INDUCTION OF ACETYLCHOLINE RECEPTOR-SPECIFIC SUPPRESSION

An In Vitro Model of Antigen-specific Immunosuppression in Myasthenia Gravis

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Myasthenia gravis is an autoimmune disease characterized by circulating autoantibodies against the acetylcholine receptor (AChR)[1] present on the neuromuscular endplate. It is generally believed that these antibodies are responsible for an accelerated rate of AChR degradation, leading to muscular weakness and fatiguability (1). To date, treatment has involved either the administration of anticholinesterases or nonspecific immunosuppressive measures. Ideally, one would like to specifically eliminate the anti-AChR autoimmune response while not affecting the host's general immune status. Attempts to specifically suppress the anti-AChR immune response by nonmyasthenic derivatives of AChR (2) and by induction of antiidiotype (3) have been reported. Moreover, a recent report indicates that anti-AChR antibody levels in mice can be suppressed if anti-Ia monoclonal antibodies are administered (4).

It has been suggested that the underlying defect in myasthenia gravis (as well as other autoimmune diseases) is a deficiency of suppressor T cells (5).Therefore, specific treatment of myasthenia gravis might be achieved if a suitable source of antigen (AChR)-specific suppressor T cells and their secreted factors could be developed. To date, however, no model system has yet been developed demonstrating a means for induction of AChR-specific suppressor T cells. In light of its potential importance to the human disease, we have established the conditions to induce AChR-specific suppressor T cells capable of suppressing the in vitro anti-AChR response. Moreover, we demonstrate that this suppressive effect can be mediated by a secreted factor found in the cell-free supernatant.

A key aspect of this work involved the development of an appropriate in vitro anti-AChR assay. The AChR-induced lymphoproliferative assay (6) was found unsuitable for the work described here. Therefore, we applied the assay described by Mishell and Dutton (7) to the AChR antigenic system. This assay is relatively well-characterized in other systems and permits analysis of immunoregulatory T

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[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; CFA, complete Freund's adjuvant; FCS, fetal calf serum; HGG, human γ-globulin; KLH, keyhole limpet hemocyanin; 2-ME, 2-mercaptoethanol; NIP, 4-hydroxy-5-iodo-3-nitrophenyl acetic acid hapten; PFC, plaque-forming cell; SE, sheep erythrocytes; Ts, suppressor T cell; TsF, suppressor T cell factor.

cells and their secreted factors. In this paper we report the details of the assay and its application for measuring AChR-specific suppressor T cell (Ts) and T cell factors (TsF). We believe that this is the first report of a suppressor factor to a physiologically and pathologically important autoantigen whose role in a human autoimmune disease is well-defined.

Materials and Methods

Mice and Immunizations. C57BL/6J female mice 6 wk of age were obtained from The Jackson Laboratory, Bar Harbor, ME. They were rested at least 1 wk before immunization with 10–15 μg AChR emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI) administered to the two hind-foot pads. These mice were used 10–30 d later without boosting for the Mishell-Dutton assay.

Isolation of AChR. AChR was isolated from the electric organs of *Torpedo californica* according to the procedure of Aharanov et al. (8) with several modifications. Elution of AChR from the Sepharose-Naja-naja siamensis toxin column was done in 0.025% Triton X-100 instead of 0.1%, except for the batch conjugated with nitroiodophenol (NIP). Also, the dialysis after elution was done without EDTA and sodium azide and with 0.025% Triton X-100 in Tris-buffered saline using twice-distilled water.

Coupling of Nitroiodophenol (NIP) to AChR. 20 μl of 4-hydroxy-5-iodo-3-nitrophenyl (NIP)-*O*-succinimide (20 mg/ml) dissolved in dimethylformamide was added to 10 mg AChR dissolved in 5 ml of 0.12 M carbonate buffer pH 9.2, with 0.1% Triton X-100. The mixture was incubated for 20 min at room temperature and then dialyzed to remove nonreacted NIP-*O*-succinimide. Absorbance at 430 nm indicated a substitution ratio of 20 NIP groups per AChR. By SDS-PAGE as well as ^{125}I-bungarotoxin binding, the NIP-AChR was indistinguishable from the unmodified AChR. Moreover, all education procedures in vivo or in vitro always employed the unmodified AChR, whereas NIP-AChR was used only to trigger a secondary response in vitro.

