JCI The Journal of Clinical Investigation

Specific tolerance to an acetylcholine receptor epitope induced in vitro in myasthenia gravis CD4+ lymphocytes by soluble major histocompatibility complex class II-peptide complexes.

M W Nicolle, … , D J Ferguson, J Newsom-Davis

J Clin Invest. 1994;93(4):1361-1369. https://doi.org/10.1172/JCI117112.

Research Article

In autoimmune disorders, inactivation of pathogenic antigen-specific T cells, rather than global immunosuppression, would be highly desirable. One way to achieve this would be to deliver the first antigen-specific signal to the T cell in the absence of the second costimulatory signal. Myasthenia gravis (MG) is a well-characterized autoimmune disease in which T cell-dependent autoantibodies are directed against the acetylcholine receptor (A ChR) at the neuromuscular junction. AChR-specific T cells have been cloned from MG patients, and in this study, we have induced long-lasting tolerance in vitro in one particular clone (PM-A1) with a known peptide epitope (alpha 144-163) and MHC class II restriction (DR4 Dw14.2 or 4.2) by using soluble MHC-class II peptide complexes. Preincubation of PM-A1 T cells with such complexes induced death by apoptosis in < or = 40-50% of the AChR-specific cells. Surviving cells remained refractory to stimulation with AChR-derived synthetic peptides or recombinant polypeptides for < or = 38 d after complex treatment. These effects were highly specific, dose-dependent and required > 2 h preincubation. The T cells could be protected from the tolerizing effects of complex by coincubation with DR-matched or -mismatched antigen-presenting cells. This work shows that antigen-specific T cells can be selectively killed or anergized using soluble MHC class II: peptide complexes. Such an antigen-specific therapy offers a rational approach to the […]

Specific Tolerance to an Acetylcholine Receptor Epitope Induced In Vitro in Myasthenia Gravis CD4+ Lymphocytes by Soluble Major Histocompatibility Complex Class II–Peptide Complexes

Michael W. Nicolle,* Bishwajit Nag,§ Somesh D. Sharma,§ Nicholas Willcox,* Angela Vincent,* David J. P. Ferguson,‡ and John Newsom-Davis*

**Neurosciences Group, Institute of Molecular Medicine, and ‡Electron Microscope Unit, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom OX3 9DU; and §Anergen Inc., Redwood City, California 94063*

Abstract

In autoimmune disorders, inactivation of pathogenic antigen-specific T cells, rather than global immunosuppression, would be highly desirable. One way to achieve this would be to deliver the first antigen-specific signal to the T cell in the absence of the second costimulatory signal. Myasthenia gravis (MG) is a well-characterized autoimmune disease in which T cell–dependent autoantibodies are directed against the acetylcholine receptor (AChR) at the neuromuscular junction. AChR-specific T cells have been cloned from MG patients, and in this study, we have induced long-lasting tolerance in vitro in one particular clone (PM-A1) with a known peptide epitope (α144-163) and MHC class II restriction (DR4 Dw14.2 or 4.2) by using soluble MHC–class II peptide complexes.

Preincubation of PM-A1 T cells with such complexes induced death by apoptosis in $\leq$ 40–50% of the AChR-specific cells. Surviving cells remained refractory to stimulation with AChR-derived synthetic peptides or recombinant polypeptides for $\leq$ 38 d after complex treatment. These effects were highly specific, dose-dependent and required > 2 h preincubation. The T cells could be protected from the tolerizing effects of complex by coincubation with DR-matched or -mismatched antigen-presenting cells.

This work shows that antigen-specific T cells can be selectively killed or anergized using soluble MHC class II:peptide complexes. Such an antigen-specific therapy offers a rational approach to the immunotherapy of autoimmune or allergic disease in vivo. (*J. Clin. Invest.* 1994. 93:1361–1369.) Key words: autoimmune disease • immunotherapy • apoptosis • anergy

Introduction

A desirable therapeutic goal in autoimmune disease is to correct the aberrant immune response selectively, rather than to impose global immunosuppression with its often hazardous side effects. In T cell–dependent, antibody-mediated autoimmune diseases, such as myasthenia gravis (MG)[1] (reviewed in reference 1), selective inactivation of disease-specific CD4+ inducer T lymphocytes that provide help to autoreactive B cells (2) should interrupt the sequence necessary for autoantibody production. Similarly, inactivation of cytotoxic T cells could directly prevent target cell damage in other diseases. Although functional inactivation (tolerance) of disease-specific cells might be adequate, specific and permanent removal of these cells would be preferable.

Previous work (3, 4; reviewed in references 5, 6) suggests that at least two signals are required for effective T cell activation. The first, specific signal is delivered to the T cell by interaction of the T cell receptor (TCR) with peptide antigen bound in the cleft of MHC molecules on the surface of antigen-presenting cells (APCs). Second, nonspecific costimulatory or accessory signals are also required for full activation. They are delivered by APC-derived cytokines (6, 7) interacting with lymphocyte receptors and/or by interaction of pairs of homologous or heterologous surface molecules present on both APCs and lymphocytes (8–10). Provision of the first specific signal in the absence of these costimulatory signals results in functional inactivation or tolerance of lymphocytes. Using this approach, tolerance has been achieved with murine systems in vitro using MHC molecules anchored in planar membranes devoid of accessory molecules (11) or chemically fixed APCs in which the function of accessory molecules is impaired (12, 13). A logical extension of this approach, and one which is potentially more applicable in vivo, would be to present purified soluble MHC class II containing the relevant peptide.

In MG, the target antigen, AChR, and the primary sequence of its five subunits, including the apparently immunodominant α subunit, are now known (14, 15). This has enabled us to raise T cell lines with α subunit recombinant polypeptide antigen, and to define their epitopes using a panel of synthetic peptides. Their MHC class II restrictions were established by using anti-DR, -DP, and -DQ blocking antibodies, and after assessing presentation by MHC class II-sharing or donor-mismatched APCs.

As a model system for inducing specific tolerance, we have used one such CD4+ T cell clone, raised from the hyperplastic thymus of a 15-yr-old MG patient (16), and likely to have disease relevance in vivo, since it responds vigorously to pro-

Part of this work was presented at the New York Academy of Science's Myasthenia Gravis and Related Disorders Meeting, April 1992, and at the American Neurological Association's annual meeting, October 1992, Toronto, Canada.

Address correspondence to Professor John Newsom-Davis, Neurosciences Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom OX3 9DU.

Received for publication 11 August 1993 and in revised form 19 October 1993.

J. Clin. Invest.

0021-9738/94/04/1361/09 $2.00
Volume 93, April 1994, 1361–1369

1. *Abbreviations used in this paper:* α, α subunit; AChR, acetylcholine receptor; Ag, antigen; APCs, antigen-presenting cells; BAPC, "bystander" APC; CAE, cellulose acetate electrophoresis; Cx, soluble MHC class II:peptide complex; MG, myasthenia gravis; p, synthetic peptide; r, recombinant; TCR, T cell receptor; TLC, thin layer chromatography.

cessed intact human AChR (16, 17). To induce tolerance, we used a soluble MHC class II:peptide complex containing both the AChR α subunit epitope peptide (p138-167 or p144-163) and a restricting class II molecule (DR4 Dw4.2). Here, we show that preincubation with these complexes inhibits subsequent proliferation of the autoreactive $CD4^+$ T cells, and demonstrate that the mechanism, which involves both cell death and functional tolerance, depends at least partly on the absence of costimulatory molecules.

