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B7-1 and B7-2 Costimulatory Molecules Activate Differentially the Th1/Th2 Developmental Pathways: Application to Autoimmune Disease Therapy

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Summary

CD4 T helper precursor cells mature along two alternative pathways, Th1 and Th2. Here we show that these pathways are differentially activated by two costimulatory molecules, B7-1 and B7-2. Using anti-B7 antibodies, this developmental step was manipulated both in vitro and in vivo in experimental allergic encephalomyelitis (EAE). Anti-B7-1 reduced the incidence of disease while anti-B7-2 increased disease severity. Neither antibody affected overall T cell induction but rather altered cytokine profile. Administration of anti-B7-1 at immunization resulted in predominant generation of Th2 clones whose transfer both prevented induction of EAE and abrogated established disease. Since cotreatment with anti-IL-4 antibody prevented disease amelioration, costimulatory molecules may directly affect initial cytokine secretion. Thus, interaction of B7-1 and B7-2 with shared counterreceptors CD28 and CTLA-4 results in very different outcomes in clinical disease by influencing commitment of precursors to a Th1 or Th2 lineage.

Introduction

$CD4^+$ T helper (Th) cells, upon antigenic stimulation, differentiate into two distinct subpopulations, each producing its own set of cytokines and mediating separate effector functions (Mosmann and Coffman, 1989; Seder and Paul, 1994). Type 1 Th cells (Th1) produce interleukin-2 (IL-2), tumor necrosis factor β (TNFβ), and interferon-γ (IFNγ), thereby activating macrophages and inducing delayed-type hypersensitivity responses. Type 2 Th cells (Th2) produce IL-4, IL-5, and IL-10, stimulating production of mast cells, eosinophils, and immunoglobulin G1 (IgG1) and IgE antibodies and possibly suppressing cell-mediated immunity (Powrie and Coffman, 1993; Mosmann and Coffman, 1989). Because their respective cytokines act antagonistically, these two cell populations mutually regulate the function of the other (Seder and Paul, 1994).

The differentiation of Th precursor (Thp) cells into Th1 or Th2 cells has important biologic implications in terms of susceptibility or resistance to a particular disease. In Leishmania major parasitic infections, there is reciprocal expression of IFNγ and IL-4 in mice of different backgrounds that correlates with resolution or progression of disease (Heinzel et al., 1989). Further, human immunodeficiency virus-infected individuals switch from a Th1 to a Th2 phenotype as symptoms worsen (Cohen, 1993), while patients with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon et al., 1994). Several organ-specific autoimmune diseases, including experimental allergic encephalomyelitis (EAE), are induced by autoreactive Th1 cells (Kuchroo et al., 1993; Baron et al., 1993). Furthermore, regulatory T cells that suppress the development of EAE produce Th2 cytokines (Chen et al., 1994), and recovery from EAE in mice and rats is associated with an increase in Th2 cells and cytokines in the central nervous system (CNS) (Khoury et al., 1992). These findings, along with the observation that Th2 cytokines can inhibit the actions of the inflammatory Th1 cytokines, suggest that the modalities that induce and activate Th2 cells and cytokines may prevent EAE and other autoimmune diseases mediated by Th1 cells.

In spite of the importance of Th lineage commitment in disease, mechanisms that determine whether an immune response will be dominated by Th1 or Th2 cells are not well understood (Seder and Paul, 1994). The dominant factors that control the differentiation of Th1 and Th2 cells from Thp cells may be cytokines. Thus, IFNγ and IL-4 reciprocally autoregulate the differentiation of Thp into mature Th1 and Th2 effector cells; IFNγ enhances Th1 while inhibiting Th2 development, while IL-4 has the opposite effect. However, the initial T cell signaling events that determine whether Thp cells produce IL-4 or IFNγ and thus initiate Th1 versus Th2 differentiation are unknown, although secretion of IL-12 from macrophages and IL-4 from $CD4^+$ NK-like cells may be important (Trinchieri, 1993; Yoshimoto and Paul, 1994).

Induction and activation of T lymphocytes require two signals from antigen-presenting cells (APCs). The first signal, the binding of the T cell receptor (TCR) to its antigen–major histocompatibility complex (MHC) ligand, provides specificity. The second signal is provided by costimulatory molecules expressed on APCs. Of the known costimulatory molecules, the family of proteins termed B7 appears to be the most potent. The B7 costimulatory pathway in-

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volves at least two molecules, B7-1 (CD80) (Freeman et al., 1991) and B7-2 (CD86) (Azuma et al., 1993; Freeman et al., 1993a, 1993b), both of which can interact with their counterreceptors, CD28 and CTLA-4, on T cells (Linsley et al., 1991; Freeman et al., 1993a, 1993b). Blocking B7-1 interactions during T cell activation induces functional inactivation in Th1 cells, leading to a state of hyporesponsiveness or anergy (Schwartz, 1992; Chen and Nabavi, 1994), and B7-1 knockout mice have significantly reduced immune responses (Freeman et al., 1993a). Although B7-2 is predicted to have a function similar to that of B7-1, the difference in the temporal expression of these two costimulatory molecules on T cells and APCs (Nabavi et al., 1992; Chen et al., 1994; Hathcock et al., 1994) suggests that these molecules may have different functions.

In this report we describe the role of B7 costimulatory molecules (B7-1 and B7-2) in the development of Th1 and Th2 cells from Thp cells. Using an in vitro T cell differentiation assay of T cells from a myelin basic protein (MBP) transgenic mouse, we show that anti-B7-1 increases the production of IL-4, whereas anti-B7-2 enhances the production of IFNγ. Furthermore, the majority of Th clones derived from anti-B7-1-injected mice were of the Th2 phenotype. This information was used to manipulate the two subsets selectively in vivo to affect the induction of the autoimmune disease EAE. Administration of anti-B7-1 antibody significantly reduced the incidence of EAE, an effect prevented by simultaneous administration of anti-IL-4 monoclonal antibody (MAb), whereas injection of anti-B7-2 antibody substantially increased disease severity. Injection of anti-B7 antibody did not significantly affect the induction of T cells to the immunizing antigen, but rather altered the cytokine profile of the responding T cells. Indeed, transfer of proteolipid protein (PLP)-specific Th2 clones prevented induction of EAE and abrogated established disease. These data suggest that treatment with anti-B7 antibodies can alter the course of autoimmune disease by differentially influencing the development of mature Th1 and Th2 effector cells from uncommitted Thp cells and raise therapeutic options for the immunoselective treatment of human organ-specific autoimmunity.