In Vitro Secondary Anti-AChR Response. AChR-immune animals were used 10–30 d after one immunization. Poor responses were obtained after longer intervals or after boosting. Cells from draining lymph nodes and spleen were washed twice and resuspended in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) (batch #607; Biolab, Jerusalem), 2 mM glutamine, 10 mM Hepes, 5×10^{-5} M 2-ME, 1 μM sodium pyruvate, and antibiotics. 10^6 viable lymphocytes were placed in each well of a 96-well Microtitre II tray (Falcon #3040). Each culture consisted of 3–6 replicate wells in a final volume of 0.2 ml. NIP-AChR was added to the cell suspension at a concentration of 0.2 μg/ml, unless otherwise indicated. At this concentration, toxicity due to the Triton X-100 detergent was negligible. Cells were cultured at 37°C, 5% CO_2 in air for 5 d and were not given any feeding during that time. On day 5, individual groups were collected and washed and indirect plaque-forming cell (PFC) responses were assayed by the Cunningham modification of the Jerne plaque assay (9). Four replicate counts were done per group and the mean ± standard error calculated. In this assay, therefore, we are measuring a primary anti-NIP B cell response (hapten) induced by a secondary T cell response to the AChR (carrier).

Primary Induction of AChR-specific Ts Cells. Splenocytes from naive C57BL/6 mice were cultured in RPMI 1640 with 10% FCS (Gibco Laboratories, Grand Island, NY) and supplements as described above. The batch of FCS was critical in order to minimize nonspecific (serum-specific) suppression (10). Cells were aliquoted 5×10^6 lymphocytes/well to a 24-well plate (Costar #3524), 1 ml per well. AChR was added to a concentration of 4 μg/ml and the culture incubated for 4–5 d. Cells were then collected, washed, counted, and replated in an anti-AChR in vitro assay. Usually, 50–60% of the cells were recovered after this culture with many blast cells present.

Production of Ts Factor from In Vitro-educated AChR-Ts Cells. In vitro-educated Ts cells were harvested on day 4, washed twice, and resuspended in RPMI-1640 plus supplements but without serum. 5×10^6 viable cells were aliquoted per well of a Costar 24-well plate in 1 ml and NIP-AChR added to 0.2 μg/ml. The following day supernatants were collected,

filtered, and stored at −20°C.

In Vivo-educated Ts Cells and Factor. Mice were injected with 100 μg AChR in PBS i.p. and used 1 wk later or boosted with an identical dose at 2 wk and used the following week. TsF was obtained by culturing splenocytes in RPMI 1640 with supplements (as in the assay), 10^7 cells per well in 1 ml in a Costar 24-well plate. Cells were incubated for 2 d, at which time the supernatants were collected, filtered, and stored at −20°C.

Affinity Columns. Sepharose-AChR and Sepharose-FCS columns were prepared according to techniques already described (11). Briefly, cyanogen bromide-activated Sepharose was mixed either with AChR (2 mg/g Sepharose) or FCS (1 ml, dialyzed into 1 M $NaHCO_3$ per 5 g Sepharose). Supernatants containing TsF were passed through such columns and binding allowed to take place for 30 min at room temperature. Columns were extensively washed with PBS and the elution done in 0.2 M NH_4OH. Eluates were quickly brought to pH 8–9 by addition of $NaHCO_3$ and were dialyzed against RPMI medium.

Enrichment of AChR-specific Proliferating Cells. Cells from draining lymph nodes were washed and resuspended in RPMI 1640 supplemented with 2.5% horse serum, 10 mM Hepes, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics. Cells were aliquoted, 5.5×10^5 cells per well in a 96-well tray, and AChR was added to a concentration of 2 μg/ml. After a 3-d culture, viable cells were separated by Lymphoprep centrifugation (Nyegaard and Co., density = 1.077 g/cm^3), washed, and resuspended in medium for use in the Mishell-Dutton assay as indicated in the text.

