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Inhibition of Experimental Autoimmune Uveitis by Amino Acid Copolymers

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Abstract

Glatiramer acetate (GA), a synthetic random amino acid copolymer, poly(Y, E, A, K)_n, is widely used for treatment of multiple sclerosis. It inhibits experimental autoimmune encephalomyelitis (EAE) in mice by competition with the antigen and by induction of regulatory T cells. A novel copolymer, poly (F, Y, A, K)_n, designated FYAK, was more effective than GA in its immunomodulatory activity in EAE. Here, FYAK and GA were compared in the amelioration of another disease model in mice, experimental autoimmune uveoretinitis (EAU). When tested by co-immunization with a uveitogenic antigen, FYAK was superior to GA in its capacity to inhibit EAU induction, as well as immune processes related to this condition. Further, regulatory T cell lines specific to FYAK were more immunosuppressive than GA-specific lines in the EAU model. The superiority of FYAK-specific lines was accompanied by higher production of Th2 cytokines. These data thus demonstrate that FYAK, a novel copolymer, is superior to GA in its capacity to inhibit immunopathogenic processes in a non-central nervous system tissue.

Keywords

Autoimmunity; Copolymers; Cytokines; EAU; glatiramer acetate

1. Introduction

Glatiramer acetate [GA, poly(Y,E,A,K)_n, Copolymer 1, Copaxone] is a synthetic random basic amino acid copolymer that is widely used for treatment of relapsing-remitting multiple sclerosis (MS). The efficacy of GA was first demonstrated in rodents developing experimental autoimmune encephalomyelitis (EAE), the animal model for MS and much of our knowledge about GA has been accumulated by use of this rodent model for MS (Arnon and Aharoni, 2004; Farina et al., 2005). The mode of action of GA is complex and may include both competitive binding to MHC molecules on antigen presenting cells (APC) (Arnon and Aharoni, 2004; Fridkis-Hareli et al., 1999; Fridkis-Hareli and Strominger, 1998; Fridkis-Hareli et al., 1994) and the induction of immunosuppressive cytokine-secreting

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copolymer-specific T-regulatory cells (Aharoni et al., 2000; Arnon and Aharoni, 2004; Duda et al., 2000; Farina et al., 2005; Stern et al., 2008). Recent studies have suggested also the involvement of dendritic cells and type II monocytes in the process (Hussien et al., 2001; Kim et al., 2004; Vieira et al., 2003; Weber et al., 2007).

Since binding to MHC molecules plays a role in the suppressive activity, additional copolymers with higher affinity for HLA-DR2, the MHC molecule strongly associated with MS, were synthesized (Fridkis-Hareli et al., 2002). Two of these novel copolymers, designated FYAK [poly(F,Y,A,K)_n, CO-14, P1-2301] and VWAK [poly(V,W,A,K)_n] were superior to GA in several aspects, including their affinity for HLA-DR2, their capacity to inhibit EAE and their induction of immunosuppressive cytokine-secreting T regulatory cells (Fridkis-Hareli et al., 2002; Illes et al., 2004; Stern et al., 2004; Stern et al., 2008). The immunosuppressive activity of GA, as well as that of FYAK, is not restricted to immune-mediated central nervous system (CNS) conditions (Arnon and Aharoni, 2004; Stern et al., 2008) and we have previously reported that treatment with this copolymer also inhibits development of an ocular inflammatory disease, experimental autoimmune uveoretinitis (EAU) (Zhang et al., 2000). EAU is induced in mice by immunization with a retinal-specific antigen, interphotoreceptor retinoid-binding protein (IRBP), and closely resembles EAE in various aspects (Caspi, 2002).

In the present study, GA and FYAK, one of the two novel copolymers, have been compared in their capacity to inhibit induction of EAU, as well as several parameters related to the immunopathogenic process of this ocular disease. FYAK was more effective than GA in all tested parameters. Of particular interest is the observation that regulatory cell lines specific for FYAK produced immunosuppressive cytokines at levels higher than those made by GA-specific lines.

2. Materials and Methods

2.1 Mice

Female B10.A (H-2^a), B10.RIII (H-2^f) and B10.BR (H-2^k) mice, 6-8 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were housed in a pathogen-free facility and all procedures involving animals were performed in compliance with the NIH Guidelines on Use of Animals in Research.