Results

Anti-B7-1 and Anti-B7-2 Antibodies Differentially Affect T Cell Development In Vitro

To test whether B7-1 and B7-2 molecules may affect the generation of Th1 versus Th2 cells/cytokines in peripheral lymphoid tissue, we studied the differentiation of naive T cells from two separate MBP TCR transgenic mouse lines in the presence of anti-B7-1 or anti-B7-2 antibodies in vitro (Lafaille et al., 1994; Goverman et al., 1993). That these resting MBP TCR transgenic T cells were indeed naive or precursor cells was suggested by their phenotype of lower level expression of two T cell markers, CD44 (pgp-1) and IL-2 receptor (CD25), more characteristic of mature/memory T cells than TCR transgenic T cells from MBP-activated cultures (data not shown). Spleen cells from a separate MBP-specific TCR transgenic mouse line (Goverman et al., 1993) were obtained, equally divided into four aliquots,

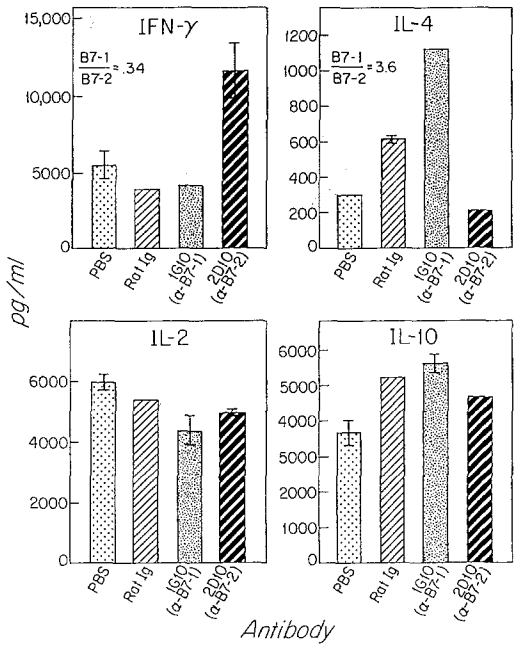

Figure 1. Effect of Anti-B7 Antibodies on In Vitro Differentiation of Th Cells

Spleen cells from a naive MBP-specific TCR transgenic mouse were activated with MBP Ac(1–11) and various anti-B7 antibodies or with control rat immunoglobulin. After 21 days of resting, the T cells from each culture were purified and then reactivated with antigen and syngeneic APCs in the presence of the respective anti-B7 or control antibodies. The amount of cytokine produced by each culture after 48 hr of activation with MBP Ac(1–11) or control peptide PLP(139–151) was determined by ELISA.

and activated in the presence of MBP Ac(1–11) peptide with the addition of anti-B7 or control reagents. Cells were then rested for 21 days in vitro, harvested, and activated again in the presence of antigen and anti-B7 antibodies (secondary stimulation), and cytokine production was determined. The data presented in Figure 1 show quantitative differences in IFNγ and IL-4 production but not in IL-2 and IL-10 production in culture supernatants following secondary stimulation, but not after primary stimulation that produced only Th1 cytokines (data not shown). A dramatic increase in IFNγ production in cultures treated with anti-B7-2 antibody (11.7 ng/ml) was observed when compared with phosphate-buffered saline (PBS) (5.5 ng/ml) or rat immunoglobulin (3.8 ng/ml) controls. The amount of IFNγ produced in the presence of anti-B7-1 was similar to that seen in the control cultures. Conversely, T cell lines derived in the presence of anti-B7-1 produced 4- to 5-fold more IL-4 than T cells from cultures treated with PBS or anti-B7-2. There was a small increase in IL-4 production in the presence of rat immunoglobulin, but this was significantly lower than with anti-B7-1. The data can be expressed in terms of ratios of Th1 to Th2 cytokines in B7-1- versus B7-2-treated cultures. Thus, IFNγ ratios are B7-1/B7-2 of 0.34 while IL-4 ratios are B7-1/B7-2 of 3.6. This results in a skewing of Th1/Th2 cytokines of 10.6-fold. There was no significant decrease in IFNγ production in cultures treated with anti-B7-1 antibody despite an in-

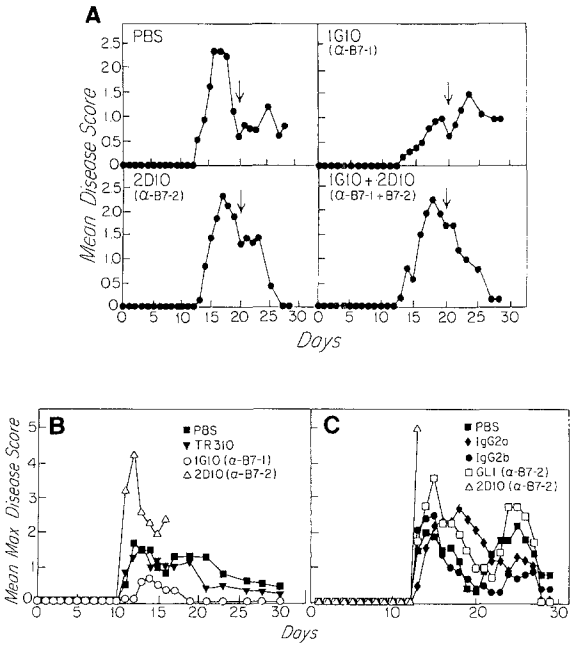

Figure 2. Effect of Injection of Anti-B7 and Control Antibodies on Clinical EAE

(A) Groups of ten animals each were immunized with PLP(139–151) peptide in CFA (50 μg/mouse) and injected intraperitoneally with 100 μg/mouse of anti-B7 antibodies (anti-B7-1 or anti-B7-2), with a mixture of both anti-B7 antibodies, or with PBS every other day. The treatment was stopped at day 20, as indicated by the arrow. The data are presented as the mean disease score in each group on different days of observation.