Antisera and Complement Treatment. Two monoclonal IgM rat-mouse hybridomas were used for cell fractionation, anti-Thy-1.2 (AT83A) and anti-Lyt-2 (3.155). Both were the generous gift of Dr. Frank Fitch, Department of Pathology of The University of Chicago. Lymph node cells were resuspended in a 1:3 dilution of hybridoma supernatants, 5×10^6 cells/ml, and incubated for 30 min at 4°C. Cells were then spun down and resuspended in a 1:10 dilution of guinea pig complement (Gibco). Complement was detoxified by sequential absorptions on sheep erythrocytes (SE) (5 ml complement to 1 ml packed SE) and naive splenocytes (1 ml complement to 1 spleen), each for 30 min at 4°C. Afterwards, cells were washed, counted, and added to the Mishell-Dutton assay in a volume of 50 μl.

Panning of Cells. In order to enrich or deplete certain cell populations, a procedure based on that used by Mage et al. (12) was used. Adherent cells and B cells were depleted by incubation of cells for 1 h at room temperature on a goat anti-mouse Ig (300 μg/ml, affinity purified) coated plate (60 × 15 mm, Falcon #3002). Cells bearing receptors for proteins present in FCS were fractionated by an additional incubation in a FCS-coated plate (20% FCS in PBS). Adherent cells were recovered by chilling the plate at 4°C for 15 min and vigorous pipetting.

Results

Secondary Stimulation of AchR-Immune Helper T Cells—Assay Parameters. Lymph node cells from AChR-immune mice (injected into the hind-foot pads) demonstrated strong helper activity when cultured in vitro with B cells and NIP-AChR. These helper T cells were able to stimulate NIP-specific B cells present in the lymph nodes and spleen to produce antibody which could be measured in a hemolytic plaque assay using NIP-coated SE. This assay thus represents a secondary response to the carrier (AChR), whereas the B cell hapten-specific response (anti-NIP) is primary. Several parameters were found to be important, i.e., antigen (NIP-AChR) concentration, cell density, and culture kinetics. These parameters were calibrated (Table I) and the optimal conditions were found to be 0.2 μg/ml NIP-AChR, 1×10^6 cells/well for a culture period of 5 d. These conditions were always used unless otherwise indicated. The cause of the dramatic drop in PFC responses on day 6 in the kinetics experiment is due to cell death.

Role of AChR-specific "Proliferating" Cell. In order to characterize the AChR-specific helper T cell and its relationship to the "proliferating cell" measured in

TABLE I
Parameters of Anti-AChR Assay

NIP-AChR	Cells/well	Day of assay	PFC/Culture
μg/ml			
2.0	1×10^6	5	2,328 ± 216
0.2*			3,306 ± 384
0.02			3,120 ± 282
0.002			1,704 ± 204
0.0002			1,638 ± 156
None‡			252 ± 57
0.2	2.5×10^5	5	190 ± 78
None			530 ± 110
0.2	5×10^5		1,036 ± 198
None			414 ± 58
0.2	7.5×10^5		1,805 ± 102
None			766 ± 54
0.2	1×10^{6}*		2,355 ± 240
None			489 ± 23
0.2	1×10^6	3	3,540 ± 360
None			1,900 ± 335
0.2		4	3,798 ± 666
None			1,638 ± 138
0.2		5*	3,306 ± 384
None			252 ± 57
0.2		6	252 ± 72
None			140 ± 37

* Optimal conditions of this parameter.
‡ An irrelevant antigen (NIP-HGG, 0.5 μg/ml) was also checked and found to be nonstimulatory. PFC = 930 ± 96 (data not shown).

TABLE II
Characteristics of AChR-specific Helper T Cells

Treatment of AChR-immune cells	NIP-AChR added in vitro	PFC responses	
		Expt. 1	Expt. 2
None	+	2,055 ± 87	1,950 ± 250
None	−	450 ± 27	200 ± 15
Anti–Thy-1.2 + complement	+	480 ± 57	ND
Anti–Lyt-2 + complement	+	2,640 ± 93	ND
Medium alone + complement	+	2,610 ± 126	ND
Irradiation* (750 R)	+	555 ± 81	500 ± 35

* Cobalt radiation source used.