2.2 Reagents

FYAK (also designated CO-14 or PI-2301) was provided by Peptimmune, Inc. (Cambridge, MA), while GA was purchased from TEVA Neuroscience Inc. (Kansas City, MO). Bovine IRBP was prepared as described by Pepperberg et al. (1991) and human IRBP peptide 161-180 was purchased from AnaSpec Inc. (San Jose, CA). Pertussis toxin (PTx) was obtained from Sigma (St. Louis, MO) and purified protein derivative (PPD) was from Parke-Davis (Morris Plains, NJ). Complete Freund's adjuvant (CFA) was prepared by adding *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) to incomplete Freund's adjuvant (Difco), to a concentration of 2.5 mg/ml.

2.3. Induction of EAU and Copolymer administration

EAU was induced as described by Silver et al. (Silver et al., 1999) with minor modifications. Mice were immunized with 50µg IRBP (B10.A or B10.BR), or 10µg IRBP peptide 161-180 (B10.RIII) emulsified in CFA. The emulsion was injected into the base of the tail and two thighs in a total volume of 0.2 ml, and the B10.A or B10.BR mice were also concurrently injected with 0.5µg of PTx, intraperitoneally. GA or FYAK were incorporated in the aqueous portion of the emulsion (co-immunization) at the indicated doses. Eyes were

collected 14 days post injection (p.i.) and ocular sections were prepared as described elsewhere (Takase et al., 2005). Severity of disease, on a scale of 0 to 4 in half-point increments, was scored as detailed elsewhere (Takase et al., 2005).

2.4. Cytokine production

Draining lymph node cells of immunized mice were cultured in 24-well plates at 5×10^6 cells in 1 ml of RPMI-1640 medium, containing HL-1 serum replacement (Cambrex Bioscience, Walkersville, MD), with or without stimulants. Supernatants were collected after incubation for 48 hours (hr). Production of cytokines by copolymer-specific cell lines was measured in culture supernatants of day 2 of the 3rd or 4th stimulation cycle (see below) with the corresponding copolymer. Cytokine levels were determined by Multiplex SearchLight Technology (Pierce Biotechnology, Woburn, MA) and major differences between samples were verified by using ELISA kits from R&D Systems (Minneapolis, MN).

2.5. Generation of copolymer-specific CD4 - cell lines

Copolymer-specific CD4 T-Cell Lines were generated as described (Stern et al., 2008), with minor modifications, as follows. Spleen and draining lymph node cells from mice immunized with 200 μg of copolymer were collected 10 days p.i. and CD4 T cells were isolated as described in detail elsewhere (de Vos et al., 2000; Foxman et al., 2002). CD4 cells were then stimulated with the corresponding copolymer (10 $\mu\text{g}/\text{ml}$) at $2.5 \times 10^6/\text{ml}$ in 24-well plates, for 3 days, in the presence of APC (irradiated syngeneic naïve splenocytes) at the ratio of 1:1. Activated CD4 T-cells were then put into a resting stage by culturing with complete DMEM medium containing 10% fetal bovine serum and IL-2 (20 ng/ml) for 5-7 days. The stimulation and resting procedures were repeated for 2 or 3 additional times.

The line cells were also tested for their responsiveness toward the corresponding copolymer. The cells were obtained from cultures of cell lines, on day 2 after re-stimulation, washed and incubated in 96 well plates, along with irradiated syngeneic APC, at the ratio of 1:1, with the corresponding copolymer at different concentrations. The proliferation response was measured by ^3H -thymidine incorporation, with a pulse of 0.5 $\mu\text{Ci}/\text{well}$, during the last 16 hr of 4 day total incubation.

2.6. Testing the immunosuppressive activity of copolymer-specific cell lines in vivo

Copolymer-specific cell lines specific to GA or FYAK, established as described above, using cells from B10.R.III, B10.A, or B10.BR, were injected into naïve mice of the corresponding strain, at 5×10^6 or 10^7 , and the recipient mice were immunized one day later with IRBP peptide 161-180 (B10.R.III), or the IRBP protein (B10.A and B10.BR), as detailed above. EAU development was measured 14 days later, as described above.

2.7. Testing the immunosuppressive activity of copolymer-specific cell lines in vitro

Copolymer-specific T line cells of B10.A origin were added, at 5×10^4 , along with the corresponding copolymer, at 10 $\mu\text{g}/\text{ml}$, to cultures made of 5×10^4 CD4 T cells from syngeneic mice, immunized 10 days earlier with IRBP, and 20×10^4 irradiated APC. 5×10^4 naïve CD4 T cells from syngeneic untreated mice were added to control cultures. IRBP was added to all cultures except control, at 10 or 30 $\mu\text{g}/\text{ml}$, and the proliferation level was determined after 90 hr incubation, with a ^3H -thymidine pulse, at 0.5 μCi per well, given for the last 16 hr.