Methods

Characteristics of the PM-A1 clone. The $CD4^+$ T cell clone PM-A1 was derived from the thymus of a 15-yr-old MG patient (anti-AChR antibody titer 180 nmol, MHC class II DR3/DR4 Dw14.2) raised from the activated low density fraction of a thymic cell suspension, using recombinant (r) human AChR α subunit polypeptide r37-429, and subsequently cloned from this parent line by limiting dilution (for details, see reference 16). The restricting epitope was mapped using a series of overlapping AChR α subunit–derived recombinant polypeptides and synthetic peptides (p). MHC class II restriction was established using a panel of irradiated PBMC from MHC sharing or mismatched donors. These cells were maintained in 2-ml well plastic plates (Nunclon, Roskilde, Denmark) in culture media consisting of RPMI 1640 with L-glutamine (Gibco, Life Technologies Ltd., Paisley, Scotland), supplemented with 5% human A^+ heat-inactivated serum, 100 U/ml penicillin, and 40–100 μg/ml streptomycin. The cells were maintained by stimulation every 12–14 d with DR4 Dw4.2- or DR4 Dw14.2-irradiated (2,000–2,500 rad/20–25 Gy) PBMC and were given IL-2 20 U/ml (Biotest Lymphocult-T-HP, Solihull, United Kingdom) on days 3, 6, and 9 after stimulation.

Complex preparation. The MHC class II:peptide complexes used for clone PM-A1 were composed of solubilized DR4 Dw4.2 bound to AChR α subunit–derived peptides p138-167, p144-163, p144-156, or DR3 bound to p309-344. Peptides were synthesized using f-moc chemistry (16). As controls, DR4 without added peptide (DR4:Nil) or DR4 bound to myelin basic protein peptide 1–14 were used.

Complex preparation is described in part elsewhere (18–20). Briefly, large numbers of EBV-transformed lymphoblastoid cells (e.g., GM 06821A; National Institute of General Medical Sciences, Coriell Institute of Medical Research, Camden, NJ) bearing the appropriate class II type (e.g., DR4 Dw4.2 homozygous) were lysed, and a high speed (100,000 *g*) membrane fraction was detergent extracted (19, 20). Class II molecules were affinity purified from extracted membrane proteins by recycling for 4 h over a preequilibrated anti-DR (protein A–purified mAb L243; American Tissue Type Collection, Rockville, MD) coupled Sepharose 4B column. After washing, MHC class II molecules were eluted (19, 20) and characterized using 12% SDS-PAGE and silver staining. They were then incubated at 50 μg/ml with a fivefold molar excess of p138-167 (or other peptide where indicated) at pH 5.0 and 37°C for 24 h. Unbound peptide was removed by extensive dialysis for 36 h (18–20).

Peptide binding was analyzed by thin layer chromatography (TLC) and by cellulose acetate electrophoresis (CAE) (18). For TLC, 1 μl of complex was applied in triplicate onto a 5-cm silica gel TLC plate and run in a solvent system of 50% methanol and 5% ammonium acetate. After drying, the distribution of radioactivity was estimated at relative front (R_f) 0–0.2 for calculating the percent of DR4 occupied with labeled peptide. CAE was also used to measure peptide binding (18). Final protein concentrations were determined by Lowry assay.

FACS® analysis and monoclonal antibodies. Cell surface markers were characterized by staining with mouse mAb at 4°C for 20–30 min, after which they were washed with ice-cold PBS/azide 0.02% and stained with FITC or PE-conjugated second layers for 20–30 min. They were kept on ice until analysis. mAbs used for FACS® staining (Becton Dickinson Immunocytometry Systems, Mountain View, CA) included mouse anti-human CD4 (RFT4, IgG_1), CD8 (RFT8, IgM), and anti-DR (RFDR1, IgM) (all kind gifts from Prof. G. Janossy, Royal Free Hospital, London, United Kingdom), and anti-TCR Vβ5.1 (clone LC4, kind gift of Prof. R. Levy, Stanford University, Palo Alto, CA). Second layers included FITC-conjugated goat anti–mouse IgG and PE-conjugated goat anti–mouse IgM (Southern Biotechnology Associates, Birmingham, AL).

Proliferation assays. Proliferation assays were performed in 200-μl U-bottomed 96-well microtiter plates (Nunclon). $1–2 \times 10^4$ T cells were stimulated with $1–2 \times 10^5$ APCs with or without antigen. APCs were prepared by irradiating (2,000–2,500 rad/20–25 Gy) PBMC collected over a Ficoll gradient. 72 h after stimulation, T cell proliferation was measured by adding 1 μCi ^{3}[H]thymidine (185 Gbq/5 Ci per mmol; Amersham Life Science, Little Chalfont, United Kingdom) and 18 h later, the cells were harvested (1295–001 cell harvester; LKB-Wallac, Milton Keynes, United Kingdom) and the incorporated radioactivity was measured using liquid scintillation counting (1205 Betaplate; LKB-Wallac).

For experiments in which T cells were preincubated with soluble MHC class II:peptide complex (Cx), the standard protocol was an 18-h preincubation (except where noted) of T cells with or without Cx, followed by challenge with a supramaximal stimulus of irradiated MHC class II sharing ($DR4^+$) APCs with or without antigen. Where noted, the complex was washed away after preincubation and before stimulation with APCs. In preliminary experiments, dialysis buffer from the complex preparation was added to the control cells, and no inhibition of proliferation was seen. For subsequent experiments, tissue culture medium was used as a control. Stimulating antigens used included AChR-derived synthetic peptides p138-167 or p144-163 (1–5 μg/ml), or recombinant polypeptides r37-181 or 1-437 ($\sim$ 0.1–0.5 μg/ml). These doses were $\geq$ 10-fold greater than those previously shown to result in maximal stimulation (data not shown).

Long term effects of Cx incubation. After PM-A1 cells were stimulated in the usual fashion with r1-437 and APCs on day 0, they were exposed to medium or Cx138 1.7×10^{-7} M on day 4. They were then given IL-2 in parallel at three day intervals. In accordance with their usual stimulation cycle, on days 14, 28, and 42, they were washed extensively and equal numbers of viable cells from each group were restimulated identically. At each stimulation, cumulative cell yields were calculated per million input (day 0) cells. On day 42, equal numbers of viable cells were stimulated in a proliferation assay.

Other T cell lines and clones. To assess the specificity of the effects of complex, other T cell lines and clones were raised (see Table II). Generally, for AChR-reactive lines, the protocol was similar to that used for clone PM-A1, the initiating cells having been obtained from PBL. Clone NWKF5 was raised from a healthy control's PBL against native keyhole limpet haemocyanin (Sigma Immunochemicals, St. Louis, MO) and subsequently cloned by limiting dilution. Line NWTTX was raised against tetanus toxoid MWC5479 (Wellcome, Beckenham, United Kingdom).

Bystander APC (BAPC) preparation. BAPCs were prepared by irradiating (2,000 rad; 20 Gy) non-$DR4^+$ (DR 1/8) PBMC, and in some cases these were then fixed with varying concentrations of glutaraldehyde (0.001–0.5%) for 30 s followed by addition of an equal volume of 0.4 M L-lysine and extensive washing in RPMI containing 10% human serum. These BAPCs alone, with or without antigen, did not cause proliferation of PM-A1 cells.

Staining of cells to evaluate cell death. An equal volume of acridine orange and ethidium bromide (6 μg/ml and 20 μg/ml, respectively, in 0.04% ethanol) was added to an aliquot of cells. Dead and viable cells were counted using fluorescence microscopy (21).

Electron microscopy. After treatment with either medium (untreated, control) or Cx for varying periods of time, samples were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in osmium tetroxide, dehydrated, treated with propylene oxide, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate. For each sample, a minimum of 1,000 cells were assessed (Ferguson, D. J. P.; blinded to treatment group or duration of treatment) and evaluated as either living, apoptotic, or necrotic.