the lymphocyte transformation assay, we performed two additional experiments. The results of these two experiments (Table II, Fig. 1) clearly indicate that a Lyt-2$^-$ proliferating T cell provides AChR-specific help. In the first experiment, AChR-immune lymph node cells were either treated with antibody plus complement or irradiated and recounted, and 2.5×10^5 of these cells were mixed with

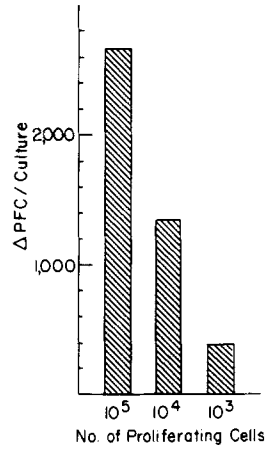

FIGURE 1. AChR-specific proliferating cell provides AChR-specific help. AChR-induced proliferating cells were collected from a 3-d culture and blast cells were enriched on Lymphoprep. Varying numbers of these cells were co-cultured with 1×10^6 naive splenocytes with either NIP-AChR or an irrelevant antigen (NIP-(T,G)-A--L). ΔPFC responses represent the difference of the two groups for each cell concentration.

7.5×10^5 naive splenocytes in a Mishell-Dutton assay. As indicated in Table II, helper T cell responses are sensitive to anti-Thy-1.2 but not anti-Lyt-2. In order to assess the relationship of AChR-specific T cell proliferation to AChR-specific T cell help, proliferation was prevented by irradiation. As can be seen, irradiation with 750 rad completely blocked helper function.

Instead of deleting the helper T cells, we also tried the opposite experiment, namely, positive selection of AChR-specific proliferating cells and their subsequent functional analysis. AChR-primed lymph node cells were placed in a proliferative assay for 3 d and collected, and blast cells were further enriched by centrifugation on Lymphoprep. After the 3-d culture, ~25% of the cells were viable and 11% were recovered after Lymphoprep gradient centrifugation. These cells were then added to naive splenocytes in different ratios in Mishell-Dutton assay conditions. NIP-AChR or an irrelevant antigen (NIP-(T,G)-A--L, 0.005 μg/ml) were added to the cultures and PFC responses were counted 5 d later. The results (Fig. 1) show that these "proliferating" cells provide substantial AChR-specific help.

In Vivo-educated AChR-specific Suppression. Injection of soluble antigen has traditionally been one method used for induction of tolerance and Ts cells. Although injection of soluble AChR did not induce tolerance even if deaggregated (data not shown), we found that Ts cells were induced by this immunizatior regimen. This suppression could be demonstrated with both whole cells from AChR-immune mice (primed with soluble AChR as described in Materials and Methods) or with a secreted cell product from these AChR-specific suppressor cells. The AChR-specific TsF was obtained in the supernatant of a 2-d culture of AChR-immune splenocytes that were cultured without any antigen (AChR). Table III shows our results using the TsF in an AChR, as well as in a NIP-HGG assay system. Moreover, we checked the effect of supernatants from naive splenocytes. As seen in the Table, the TsF suppressed only the anti-AChR PFC

TABLE III
In Vivo-educated Ts Factor

Antigen added to assay	Factor (dilution)	AChR system: Expt. 1 PFC/culture	AChR system: Expt. 1 Suppression*	AChR system: Expt. 2 PFC/culture	AChR system: Expt. 2 Suppression	NIP-HGG System PFC/culture	NIP-HGG System Suppression
			%		%		%
+	None	1,950 ± 250	—	2,055 ± 86	—	1,025 ± 100	—
−	None	200 ± 15	—	330 ± 60	—	450 ± 68	—
	AChR-TsF						
+	1:10	400 ± 130	89	ND	—	1,250 ± 180	<0
+	1:20	1,400 ± 60	31	600 ± 96	84	1,400 ± 135	<0
+	1:40	1,200 ± 115	43	1,440 ± 57	36	1,275 ± 153	<0
+	1:80	1,300 ± 220	37	1,290 ± 57	44	ND	—
	Control supernatant‡						
+	1:10	1,950 ± 210	0	ND	—	ND	—
+	1:20	2,500 ± 430	<0	ND	—	ND	—
+	1:40	2,000 ± 165	<0	ND	—	ND	—

* Percent suppression was calculated as: (PFC assay positive control − PFC suppressor cell group)/total assay ΔPFC. Assay positive control is the group containing assay cells plus antigen but without Ts cells. Total assay ΔPFC is the difference of the PFC counts of assay cells cultured with and without antigen.

