Induction of Suppressor Cells Specific for AChR in Experimental Autoimmune Myasthenia Gravis

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Suppressor cells specific for acetylcholine receptor (AChR) were induced in a population of lymphocytes previously sensitized to AChR, obtained from rats with experimental autoimmune myasthenia gravis (EAMG). The lymphocytes were cultured with the immunosuppressive drug cyclosporin A plus purified AChR for 7 days. These cells, when mixed with lymphocytes from rats with EAMG in vitro, strongly suppressed the antibody response to AChR. They did not inhibit antibody responses to an unrelated antigen, an indication that suppression was specific for AChR. This approach should be a useful way to induce specific suppressor cells from sensitized populations of lymphocytes and may be applicable in the treatment of autoimmune diseases such as myasthenia gravis.

THE BASIC ABNORMALITY IN MYASthenia gravis is a reduction in the number of acetylcholine receptors (AChR's) at neuromuscular junctions (*1–3*) brought about by an antibody-mediated autoimmune attack (*4–6*). Treatment of myasthenia gravis with immunosuppressive drugs, although clinically effective, often has adverse side effects, including inhibition of necessary (as well as pathological) immune responses (*7*). Ideally, treatment of myasthenia gravis should eliminate only the immune response against the autoantigen AChR without otherwise impairing immune function. If suppressor lymphocytes specific for AChR could be induced, they might provide such a therapeutic agent. However, this poses a problem in autoimmune diseases, such as myasthenia gravis, in which lymphocytes are already sensitized to antigen; their reexposure to antigen to induce suppressor cells would instead result in an overwhelming immune response. We have circumvented this problem by using the immunosuppressive drug cyclosporin A (CsA). CsA inhibits immune responses while allowing antigen-induced activation of suppressor T cells (*8–11*). Our results show that incubation of lymphocytes from rats with experimental autoimmune myasthenia gravis (EAMG) with purified AChR and CsA induces a population of suppressor T cells specific for AChR. These cells strongly suppressed the antibody response to AChR of lymphocytes from rats with EAMG in vitro, but they did not inhibit unrelated immune responses.

To produce EAMG, we immunized female Lewis rats by subcutaneously injecting them with 500 pmol (~120 μg) of AChR purified from the electric organ of *Torpedo californica* (*12*) emulsified in Freund's complete adjuvant. Three to six weeks later, the draining lymph nodes were aseptically removed and dissociated mechanically into single-cell suspensions. When these "responder" cells were cultured in microwells with AChR (2.0 pmol/ml) for 7 days (*13*), they produced 50 to 300 pmol of antibody to AChR per milliliter of supernatant as measured by radioimmunoassay (*4, 14*).

Addition of CsA to the responder cells inhibited the AChR-stimulated secondary antibody response. With increasing concentrations of CsA, the antibody response to AChR progressively decreased, reaching 95 percent inhibition at 200 ng/ml (Fig. 1). At low concentrations of CsA (2.0 ng/ml), a small but consistent increase (28 percent) in the antibody response occurred; a similar paradoxical enhancement at low CsA doses has been reported (*15*). At all concentrations of CsA, the viability of the cultured cells was greater than 95 percent as determined by the ability of the viable cells to exclude trypan blue dye.

To determine whether incubation with CsA plus AChR induces persistent inhibition of the antibody response of lymphocytes (that is, after removal of CsA), we carried out restimulation experiments. Rats were immunized by intraperitoneal injection with 500 pmol of AChR absorbed on bentonite (*16*). Three to six weeks later, lymph node or spleen lymphocytes from these rats were removed and incubated in bulk cultures (*17*) with CsA plus AChR for 7 days as above. Control lymphocytes from rats with EAMG were cultured with CsA alone. At the end of the culture period, the cells were washed thoroughly to remove CsA (*18*). They were then cultured again with fresh AChR (2.0 pmol/ml) for seven additional days, and antibodies to AChR in the supernatants were measured by radioimmunoassay. The antibody response of lymph node cells was decreased by 88 percent, and that of spleen cells by 84 percent, in comparison to the control lymphocytes (Fig. 2). These results indicate that treatment of sensitized lymphocytes with CsA plus AChR induces a state of tolerance to the antigen AChR. Unresponsiveness was not due to "antigen blockade" of receptors; cells that were cultured with AChR alone (2.0 pmol/ml) for 7 days, washed, and stimulated with fresh AChR produced vigorous antibody responses.

We next assayed tolerant cultures for the presence of functional suppressor cells.