(B and C) Groups of 5–15 mice were immunized with PLP(139–151) in CFA (25 μg/mouse) and injected intraperitoneally every other day for 30 days with 100 μg/mouse of anti-B7 antibodies or isotype-matched rat antibody as control. The data are presented as mean disease scores in each group on different days of observation.

crease in IL-4 production. It is possible that the T cells from the MBP TCR transgenic mice that have differentiated to Th1 cells in vivo do not revert and continue to produce IFNγ in vitro following antigen-specific stimulation. Indeed, in an experiment (data not shown) in which we failed to drive Th differentiation with anti-B7 antibodies, concurrent fluorescence-activated cell sorting analysis of resting cells showed extremely high levels of CD44 and IL-2 receptor expression, as well as high levels of IFNγ secretion, suggesting that these T cells were no longer Thp cells and, thus, could not be further differentiated. In the experiment in Figure 1, there were no significant differences in IL-10 production in various treatment groups, suggesting that it may be regulated differently from IL-4. However, in additional experiments using cells from three separate TCR transgenic lines, a substantial Th1/Th2 shift was achieved by the dramatic effect of anti-B7-2 in decreasing or abrogating production of IL-4, IL-10, or both. IL-2 also decreased in two of the three experiments with anti-B7-2. In these experiments, anti-B7-1 did not increase IL-4 or decrease IFNγ production (A. M. R. and L. H. G., unpublished data). Overall, these data demonstrate that antigen-specific activation of T cells in the presence of either anti-B7-1 or B7-2 results in the polarization of Th cells to T cells that predominantly produce Th1 or Th2 cytokines.

MAbs to Costimulatory Molecules B7-1 and B7-2 Have Opposite Effects on the Development of EAE

We then used this information to manipulate Th cell differentiation in vivo in EAE. To test the effect of anti-B7 antibodies on the development of EAE, we immunized SJL mice with various amounts of peptide PLP(139–151) in complete Freund's adjuvant (CFA). The mice were also injected with anti-B7-1, anti-B7-2, or control antibodies. In the first series of experiments, SJL mice were immunized with 50 μg of PLP(139–151) in CFA and injected every other day with 100 μg/mouse of anti-B7-1, anti-B7-2, a mixture of anti-B7-1 plus anti-B7-2, or PBS. The antibody treatment was continued up to day 20. The data in Figure 2A give the mean disease scores of each group over a period of 1 month. In the PBS-injected control group, mice started showing signs of EAE on day 12 with peak disease (mean disease score of 2.4) on day 15. By days 17 and 18, disease began to remit, and the majority of the mice in this group were free of disease by day 20. In the anti-B7-1 (1G10)-treated group, there was a delay in the onset of disease with peak disease (mean score of 1) on day 19; by day 20 mice that were sick began to remit. Interestingly, at day 20, when the antibody treatment was halted, mice that had not previously shown signs of disease became symptomatic, and a second peak of disease (mean disease score of 1.5) appeared on day 24. Treatment with anti-B7-2 (2D10) alone did not have any ameliorating effect on disease. Similarly, treatment with a mixture of anti-B7-1 and anti-B7-2 had only a slight effect on delaying disease onset, and disease peaked on day 18 after immunization. The results from this initial experiment suggested that injection of anti-B7-1 alone had the most effect in ameliorating disease. The data further suggested that this was directly related to the antibody treatment, since stopping treatment on day 20 resulted in the appearance of disease in the remainder of the animals.

Accordingly, in the next experiment, the antigen dose was reduced (25 μg/mouse of PLP peptide in CFA) to induce milder disease, and mice were injected intraperitoneally with 100 μg/mouse of anti-B7 or control antibodies every other day for the entire length of the experiment. The data (Table 1; Figures 2B and 2C) demonstrate that, when compared with PBS or control antibody (TR-310), administration of anti-B7-1 antibody significantly inhibited EAE induction. A significant decrease in the incidence of disease (43% as compared with 100% in PBS controls) was observed, but there was no difference in the day of onset or the severity of disease in those mice that did develop EAE (experiment 1 in Table 1). Conversely, the same dose of two different anti-B7-2 antibodies (2D10 and GL-1) significantly increased disease severity. Isotype-matched antibody controls (rat IgG2a and IgG2b) did not significantly alter disease course or severity (experiments 1 and 2 in Table 1) when compared with PBS. The course of disease shown as cumulative clinical disease scores in different groups of mice on each day over the course

Table 1. Effect of Anti-B7-1 and Anti-B7-2 Antibody Treatment on EAE

	Clinical Disease			Histological Lesions		
					Number of Foci in Mice	
Treatment[a]	Incidence	Day of Onset	Severity[b]	Incidence	With Disease	Without Disease
First Experiment						
PBS	13 of 13	14.2 ± 0.7	2.8 ± 0.2	13 of 13	61.9 ± 16.2	NA
TR-310	14 of 15	13.2 ± 1.1	2.8 ± 0.3	15 of 15	52.8 ± 10.5	NA
Anti-B7-1 (1G10)	6 of 14[c]	14.5 ± 1.0	2.7 ± 0.6	12 of 14	64.4 ± 22.9	7.8 ± 2.2
Anti-B7-2 (2D10)	9 of 9	12.6 ± 0.3	4.7 ± 0.1[c]	6 of 6	95.0 ± 10.4	NA
Second experiment						
PBS	5 of 6	13.8 ± 0.6	3.1 ± 0.5	ND	ND	ND
IgG2a	5 of 5	13.8 ± 0.2	3.5 ± 0.4	ND	ND	ND
IgG2b	5 of 6	13.0 ± 0.0	3.2 ± 0.7	ND	ND	ND
Anti-B7-2 (2D10)	5 of 5	13.0 ± 0.0	5.0 ± 0.0[c]	ND	ND	ND
Anti-B7-2 (GL-1)	4 of 5	13.5 ± 0.3	4.5 ± 0.3[c]	ND	ND	ND

Abbreviations: ND, not determined; NA, not applicable.
[a] Animals were immunized with PLP(139–151) in CFA and injected intraperitoneally with 100 μg/mouse of various antibodies or PBS.
[b] Data are presented as mean clinical scores for the animals showing clinical disease.
[c] Statistically significant; $P < 0.001$.

of an entire month is shown in Figures 2B and 2C. In an additional experiment, animals immunized with a larger dose of antigen (100 μg/mouse of PLP[139–151] in CFA) and injected with anti-B7-1 or anti-B7-2 antibodies developed a severe EAE that could not be suppressed by the anti-B7 antibody treatment.