2.8. Flow cytometric identification of Foxp3+CD25+ Treg cells

One million copolymer-specific cell lines were first stained with anti-mouse CD4 and CD25 (BD Pharmingen, San Diego, CA), for 30 min, on ice, in phosphate-buffered saline (PBS)

containing 0.2% bovine serum albumin (BSA), then were stained for intracellular Foxp3 using allophycocyanin-conjugated anti-mouse/rat Foxp3 Staining Set (BD Pharmingen), following the producer's protocol. Cells were fixed using Fix/Perm buffer for 0.5 hr, then incubated with the anti-Foxp3 antibody (FJK-16) for 30 min at 4°C. Samples were re-suspended in PBS containing 0.2% BSA and data were acquired on a FACSCalibur (BD Biosciences, San Diego, CA), and analyzed by Flowjo software (Tree Star Inc., Ashland, OR).

2.9. Statistical analysis

Data were analyzed using statistical software Prism 5. Mann-Whitney U test was used for comparisons among groups. 2-way ANOVA was used for comparisons of cytokine production induced by the copolymers in EAU. Probability values less than 0.05 were considered statistically significant.

3. Results

3.1 Comparison of FYAK and GA in suppression of EAU development

To compare FYAK and GA for their capacity to inhibit the development of EAU we used the co-immunization procedure, in which the copolymer is incorporated into the uveitogenic antigen/CFA emulsion. Mice co-immunized with PBS and antigen/CFA were used as controls. Data from repeated experiments, with two strains of mice, B10.A (H-2^a) and B10.RIII (H-2^f), are summarized in Fig. 1. B10.A mice were immunized with whole IRBP and were also treated with PTx. However, B10.RIII were immunized with peptide 161-180 derived from the IRBP sequence; no treatment with PTx is necessary for disease development in these latter mice (Silver et al., 1999).

Both copolymers inhibited EAU induction in the two mouse strains. FYAK was remarkably more suppressive than GA in both strains. The inhibitory effects of the copolymers were particularly apparent in mice of the B10.RIII strain in which FYAK fully inhibited disease development even at the low dose of 0.05 mg per mouse, whereas GA was fully inhibitory in these mice only at a ten times higher dose, 0.5 mg. In B10.A mice the disease was only partially suppressed by both copolymers, but FYAK was again more inhibitory than GA at all tested doses.

3.2. Reduction of pro-inflammatory cytokine production induced by the copolymers

Inflammatory processes such as EAU are mediated by cytokines produced by lymphoid cells. Of particular importance are interferon (IFN)- γ and IL-17, produced by Th1 and Th17 cells, respectively (Bettelli et al., 2007; Steinman, 2007). We compared, therefore, the effects of copolymer treatment on the secretion of these two pro-inflammatory cytokines by draining lymph node cells when stimulated in culture with IRBP. Treatment with GA had little or no effect on the release of the tested cytokines. In contrast, treatment with FYAK inhibited the release of both IFN- γ and IL-17 (Fig. 2). The culture supernatants were also examined for Th2 cytokines, IL-4, IL-5 and IL-10, but the levels of these cytokines were marginal and no differences could be detected between control and copolymer-treated cultures (data not shown).

3.3. Inhibition of EAU development by copolymer-specific T-cell lines

A major mechanism of action of GA and FYAK is induction of immunosuppressive cytokine-secreting T-regulatory cells (Arnon and Aharoni, 2004; Farina et al., 2005; Stern et al., 2008). To compare the two copolymers for their capacity to generate regulatory cells in our experimental system we established cell lines specific to GA or FYAK and tested their effect on EAU development by adoptively transferring the line cells into syngeneic

recipients one day prior to induction of EAU. The line cells were tested at 5×10^6 or 10^7 cells per mouse and data collected with mice of three strains are recorded in Fig. 3. FYAK-specific line cells inhibited EAU development in all three mouse strains, whereas treatment with GA-specific lines was effective only with the B10.RIII strain and only at the high cell number of 10^7 per mouse (Fig. 3). Control syngeneic line cells, specific to an unrelated protein, hen egg lysozyme, or of cells stimulated by concanavalin A, were tested in control mice similarly immunized by IRBP. These control line cells did not exert any effect on EAU development (data not shown).