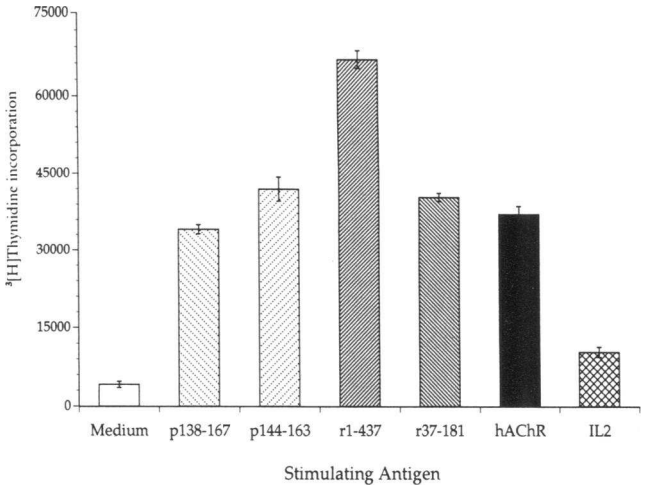

Figure 1. Proliferation of PM-A1 cells in response to processing and presentation of AChR α subunit–derived antigens or intact AChR by irradiated DR4-matched APCs. Antigens used included none (*Medium*), synthetic α subunit peptides p138-167 or p144-163, recombinant α subunit 1-437 or 37-181, and affinity-purified intact human AChR (*hAChR*). The response to IL-2 in the presence of APCs is also shown.

Results

Characteristics of the clone PM-A1. These cells proliferate vigorously when stimulated with any peptide or preparation of purified native or recombinant human AChR α subunit containing its epitope p144-163 (16, 17) in the presence of DR4 Dw4.2 or DR4 Dw14.2 APCs (Fig. 1). FACS® analysis showed clonal cells to be > 97% $CD4^+$, < 4% $CD8^+$, and > 96% DR^+ (not shown). PCR analysis showed that the TCR usage was Vβ5.1 Vβ5.1 (Moody, A.-M., unpublished observations). Cytokine analysis showed both IL-2 and IFN-γ production, without IL-4 production, in keeping with a "T_H1" phenotype (Hawke, S., unpublished observations).

Complex preparation and characterization. DR4 molecules were purified to > 98% purity as shown by SDS-PAGE and silver staining (Fig. 2 *A*). Approximately 4 μg of purified DR was obtained per 10^6 EBV cells. Optimal conditions for peptide binding to DR4 were established initially by using ^{125}I-Tyr-α138-167 (Fig. 2, *B–D*). For subsequent complex preparation, the conditions consisted of 24-h incubation of DR4 with a fivefold molar excess of p138-167 at pH 5.0. Under these conditions, 40–50% of the DR4 was occupied with p138-167 (Fig. 2 *E*) or 35% with p144-163 (not shown). Several independent preparations were used for the following experiments.

Immediate effects of the complexes on PM-A1 cells. In the absence of added APCs or antigen, addition of Cx138 produced a small and variable degree of T cell proliferation. This proliferation was < 5% of that seen when APCs and antigen were added in parallel to medium-incubated cells, and did not correlate with the ability of the Cx to induce tolerance (see below). If specific complexes (e.g., Cx144) were not washed away before addition of MHC II–matched APCs, the T cells proliferated to some extent regardless of whether further antigen was added (data not shown). This was at least partially because the Cx was taken up, processed, and presented by the added APCs to any remaining viable, nontolerized T cells (Nicolle, M. W., unpublished observations).

Induction of tolerance in PM-A1 cells. To induce tolerance, PM-A1 cells were preincubated with a soluble complex of DR4 Dw4.2 bound to an epitope peptide p138-167 (DR4:p138-167; Cx138) for 48 h followed by extensive washing. The cells were then challenged with a known supramaximal stimulus of antigen, either peptide or recombinant AChR r37-181, and $DR4^+$

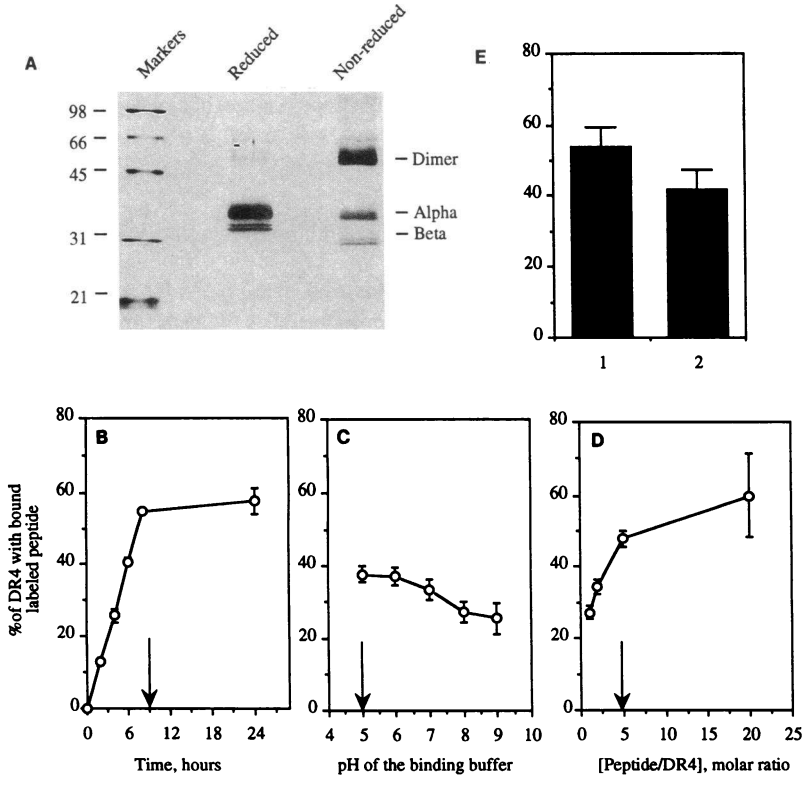

Figure 2. Preparation and optimization of DR4 Dw4:p138-167 complexes. (*A*) 1 μg of purified HLA-DR4 Dw4 from GM-6821A lymphoblastoid cells run under reduced and nonreduced conditions on SDS-PAGE followed by silver staining. (*B–D*) Optimal conditions for binding of p138-167 to DR4.Dw4 were established using ^{125}I-Tyr-138-167: (*B*) time course of peptide binding; (*C*) optimal pH for peptide binding; and (*D*) molar ratio of peptide to DR4 for binding. *Arrows,* conditions used for subsequent complex preparation. (*E*) Binding of ^{125}I-Tyr-138-167 to a typical preparation using TLC (*1*) or CAE (*2*), under optimal conditions. All results are mean of three determinations.

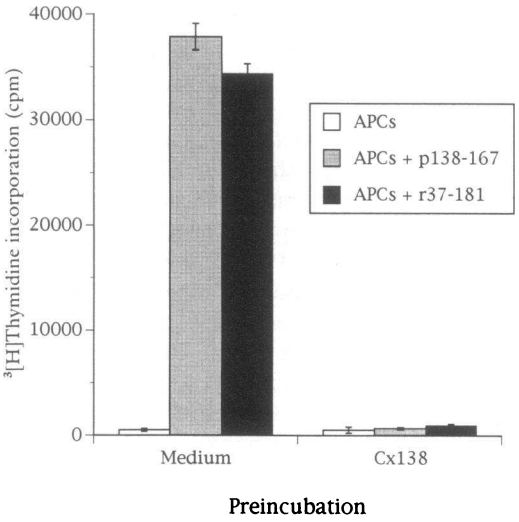

Figure 3. Cx-induced inhibition of PM-A1 proliferation. PM-A1 cells were preincubated for 48 h without (*Medium*) or with Cx138, 1.7 $\times 10^{-7}$ M. After extensive washing, equal numbers of viable cells were replated for a proliferation assay. DR4$^+$ APCs were added without (*APCs*) or with stimulating antigens (p138-167, r37-181), and T cell proliferation was assessed by ^{3}[H]thymidine incorporation.