Histopathological analysis of the brains and spinal cords of the mice was also performed. In general, there was a close correlation between clinical scores and the number of inflammatory foci in brain and spinal cords (experiment 1 in Table 1). In particular, 1G10-treated mice that did not develop EAE had few or no lesions while those that did develop disease had significantly fewer lesions than 2D10-treated and control-treated (PBS or TR-310 antibody) mice (Figure 3).

Cytokine-specific polymerase chain reaction (PCR) from brain-derived cDNA revealed a uniform distribution of Th1- and Th2-specific cytokines in all mice with the exception of one disease-free animal from the 1G10 group, which expressed IL-4 but negligible amounts of other cytokines by PCR, consistent with the presence of Th2 cells (data not shown). Careful quantitative determinations of cytokine levels at multiple timepoints over the course of disease progression would be required to detect differences in Th1/Th2 ratios in control versus anti-B7-treated animals.

Anti-B7 Antibodies Do Not Inhibit the Induction of T Cells In Vivo but Influence Cytokine Profile

To study the immunological basis for the opposing effects of anti-B7 antibodies on disease induction, we tested for specific proliferative responses and production of various cytokines (IL-2, IFNγ, IL-4, and IL-10) following in vitro stimulation with PLP using lymph node cells (LNCs) from mice immunized with peptide PLP(139–151) and treated with anti-B7 or control antibodies. The LNCs from each group showed significant proliferative responses to the immunizing antigen and not to the control peptide PLP(190–209) (data not shown). The data, presented as stimulation indices for each group (Figure 4A), showed mean stimulation indices of 10.8 ± 3.4, 12.1 ± 3.5, 8.4 ± 1.8, and 10.4 ± 2.9, respectively, for the PBS-treated, TR-310-treated, anti-B7-1-treated, and anti-B7-2-treated groups.

LNCs from PLP-immunized and antibody-treated mice were also cultured with media alone, with immunizing peptide PLP(139–151), or with control peptide PLP(190–209). Culture supernatants were harvested 24 and 48 hr later, and the amount of cytokine present was measured by cytokine-specific enzyme-linked immunosorbent assay (ELISA). Data presented in Figure 4B show no difference in IL-2 production in PBS-treated (470 ± 170 pg/ml), TR-310-treated (535 ± 230 pg/ml), or anti-B7-1-treated (491 ± 180 pg/ml) groups. There was a slight but not significant increase in IL-2 production (1045 ± 460) in the anti-B7-2-treated group. The amount of IFNγ in the same supernatants, however, varied widely among individual animals. Animals injected with PBS and control antibody TR-310 produced, on average, similar amounts of IFNγ (PBS at 1750 ± 730 versus TR-310 at 1770 ± 490 pg/ml; Figure 4C). Animals injected with anti-B7-1 (1G10) generally produced little or no IFNγ. However, LNCs from 1G10-treated mice that did develop EAE produced large quantities of IFNγ following antigen activation (up to about 8200 pg/ml), similar to that seen in the control groups. The mean IFNγ production in the anti-B7-1-treated group was about half (850 ± 540 pg/ml) that seen in the control groups. In contrast, LNCs from anti-B7-2-treated mice generally showed substantially increased production of IFNγ: the mean IFNγ (4860 ± 1240 pg/ml) production in this group was approximately 3- to 4-fold higher than control groups. No detectable levels of IL-4 or IL-10 were observed, consistent with previous difficulties in detecting Th2 cytokines in bulk lymph node cultures (Kuchroo et al., 1993; data not shown). In a separate experiment, we determined by depletion that the cytokines measured were being produced by $CD4^+$ T cells and not by $CD8^+$ or NK cells in the culture (data not shown).