‡ Control supernatant was generated in an identical manner as AChR-TsF except that the mouse used was not immunized.

response and did not suppress an unrelated (NIP-HGG) system. Moreover, naive supernatants also had no effect on the anti-AChR PFC response. Therefore, we conclude that our TsF is specific for the AChR.

In Vitro-educated Ts and TsF. In parallel to the studies on in vivo-educated suppression, we also developed the means to induce AChR-specific Ts cells and TsF from naive cells in vitro. Although the in vitro procedure is more difficult due to serum-related background suppression (see below), it carried an important advantage. It serves as a model for Ts cell induction in systems where immunization is not possible, namely, for potential induction of human AChR-specific Ts cells.

We found that in vitro induction of AChR Ts cells was dependent on the dose of AChR used for education as well as the number of cells transferred to the assay. Our procedure as presented in Materials and Methods was based on the methods used in the SE (13) and keyhole limpet hemocyanin (KLH) (14) systems. Naive splenocytes were cultured with AChR for 4 d, at which time 1×10^5 of these educated cells were added to 1×10^6 assay cells in a Mishell-Dutton culture. For a comparison, a group of 1×10^6 assay cells alone was also included. Fig. 2 shows the role of AChR concentration as a function of the subsequent PFC response. The results indicate virtually complete suppression of the anti-AChR immune response in vitro. Eardley and Gershon (13) found that removal of 2-ME was helpful during SE-specific Ts induction. In our AChR system, however, we found that medium with 2-ME was superior (Fig. 2).

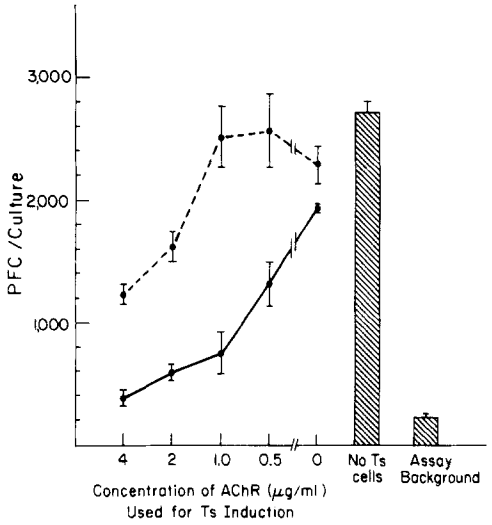

FIGURE 2. Role of the AChR concentration used for Ts induction. Naive splenocytes were incubated with the indicated AChR concentrations as described in Materials and Methods. Ts induction was done in the presence (●——●) or absence (●---●) of 5×10^{-5} M 2-ME. 1×10^5 of these precultured cells were then added to a Mishell-Dutton assay and PFC responses counted 5 d later. The shaded bars are the positive and negative controls of the assay, representing the PFC counts of 1×10^6 assay cells with and without antigen, respectively.

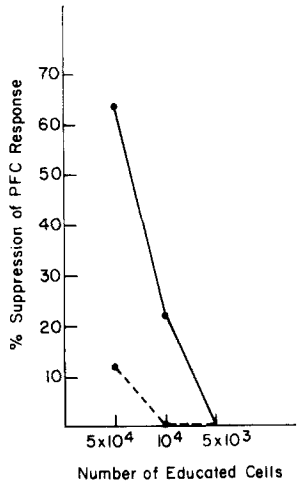

FIGURE 3. Role of the number of precultured cells transferred to the Mishell-Dutton assay. Varying numbers of cells cultured with (●——●) or without (●---●) AChR (4 μg/ml) were added to a Mishell-Dutton assay. Percent suppression was calculated as in Table III. Total ΔPFC = 2,850.