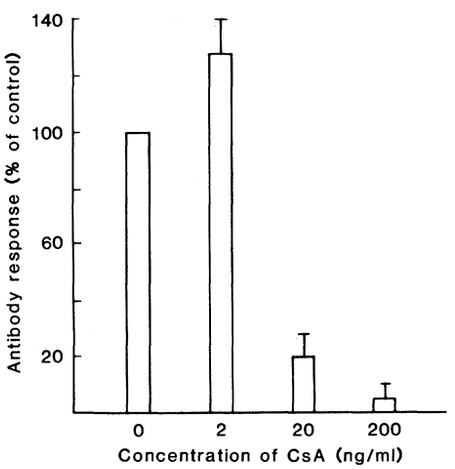

Fig. 1. Dose-response curve for CsA. Responder cells were cultured with AChR (2.0 pmol/ml) and various concentrations of CsA as indicated (*13*). After 1 week of culture, supernatants were harvested, and antibodies to AChR were measured by radioimmunoassay. The results show mean responses of four experiments and brackets show SEM's. At a CsA concentration of 200 ng/ml, the antibody response to AChR was virtually abolished.

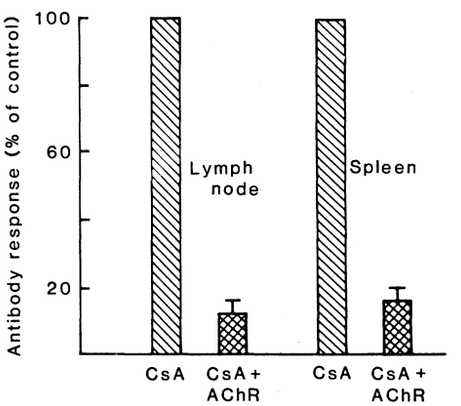

Fig. 2. Unresponsiveness of cells to AChR after treatment with CsA plus AChR. Lymph node cells and spleen cells from rats with EAMG were used in these experiments. Cells were cultured in bulk with CsA (200 ng/ml) plus AChR (2.0 pmol/ml) or with CsA alone (controls) (*17*). After 1 week in culture, the cells were harvested and washed thoroughly to remove CsA (*18*). They were then stimulated with fresh AChR (2.0 pmol/ml) for 1 week in microtiter wells (*13*) and assayed for antibody to AChR by radioimmunoassay. Results shown are mean responses of four lymph node and six spleen cell experiments ± SEM.

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EAMG lymph node and spleen cells were treated as before by culturing with CsA plus AChR for 7 days. Control EAMG lymphocytes were cultured with CsA alone. To assay for suppressor activity, we added various proportions of these putative suppressor cells to fresh cultures of responder cells. The combined cultures were then stimulated with AChR (2.0 pmol/ml), and 7 days later antibodies to AChR were measured in the supernatants. Spleen cells cultured with CsA plus AChR had a powerful suppressive effect. At a ratio of putative suppressor cells to responder cells of 1:10 (*19*), the antibody response was inhibited by 90 percent; only 6 percent of this effect could be attributed to nonspecific suppression (Fig. 3). Lymph node cells treated with CsA plus AChR exhibited a similar but much less marked suppressive effect (33 percent total suppression minus 17 percent nonspecific suppression for 16 percent net specific suppression). Suppression was not due to carryover of AChR; cells cultured with AChR alone (2.0 pmol/ml) were not suppressive when cultured together with responder cells.

To evaluate the antigen specificity of suppressor cells, we compared their effects on responder cell cultures primed to an unrelated antigen, keyhole limpet hemocyanin (KLH), with their effects on AChR-primed lymphocytes. We produced suppressor cells by culturing spleen cells from EAMG rats with CsA plus AChR for 7 days. These cells or control cells (cultured previously with CsA alone) were then added to responder cell cultures specific for AChR or for KLH (*20*). Each set of cultures was stimulated with its appropriate antigen, and antibodies to AChR or to KLH were measured 1 week later by radioimmunoassay. The KLH response was suppressed by only 7 percent, whereas the same dilution of suppressor cells inhibited the AChR antibody response by 62 percent (Fig. 4). Furthermore, in the KLH cell cultures, the control cells produced virtually the same degree of suppression as the cells treated with CsA plus AChR at all ratios of precultured to responder cells tested. These results could not be attributed to a greater resistance of the KLH cells to suppression because the cells were slightly more susceptible to nonspecific suppression by control lymphocytes. Our results indicate that treatment of primed lymphocytes with CsA plus AChR induces suppressor cells that are specific for the antigen AChR.