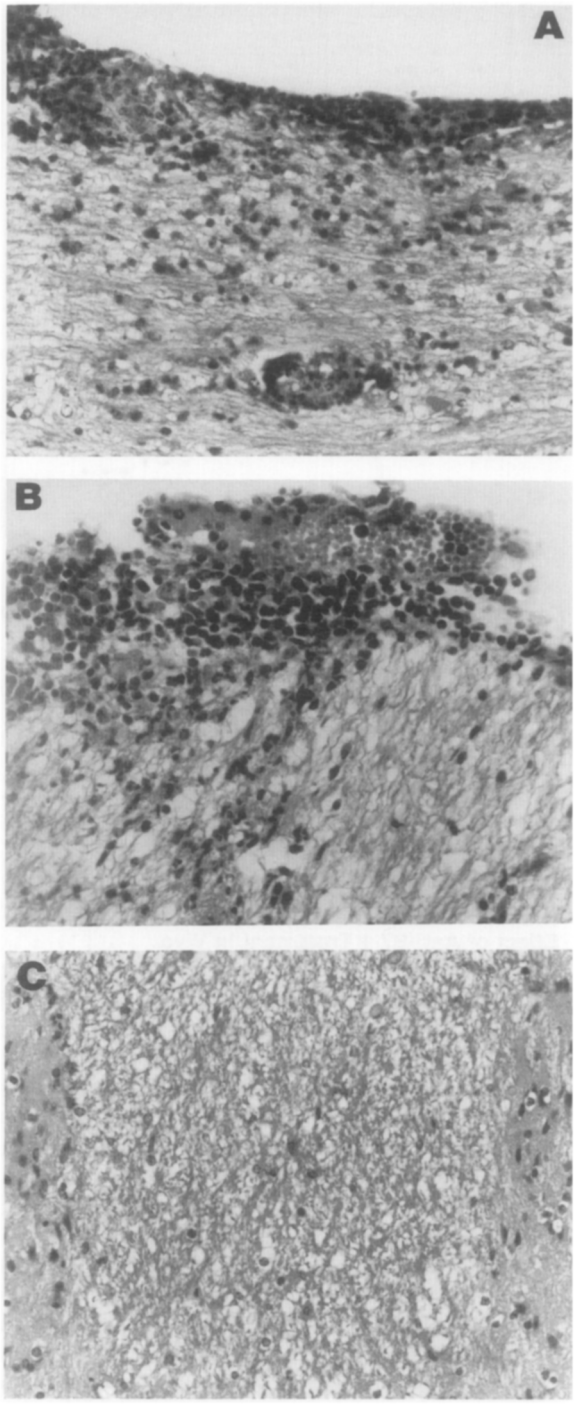

Figure 3. Histology

(A) Anti-B7-2-treated SJL mouse with severe EAE. Prominent meningeal and perivascular parenchymal mononuclear cell infiltrates are present in the spinal cord. Stained with Luxol fast blue–hematoxylin and eosin. Magnification, 83.8×.

(B) Meningeal and perivascular inflammatory infiltrate in the spinal cord of an anti-TR-310 control MAb-treated mouse with severe EAE. Stained with Luxol fast blue–hematoxylin and eosin. Magnification, 126.1×.

(C) Spinal cord white matter (central portion) and gray matter of an SJL mouse sensitized for EAE but treated with anti-B7-1 antibody. No inflammatory infiltrates are present. Stained with Luxol fast blue–hematoxylin and eosin. Magnification, 83.8×.

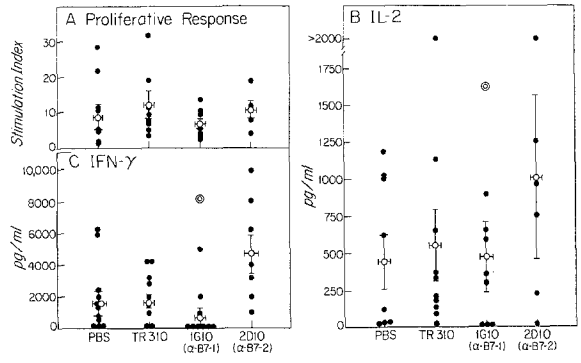

Figure 4. Proliferation and Cytokine Production by the LNCs of Mice Immunized with PLP(139–151) and Injected with Anti-B7 Antibodies

Groups of SJL mice were immunized with PLP(139–151) and injected with 100 μg/mouse of anti-B7-1 or anti-B7-2, TR-310 (isotype control), or PBS. Lymph nodes were harvested at different times during the course of the experiment or on day 10 after immunization. The LNCs were then activated in triplicate wells with 100 μg/ml PLP(139–151) or a control antigen. Proliferation (A) was measured in a 72 hr activation assay, and 1 μCi of [^{3}H]thymidine was added during the last 16 hr of culture. The data were calculated as mean radioactivity incorporated in triplicate wells. The data have been presented as a stimulation index: mean counts per minute in test wells divided by mean counts per minute in wells with media. Levels of specific cytokines (IL-2 [B] and IFNγ [C]) were determined in 48 hr culture supernatant of the LNCs following antigen-specific activation. Means and standard errors of each group are shown.

Anti-B7-1 Treatment In Vivo Induces IL-4-Producing Th2 Cells

The data suggested that anti-B7-1 did not mediate its effects by inhibiting the overall generation of T cells. Since there was a reduction in the production of IFNγ in the bulk cultures, we hypothesized that anti-B7-1 may ameliorate disease by either directly reducing the number of IFNγ-producing cells or, alternatively, by promoting the generation of Th2 cells that may in turn inhibit IFNγ production. To address this issue, we initially plated purified lymph node T cells from the PLP(139–151)-immunized antibody- or control-treated mice, at 10^4 and 10^5 cells/ml (100 μl/well). The number of wells producing IFNγ and IL-4 was determined after restimulation with specific antigen in each treatment group. Interestingly, PBS-, TR-310-, and anti-B7-2-treated groups had approximately the same frequency of IL-4-producing cells (1×10^6 to 2×10^6 T cells) while there were five times more wells (5×10^6 to 6×10^6 cells) that produced IL-4 following in vivo administration of anti-B7-1. This limited analysis suggested that, following anti-B7-1 treatment in vivo, T cells producing Th2 cytokines are induced, but these cytokines are not detected in the lymph node bulk cultures, possibly owing to the dominant inhibition by IFNγ.

To address directly whether Th2 cells are preferentially generated following in vivo administration of anti-B7-1 antibody, we derived T cell clones from the PLP(139–151)-immunized, anti-B7-1-treated mice. A total of ten stable PLP(139–151)-specific clones were established from the anti-B7-1-treated mice, and the clones were classified by cytokine analysis as Th1 (IFNγ) or Th2 (IL-4 and IL-10) phenotypes. Table 2 summarizes the data on the cytokine

Table 2. Cytokine Profile of PLP(139–151)-Specific T Cell Clones Generated from Mice Injected with Anti-B7-1 MAb or Control Immunoglobulin

	Cytokine Concentration (ng/ml)			
Clone	IFNγ	IL-4	IL-10	Subset
Anti-B7-1				
1A8	0.04	**5.00**	0	Th2
1C2	0.20	**5.00**	0	Th2
2F12	0.10	**1.60**	0	Th2
1F4	0.60	**1.30**	**2.60**	Th2
2E10	0.30	**1.60**	0	Th2
1E3	0.04	**>5**	**>5**	Th2
1B3	0.50	0.50	0	Th2
1E6	0.00	0.20	**>5**	Th2
2A2	**2.20**	0.10	0	Th1
1A4	**5.00**	0.30	0	Th1
Control immunoglobulin				
1B7	**5.40**	0	0	Th1
1C3	**1.00**	0	0	Th1
1C4	**1.00**	0	0	Th1
1C7	**>5**	0	0	Th1
1D7	**5.80**	0	0	Th1
1F7	**3.20**	0	0	Th1
3F5	**5.70**	0	0	Th1
4C3	**2.10**	0	0	Th1
4F3	**2.90**	0	0	Th1
4G3	**3.10**	0	0	Th1
1F7	**2.70**	0	0	Th1
2C7	**1.80**	0	0	Th1
4G2	**2.70**	0	0	Th1
3E6	0.00	**1.10**	0	Th2
3E5	0.00	**1.25**	0	Th2
HEL	ND	**4.70**	1	Th2