3.4. FYAK-specific T-cell lines inhibit T-cell proliferation more efficiently than GA-specific lines

In addition to testing their immunosuppressive activity in vivo, we also examined the FYAK- and GA-specific cell lines for their ability to inhibit proliferation in vitro of IRBP-specific T cells in culture (Fig. 4). Copolymer-specific T cell lines were mixed with the IRBP-specific T-cells at a 1:1 ratio. The response of control cultures to which naïve CD4 T cells were added was measured for comparison. Both FYAK-specific and GA-specific T cell lines suppressed the response at the two tested concentrations of IRBP and again, the FYAK line was more inhibitory than the GA line (Fig. 4).

In contrast to the difference in their inhibitory capacity on the IRBP-specific T-cells, the copolymer-specific line cells proliferated remarkably similarly to the corresponding copolymer. Data of a representative experiment are shown in Fig. 5, in which line cells specific to FYAK or GA were cultured with the corresponding copolymer at several concentrations and their response was measured by ^3H -thymidine incorporation. Similar levels of proliferation were measured for the two lines in this and in two additional experiments.

3.5. Secretion of Th2 cytokines by copolymer-specific Treg cells

The suppressive capacity of GA- and FYAK-specific T-cells has been attributed to the release of Th2 cytokines, in particular IL-10 (Arnon and Aharoni, 2004; Stern et al., 2008). To investigate the Th2 cytokine production by the immunosuppressive cell lines we developed against GA or FYAK from the three tested mouse strains, we measured the levels of four of these cytokines, IL-4, IL-5, IL-10 and IL-13, in their supernatants. Representative data are summarized in Table 1; similar patterns of cytokine production were obtained in repeated experiments, using supernatants collected from the cell lines after three or four cycles of activation. Remarkably, the levels of Th2 cytokines were repeatedly higher in the FYAK-specific cultures than in the GA-specific ones. The levels of IFN- γ and IL-17 were relatively low in these cultures, <2.5 ng/ml and <0.05 ng/ml, respectively, and unlike the pattern with the Th2 cytokines, the levels of IFN- γ were similar or even higher in the GA-specific lines than in the FYAK-specific lines (data not shown).

3.6. Expression of immunoregulatory markers by copolymer-specific T-cell lines

Although the immunosuppressive capacity of copolymer-specific lines was attributed mainly to their release of anti-inflammatory cytokines (Arnon and Aharoni, 2004; Hong et al., 2005; Stern et al., 2008), Some data suggest that copolymers may also stimulate suppressive Foxp3+ Treg cells (Hong et al., 2005). Therefore, we examined the FYAK- and GA-specific lines for expression of the markers for these Tregs, i.e., CD25 and Foxp3. Data of a representative experiment are recorded in Fig. 6 and show that a relatively small percent of the cells of both FYAK- and GA-specific line cells express both CD25 and Foxp3, with the percentage of cells expressing these markers being higher in FYAK-specific line cells.

4. Discussion

The present data provide new information on the use of random amino acid copolymers in a non-CNS disease model. The study also compares the immunosuppressive activities of a novel copolymer, FYAK [poly (F,Y,A,K)_n] with those of GA [poly(Y,E,A,K)_n], a copolymer widely used for treatment of MS (Arnon and Aharoni, 2004; Farina et al., 2005; Stern et al., 2008). The immunosuppressive capacities of the two copolymers have been previously compared in the EAE system (Illes et al., 2004; Stern et al., 2004; Stern et al., 2008). In the present study, they were compared in their immunosuppressive ability in the ocular disease model, EAU. EAU serves as a model for intraocular inflammatory eye conditions grouped under the term “uveitis”, including Behcet's disease, Vogt-Koyanagi Harada (VKH) syndrome, birdshot retinochoroidopathy and sympathetic ophthalmia (Adamus and Chan, 2002; Forrester et al., 1990; Gery et al., 2002). It is assumed that autoimmunity plays a major role in the pathogenesis of these eye diseases, a notion supported by the similarity between these human conditions and EAU, a disease induced by immunization with ocular specific antigens (Adamus and Chan, 2002; Caspi, 2002; Gery et al., 2002).

FYAK ameliorated EAU as well as it inhibits EAE and was superior to GA in all assays employed in the present study. The effect on EAU induction was tested with two mouse strains, B10.A and B10.RIII (Fig. 1). GA inhibited moderately the development of EAU in B10.A mice, as was found previously (Zhang et al., 2000), but was found here to be remarkably more inhibitory in the highly susceptible B10.RIII mice. In both strains, however, treatment with FYAK was clearly more effective than with GA, reducing the disease severity in B10.A mice to lower levels and completely inhibiting disease development in B10.RIII mice at doses at which GA achieved only a partial effect. These data and those in Fig. 3 also show that the effectiveness of FYAK and the difference between it and GA is relatively broad and not restricted to a single haplotype.