APCs (16). Non–Cx-treated cells showed antigen-specific ^{3}[H]thymidine incorporation of > 34,000 cpm compared to < 1,000 cpm in Cx138-treated cells (Fig. 3). Although such reductions were consistently seen after preincubation with complex, the degree of inhibition varied slightly from one preparation to the next, presumably because of variation in the amount of the AChR-specific peptide bound to the MHC class II molecules in the complex. Mean results are shown in Table I.

Dose and time dependence of complex-mediated inhibition. Both of the PM-A1–specific complexes, Cx138 and Cx144, were effective at concentrations above 10^{-10} M, with a plateau at concentrations above 10^{-8} M (Fig. 4 *A*). Preincubation with free peptide (p138-167) mimicked the inhibition seen with Cx but only at 100–1,000-fold greater concentrations. DR4 molecules without added peptide were ineffective. Inhibition required > 2 h preincubation and was maximal at 10–20 h (Fig. 4 *B*); 18 h was chosen as the standard preincubation time thereafter.

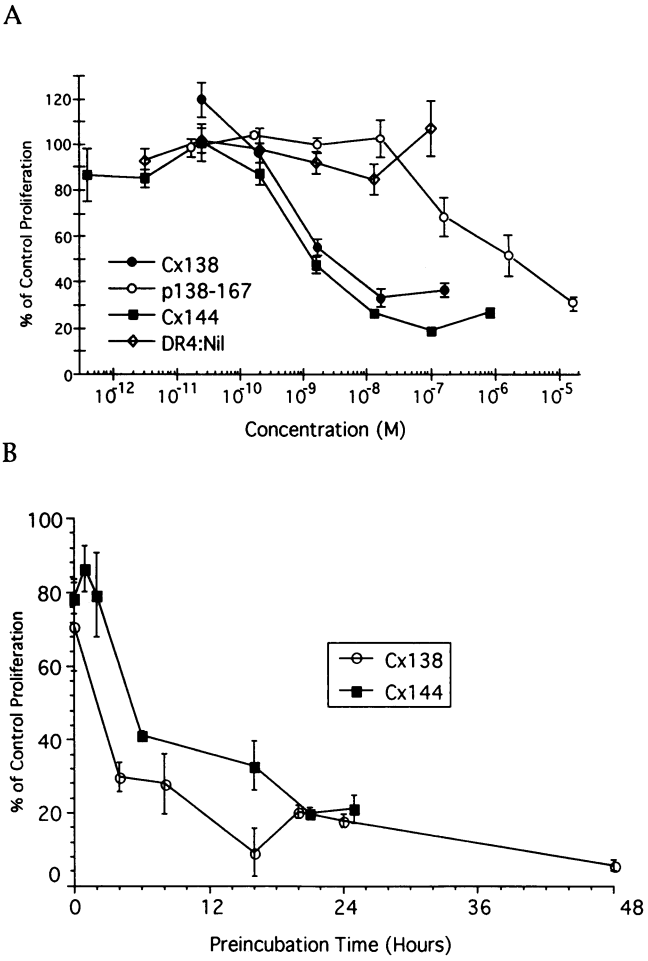

Figure 4. Dose and time dependence of the inhibitory effects of the complex. (*A*) PM-A1 cells were preincubated for 18 h with varying concentrations of either Cx138 or Cx144, or with DR4 without added peptide (DR4:nil) or with free peptide p138-167. After preincubation, DR4$^+$-matched APCs and stimulating antigen r37-181 were added. (*B*) PM-A1 cells were preincubated with Cx138 or Cx144, at 1.7 $\times 10^7$ M for varying times before addition of DR4$^+$-APCs and r37-181. In *A* and *B*, results are given as percentages of proliferation seen in control (medium-preincubated) cells.

Table I. Effect of Different Class II: Peptide Complexes on PM-A1 Cell Responses

	Complex used	^{3}[H]Thymidine incorporation (% of control)
A	DR4:p138–167	22.00±9.5 (6)
B	DR4:p144–163	19.70±2.9 (6)
C	DR4:p144–156	29.25±1.9 (2)
D	DR4:Nil	88.40±12.0 (2)
E	DR4:MBP p1–14	96.70±4.0 (3)
F	DR3:p309–344	112.50±7.8 (2)

PM-A1 cells were preincubated for 18 h with equimolar amounts of various soluble complexes containing the appropriate MHC class II (DR4) and peptide epitope (A–C), DR4 without exogenously added peptide (D), DR4 with an irrelevant peptide (E), or both an irrelevant MHC class II and peptide epitope (F). After addition of DR4$^+$ APCs and r37-181, ^{3}[H]thymidine incorporation was assessed. Results are shown as percentages of mean ± SD of media-preincubated (control) response.

Long term effects of exposure to complex. To see whether the complex reduced the number of viable cells, and to assess the antigen-specific proliferation of the remaining cells, PM-A1 cells were studied longitudinally during three consecutive 14-d cycles of stimulation after Cx addition on day 4 and washing of the cells at each stimulation. By day 42, the yield of the Cx-treated cells was < 1% of control (Fig. 5 *A*). Moreover, when equal numbers of surviving cells were assessed, now 38 d since complex was added, the antigen-specific proliferation of Cx-treated cells was virtually absent, whereas IL-2 responsiveness was largely preserved (Fig. 5 *B*).

Specificity of tolerance induction. The induction of unresponsiveness by soluble Cx was peptide- and DR allele-specific and required simultaneous recognition of both these elements by the T cells, as shown in Tables I and II. Complexes of DR4 with each of three different peptides containing the core epitope (p144-156) for PM-A1 were effective (Table I, *A–C*).

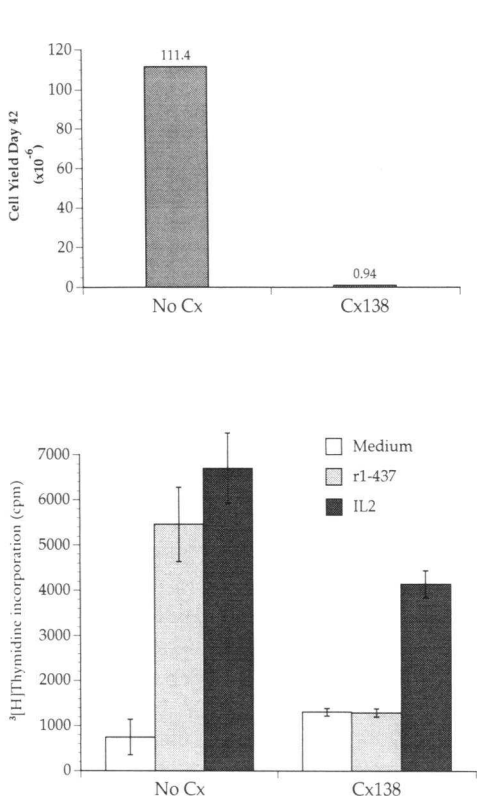

Figure 5. Long-term effects of Cx incubation. Cell yields (*A*) and proliferation of remaining viable cells (*B*) were assessed on day 42, 38 d after Cx addition on day 4. (*A*) After a modest initial increase in number of cells as measured on day 14 (not shown), the number of Cx138-treated cells subsequently decreased, resulting in dramatically reduced cell yields compared to those of control cells (*No Cx*). (*B*) The responsiveness of the cells remaining on day 42. Equal numbers of viable cells from the two groups were incubated with APCs and medium, r1-437 or IL-2.