Cytokine production by PLP(139–151)-specific T cell clones generated in the presence of anti-B7-1 antibody or control hamster IgG. T cell clones were derived from SJL mice immunized with PLP(139–151) and injected in vivo with anti-B7-1 antibody or control hamster IgG. T cell clones specific for the antigen derived from anti-B7-1-treated mice were tested for the production of IL-4, IL-10, and IFNγ. Data are presented as the concentration of various cytokines produced by each individual clone. HEL is a hen–egg white lysozyme–specific T cell line.

profile of these clones. Of the ten clones, seven produced IL-4 but not IFNγ, and two of the clones produced IFNγ but not IL-4. Of the ten clones, three produced IL-10. In summary, of the ten clones generated, two produced IFNγ, one produced IL-10, five produced only IL-4, and two produced both IL-4 and IL-10. None of the clones produced both IFNγ and IL-4 and IL-10. In contrast, under the same culture conditions, T cell clones derived from SJL mice following immunization with PLP(139–151) in CFA and control IgG led to the generation of predominantly Th1 cells (13 of 15) (Table 2). These data suggest that blocking the costimulatory molecule B7-1 in vivo by injection of anti-B7-1 antibody during induction of an immune response forces T cells into a Th2 pathway despite a microenvironment that strongly favors induction of Th1 cells. In vivo studies with two of the clones producing IL-4, IL-10, or both further suggest that these Th2 clones suppress clinical EAE when transferred into naive mice (see below).

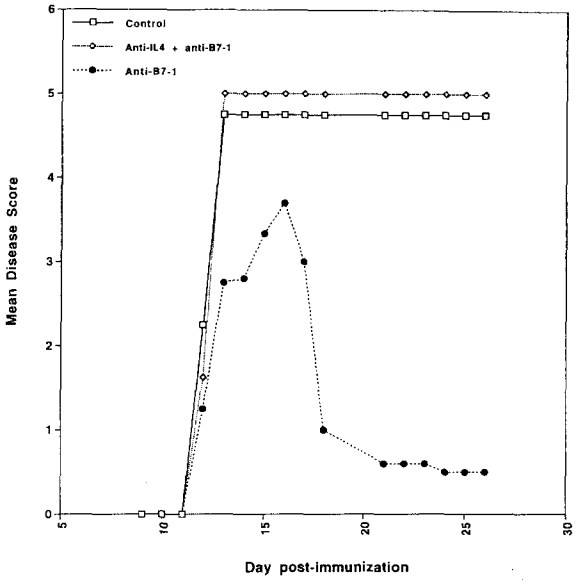

Figure 5. Anti-IL-4 MAb Treatment Abrogates the Protective Effect of Anti-B7-1 Treatment In Vivo

Three groups of four mice each were immunized with PLP(139–151) in CFA (50 μg/mouse) and injected as follows: group 1, Vβ3 as control (10 mg) on day 0; group 2, 11B11 anti-IL-4 MAb (10 mg) on day 0 plus 1G10 anti-B7-1 MAb (100 μg) every other day; group 3, anti-B7-1 MAb (100 μg) every other day.

Anti-IL-4 MAb Treatment Abrogates the Protective Effect of Anti-B7-1 Treatment In Vivo

It has been demonstrated that cytokines themselves can positively or negatively affect Th cell differentiation, as discussed earlier. It was possible, therefore, that antibodies to the B7-1 and B7-2 costimulatory molecules influence Th cell commitment by directly or indirectly causing cytokine release. Two approaches to explore this issue further, both using the in vivo EAE model, were taken. In the first approach, we asked whether preventing or neutralizing the secretion of IL-4 could abrogate the protective effect of the anti-B7-1 antibody on clinical disease. Mice immunized with PLP were, therefore, treated according to one of three protocols: a control group received control anti-Vβ3 antibody; the second group received anti-B7-1 MAb at 100 μg every other day, as described earlier; and a third group received the anti-B7-1 MAb plus a single dose of 10 mg of anti-IL-4 MAb on day 0. The mice were then monitored for clinical symptoms over 25 days. The results were clear cut. All control mice developed severe disease (average disease score at day 14 was 4.9), while anti-B7-1-treated mice, as before, developed much less severe disease (mean score at day 14 was 2.9). In contrast, mice given both anti-B7-1 and anti-IL-4 MAbs developed disease as severe as the mice in the control group (mean score at day 14 was 5.0) (Figure 5). We conclude that the protective effect of the anti-B7-1 MAb can be overcome by preventing or neutralizing the initial secretion of IL-4.

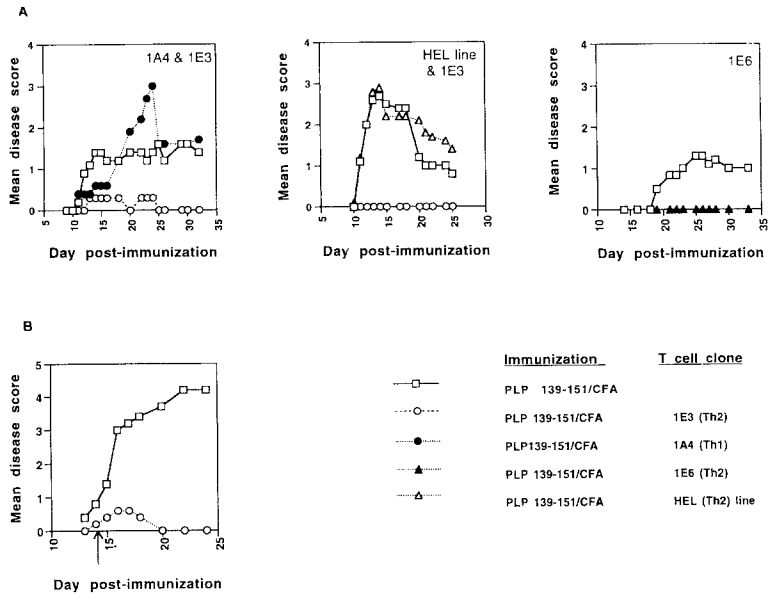

Figure 6. Adoptively Transferred Th2 Clones Protect Mice from Development of EAE and Abrogate Established Disease

Groups of five mice each were immunized with PLP(139–151) in CFA and injected with 5 × 10^6 Th1 or Th2 clones at the time of immunization (A) or at the first signs of disease (B).