The difference between B10.A and B10.RIII mice in their response to the inhibitory effect of the copolymers on EAU induction is of note. The two copolymers were tested in this system by co-immunization with the uveitogenic antigen. It is conceivable, therefore, that the 20 amino acid long 161-180 peptide used for induction of disease in the B10.RIII mice was an easier competition target for binding to mouse MHC proteins than the large IRBP protein (~140 kDa) used in the B10.A mice. A major effect of both FYAK and GA appears to be due to the generation of regulatory T cells that secrete immunosuppressive cytokines (Aharoni et al., 2000; Arnon and Aharoni, 2004; Duda et al., 2000; Farina et al., 2005; Stern et al., 2008). However, at least a part of the copolymers' activity in this model is attributable to competition with the uveitogenic antigen when administered by co-immunization in CFA.

Treatment with GA induces a shift toward Th2 in both animals and humans (Aharoni et al., 2000; Arnon and Aharoni, 2004; Dhib-Jalbut et al., 2003; Duda et al., 2000; Farina et al., 2005; Neuhaus et al., 2000) and a similar observation was made in mice treated with FYAK (Stern et al., 2008). Data recorded in the present study are in line with these observations and, remarkably, we found that FYAK-specific cell lines were superior to GA-specific lines in their production levels of Th2 type cytokines, IL-4, IL-5, IL-10 and IL-13.

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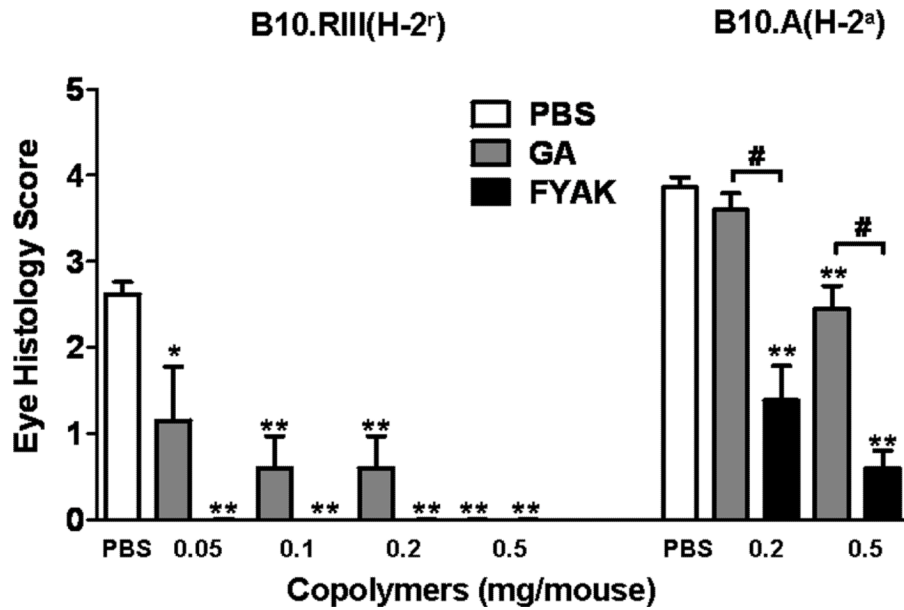


Fig. 1. Comparison of GA and FYAK in inhibiting EAU development. The immunizing antigens and copolymers were administered, at the indicated doses, by the co-immunization route as described in Materials and Methods. EAU was induced in B10.A mice with whole IRBP and in B10.RIII mice with peptide 161-180. Severity of eye disease was determined on day 14 p.i., by histological examination and the data are means \pm SEM of three experiments with each mouse strain. * $p < 0.01$, ** $p < 0.0001$ when the response is compared to PBS controls; #, $p < 0.0001$ when the indicated columns are compared.