DR4 molecules with no added peptide (DR4:Nil) (Fig. 4 *A*; Table I *D*), or bound with an irrelevant peptide, MBP p1-14 (Table I *E*), were ineffective, as was a complex with neither of the correct elements (Table I *F*).

We also assessed the effects on other cell lines. Line HBC was raised from a healthy DR4 Dw4.2 donor and recognized p138-167, but not the smaller peptide, p144-163; inhibition was seen with Cx138 but not with Cx144 (Table II), demonstrating the extremely fine specificity of this inhibition. Another cell line, MHP, raised against the same peptide 138-167, but from a non-DR4 donor (DR12/15), was unaffected by the DR4:138-167 complex. A soluble DR3:p309-344 complex inhibited proliferation of a DR3-restricted line, raised against p309-344 from the same patient as the PM-A1 clone, whereas Cx144 was ineffective. Other lines, with neither DR4 restriction, nor an AChR epitope, were unaffected by Cx138 (Table II).

Mechanisms of action of the complex

Inclusion of 25% culture supernatant obtained from maximally stimulated PM-A1 cells, or IL-2 20 U/ml, during Cx preincubation did not significantly protect cells from the effects of complex (data not shown), arguing against a role for soluble factors in costimulation for this clone. Nor did soluble factors appear to be involved in the complex-mediated inhibition; coculture of PM-A1 cells and Cx144 with other cell lines did not impair the subsequent proliferation of these lines (data not shown).

Protection from the inhibitory effects of Cx preincubation. To see whether the Cx-induced unresponsiveness resulted from a lack of APC accessory signaling (3–6, 8–13) and not simply TCR blockade (22), we restored surface accessory signals by including class II–mismatched BAPCs (23) during the incubation with Cx. Alone, these BAPCs could not present r37-181 (or other relevant antigens) to this clone (reference 16 and unpublished observations). Again, Cx-treated cells showed a reduced response to class II–matched APCs and peptide (Fig. 6 *A*). BAPCs alone, without Cx, did not significantly alter the response of the untreated cells to APCs and antigen (not shown). However, when BAPCs were included during the Cx preincubation, the Cx-treated cells now proliferated without addition of matched APCs, and regardless of whether further antigen was added, though the response was submaximal. This BAPC protection was dose dependent (not shown) and diminished progressively with delayed addition of BAPCs (Fig. 6 *B*).

Table II. Effect of Various Class II: Peptide Complexes on T Cell Lines of Different Specificity

			^{3}[H]Thymidine incorporation (% of control)		
			Complex used:		
Cell line	Inducing antigen	MHC II restriction	DR4:p138–167	DR4:p144–163	DR3:p309–344
HBC	AChR α138-167	DR4 Dw4	29.2±10.0 (2)	96.0 (1)	nt
MHP	AChR α138-167	DR12/15	116.0±16.9 (2)	nt	nt
PM-P309	AChR α309-344	DR3	nt	82.0 (1)	30.0 (1)
NWKF5	KLH	DR3/DR6 (14)	100.2±6.8 (2)	nt	nt
NWTTx	T-Tox	DR3/DR6 (14)	101.8±15.8 (2)	nt	nt

Cell lines were raised against the "inducing" antigen shown. MHC class II restrictions (if definitely known) or the MHC class II type of the donor are shown. All lines were DR-restricted as shown by inhibition of proliferation with anti-DR mAb. Cells from each of these lines were preincubated for 18 h with the indicated Cx before appropriate MHC class II–matched APCs and antigens were added, and proliferation was assessed (see Fig. 1). KLH, keyhole limpet haemocyanin; TTox, tetanus toxoid; nt, not tested.

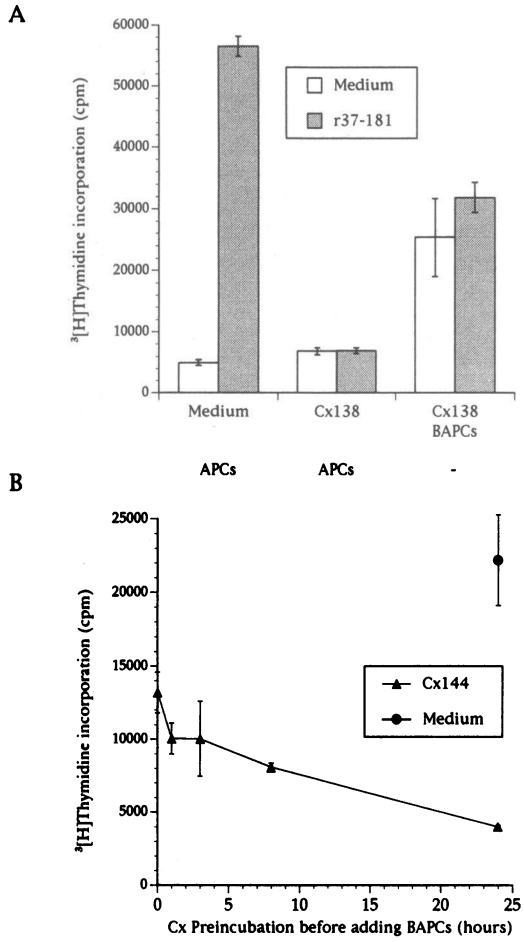

Figure 6. Effect of coincubation of Cx with BAPCs. (*A*) 2×10^4 PM-A1 cells were preincubated with either medium alone, Cx138 (1.7×10^{-7} M) alone, or Cx138 and 5×10^5 non-DR4$^+$ (DR1/8) BAPCs. After 18 h, DR4$^+$ APCs with or without r37-181 were added, or medium with or without r37-181 to the Cx138 and BAPCs, and proliferation assessed. (*B*) 1×10^5 BAPCs were added to 2×10^4 PM-A1 cells at increasing intervals after Cx144 addition, and before stimulation with DR4$^+$ APCs and antigen r37-181. The maximum period of preincubation with Cx before addition of BAPCs was 24 h, the time at which all the cells were stimulated. PM-A1 cells preincubated for 24 h with medium alone gave 23,000 cpm incorporation (*Medium*).

Fixation of the BAPCs with > 0.1% glutaraldehyde reduced their ability to protect from the effects of Cx, compared to nonfixed BAPCs (not shown).

Apoptosis in Cx-treated cells. To look for Cx-induced cell death, we used a variety of methods. Staining with trypan blue or ethidium bromide/acridine orange gave similar results (Fig. 7 *A*); incubation with Cx caused the death of T cells, evident at 2 h and maximal at 24 h (40–50% dead; Fig. 7 *A*). Electron microscopy at 2 h (the earliest time point studied) showed apoptosis of complex-treated cells (Fig. 7 *B*). 4 h after Cx addition, there were 24.1%±5.8 ($n = 2$) apoptotic cells compared to 0.5%±0.45 in control cells. This was more difficult to quantitate at later times, owing to the problems in assessing the large numbers of membrane bound bodies seen. Although increased ^{51}Cr release was also seen in Cx-treated cells, this was not evident until at least 9–15 h after Cx addition (not shown). Thus ^{51}Cr release, indicating necrosis, followed the electron microscopy evidence of apoptosis.