Adoptive Transfer of PLP-Specific Th2 Clones Both Prevents EAE and Abrogates Established Disease

In the second approach, we directly tested the effects of Th2 clones generated by blocking the B7-1 molecule in vivo on EAE. In this experiment, we transferred Th2 clones to mice at the same time that they were immunized for the development of EAE or at the first appearance of clinical signs of EAE. Clones transferred into mice (1A4, 1E3, and 1E6) were PLP(139–151) specific and were I-A^S restricted, as determined by blocking with MAb 10.2.16 (data not shown). Th2 clones (1E3 and 1E6) transferred into mice on the day of immunization with PLP(139–151) significantly inhibited EAE. Mice injected with clone 1E3 (an IL-4 and IL-10 producer) showed little (mean score of 0.3) or no disease as compared with controls (Figure 6A, left and middle panels), whereas a Th1 clone (1A4, an IFNγ producer) that was transferred into mice increased the severity of disease above that of the controls, with a mean maximal score of 3 (Figure 6A, left panel). The transfer of a control Th2 line (HEL specific) did not ameliorate disease that was just as severe as in mice immunized with peptide alone, with a mean maximal score of 3 (Figure 6A, middle panel). When a second Th2 clone, 1E6, was introduced into mice again, no signs of disease at all were observed (Figure 6A, right panel). We conclude that transfer of Th2 clones generated following treatment with anti-B7-1 MAb can prevent the induction of disease in an antigen-specific manner.

To determine the possible therapeutic efficacy of this approach, we wished to determine whether established disease could be ameliorated or abrogated by Th2 clone transfer. Therefore, the Th2 clone 1E3 was transferred into mice after onset of initial signs of disease (limp tail or weight loss). This clone was injected on day 14, and the incidence of disease in the clone-injected group was 40% compared with the controls (100%) (Figure 6B). In the clone-injected mice, the disease peaked (mean disease score of 0.6) on day 16–17, whereas in the control group, all the animals showed complete paralysis of fore and hind limbs (mean score of 4). These data suggest that an antigen-specific Th2 clone that produces IL-4, IL-10, or both is capable of treating mice that manifest clinical disease when introduced after the onset of disease. These data (Figures 5 and 6) taken together suggest that initial secretion of IL-4 by Th2 or $CD4^+$ $NK1.1^+$ cells may directly enhance Th2 generation in an autocrine fashion and may also inhibit the generation of encephalitogenic Th1 cells.

Discussion

We have examined the role of the costimulatory molecules B7-1 and B7-2 in the differentiation of Thp cells to mature Th1 and Th2 effector cells and in the induction of an autoimmune disease, EAE. Utilizing MAbs specific for these two costimulatory molecules, we have shown that anti-B7-1 drives naive MBP-specific Thp cells along a Th2 pathway while anti-B7-2 favors Th1 development. Administration of anti-B7-1 antibody ameliorated an organ-specific autoimmune disease, EAE, whereas injection of anti-B7-2 antibody significantly worsened clinical and histological disease. Blocking B7 molecules in vivo did not inhibit the generation of antigen-specific T cells, consistent with the in vitro differentiation results, but affected the cytokine profile of the responding T cells. Our results further suggest that the ability of anti-B7 antibodies to inhibit or enhance EAE relates to the capacity of these antibodies differentially to activate Th1 or Th2 cells/cytokines upon contact with Thp cells in the peripheral lymphoid compartment. Indeed, prevention of the initial secretion of IL-4 abrogates the protective effect of anti-B7-1. In addition, we provide evidence that transfer of antigen-specific Th2

clones generated by blocking the B7-1 molecule can prevent an organ-specific autoimmune disease and can abrogate established disease.

B7-Based Immunotherapy

Interactions with costimulators expressed on APCs are required for T cell activation and cytokine secretion and in the prevention of tolerance (Harding et al., 1992; Lenschow et al., 1992). A number of studies have suggested that manipulating the interaction of B7 molecules with their counterreceptors on T cells provides effective immunotherapy in vivo. For example, a soluble CTLA-4 immunoglobulin fusion protein prolonged allograft rejection (Turka et al., 1992) and prevented xenograft rejection (Lenschow et al., 1992). Furthermore, CTLA-4 immunoglobulin inhibited antibody production (Linsley et al., 1992) and ameliorated murine lupus (Finck et al., 1994). Conversely, we and others have recently demonstrated that provision of B7-1 to malignant tumor cells results in their rejection and can reduce tumor burden and inhibit tumor metastasis (Baskar et al., 1995; Townsend and Allison, 1993; Chen et al., 1992) and that overexpression of B7-1 in pancreatic islets results in autoimmune insulitis (Guerder et al., 1994). Here we have extended these applications to include the treatment of an organ-specific autoimmune disease.

EAE has been used as an experimental model of an autoimmune disease to study basic immune mechanisms and to develop immunotherapeutics to prevent or treat autoimmunity. A number of immunotherapeutic strategies have been successfully used in the treatment of EAE (reviewed by Zamvil and Steinman, 1990). These include anti-MHC class II, anti-CD4, and specific anti-TCR Vβ antibodies. These treatment strategies were aimed at blocking the generation of all Th cells or at least the specific T cells involved in the induction of EAE. In contrast, our approach was aimed at skewing the T cell response to a nonpathogenic Th2 pathway. Both approaches inhibit the acute disease; however, it is possible that they may have different effects on the incidence and intensity of relapsing disease (Jiang et al., 1992). Of particular interest is the observation that only anti-B7-1 and not anti-B7-2 inhibited EAE. Indeed, anti-B7-2 treatment worsened disease signs and increased the amount of CNS inflammation. If the B7-1 costimulatory molecule is essential for the induction of encephalitogenic Th1 cells, then its blockade would inhibit the generation of or induce anergy in these encephalitogenic T cells. This would also result in a compensatory increase of Th2 cells with the end result being an overall reduction in the production of Th1 cytokines, including IFNγ. However, this alone cannot account for the worsening of EAE and increase in the production of Th1 cells/cytokines (IFNγ) by the administration of anti-B7-2 antibody. An explanation might be provided by the temporal patterns of expression of these molecules during the development of a given immune response or by a preferential inhibition of IL-4 secretion by the anti-B7-2 MAb (see below).