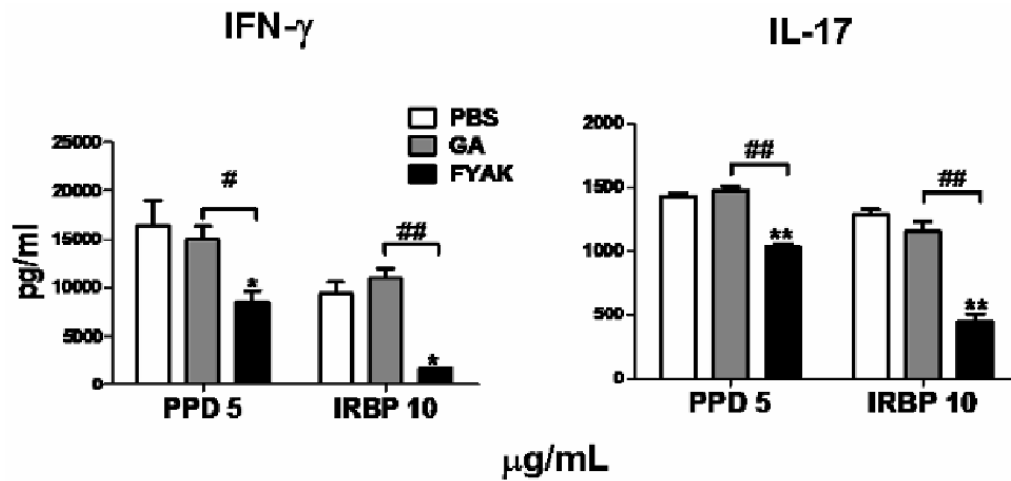


Fig. 2. Comparison of the suppressive effects of FYAK and GA on cytokine release by T cells from treated mice. Draining lymph node cells from B10.A mice, detailed in the legend for Fig. 1, were tested for the release of Th1 and Th17 specific cytokines (IFN- γ and IL-17, respectively), following 48 hr incubation with the indicated antigens. Data are means \pm SEM of duplicated assays from representative experiments. Similar patterns of cytokine release inhibition were observed in two other independent experiments. * $p < 0.05$, ** $p < 0.01$ when the response is compared to PBS controls; #, $p < 0.05$, ##, $p < 0.01$ when the indicated columns are compared.

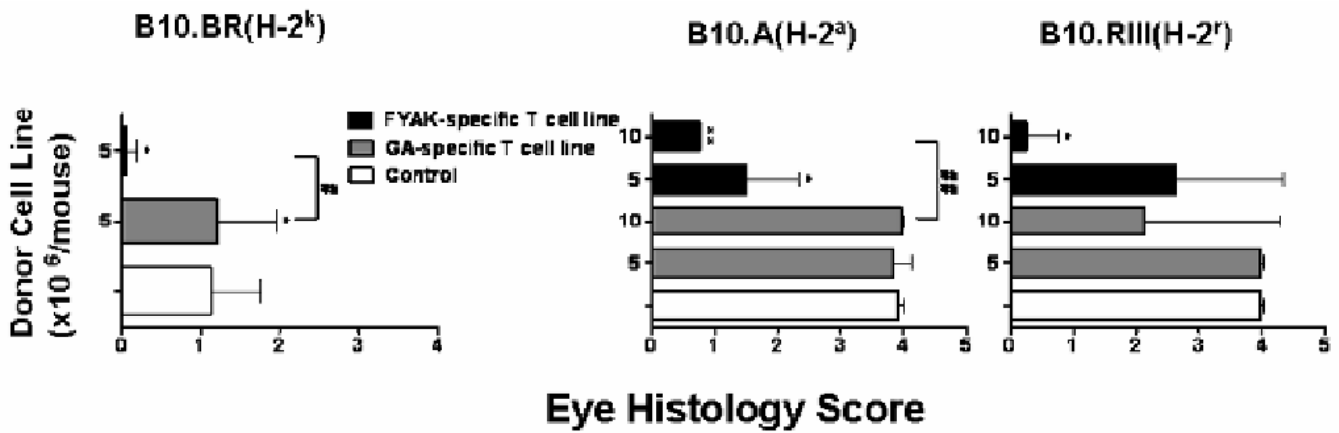


Fig. 3. T cell lines specific to FYAK or GA inhibit EAU development. T cells of lines specific to FYAK or GA, established as detailed in Materials and Methods, were adoptively transferred intravenously at the indicated numbers to recipients of the corresponding strain. One day later recipients were immunized with IRBP, (B10.A and B10.BR), or peptide 161-180 (B10.RIII), as detailed in Materials and Methods. Eyes were collected on day 14 p.i. and analyzed for histological changes. The recorded data are means \pm SEM of 3-5 mice of each group. *, $P \leq 0.05$, **, $P \leq 0.0001$ when compared to the PBS group; #, $P \leq 0.05$, ##, $P \leq 0.0001$ when the indicated two groups are compared.