Discussion

We have demonstrated induction of specific tolerance in vitro in a potentially pathogenic autoreactive T cell clone using soluble MHC class II–peptide complexes. This effect is highly specific, requiring a restricting MHC class II (DR4 Dw4.2) molecule bound to a relevant peptide (p138-167 or p144-163), and results from death by apoptosis, as well as unresponsiveness in the surviving cells. In addition, appropriate soluble complexes inhibited two other clones raised against AChR-derived peptides. For one of these, we used a complex with different class II and peptide elements, showing that the principle extends to other clones and epitopes.

The concept that two signals are required for T cell activation has been exploited in the past to induce tolerance by providing only the first antigen-specific signal to the T cell (8, 10–13). The present study targets a human autoantigen-reactive clone (16), and uses a soluble delivery system that would be more applicable in vivo than cell or membrane-based systems. TCR engagement and tolerance can also be achieved with TCR-specific monoclonal antibodies (24). However, their application in vivo would depend on very restricted heterogeneity in TCR usage by the pathogenic T cells between individuals, not demonstrated in MG (25), whereas our approach exploits the least diverse part of the ternary complex: the pathogenic epitopes and presenting class II molecules. The combined recognition of both these elements greatly refines the selectivity of this strategy.

The potential pathogenicity of this clone is suggested by several findings. First, the cells proliferate when stimulated by minute quantities of intact human AChR (17), an important characteristic not often seen in clones raised in vitro using synthetic peptides (26, 27). Moreover, the epitope for PM-A1 is also generated after natural processing of endogenous AChR by TE671 muscle cells transfected with MHC class II (28). Second, this clone originated from the activated (low density) fraction of an MG patient's hyperplastic thymus. The thymus is implicated in the pathogenesis of MG by virtue of its frequent pathological involvement (29) (> 80% of MG patients have either hyperplasia or thymomatous involvement), AChR content (30–32), and spontaneous anti-AChR antibody production (33–35). Third, several years after the isolation of this clone, cells sharing the same TCR Vβ usage and peptide epitope were shown to persist in this patient's peripheral blood (Plebanski, M., unpublished observations). Moreover, the α144-163 sequence recognized by PM-A1 contains a pathologically relevant and dominant epitope involved in experimental MG in C57Bl/6 mice (36).

The cytokine profile (production of IL-2 and IFN-γ, but not IL-4) of PM-A1, and its ability to kill a DR4-transfected muscle line (28) are in keeping with a T_H1 phenotype (37). In vivo, cells with a similar phenotype might initiate or perpetuate the response by damaging AChR$^+$ thymic myoid cells (31–32, 38) or peripheral motor end plates (39) leading to further T cell activation and antibody production. However, the correlation of in vitro phenotype with function is unclear: the culture microenvironment may artificially bias development away from that occurring in vivo (40–42), and in vivo the same TCR specificities have been found in cells of either phenotype (43). Moreover, there is evidence that T_H1 cells can provide help for antibody production. In mice, either T_H1 or T_H2 cells can provide help for certain Ig classes or subclasses (2, 44), and T_H1-

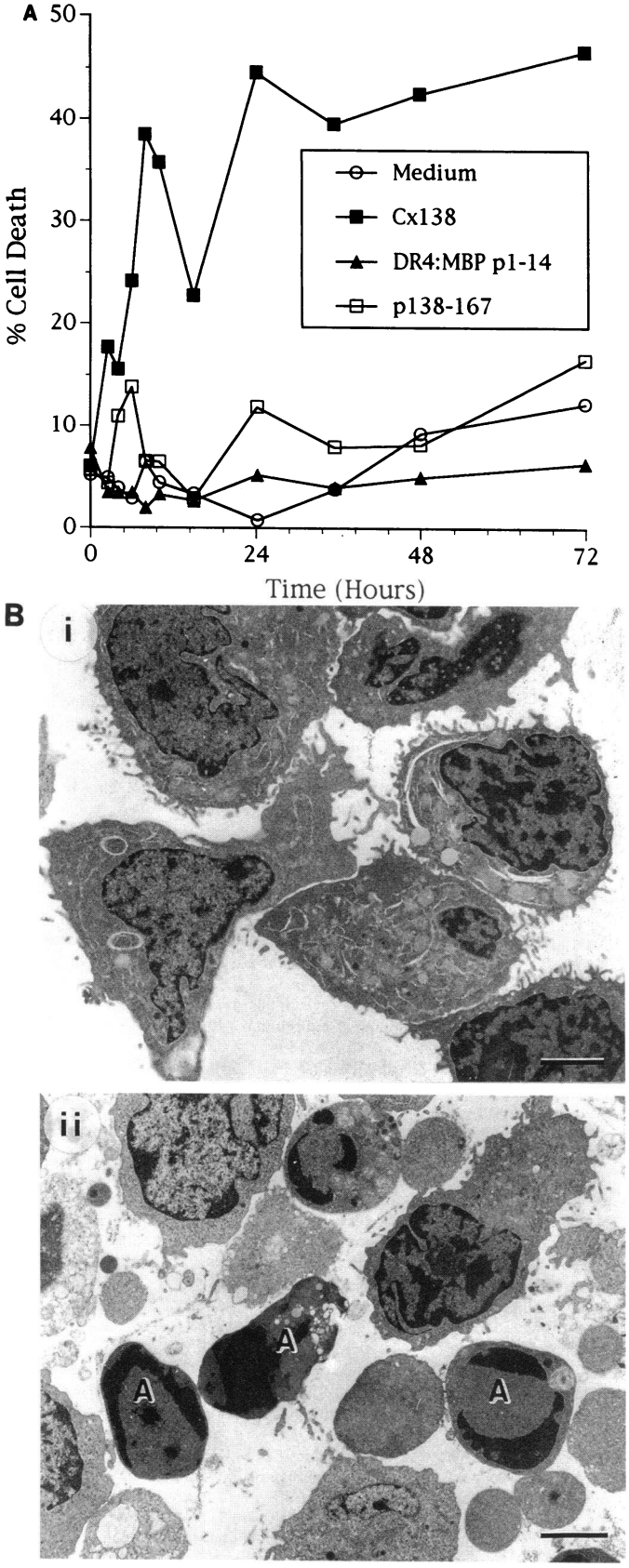

Figure 7. Apoptosis of T cells induced by complex. (*A*) Medium, Cx138, DR4:MBP p1-14 or free p138-167 (all at 1.7×10^{-7} M) were added to PM-A1 cells. At intervals, aliquots of cells were stained with acridine orange and ethidium bromide. Dead and viable cells were counted, and cell death calculated at each time point. (*B*) Electron micrographs of (*i*) untreated or (*ii*) Cx144-treated PM-A1 cells, 4 h after Cx144 addition. At 2, 4 and 6 h, apoptotic cells were counted. Control cells (*i*) remained mostly viable with apoptosis or necrosis rarely seen. In Cx-treated cells (*ii*), at 4 h, apoptotic cells (*A*) were seen, characterized by a decrease in cell size, condensation of nuclear material, and relative preservation of cytoplasmic structure. The degree of apoptosis appeared less at 6 h, possibly because of fragmentation of apoptotic cells. Necrosis (not shown), characterized by early cytoplasmic changes and relative sparing of nuclear structure, was seen occasionally in Cx-treated cells, and rarely in control cells. *Bar*, 2 μm.

mediated help has been shown in humans (45). The antibody heterogeneity in MG, with representation from several subclasses (see reference 29), suggests that help might not be restricted solely to T_H2 cells.

