. The peptide sequences were derived from the sequence of bovine IRBP.30 The peptides we used were peptide R4, which occupies sequence 1158–1180 (HVDDTDLLYLTIP-TARSVGADDG); peptide R16, at sequence 1177–1191 (ADGSSWEGVGVVPDV), and peptide W3, at sequence 271–283 (SQTWEGSGVLPCV). The peptides were dissolved in phosphate-buffered saline (PBS) and filtered through a 0.22-μm pore filter before use.

**IV Injection and Immunization**

The rats were injected IV with various amounts of the peptides in a volume of 0.2 ml on days −7 and 0. Control rats similarly were injected with PBS. All rats were challenged for disease induction on day 0 by a single injection of IRBP or the peptides, emulsified in complete Freund’s adjuvant (CFA), containing *Mycobacterium tuberculosis* H37Ra at 2.5 mg/ml (Difco, Detroit, MI). The emulsion was injected into one footpad in a volume of 0.1 ml containing different doses of the antigens.

**Disease Monitoring and Assessment**

Immunized rats were examined daily for clinical ocular changes.4 Disease occurrence and severity were verified by conventional histologic examination. The severity of disease was graded according to the intensity of the clinical and histopathologic changes, using scales containing 0–4 points.4

**Statistics**

The data were analyzed statistically using Student’s t-test. P values less than 0.05 were considered significant.

**Results**

**IV Injection of Peptide R16 Inhibits the Development of R16-Induced EAU**

Figures 1–3 summarize the data of a series of experiments in which IV treatment of rats with peptide R16 in aqueous solution was tested for its modulatory effect on the development of EAU induced by a challenge with R16 in CFA. The IV treatment inhibited the disease completely in some rats. In most animals, the effect was partial and was assessed by three parameters: (1) the delay of disease onset and reduction of severity as measured by both (2) clinical and (3) histologic changes. The latter parameter can be measured more accurately, and therefore, it was the one used for statistical analysis of the treatment effect. A good correlation usually was seen among these three parameters.

Different doses of peptide R16 were tested for both the IV treatment and for the EAU-inducing challenge; the efficacy of disease inhibition depended on both the treatment and challenge doses. Thus, IV treatment with R16 at 44, 133, or 400 nmol completely inhibited EAU development in most rats challenged with the low dose of 0.2 nmol (Fig. 1), but it had only a partial inhibitory effect in most animals challenged with 2.0 nmol (Fig. 2). Furthermore, only partial inhibition of EAU was observed in rats challenged with the 40-nmol dose. Animals treated with 400 nmol were the only ones to show a significant reduction in the disease induced by this dose of R16 (Fig. 3).
Fig. 1. Inhibitory effect of intravenous (IV) treatment with peptide R16 on the development of EAU induced by this peptide at 0.2 nmol/rat. The panel on the left records the mean clinical scores (±SE) of eyes of rats of each treatment group, at different time intervals after the challenge with the peptide. The panel on the right summarizes the mean scores (±SE) of the histologic changes and records the incidence of diseased eyes. The IV treatment consisted of two injections, on days -7 and 0. The recorded values are the total amounts injected to rats of each group, as indicated. The challenge, given on day 0, consisted of the peptide, emulsified in complete Freund's adjuvant. Significance of the treatment effect was calculated by comparing each of the treatment groups with the control group injected with phosphate buffered saline ("none"). *P < 0.05; **P < 0.01.

Our data were collected from repeated experiments, with varying degrees of susceptibility to disease induction and to the treatment effect among rats of the different experiments. Despite this variability, however, our results showed that significant inhibition of EAU was achieved when the dose of R16 in-
JECTED IV was 2–20-fold higher than that used for the challenge. Thus, EAU development was inhibited significantly by IV injection of R16 at a dose of 5 nmol in rats challenged with 0.2 or 2 nmol (Figs. 1 and 2, respectively), and only treatment with a dose of 400 nmol was effective in animals challenged with 40 nmol (Fig. 3).

Also interesting was the finding that IV treatment with the peptide R16 at the nonsuppressive low doses of 0.05 or 0.5 nmol usually exacerbated the disease in rats challenged with the peptide at 0.2 or 2 nmol. Both the clinical and histologic changes were more severe in these rats than in the control animals injected with PBS. In addition, rats with this exacerbated disease showed cellular immune responses higher than those of the control animals (data not shown).