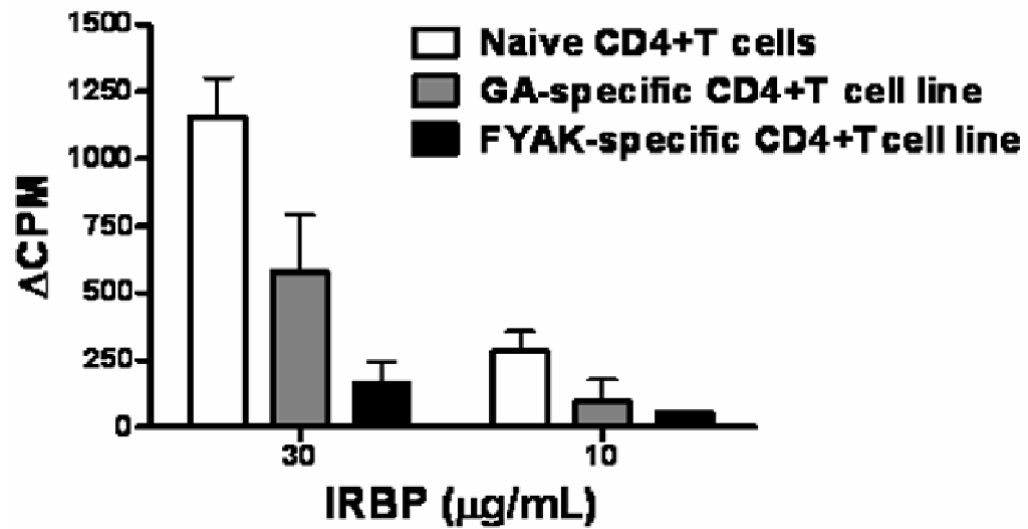


Fig. 4. FYAK-specific lines are superior to GA-specific lines in their capacity to inhibit lymphocyte proliferation in vitro. B10.A line cells specific to FYAK or GA, or naïve CD4 cells, were added to cultures of syngeneic lymphocytes sensitized against IRBP, as detailed in Materials and Methods. The ^3H -thymidine incorporation was measured after 90 hr of incubation and recorded as delta CPM. The recorded data are means \pm SEM of replicates of 4 wells for each culture from a representative experiment; similar data were collected in another independent experiment. Control cultures containing FYAK-specific line cells incorporated 97 ± 14 CPM when incubated with no IRBP or copolymer and 1891 ± 204 CPM when incubated with only the copolymer. Control cultures containing GA-specific line cells incorporated 113 ± 31 with no addition and 1791 ± 208 CPM in the presence of the copolymer. These control CPM values were subtracted from the values recorded by the bars in the Figure, that show, therefore, the net CPM stimulated by the added IRBP in these cultures.

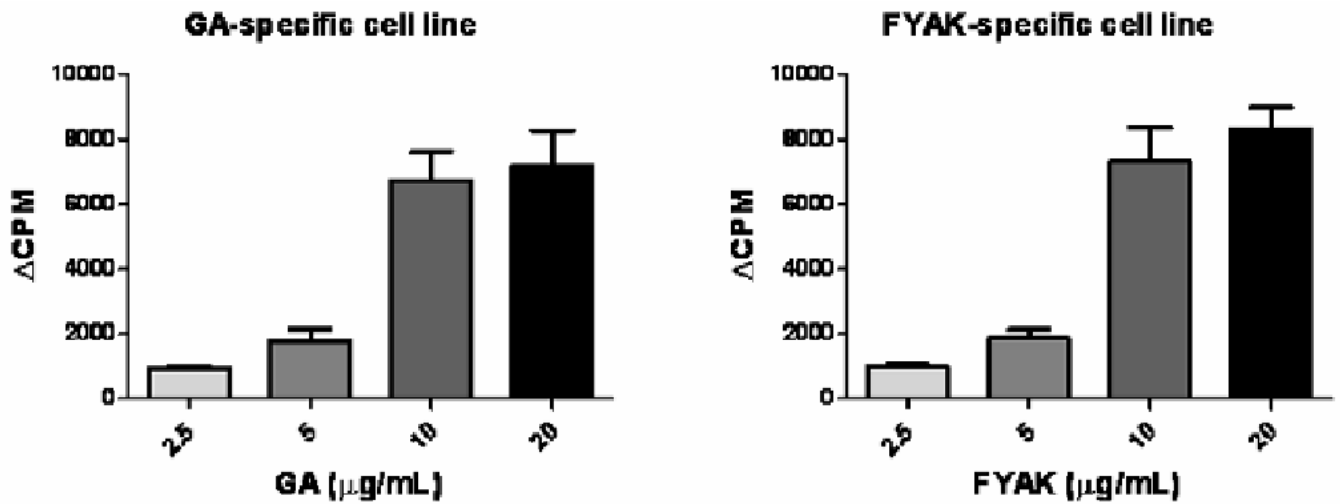


Fig. 5.