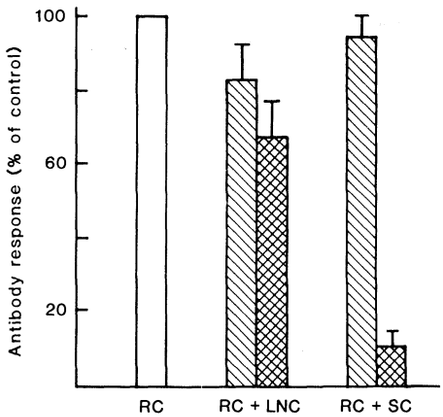

Fig. 3. Suppressor cell activity induced by CsA plus AChR. Primed lymph node cells (LNC) or spleen cells (SC) were incubated with CsA plus AChR in bulk cultures (cross-hatched bars) or with CsA alone (controls, single hatched bars) as described in the legend to Fig. 2. One week later the cells were harvested and washed thoroughly to remove CsA. Various numbers of these putative suppressor cells were added to 5×10^5 responder cells (RC) in microwells, and the cocultures were stimulated with fresh AChR (2.0 pmol/ml). One week later antibodies to AChR in the culture supernatants were measured by radioimmunoassay. Antibody responses are expressed as the percentage of the RC antibody response. Four LNC and eight SC experiments were performed (error bars show SEM). When nonspecific suppression by control cells is subtracted, SC treated with CsA plus AChR suppressed the antibody response to AChR by 84 percent, and treated LNC suppressed the response by 16 percent. The degree of suppression was determined as described in the text (*19*).

Our results demonstrate, first, that CsA in concentrations ranging from 20 to 200 ng/ml inhibited the secondary antibody response of AChR-primed lymphocytes to antigen stimulation in vitro. These results support our findings in vivo, which demonstrated that treatment of EAMG rats with CsA during an AChR boost prevented the secondary antibody response (*21*). Second, we found that prolonged incubation of primed lymphocytes with CsA plus AChR induced a state of tolerance; that is, treated cells were unresponsive to further stimulation with AChR. We found that lymphocytes from both lymph nodes and spleen were tolerized to a similar degree. Suppressor activity, however, was much more marked in the tolerized spleen cell population. This is probably due to the larger proportion of suppressor cell precursors found in spleen compared to lymph nodes (*22*). It is possible that the lymph node cell population, when treated with CsA plus AChR, yields a sufficient number of suppressor cells to prevent restimulation to AChR but not enough suppressor cells to suppress the additional number of responder cells in cocultures. Alternatively, it is possible that other mechanisms of tolerance-induction, such as clonal deletion (*23*) or blockade of antigen receptors (*24*), occurred in the lymph node cell population.

In these studies, normally immunogenic doses of AChR were used together with CsA to induce the suppressor cells. These cells had the capacity to suppress the induction of a secondary antibody response. This is important in view of the evidence that "memory cells" (that is, lymphocytes previously sensitized to a given antigen) are much more resistant to suppression than naïve cells (*25*). Studies by other investigators have shown that alloantigen-specific suppressor cells were generated during the course of a mixed lymphocyte reaction when CsA was added to the cultures (*9, 26*). These cells were shown to be T lymphocytes that were adherent to nylon wool (*26*). The suppressor cells in our system may be similar; they are also antigen specific, and they appear to be suppressor T lymphocytes. We have recently shown that, by removing rat T cells bearing the suppressor phenotype (OX8 positive cells) (*22*) before culturing them together with responder cells, we can remove most of the suppressor activity in the population.

Ultimately, suppressor cells induced by this strategy could be used to control myasthenia gravis or other autoimmune diseases for which the antigen is available. This therapeutic strategy has many potential advantages for treatment of myasthenia gravis: it should be highly specific for the immune response to AChR; it is completely physio-