**Effect of IV Treatment With Peptide R4 on EAU Induced by This Peptide**

Peptide R4, which is nondominant (or “cryptic”) produces EAU only at doses much higher than those of the immunodominant peptide R16. The dose of R4 we used to induce EAU was 40 nmol. Even at this dose, disease onset was delayed (day 12 or 13), and its severity (Fig. 4) was lower than that induced by R16 at doses 20- or 200-fold lower (Figs. 1, 2). However, IV treatment with R4 was effective in inhibiting the EAU induced with this peptide. As shown in Figure 4, the disease was inhibited completely in all rats treated with the peptide at doses of 133, 400, or 800 nmol, as it was in most animals injected with the 44-nmol dose. A marginal inhibitory effect (ie, a delay of onset by 1 day) was seen in rats treated IV with R4 at a dose of 15 nmol.

**Effect of IV Treatment With the Peptides on the Development of EAU Induced by Whole IRBP**

The difference between the immunodominant (R16) and cryptic (R4) peptide determinants of IRBP was established further in experiments in which rats were injected IV with either one of these peptides and challenged for EAU induction with whole IRBP in CFA. As shown in Figure 5, IV treatment with peptide R4 had no effect in this system. Rats injected IV with R4 developed EAU similarly to the control animals injected with PBS. By contrast, IV treatment with peptide R16 was effective in this system. In rats challenged with IRBP at a dose of 2 μg (Fig. 6), a complete inhibition of EAU was achieved in all rats treated with R16 at a dose of 400 nmol and in one half of those injected IV with the peptide at a dose of 200 nmol. Treatment with R16 was less effective in rats challenged with 20 μg of IRBP (Fig. 7). The inhibition was partial in most animals treated with peptide R16.

**Specificity of the IV Treatment Effect**

The specificity of the unresponsiveness induced by IV treatment was examined by testing the development of EAU in rats treated with peptide R16 and
challenged with peptides R4 or W3. Peptide R4 does not cross react with R16. W3 (sequence, 271–283), which is a “repeat” of sequence 1179–1191, cross reacts weakly with R16. Our findings are shown in Table 1. The IV treatment with R16, which effectively inhibited the disease induced by this peptide, had little or no effect on the development of EAU induced by R4 or W3.

Discussion

Our results show that IV injection of uveitogenic peptides is a powerful immunomodulating treatment that effectively inhibits the development of EAU induced by the same peptides in rats. Thus, this treatment was able to inhibit completely the disease induced by the two IRBP-derived peptides we adminis-

Fig. 4. Inhibitory effect of IV treatment with peptide R4 on the development of EAU induced by this peptide, at 40 nmol/rat. See Figure I for additional detail.

Fig. 5. Inhibitory effect of IV treatment with peptide R4 on the development of EAU induced by a challenge with whole interphotoreceptor retinoid-binding protein (IRBP) at 2 μg/rat. See Figure I for additional information.
tered (R4 and R16). The effectiveness of the treatment was dose dependent, and the experiments with peptide R16 showed the dependence on both the treatment dose injected IV and that used for the challenge. In this regard, increasing doses of the peptide were required for IV treatment to inhibit EAU in rats challenged with the higher doses of R16 (Figs. 1-3).

The direct relationship between the peptide doses needed for EAU induction and for its inhibition by IV treatment was indicated further by the finding that the ratios between these doses remained within the range of 2-20 for peptide R16 (Figs. 1-3) and was approximately 1 for peptide R4 (Fig. 4). The same range of ratios also was found for systems in which

Fig. 6. Inhibitory effect of IV treatment with peptide R16 on the development of EAU induced by whole IRBP at 2 μg/rat. See Fig. 1 for more detail.

Fig. 7. Inhibitory effect of IV treatment with peptide R16 on the development of EAU induced by a challenge with whole IRBP at 20 μg/rat. See Figure 1 for additional information.
whole proteins were used. The cellular immune response to 100 μg of bovine gamma globulin was inhibited by an IV injection of 2 mg, and thyroiditis induced by thyroglobulin at the dose of 80 μg was inhibited by treatment with 50 μg of this protein.