Fig. 3 demonstrates the relationship of the number of educated cells transferred as a function of suppression. In this experiment, naive splenocytes were educated with or without AChR (4 μg/ml) for 4 d and varying numbers of these precultured cells were added to a Mishell-Dutton assay. As can be seen, although 10^5 cells yield some nonspecific suppressive background (from Fig. 2), it rapidly disappears with lower cell doses. AChR-specific suppression is at least fivefold

greater than this background.

In order to characterize the AChR-specific Ts and to distinguish its characteristics from those of the nonspecific Ts cells causing the background level of suppression, we performed two further experiments (Table IV; Fig. 4). Table IV describes two surface markers of the AChR-specific Ts cells. In this experiment educated Ts cells were treated with the indicated antibodies and complement and 5×10^4 of these cells were added per well to a Mishell-Dutton assay. The results indicate that the AChR-specific Ts cell bears both the Thy-1.2 as well as Lyt-2 cell differentiation markers.

We then attempted to determine whether the background suppression observed with high numbers of cells precultured without antigen could be due to cells with specificities to FCS proteins. If so, it would clearly suggest that such cells are also induced in a manner similar to the AChR-specific cells, but are specific to serum antigens, not the AChR. For that, "control" cells which were cultured without AChR for 4 d were collected and incubated first on a goat anti-mouse Ig plate as described in Materials and Methods, and then plated in an FCS-coated plate. Of the cells obtained after the goat anti-mouse Ig plate (nonadherent), 9% were in the FCS-adherent fraction and 91% in the FCS nonadherent fraction. These two fractions, as well as the FCS-unfractionated

TABLE IV

Characteristics of In Vitro–educated AChR-specific Suppressor Cells

Treatment of AChR-specific Ts cells	NIP-AChR added to assay	PFC/culture	Suppression*
			%
No Ts cells	+	3,990 ± 144	—
No Ts cells	−	1,140 ± 102	—
Medium + complement	+	1,530 ± 69	86
Anti–Thy-1.2 + complement	+	3,720 ± 342	9
Anti–Lyt-2 + complement	+	4,020 ± 138	<0

* Percent suppression was calculated as in Table III.

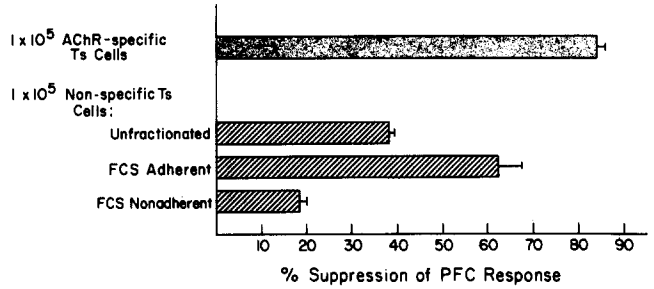

FIGURE 4. 4-d "control" cells (no AChR in vitro for Ts induction) were depleted of adherent and B cells. 1×10^5 of these cells were added to a Mishell-Dutton culture (FCS unfractionated) and the remainder to a FCS-coated plate. Adherent and nonadherent fractions were assayed for suppressive activity, 1×10^5 cells per well. For comparison, AChR-educated Ts cells derived from the same spleen cell population were also checked. Percent suppression was calculated as in Table III. Total ΔPFC = 2,745.

TABLE V

Specificity of In Vitro-educated Ts Factor

Antigen in assay	Factor	AChR-system		NIP-HGG system	
		PFC/culture	Suppression*	PFC/culture	Suppression
			%		%
+	None	1,950 ± 250	—	1,080 ± 104	—
–	None	200 ± 15	—	225 ± 81	—
+	TsF-AChR: unfractionated	750 ± 100	69	405 ± 104	79
+	effluent from Sepharose-FCS‡	1,050 ± 115	51	990 ± 126	11
+	effluent from Sepharose-AChR§	2,200 ± 115	<0	ND	—
+	eluate from Sepharose-AChR	1,150 ± 180	46	1,080 ± 32	0
+	TsF-Control: unfractionated	530 ± 30	81	ND	—
+	effluent from Sepharose-FCS	1,533 ± 77	22	ND	—

* Percent suppression was calculated as in Table III.

‡ The dilution of all fractions was 1:40 based on the original volume of the supernatant.

