In vitro functional blocking of myelin basic protein-specific cytolytic human T lymphocyte clones by immunosuppressive drugs and monoclonal antibodies

Wim E.J. Weber 1,2 and W.A. Buurman 3

1 Department of Immunology, Dr. L. Willems Institute, Diepenbeek, Belgium, and departments of 2 Neurology and 3 General Surgery, Academic Hospital Maastricht, University Limburg, Maastricht, The Netherlands

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Summary

The in vitro effects of cyclosporin A, prednisolone, and anti-CD4 monoclonal antibody WW.T4 on myelin basic protein-specific human CD4+ cytolytic T lymphocyte clones were studied. Functional assays of antigen-specific proliferation, induction of specific lysis, cytolysis itself, and interferon-γ production were done. Prednisolone decreased secretion of interferon-γ by the clones and blocked specific proliferation; the latter could, however, be overcome by the addition of exogenous interleukin 2. It did not influence cytolytic properties. In contrast, cyclosporin A and WW.T4 blocked the four antigen-specific functions of the autoimmune myelin basic protein-specific human T cell clones measured.

Introduction

Cellular immune responses against myelin basic protein (MBP), a major protein constituent of the neuronal myelin sheath (Lees and Brostoff, 1984) are held responsible for the occurrence of postinfectious and postvaccination encephalomyelitis (Johnson et al., 1984; Hemachudha et al., 1987). In animals, e.g. SJL/J mice and Lewis rats, experimental autoimmune encephalomyelitis (EAE) has been proven to be transferable by MBP-specific helper-independent major histocompatibility complex (MHC) class II-restricted cytotoxic T lymphocyte clones (Ben-Nun et al., 1981, 1982; Mokhtar et al., 1984; Zamvil et al., 1985a, b, 1986, 1987; Sun and Wekerle, 1986). MBP-sensitized T lymphocytes have also been implicated in the pathogenesis of multiple sclerosis (MS) (Frick et al., 1972; Colby et al., 1977; Czlonkowska et al., 1980; Vandenbark et al., 1980; Iivanainen, 1981; Wicher et al., 1981; Brinkman et al., 1982).

We have recently isolated and generated long-term cell lines of MBP-specific CD4+ cytolytic T
lymphocyte (CTL) clones expressing a number of MBP-specific T cell functions, including proliferation, cytolysis, and production of interferon-γ (IFN-γ), from several MS patients (Weber and Buurman, 1988a, b). These human autoimmune CTL clones provided an excellent opportunity to study in vitro the therapeutic potential of immunosuppressive drugs and anti-T cell monoclonal antibodies (MAbs). In the present study we report the effects of prednisolone, cyclosporin A, and anti-T lymphocyte monoclonal antibodies on the functions of two autoimmune MBP-specific CTL clones derived from two different MS patients.

Materials and methods

Reagents

Cyclosporin A (CsA) was a kind gift of Mr. J. Stokvis, Sandoz, The Netherlands. CsA was dissolved in an ethanol (96%) and Tween 80 mixture at 4:1. The desired concentration of CsA was obtained by additional dilution in the ethanol/Tween mixture followed by dilution in RPMI medium in such a way that all cultures contained the identical ethanol/Tween concentration. Prednisolone was purchased from Centra-Chemie, Etten-Leur, The Netherlands, and diluted in sterile phosphate-buffered saline (PBS) just prior to use. Further dilutions were made in RPMI 1640 medium.

Human myelin basic protein (MBP) was prepared from normal human cerebral white matter, according to standard methods (Deibler et al., 1972) and was more than 95% pure as measured on a sodium dodecylsulfate-polyacrylamide gel.

WW.T4 is a monoclonal antibody (MAb) directed against CD4 antigen of human T cells. It is secreted by a murine hybridoma cell line, derived by fusion (Kohler and Milstein, 1975) of Sp2/0 myeloma cells with splenocytes from BALB/c mice immunized with a human CD4+ T lymphocyte clone. The hybridoma was subcloned twice and malignant ascites was made, which was purified by affinity chromatography on a Protein A-Sepharose CL4B column (Pharmacia Fine Chemicals, Uppsala, Sweden). WW.T4 stains normal human peripheral blood T lymphocytes in a similar mode as OKT4 (Ortho Diagnostics, Beerse, Belgium) and anti-Leu 3 (Becton-Dickinson, Erembodegem, Belgium). The MAb blocked antigen-specific proliferation by CD4+ T cells and immuno-precipitated a 62 kDa human T cell surface molecule which was shown by protease mapping to be identical to the CD4 molecule. OKT8 was purchased from the American Type Culture Collection (ATCC) and subcloned twice. Malignant ascites was produced and purified by affinity chromatography.