Particularly interesting were our results concerning the comparison between the immunodominant (R16) and cryptic (R4) peptides with regard to their capacity to induce unresponsiveness. R16 induced unresponsiveness at doses lower than those of R4 (4 and 44 nmol/rat, respectively), but this effect of R16 was achieved only in rats challenged with the low dose of 0.2 nmol. However, when the two peptides were tested in rats challenged with the same dose (40 nmol/rat), which is the minimal uveitogenic dose of R4, peptide R16 was inferior to R4 in its capacity to inhibit the disease (Figs. 3, 4). Little is reported in the literature concerning the relationship between immunodominance of peptides and their capacity to induce tolerance. In a recent publication, it was reported that an immunodominant peptide was superior to a nondominant one in its capacity to induce tolerance when the two peptides were tested while linked covalently. These authors did not report, however, on the tolerogenicity of the two peptides when tested separately. Our study thus sheds new light on the relationship between the immunodominance of peptides and their capacity to induce unresponsiveness. We found that, when the ratios between the doses needed for disease induction and inhibition were calculated, the nondominant peptide was at least as effective as the dominant one. In addition, our data suggest that the process of unresponsiveness initiated by the low doses of the dominant peptide, R16, was overcome completely by the powerful immunopathogenic responses induced by this peptide at the high doses. By contrast, the pathogenic response induced by R4 was relatively weak and could be modulated readily even by unresponsiveness processes of limited intensity.

Our study also documented the effects of IV injection of R4 and R16 on the development of EAU induced by whole IRBP. As expected, R4, which is not recognized by lymphocytes sensitized to the whole protein, had no effect on the induced disease in rats challenged with whole IRBP (Fig. 5). Conversely, treatment with peptide R16 effectively inhibited the disease induced by the whole protein (Figs. 6, 7). This finding underscored the prominence of the R16 site by showing that, in addition to its immunodominance in the cellular response to whole IRBP, this determinant also plays a major role in the pathogenic process initiated by IRBP in Lewis rats. In addition, other uveitogenic sites of this large protein play only a minor role in the immunopathogenic process of IRBP-induced EAU. The finding that IV treatment with peptide R16 had little or no effect on the development of EAU induced by R4 or even by peptide W3, which is a weakly cross-reacting “repeat” of peptide R16 (Table 1).

Our finding that IV treatment with peptide R16 inhibited EAU induced by whole IRBP agreed with the results of others that EAU induced by S-antigen can be inhibited by feeding rats the peptide 341–360, which is an immunodominant determinant of this protein. The dose of this peptide that was used to inhibit the disease by feeding (approximately 300 nmol/rat) was in the same range as that of peptide R16 injected IV to inhibit the IRBP-induced EAU (200 nmol/rat). More studies currently are ongoing to compare these two procedures further for their induction of unresponsiveness.

The finding that induction of unresponsiveness toward an immunodominant peptide may inhibit the disease provoked by the whole protein suggests that this procedure might be considered a technique adaptable for humans (to suppress pathogenic autoimmune processes affecting organs such as the eye). A prerequisite for the potential usefulness of this procedure is the identification of the epitope(s) that serve(s) as a target for the pathogenic process. Our preliminary data in patients with uveitis and accumulating information of studies on those with multiple sclerosis indicate that different determinants of S-antigen or myelin basic protein, respectively, serve as the target epitopes in those with autoimmune responses to these proteins.

The mechanism of unresponsiveness induced by the IV administration of peptides is still unknown.
However, because the process was done in adult rats, it could be assumed that clonal deletion in the thymus plays a minor role, if any, in this process. Two other mechanisms could be considered, therefore, as potential explanations for the treatment effects (namely, clonal anergy\textsuperscript{36} and involvement of suppressor cells\textsuperscript{13,37}). These two mechanisms are not mutually exclusive, and their possible participation in the unresponsiveness phenomenon currently is being investigated.

Key words: experimental autoimmune uveoretinitis (EAU), interphotoreceptor retinoid-binding protein (IRBP), IRBP-derived peptides, immunodominance of peptides, immunomodulation/immunotolerance

References