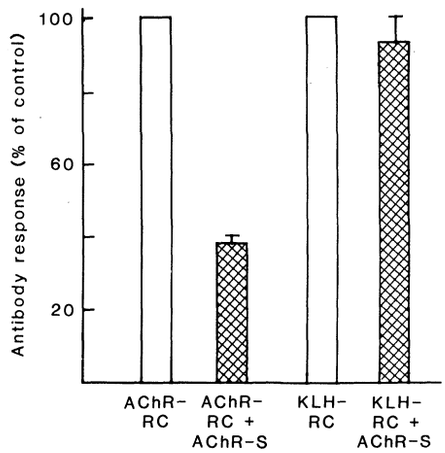

Fig. 4. Antigen specificity of suppressor cells. Suppressor cells (AChR-S) were induced in bulk cultures with CsA plus AChR as described in the legend to Fig. 2. Various numbers of suppressor cells or control cells (cultured previously with CsA) were cultured together with responder cells (RC) primed to AChR or to KLH (*20*). Cocultures were stimulated with the appropriate antigen; responder cells to AChR (AChR-RC) cultured with an optimal concentration of AChR (2.0 pmol/ml), and responder cells to KLH (KLH-RC) were stimulated with an optimal concentration of KLH (0.02 μg/ml). Seven days later antibodies to AChR and KLH were measured by radioimmunoassay. Suppression was calculated as described in the text (*19*). Results shown are the mean responses of three experiments ± SEM. Suppression by cells treated with CsA plus AChR was specific for AChR.

logical, since the individual's own lymphocytes would be used to down-regulate the abnormal immune response; and it requires relatively small amounts of antigen. The usefulness of this approach depends on the ability of the cells to suppress the autoimmune response in vivo and on the duration of the putative suppressive effect.

REFERENCES AND NOTES

1. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *Science* **182**, 293 (1973).
2. Y. Ito, R. Miledi, A. Vincent, *Brain* **101**, 345 (1978).
3. A. G. Engel, J. M. Lindstrom, E. H. Lambert, V. A. Lennon, *Neurology* **27**, 307 (1977).
4. J. M. Lindstrom, M. E. Seybold, V. A. Lennon, S. Whittingham, D. D. Duane, *ibid.* **26**, 1054 (1976).
5. K. V. Toyka, D. B. Drachman, A. Pestronk, I. Kao, *Science* **190**, 397 (1975).
6. D. B. Drachman, R. N. Adams, E. F. Stanley, A. Pestronk, *J. Neurol. Neurosurg. Psychiatry* **43**, 601 (1980).
7. R. M. Pascuzzi, H. B. Coslett, T. R. Johns, *Ann. Neurol.* **15**, 291 (1984).
8. A. D. Hess and P. J. Tutschka, *J. Immunol.* **124**, 2601 (1980).
9. G. A. Dos Reis and E. M. Shevach, *ibid.* **129**, 2360 (1982).
10. H. J. Deeg *et al.*, *Exp. Hematol. Suppl.* **9**, 94 (1981).
11. I. F. Hutchinson *et al.*, *Transplant. Proc.* **13**, 412 (1981).
12. J. Elliot *et al.*, *Biochem. J.* **185**, 667 (1980).
13. Responder cells were cultured at 5×10^5 cells per microwell with purified AChR (2.0 pmol/ml) for 7 days in a humidified atmosphere of 95 percent air and 5 percent CO_2. The cultures were performed in triplicate in 96-well flat-bottomed microtiter plates (Corning). Cells were cultured in RPMI 1640 medium with glutamine (Gibco) supplemented with $5 \times 10^{-5} M$ 2-mercaptoethanol, 1:100 dilutions of 100× solutions of sodium pyruvate, nonessential amino acids, and penicillin-streptomycin plus Fungizone (all concentrated stock solutions, Gibco), 1 percent rat serum, and 1 percent horse serum (Gibco).
14. A. Pestronk, D. B. Drachman, R. N. Adams, *Muscle Nerve* **5**, 79 (1982).
15. S. Cammisuli, in *Cyclosporin A*, D. J. G. White, Ed. (Elsevier, Amsterdam, 1982), pp. 243–259.
16. M. B. Rittenberg and K. L. Pratt, *Proc. Soc. Exp. Biol. Med.* **132**, 575 (1969).
17. Lymphocytes were cultured in bulk with CsA (200 ng/ml) plus AChR (2.0 pmol/ml) or with CsA alone (controls). Cultures were performed in a total volume of 5 ml in six-well cluster plates (Costar) at a density of 5×10^6 cells per milliliter.
18. Cells were washed to remove CsA as follows: one wash in RPMI 1640 medium, one spin through horse serum, and two additional washes in RPMI 1640.
19. Various ratios of putative suppressor cells to responder cells or control (CsA-treated) cells to responder cells were used in each suppressor assay. The same ratio that gave the minimal level of "nonspecific" suppression with control cells was used to determine suppression by cells treated with CsA plus AChR. In the spleen cell experiments, nonspecific suppression was 31 percent at a ratio of 1:5 and decreased to 6 percent at a ratio of 1:10 (Fig. 3). In initial experiments, lower ratios (1:20 and 1:40) of control cells to responder cells gave no further decrease of "nonspecific" suppression. In the lymph node experiments, nonspecific suppression was 20 percent at a ratio of control cells to responder cells of 1:1 and 17 percent at a ratio of 1:2. At lower ratios (1:10 and 1:20), no further reduction of "nonspecific" suppression occurred.
20. Responder cells to KLH were prepared as described for AChR except that the rats were immunized with 1 mg of KLH (Sigma) emulsified in Freund's complete adjuvant.
21. D. B. Drachman, R. N. Adams, K. McIntosh, A. Pestronk, *J. Clin. Immunol. Immunopathol.* **34**, 174 (1985).
22. R. J. Brideau *et al.*, *Eur. J. Immunol.* **10**, 609 (1980).
23. P. J. Conlon, J. W. Moorhead, H. N. Claman, *Nature (London)* **278**, 257 (1979).
24. T. Hamaoka, U. Kamashita, T. Takami, M. Kitagawa, *J. Exp. Med.* **141**, 1308 (1975).
25. N. E. Goeken, *J. Immunol.* **128**, 2081 (1982).
26. A. D. Hess, P. J. Tutschka, G. W. Santos, *ibid.* **126**, 961 (1981).