§ The effluent of the Sepharose-FCS column was applied to the Sepharose-AChR column.

cell population were added to an anti-AChR Mishell-Dutton assay, 1×10^5 precultured cells to each well. Fig. 4 summarizes the results. The background level of suppression that is found at high cell doses (38% in this experiment) was greatly enriched on the FCS-coated plate, clearly suggesting that there are FCS-specific cells involved in this background effect. AChR-specific suppression at this cell dose in this experiment was 84%.

Suppressor T cell supernatants from in vitro-educated Ts cells were also found to contain an AChR-specific TsF. This factor was antigen-specific since it absorbs to and is eluted from a Sepharose-AChR column. Moreover, the eluate was found to have no effect in an irrelevant (NIP-HGG) assay system (Table V). Similar to our results in using whole cell preparations, control supernatants (no AChR in vitro) were also suppressive. However, as seen in Table V, most of the nonspecific suppression in the control supernatant effluent was removed by a Sepharose-FCS column. In addition, the nonspecific suppressive effect contained in the supernatant of AChR-educated Ts cells (TsF-AChR) was removed by passage through a Sepharose-FCS column. The effluent from such a column retained its AChR-specific suppressive activity, whereas it was ineffective in an irrelevant NIP-HGG system (Table V).

Discussion

During the past decade, the understanding of immunoregulatory events following injection with experimental antigens has leaped forward at an impressive rate. Much of this progress is undoubtedly due to the development of suitable assay systems such as those described by Mishell and Dutton using SE as an antigen (7). Application of this assay to the study of autoimmunity is thus a desirable goal and was made possible in our system because of the fortuitous availability of high concentrations of AChR in the electric organ of the electric fish that can be purified by affinity chromatography techniques (8).

The present report describes an in vivo and in vitro model system of AChR-

specific suppressor T cell and T cell factor induction. The results of this work are of special importance since they relate to an antigen directly involved in the human autoimmune disease myasthenia gravis. Although human antigen (AChR)-specific immunotherapy may be still far off, we believe that this work represents the required first step—development of the appropriate in vitro model systems.

We also describe a reliable and reproducible in vitro assay system that models the anti-AChR immune response on a T cell level. This assay is essential for the measurement of AChR-specific suppressor T cell and T cell factors. As already mentioned, the lymphoproliferative assay was unsuitable for this type of work. For example, we found that when AChR-specific suppressor T cell preparations were placed in a lymphoproliferative assay, they themselves proliferated. If these suppressor T cells were irradiated in order to prevent their growth, no suppression resulted since suppressor T cells have been shown to be radiosensitive (13). Suppressor T cell factors were also nonspecifically mitogenic due to the FCS.

Our first attempts for the adaptation of the Mishell-Dutton assay to the AChR system involved exposing AChR-primed lymphocytes to AChR (not NIP-AChR) and measuring the anti-AChR activity in the supernatants following removal of the added antigen. We found that, while different doses of AChR in vitro could modulate anti-AChR activity, as was also reported by De Baets et al. (15), background levels of antibody (no AChR added in vitro) were highest of all. Presumably, plasma cells generated in vivo following immunization with AChR in CFA continued to secrete antibody in vitro. Therefore, we adopted the hapten-carrier approach whereby we measure a primary anti-NIP B cell response. This has the advantage of low backgrounds and large responses in positive cultures, usually of a 5–10-fold magnitude. In this manner, we can be sure of studying a secondary AChR-specific T cell in vitro response rather than events which occurred in vivo. The finding that we could obtain a primary anti-NIP response while the T cell response was secondary in nature is in complete agreement to the results of Schreir (16). In an analysis of the in vitro antibody response, he found that T cells, not B cells, were the limiting cell type in a primary in vitro immune response.

Using the Mishell-Dutton assay, we have shed light on the function of the "proliferating" cell measured in the lymphoproliferative assay. By both positive and negative selection techniques, we show that the Lyt-2$^-$ proliferating T cell provides AChR-specific help. It is still possible that two (or more) cell types, both Lyt-2$^-$, are necessary in providing help and only one is inactivated by irradiation. In this regard, Agarossi et al. (17) found two separate helper T cell subpopulations that differed in their radiosensitivity. Other groups have also found two types of helper T cells that can act independently or synergistically (18, 19). Another group described two helper T cell preparations that differed in their radiosensitivity (20). Whereas the radiosensitive one acted in an antigen-specific manner, the radioresistant preparation was largely antigen-nonspecific. We thus conclude that our lymph node preparations probably contain mainly the radiosensitive type of helper T cell.