Line cells specific toward FYAK or GA responded similarly to their corresponding copolymer. Copolymer-specific line cells were collected on day 2 of re-stimulation, washed and cultured at 1.25×10^5 /well, along with the same number of irradiated syngeneic APC, and with the corresponding copolymer at different concentrations, as indicated. The proliferation level was determined by ^3H -thymidine incorporation, measured after a pulse of $0.5 \mu\text{Ci/well}$ during the last 16 hr of the 90 hr total incubation. The data are expressed as $\Delta\text{CPM} \pm \text{SEM}$.

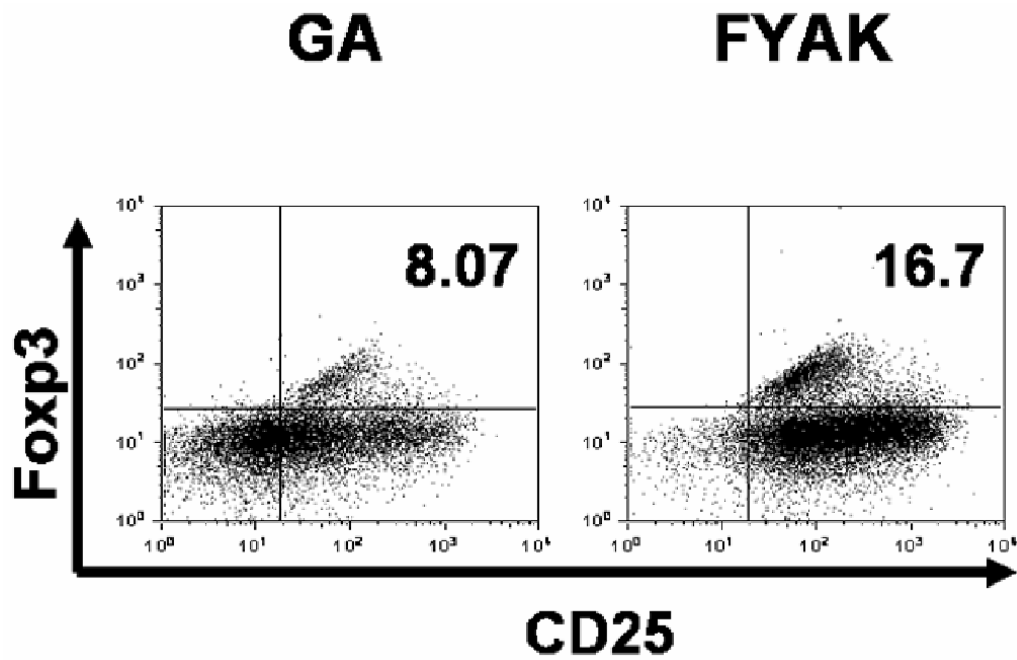


Fig. 6. CD25⁺Fcpx3⁺ Treg cells in FYAK-specific and GA-specific cell lines. Copolymer-specific cell lines of B10.A mice, collected on day 2 of the 3rd stimulation cycle, were tested by flow cytometry for the proportion of cells expressing CD25 and Fcpx3. The data shown are one of two similar experiments.

Table 1
Production of Th2-specific cytokines by CD4 T cell lines specific against GA or FYAK

Mouse Strain	Cell line specificity	Cytokine production (ng/ml)				
		IL-4	IL-5	IL-10	IL-13	
B10.RIII	GA	15.9	5.4	4.7	17.6	
	FYAK	17.3	16.3	9.3	35.5	
B10.A	GA	6.6	8.0	3.7	2.9	
	FYAK	15.1	17.5	13.3	4.6	
B10.BR	GA	1.8	9.8	3.3	1.3	
	FYAK	10.5	61.6	32.3	28.1	

Culture supernatants were collected following incubation with the corresponding copolymer and analyzed for levels of the indicated cytokines. The recorded data were obtained from the 3rd cycle cultures of B10.RIII and B10.A and the 4th cycle cultures of B10.BR. Similar patterns of cytokine production were observed in two or three additional experiments with each mouse strain.