It is too early to assess definitively the heterogeneity of the pathogenic T cells and epitopes in MG. While T cell lines raised against pooled synthetic peptides may respond to several different regions (26, 27), they often fail to recognize naturally processed antigen and may not be pathogenic. Indeed, very few T cell lines have shown convincing responses to human native AChR (26). Thus, the pathogenic T cells may prove to be more restricted, especially within an individual patient. Moreover, when sufficient antigen and professional antigen-presenting cell lines are available from patients in bulk, it should prove possible to isolate the repertoire of naturally derived peptide:class II complexes to define the complexes required. One might expect that epitope heterogeneity for responses against low molecular weight allergens or less complex autoantigens would be even less, further limiting the range of complexes needed for each individual.

The protection from the inhibitory effects of the complex provided by nonfixed DR-mismatched bystander APCs (Fig. 6 *A*) suggests that Cx engagement of the TCR alone, by supplying the antigen-specific signal in the absence of APC-dependent accessory signals, induces apoptosis or tolerance. The partial protection seen after minimal fixation of BAPCs and the lack of protection by IL-2 or culture supernatant imply that, for this clone, much of the costimulatory activity is provided by surface accessory molecules. Additionally, we could not tolerize T cells of other specificities by coculture with PM-A1 and Cx; arguing that soluble mediators are not involved in inducing tolerance in this system. Preliminary studies (Nicolle, M. W., unpublished observations) have shown that proliferation of PM-A1 is inhibited by blockade of leukocyte function–associated antigen-3 but not B7/BB1, suggesting a role for the former as a costimulatory molecule.

In a sense, protection was also produced by adding DR-matched APCs before maximum inhibition of proliferation had developed, a process normally requiring 12–24 h of complex–T cell interaction (Fig. 4 *B*). Although minimally fixed BAPCs were themselves unable to provide sufficient costimulation to induce proliferation, when acting in concert with the complex-delivered TCR signal, they were apparently able to preserve the viability of the cells, and allow subsequent proliferation in response to MHC II–matched APCs (data not shown). Presumably, fixation reduced the degree of costimulation below the necessary threshold for direct activation of the T cells (3, 4, 7). Similarly, the degree of protection provided by BAPCs was never complete. Perhaps both signals must be provided on the surface of the same cell for optimal signaling to occur.

It might be asked whether the proximity of APCs in vivo to T cells and complex would prevent the tolerance induction achieved in vitro. In fact protection required the presence of a considerable excess of APCs in the cultures, a situation that seems unlikely to occur in vivo. Moreover, this theoretical possibility has not been borne out in the in vivo experimental models of multiple sclerosis and MG in which successful induction of tolerance has been achieved (reference 46, Spack et al., manuscript in preparation) using this approach.

Free peptides could, like the complex, induce tolerance, but only at 100–1,000-fold higher concentrations. This is probably accounted for by T cell–T cell autopresentation between these strongly MHC class II^+ cells. Presumably, however, they act as nonprofessional APCs, and they are deficient in the appropriate costimulatory signals (47, 48). This phenomenon, requiring the close proximity of class II^+ pathogenic T cells capable of autopresentation, would be unlikely to occur in vivo given the low precursor frequency of autoantigen-specific T cells.

A significant mechanism of the unresponsiveness induced by complex is through apoptosis of target lymphocytes. There was also a striking long term reduction (> 99%) in T cell growth. These would be desirable effects in vivo, since viable anergized cells might eventually recover their antigen-responsiveness (3, 49). TCR-mediated activation-induced apoptosis might be occurring here (50, 51), but further work is required to define more precisely the underlying mechanisms of tolerance induction in these T cells.

Thus, we have shown in vitro that treatment with a stable soluble complex of the restricting class II molecule and epitope peptide specifically inhibited an autoantigen-reactive T cell clone, resulting from both apoptosis and prolonged proliferative unresponsiveness. This effect evidently depends on engagement of the TCR in the absence of accessory signaling, and suggests a promising basis for specific immunotherapy in human autoimmune or allergic diseases where the disease-related epitopes are known and class II–restricted ($CD4^+$) T cells play a role.

Acknowledgments

We thank Dr. B. Ong, who raised line PMA-1, L. Jacobson for peptide synthesis, Dr. D. Beeson and Miss N. Pantic for recombinant AChR synthesis, Miss A. M. Moody for T cell receptor analysis, Dr. H. Matsuo for cell line MHP, and Prof. A. McMichael for critical comments on the manuscript.

M. N. holds a Medical Research Council (Canada) Fellowship. This work was supported by the Medical Research Council, Muscular Dystrophy Group, and Myasthenia Gravis Association of Great Britain.

References

1. Vincent, A. 1980. Immunology of acetylcholine receptors in relation to myasthenia gravis. *Physiol. Rev.* 60:756–824.

2. Coffman, R. L. 1993. Mechanisms of helper T cell regulation of B cell activity. *Ann. NY Acad. Sci.* 681:25–28.

3. Schwartz R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science (Wash. DC).* 248:1349–1356.

4. Roska, A. K., and P. E. Lipsky. 1985. Dissection of the functions of antigen-presenting cells in the induction of T cell activation. *J. Immunol.* 135:2953–2961.

5. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional inactivation: a costimulatory signalling pathway determines the outcome of T cell receptor occupancy. *Annu. Rev. Immunol.* 7:445–480.

6. Weaver, C. T., and E. R. Unanue. 1990. The costimulatory function of antigen-presenting cells. *Immunol. Today.* 11:49–55.

7. Scala, G., and J. J. Oppenheim. 1983. Antigen presentation by human monocytes: evidence for stimulant processing and requirement for interleukin 1. *J. Immunol.* 131:1160–1166.

8. Tan, P., C. Anasetti, J. A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J. A. Ledbetter, and P. S. Linsley. 1993. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* 177:165–173.

9. June, C. H., J. A. Ledbetter, P. S. Linsley, and C. B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol. Today.* 11:211–216.

10. Damle, N. K., K. Klussman, and A. Aruffo. 1992. Intercellular adhesion molecule-2, a second counter receptor for CD11a/CD18 (leukocyte function-associated antigen-1), provides for a costimulatory signal. *J. Immunol.* 148:665–671.

11. Quill, H., and R. H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of long-lived state of proliferative nonresponsiveness. *J. Immunol.* 138:3704–3712.

12. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302–319.

13. Reim, J., K. McIntosh, S. Martin, and D. B. Drachman. 1992. Specific immunotherapeutic strategy of myasthenia gravis: targeted antigen-presenting cells. *J. Neuroimmunol.* 41:61–70.

14. Beeson, D., M. Brydson, H. Wood, A. Vincent, and J. Newsom-Davis. 1988. Human muscle acetylcholine receptor: cloning and expression in Escherichia coli of cDNA for the α-subunit. *Biochem. Soc. Trans.* 17:219–220.

15. Beeson, D., M. Brydson, M. Betty, S. Jeremiah, S. Povey, A. Vincent, and J. Newsom-Davis. 1993. Primary structure of the human muscle acetylcholine receptor. cDNA cloning of the γ and ϵ subunits. *Eur. J. Biochem.* 215:229–238.

16. Ong, B., N. Willcox, P. Wordsworth, D. Beeson, A. Vincent, D. Altmann, J. S. S. Lanchbury, G. C. Harcourt, J. I. Bell, and J. Newsom-Davis. 1991. Critical role for the Val/Gly86 HLA-DRβ dimorphism in autoantigen presentation to human T cells. *Proc. Natl. Acad. Sci.* 88:7343–7347.

17. Hawke, S., N. Willcox, G. Harcourt, A. Vincent, and J. Newsom-Davis. 1992. Stimulation of human T cells by sparse antigens captured on immunomagnetic particles. *J. Immunol. Methods.* 155:41–48.