MBP-specific T cell clones and Epstein-Barr virus (EBV)-transformed B cell lines

T cell clones were derived from two different MS patients, J.V.A. and A.H. (diagnosis according to criteria formulated by Poser et al., 1983) as described (Weber and Buurman, 1988a). In short: to propagate clones and subclones, MBP was added in the presence of autologous, irradiated (10000 rad from a 60Co source) presenting cells in RPMI 1640 (Gibco Europe, Ghent, Belgium) with 10% autologous heat-inactivated (30 min at 56°C) serum. Clones were subsequently expanded in RPMI 1640 (Gibco Europe, Ghent, Belgium) with 50 U/ml recombinant IL2 (r-IL2, Janssen Chimica, Beerse, Belgium) and 10% fetal calf serum (FCS, Flow Laboratories, Brussels, Belgium). These cultures were examined daily for proliferation. When proliferation under these circumstances slowed, the above-mentioned stimulation procedure was repeated, which usually resulted in re-establishment of proliferation for a number of weeks. Using this culture method, MBP-specific T lymphocyte clones have been kept in continuous culture for more than 2 years.

Autologous EBV-transformed B cell lines from peripheral blood mononuclear cells (PBMC) of patients J.V.A. and A.H. were initiated by co-culturing supernatant of B95.8 cell line with PBMC depleted of T cells (by repeated rosetting with 2-aminoethylisothiouronium bromide hydrobromide (AET)-treated sheep erythrocytes, Weber et al., 1985, 1987) and maintained in RPMI 1640 medium supplemented with nonessential amino acids, pyruvate and 10% FCS).

Proliferation assay

MBP-specific proliferation of clones was assayed as described (Weber and Buurman, 1988a).
Briefly, 10^4 clone cells were incubated with 10^5 autologous irradiated PBMC or EBV-transformed cells, with and without MBP (used in an optimal dose, 20 μg/ml), in triplicate 200 μl round-bottom microwells (Greiner, Nurtingen, F.R.G.) in RPMI 1640 medium + 10% heat-inactivated autologous serum. These microwcultures were incubated for 3–5 days, after which tritiated thymidine ([3H]TdT) uptake was measured. Drugs and MAbs were added at the beginning of culture in dosages as indicated. Where indicated, 100 U/ml r-IL2 was added at the start of each microwculture.

Cytolysis assay

Cytolysis was assayed as described (Weber and Buurman, 1988a). Briefly, MBP-pulsed targets were prepared by incubating EBV-transformed cells at 2 × 10^7 cells/ml RPMI with 1.5 mg/ml MBP. Cells were gently shaken for 1 h at 37°C, and subsequently washed 3 times in RPMI. Experiments always included nonpulsed autologous EBV-transformed B cells as controls. Target cells were washed and labelled with 51Chromium. In all blocking experiments, a fixed effector to target ratio of 20:1 was used. In these experiments, 51Cr-labelled target cells (5000/microwell) were incubated with 10^5 effector cells for 4 h, after which the supernatant was harvested and its 51Cr contents was measured in a gamma counter. Maximal release was counted in microwells containing labelled targets and a detergent; spontaneous release was counted in wells with targets and RPMI 1640 medium. Specific release was calculated as follows:

\[
\text{% specific release} = \frac{\text{Exp. release} - \text{spont. release}}{\text{Max. release} - \text{spont. release}} \times 100\%
\]

Another set of experiments included the addition of drug or MAb in the induction stage of CTL function. To this end, indicated concentrations of various reagents were added (in triplicate) to cultures of 10^5 clone cells with 20 μg/ml MBP and 10^6 irradiated autologous EBV-transformed cells, in 2 ml wells. After 7 days of culture at 37°C in a humidified atmosphere with 5% CO₂, cultures were washed several times by centrifugation. Viable clone cells were counted by trypan blue dye exclusion test and were then used as effector cells in an effector to target ratio of 20:1, in the standard cytolysis assay as described above, without addition of drugs or MAbs.

Interferon-γ production

Microcultures with clone cells and irradiated autologous presenting cells and MBP were set up as described above. After the indicated culture time, microcultures were centrifuged and 100 μl of supernatant were removed for measurement of interferon-γ (IFN-γ). Drugs or MAbs were added in indicated amounts at the beginning of culture. Supernatant samples were stored at −70°C and were all assayed simultaneously for IFN-γ concentration. IFN-γ was measured with an IFN-γ radioimmunoassay kit purchased from Centocor, Malvern, PA, U.S.A.). In all experiments, plotting of values obtained with control samples gave straight lines.