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Alloantigen Recognition Is Preceded by Nonspecific Adhesion of Cytotoxic T Cells and Target Cells

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T-cell receptors bind antigens only when the antigens are exposed on the cell surface. This can be studied best in the interaction of cytolytic T lymphocytes (CTL) with target cells because the recognition and binding event can be separated from the lytic phase. Studies with CTL clones specific for HLA-A2 and HLA-B7 demonstrated that conjugates of CTL's and target cells can be formed in the absence of specific antigen recognition. Furthermore, T-cell receptor and target antigen cannot interact unless there is conjugate formation. This indicates that nonspecific conjugate formation between CTL's and target cells precedes the recognition of specific antigen by the T-cell receptor.

THYMUS-DERIVED-LYMPHOCYTE (T-cell) recognition and action are mediated by cell-cell interactions. Such cell-cell interactions can be studied in detail with the use of cytotoxic T lymphocytes (CTL's), which play a critical role in the immune response to viral and parasitic infections and in the destruction of tumor cells. The mechanism by which CTL's lyse target cells can be separated into several stages. In the initial phase, cell-cell contact between the CTL and its target cell is established (see Fig. 1). Next, the so called programming for lysis takes place and is followed by the delivery of the "lethal hit." Once the lethal hit has been delivered the presence of the CTL is not required for completion of the lysis of the target cell (*1*).

Until now it has been assumed that the adhesion between the CTL and its target cell is initiated by the recognition of one or more target antigens by the T-cell receptor (*1*). Indeed, monoclonal antibodies directed at the T-cell receptor or the closely associated T3 antigens block cytolysis (*1*). However, these antibodies do not block the formation of adhesions between CTL's and target cells (*2*). This raises the possibility that the interaction between the T-cell receptor and antigen is not required for the formation of conjugates. We reported recently that an HLA-A2–specific CTL clone JR-2-16 was unable to lyse mouse L cells that express the HLA-A2 antigen after gene transfer (*3*). In contrast, human cells transfected with an HLA-A2–specific DNA fragment were all killed (*3*). This finding implies that the presence of specific antigen on the target cell is not sufficient for lysis. Since it has been reported that other T-cell surface structures, the "accessory molecules" T4, T8, T11, and LFA-1, are involved in cytotoxic reactions (*4*), we speculated that target cell structures other than HLA-A2 are required for lysis (*3*). Whether these determinants are the counter-structures of the accessory molecules that may be absent on the surface of L cells remains to be determined.

To study the roles of the T-cell receptor–T3 complex and of the accessory molecules, we used the human HLA-A2–specific CTL clone JR-2-16 (*5*), the HLA-B7–specific CTL clone KOR-132, and various target cells in a single cell assay (*6*). This assay allows the measurement of the number of conjugates formed between killer cells and target cells and an estimate of the number of lysed target cells present in the conjugates. In the single-cell assay, effector cells labeled with carboxyfluorescein diacetate at a concentration of 2×10^6 cells per milliliter were mixed with target cells at the same concentration, incubated for 10 minutes at 30°C, and centrifuged. The fluorescent label

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