Our results on the characteristics of the AChR-specific helper T cell are in agreement with those obtained by others using the lymphoproliferative assay in

mice (21) and rats (15). They both found that a cell type with surface differentiation markers associated with helper T cells was the cell type responsible for tritiated thymidine uptake. Namely, the Lyt-1^+ 23^- phenotype in mice and the W3/25^+ phenotype in rats were implicated in the lymphoproliferative response. The latter report also demonstrated that when the W3/25^+ subset was deleted by separation in the FACS, no anti-AChR antibody was synthesized in vitro by the remaining W3/25^- cells. Our results thus complement and extend these others, as we have both positively and negatively selected the AChR-specific proliferating cell type in an assay system designed to directly examine the function of the cell preparation.

The main topic of this paper deals with the strategies that we found successful in inducing AChR-specific Ts cells and TsF. The in vivo education method was presented because of its simplicity and freedom from nonspecific suppression. As demonstrated using the in vivo-educated TsF, the predominant cell type induced was the AChR-specific Ts cell. In vitro education of naive splenocytes was demonstrated to be another effective method for the induction of AChR-specific suppression. The batch of FCS, however, was critical in order to minimize serum-specific suppression. With many batches of FCS, we found that precultured cells that were not exposed to AChR completely suppressed in vitro immune responses after transfer into a Mishell-Dutton assay. This did not occur with the in vivo-educated TsF because of a difference in kinetics. Whereas in vivo–educated Ts cells were cultured for only 2 d to obtain their TsF, at least 3 d are required for primary induction of both specific (13) and nonspecific (10) Ts cells.

Slight levels of background suppression were also reported by other investigators (13). The cause of the background suppression, as suggested by Parish (10), was due to induction of Ts cells to proteins in the FCS. Our results from panning of FCS-binding cells as well as studies with FCS-binding TsF both lead us to the same conclusion. Ts cells may be induced to proteins in the FCS in the same manner as those induced to the AChR. These FCS proteins are presumably passively adsorbed by helper T cells in the in vitro anti-AChR assay and thus serve as targets for serum-specific suppression. In spite of this difficulty, however, there was consistently a wide difference between the AChR-specific suppression vs. background when using cell numbers below 1×10^5 (Fig. 3). With factor experiments the specific and nonspecific suppression were separable due to their differential adherence to the Sepharose-FCS and Sepharose-AChR columns (Table V).

The development of conditions to induce AChR-specific Ts cells and the means to measure them appropriately in vitro opens the way to extend these findings to the in vivo situation. Thus, it is now possible to study the state of AChR-specific suppression in experimental as well as human myasthenia. Moreover, this work enables the development of long-term T cell lines or hybridomas in the murine system, and the adaptation of the in vitro education procedure for human cells. The availability of large amounts of AChR-specific Ts factors from such lines may be of potential use for immunotherapeutic intervention in myasthenia gravis.

Summary

This report describes the in vivo and in vitro induction of murine (AChR)-specific suppressor T cells (Ts) and T cell factors (TsF), and the development of an appropriate assay system for their measurement. The assay described is based on the in vitro Mishell-Dutton culture system. Using this assay, it was shown that the AChR-specific helper cell is an Lyt-2$^-$ radiosensitive T cell. Moreover, the proliferating cell measured in the lymphocyte transformation assay was shown to provide AChR-specific T cell help. In vivo induction of Ts cells is achieved by injection of soluble AChR; potent AChR-specific suppression is found in the spleen 1 wk later. In vitro induction of Ts cells involves the primary education of naive splenocytes by culturing them with high concentrations of AChR. Both the in vivo- and in vitro-induced Ts cells were shown to secrete AChR-specific factors that mediate their suppressive effects. The possibility of specifically suppressing the AChR-immune response may be of a particular clinical importance since the AChR is the target autoantigen in the neuromuscular autoimmune disease myasthenia gravis.

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