Results

MBP-specific proliferation

As can be seen from Fig. 1, both CsA and prednisolone inhibited antigen-specific proliferation of the two autoimmune MBP-specific T cell clones tested in a 4-day assay, in concentrations ranging from 10 ng/ml to 320 ng/ml. These dosages were chosen since they were equivalent to the serum values obtained in patients given the usual oral dosages (Gillis et al., 1979a, b; Kahan et al., 1985). Both drugs were also able to inhibit proliferation of the two T cell clones when these were activated with phytohemagglutinin (PHA) (data not shown). CsA appeared to be most effective, blocking proliferation completely in concentrations over 100 ng/ml. The anti-CD4 MAb WW.T4 also blocked MBP-specific proliferation. It did not block proliferation of both MBP-specific T cell clones when these were activated with PHA, in contrast to the action of the two immunosuppressive drugs (data not shown). Addition of exogenous r-IL2 (100 U/ml) at the beginning of the culture could overcome the blocking effect of prednisolone, but had almost no effect on blocking by CsA and WW.T4. When drugs or MAbs were added at 24 h after initiation of the microcul-
Fig. 1. Effects on MBP-specific proliferation by clone JVA16.4. Proliferation was measured in microcultures with (○) and without exogenously added IL2 (■). Each point is based on the mean of quadruplicate microwells (standard deviation was < 5%). Experiments with clone AH.F4 gave similar results (data not shown).

MBP-specific cytolysis

The MBP-specific cytolytic capacities, assessed by a novel assay involving autologous MBP-pulsed EBV-transformed B cells, of the two T lymphocyte clones JVA16.4 and AH.F4 have been described extensively (Weber and Buurman, 1988a). It is of importance in this regard that autologous EBV-transformed B cells, pulsed with another negatively charged basic protein histone, were not lysed by the clones. Moreover, MBP-specific cytolysis was shown to be HLA class II-restricted (Weber and Buurman, 1988b). To assess effects by CsA and prednisolone on the cytolytic reactivity of the two MBP-specific human T cell clones, clones were harvested from long-term culture and placed in IL2-containing medium in the presence of clinically relevant serial dilutions of CsA and prednisolone. After 48 h of culture in the presence of either drug, clone cells were harvested and tested for their ability to mediate lysis of their appropriate 51Cr-labelled target cells. It appeared that culturing of the autoimmune MBP-specific
CTL clones in the presence of prednisolone did not alter the cytolytic effect (Fig. 2). In contrast, culturing the clones for the same period in the presence of CsA did result in some reduction of its specific cytotoxic capacity (Fig. 2). The blocking capacities of the MAbs were tested in a different way: the CTL clones JVA16.4 and AH.F4 were harvested from continuous culture and incubated with serial dilutions of MAbs for 30 min at 37°C. After several washings by centrifugation, these cells were used as effector cells in the cytolytic assay. WW.T4 blocked antigen-specific lysis by the two clones in a dose-dependent way, whereas OKT8 did not affect the lytic capacities of the two clones (Fig. 2).

Without activation, both MBP-specific T cell clones JVA16.4 and AH.F4 lost their specific cytolytic capacities after more than 2 weeks of culture in IL2-containing medium (Weber and Buurman, 1988a). The effect of the drugs and MAbs on the in vitro activation of MBP-specific CTL function was tested in a 7-day culture system. For this, clone cells were incubated with MBP and irradiated, autologous presenting cells (without exoge-
nously added IL2) for 7 days as described in Materials and Methods, with the indicated amounts of drugs or MAbs. Cells were harvested and washed several times, counted by trypan blue dye exclusion test and used as effector cells in the cytolyis assay at a fixed effector to target ratio, without drugs or MAbs. CsA decreased cytolytic effector functions by both T cell clones after 7 days of culture. At concentrations higher than 80 ng/ml, almost no effector cells were generated in the 7-day culture period (Fig. 3). Prednisolone did not affect the induction of CTL function in both MBP-specific T cell clones. The low values of specific $^{51}$Cr obtained when incubated with concentrations higher than 160 ng/ml were due to the fact that, because of low proliferation, only small amounts of effector cells were generated (Fig. 3), and the viable effector cells that were used were in a relatively poor condition. WW.T4, in contrast, did decrease the induction of cytotoxic function in both T cell clones, as shown in Fig. 3.