18. Nag, B., S. V. Deshpande, and B. R. Clark. 1991. Novel methods to rapidly and sensitively analyze antigenic peptide binding to MHC class II molecules. *J. Immunol. Methods.* 142:105–111.

19. Nag, B., H. G. Wada, S. V. Deshpande, D. Passmore, T. Kendrick, S. D. Sharma, B. R. Clark, and H. M. McConnell. 1993. Stimulation of T cells by antigenic peptide complexed with isolated chains of major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA.* 90:1604–1608.

20. Nag, B., D. Passmore, S. V. Deshpande, and B. R. Clark. 1992. In vitro maximum binding of antigenic peptides to murine MHC class II molecules does not always take place at the acidic pH of the in vivo endosomal compartment. *J. Immunol.* 148:369–372.

21. Parks, D. R., V. M. Bryan, V. T. Oi, and L. A. Herzenberg. 1979. Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter. *Proc. Natl. Acad. Sci.* 76:1962–1966.

22. De Magistris, M. T., J. Alexander, M. Coggeshall, A. Altman, F. C. A. Gaieta, and A. Sette. 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell.* 68:625–634.

23. Jenkins, M. K., J. D. Ashwell, and R. H. Schwartz. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol.* 140:3324–3330.

24. Acha-Orbea, H., D. J. Mitchell, L. Timmermann, D. C. Wraith, G. S. Tausch, M. K. Waldor, S. S. Zamvil, H. O. McDevitt, and L. Steinman. 1988. Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell.* 54:263–273.

25. Melms, A., J. R. Oksenberg, G. Malcherek, R. Schoepfer, C. A. Muller, J. Lindstrom, and L. Steinman. 1993. T cell receptor gene usage of acetylcholine receptor-specific T helper cells. *Ann. NY Acad. Sci.* 681:313–314.

26. Willcox, N., F. Baggi, A-P. Batocchi, D. Beeson, G. Harcourt, S. Hawke, L. Jacobson, H. Matsuo, A.-M. Moody, N. Nagvekar, et al. 1993. Approaches for studying the pathogenic T cells in autoimmune patients. *Ann. NY Acad. Sci.* 681:219–237.

27. Moiola, L., M. P. Protti, A. A. Manfredi, M.-H. Yuen, J. F. Howard, and B. M. Conti-Tronconi. 1993. T helper epitopes on human nicotinic acetylcholine receptor in myasthenia gravis. *Ann. NY Acad. Sci.* 681:198–218.

28. Baggi, F., M. Nicolle, A. Vincent, H. Matsuo, N. Willcox, and J. Newsom-Davis. 1993. Presentation of endogenous acetylcholine receptor by an MHC class II-transfected human muscle cell line to a specific $CD4^+$ T cell clone from a myasthenic patient. *J. Neuroimmunol.* 46:57–66.

29. Willcox, N., and A. Vincent. 1988. Myasthenia gravis as an example of organ-specific autoimmune disease. *In* B Lymphocytes in Human Disease. G. Bird and J. E. Calvert, editors. Oxford University Press, Oxford. pp. 469–506.

30. Wekerle, H., and U.-P. Ketelsen. 1977. Intrathymic pathogenesis and dual genetic control of myasthenia gravis. *Lancet. i*:677–680.

31. Kao, I., and D. B. Drachman. 1977. Thymic muscle cells bear acetylcho-

line receptors: possible relation to myasthenia gravis. *Science (Wash. DC).* 195:74–75.

32. Schluep, M., N. Willcox, A. Vincent, G. K. Dhoot, and J. Newsom-Davis. 1987. Acetylcholine receptors in human thymic myoid cells in situ: an immunohistological study. *Ann. Neurology.* 22:212–222.

33. Scadding, G. K., A. Vincent, J. Newsom-Davis, K. Henry. 1981. Acetylcholine receptor antibody synthesis by thymic lymphocytes: correlation with thymic histology. *Neurology.* 31:935–943.

34. Schluep, M., N. Willcox, M. A. Ritter, J. Newsom-Davis, M. Larche, and A. N. Brown. 1988. Myasthenia gravis thymus: clinical, histological and culture correlations. *J. Autoimmunol.* 1:445–467.

35. Schönbeck, S., F. Padberg, R. Hohlfeld, and H. Wekerle. 1992. Transplantation of thymic microenvironment to severe combined immunodeficiency mice. *J. Clin. Invest.* 90:245–250.

36. Shenoy, M., M. Oshima, M. Z. Atassi, and P. Christadoss. 1993. Suppression of experimental autoimmune myasthenia gravis by epitope-specific neonatal tolerance to synthetic region α-146-162 of acetylcholine receptor. *Clin. Immunol. Immunopath.* 66:230–238.

37. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clones. 1. Definition according to profile of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348–2357.

38. Kirchner, T., F. Hoppe, B. Schalke, and H. K. Muller-Hermelink. 1988. Microenvironment of thymic myoid cells in myasthenia gravis. *Virchows Arch. B Cell Pathol.* 54:295–302.

39. Masselli, R. A., D. P. Richman, and R. L. Wolmann. 1991. Inflammation at the neuromuscular junction in myasthenia gravis. *Neurology.* 41:1497–1504.

40. Scott, P. 1993. Selective differentiation of $CD4^+$ T helper cell subsets. *Curr. Opin. Immunol.* 5:391–397.

41. Seder, R. A., W. E. Paul, M. M. Davis, and B. F. de St. Groth. 1992. The presence of interleukin 4 during in vivo priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *J. Exp. Med.* 176:1091–1098.

42. Van der Pouw-Kraan, T., R. de Jong, and L. Aarden. 1993. Development of human Th1 and Th2 cytokine responses: the cytokine production profile of T cells is dictated by the primary in vitro stimulus. *Eur. J. Immunol.* 23:1–5.

43. Reiner, S. L., Z. Wang, F. Hatam, P. Scott, and R. M. Locksley. 1993. T_H1 and T_H2 cell antigen receptors in experimental leishmaniasis. *Science (Wash. DC).* 259:1457–1460.

44. Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature (Lond.).* 362:245–248.

45. Del Prete, G. F., M. De Carli, M. Ricci, and S. Romagnani. 1991. Helper activity for immunoglobulin synthesis of T helper type I (Th1) and Th2 human T cell clones: The help of Th1 clones is limited by their cytolytic capacity. *J. Exp. Med.* 174:809–813.

46. Sharma, S. D., B. Nag, X.-M. Su, D. Green, E. Spack, B. R. Clark, and S. Sriram. 1991. Antigen-specific therapy of experimental allergic encephalomyelitis by soluble class II major histocompatibility complex-peptide complexes. *Proc. Natl. Acad. Sci. USA.* 88:11465–11469.

47. LaSalle, J. M., P. J. Tolentino, G. J. Freeman, L. M. Nadler, and D. A. Hafler. 1992. Early signaling defects in human T cells anergized by T cell presentation of autoantigen. *J. Exp. Med.* 176:177–186.

48. Sidhu, S., S. Deacock, V. Bal, J. R. Batchelor, G. Lombardi, and R. I. Lechler. 1992. Human T cells cannot act as autonomous antigen-presenting cells, but induce tolerance in antigen-specific and alloreactive responder cells. *J. Exp. Med.* 176:875–880.

49. Jenkins, M. K. 1992. The role of cell division in the induction of clonal anergy. *Immunol. Today.* 13:69–73.

50. Green, D. R., R. P. Bissonette, J. M. Glynn, and Y. Shi. 1992. Activation-induced apoptosis in lymphoid systems. *Semin. Immunol.* 4:379–388.

51. Kabelitz, D., T. Pohl, and K. Pechold. 1993. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol. Today.* 14:338–339.