**Interferon-γ production**

IFN-γ production by the two MBP-specific human T lymphocyte clones was induced in an antigen-specific mode as described in Materials and Methods. After 3 days of culture, supernatant was harvested for measurement of IFN-γ concentrations. As shown in Fig. 4, IFN-γ secretion by each T cell clone was inhibited in a dose-dependent fashion by CsA, prednisolone, and WW.T4. OKT8 had no effect. CsA appeared to be most efficient in this regard. It is important to note that addition of the drugs or MAbs at more than 12 h after the initiation of the culture did not affect the amount of IFN-γ secretion (data not shown).

**Discussion**

To study the characteristics of the human cellular immune response to MBP, we isolated and generated human MBP-specific CD4+ cytolytic T lymphocyte clones as long-term cell lines (Weber and Buurman, 1988a). The effects of the immunosuppressive drugs, CsA and prednisolone, and anti-T cell MAbs, on the in vitro, antigen-specific functions of two MBP-specific T cell clones were studied, namely proliferation, induction of cytolyis, cytolyis capacity itself, and IFN-γ secretion.

Both CsA, prednisolone, and an MAb against the CD4 structure inhibited MBP-specific proliferation. The inhibition by prednisolone appeared to be due to impaired secretion of IL2, as it could be overcome by addition of exogenous IL2. The blocking effects of CsA and WW.T4 were not sensitive to the addition of exogenous IL2. Prednisolone did not affect the cytolytic functions of the MBP-specific T lymphocyte clones. In contrast, CsA and the anti-CD4 MAb each inhibited induction of CTL function, as well as cytolytic function itself. All three reagents were able to block IFN-γ secretion by the two autoimmune T cell clones, although CsA and WW.T4 were more potent in this regard.

These data are in accordance with earlier observations that glucocorticoids exert their immunosuppressive influence on T lymphocytes mainly by interfering with IL2 and IFN-γ secretion (Gillis et al., 1979a; Arya et al., 1984) and do not affect cytolytic function (Gillis et al., 1979b). In contrast, CsA inhibits all four tested antigen-specific functions of the autoimmune T cell clones, including the secretion of γ-interferon, according to our results and those of others studying allo-activated CTL (Bunjes et al., 1981; Andrus and Lafferty, 1982; Hess et al., 1982; Reem et al., 1983; Heeg et al., 1984; Buurman et al., 1986). This confirms suggestions that the drug interferes with a basic cellular mechanism of the T cells.

The WW.T4 MAb against CD4 also impaired the four antigen-specific functions of the autoimmune MBP-specific human T cell clones. These findings agree with other observations that CD4 is an essential surface structure in antigen-specific proliferation and cytotoxicity by CD4+ cytolytic T lymphocytes. Similar results were obtained by Schluesener (1986) using the encephalitogenic, MBP-specific, CTL rat lines, and by others using human and murine T cell clones and hybridomas with other specificities (Meuer et al., 1982; Biddison et al., 1984; Greenstein et al., 1984; Strassman and Bach, 1984; Shaw et al., 1985; Fleischer et al., 1986).

In the EAE models, CsA, prednisolone and MAbs against CD4 (or its equivalents in other
species) have been used successfully to prevent the induction of EAE (Hinrichs et al., 1983, 1984; Schluesener, 1986; Van Lambalgen and Jonker, 1987). However, if these agents were administered when clinical disease was already present, the therapeutic effect was greatly reduced. Apparently, these agents are most potent in blocking an in vivo cellular immune response before T cell activation takes place, and much less potent in blocking an ongoing response. We report here that functional in vitro blocking of the autoimmune T cell clones was almost completely abrogated when the immunosuppressive reagents were added some time after the cultures were set up. This finding could well account for the reported therapeutic failures of these drugs in disorders such as multiple sclerosis (Koetsier et al., 1987; Troiano et al., 1987; Hafler and Weiner, 1988; Kappos et al., 1988). Another reason for the poor therapeutic results of prednisolone in MS, could be the inability to inhibit T cell cytotoxic function, whereas the failure of CsA and WW.T4 (which do both block T lymphocyte cytotoxicity) might be explained by poor availability in vivo. The antibody does not cross an intact blood–brain barrier and CsA concentrations in the brain and cerebrospinal fluid have been reported as undetectable, or present in only 1/100 of serum values, when given at the usual oral dosages (Atkinson et al., 1983; Ried et al., 1983; Cefalu, 1985; Fazakerley, 1985; Palestine et al., 1985).

Clearly more insight is needed into the regulation of potentially autoaggressive T lymphocyte responses in multiple sclerosis, before a rational, immunospecific therapy for this disorder can be designed.

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