EAE Is Induced by Th1 Cells, and Th2 Cells Can Prevent Induction of Disease and Abrogate Established Disease

Myelin antigen (MBP or PLP)–reactive T cells that induce EAE have thus far all been shown to display a Th1 phenotype (Kuchroo et al., 1993; Baron et al., 1993). In addition, during progression of disease, Th1 cyokines (IL-2, TNFβ, and IFNγ) are present in inflammatory EAE lesions, while Th2 cytokines are absent (Khoury et al., 1992), strongly suggesting that Th1 cytokines play a role in the pathogenesis of the disease. This is consistent with the present data, since mice immunized with PLP(139–151) and injected with either PBS or the control TR-310 antibody generate T cells that produced significant amounts of Th1 (IL-2 and IFNγ) but not detectable levels of Th2 (IL-4 and IL-10) cytokines. Administration of anti-B7-2 antibody significantly enhanced the severity of EAE, and this was accompanied by a marked increase in antigen-specific IFNγ production by LNCs, suggesting that injection of anti-B7-2 antibody leads to the hyperinduction or activation of IFNγ-producing Th1 cells. On the other hand, administration of anti-B7-1 antibody leads to amelioration of disease with a commensurate decrease in the production of IFNγ, indicating that blocking the costimulatory B7-1 molecule in vivo inhibits (directly or indirectly) the generation of Th1 cells/cytokines. Thus, our data indicate that anti-B7-1 and anti-B7-2 antibodies have opposing effects in the induction of EAE and concurrently in the generation of Th1 and Th2 cells/cytokines. The presence of IL-4 alone in the brain in an asymptomatic treated animal, as well as our experiments with Th2 clones generated by the administration of anti-B7-1 antibody, further strengthens the argument that these cells/clones do not induce EAE on their own but can protect mice from the development of EAE. In other systems, regulatory T cells that suppress the development of EAE have also been shown to produce cytokines that correspond to the Th2 profile (Chen et al., 1994). Furthermore, recovery from EAE in mice and rats is associated with an increase in the presence of Th2 cells and cytokines in the CNS (Khoury et al., 1992). The present findings demonstrate that an antigen-specific Th2 clone can prevent the induction of an organ-specific autoimmune disease and can abrogate established disease. These findings, together with the observation that administration of anti-B7-1 results in the preferential induction of antigen-specific Th2 cells, strongly suggests that the protective effects of anti-B7-1 antibody may be due to activation of the Th2 pathway and thus offer the basis for novel immunotherapeutic strategies. Such strategies might rely on the manipulation of cytokines such as IL-4, since blocking the initial production of IL-4 abrogated the protective effect of the anti-B7-1 antibody.

Role of the Costimulatory Molecules B7-1 and B7-2 in Th Cell Differentiation

Differentiation of Thp cells into Th1 or Th2 cells upon antigenic stimulation in vivo has important biologic consequences. However, the processes that dictate whether an

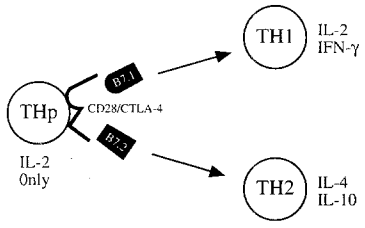

Figure 7. Interaction of B7-1 and B7-2 with the Same Counterreceptor on a Thp Cell Results in Different Th Subset Generations

immune response will be dominated by Th1 versus Th2 cells are not well understood (Paul and Seder, 1994; Seder and Paul, 1994), although initial cytokine release, antigen concentration, and the route of antigen administration may all affect the outcome. The data presented here are direct evidence that interaction of the costimulatory molecules B7-1 or B7-2, with their counterreceptors CD28 and CTLA-4, on Thp cells during antigen presentation leads to polarization of Th responses. The simplest interpretation of our data is that B7-1 preferentially acts as a costimulator for the generation of Th1 cells while B7-2 costimulates and induces Th2 cells (see model in Figure 7). Thus, blocking B7-1 will inhibit the generation of Th1 cells, while enhancing the generation of Th2 cells and blocking B7-2 will have the opposite effect. If this hypothesis is correct, then the interaction of B7-1 and B7-2 molecules with their T cell counterreceptors likely generates different intracellular signals that lead to the differentiation of Thp cells along a Th1 or Th2 pathway. The recent evidence (Nunès et al., 1994) that triggering of CD28 with anti-CD28 antibody, but not with the ligand B7-1, can activate $p21^{ras}$ is intriguing in this regard. This raises the possibility that a differential effect on $p21^{ras}$ or other molecules implicated in CD28 signaling (such as phosphatidyl inositol 3-kinase) (Prasad et al., 1994) would also be observed when interaction with B7-1 versus B7-2 ligands is compared. It is also possible that differential interaction of B7-1 and B7-2 with their two counterreceptors, CD28 and CTLA-4, may occur. Although several structural motifs are shared between the two amino acid sequences (Freeman et al., 1993a, 1993b; Azuma et al., 1993), binding studies have shown that the affinity of B7-1 for CTLA-4 is of 10- to 20-fold higher affinity than to CD28. Similar measurements have recently been published for B7-2 (Linsley et al., 1994), which demonstrate that the two costimulatory molecules do not bind equivalently to CTLA-4. Since CTLA-4 has recently been shown to function as a negative regulator of T cell activation (Walunas et al., 1994), the more rapid dissociation of B7-2 from CTLA-4 could result in the effects observed. One outcome of this differential signaling could be the initial secretion of opposing cytokines that would drive Th lineage commitment. The observations that treatment with anti-IL-4 MAb abrogates the protective effect of anti-B7-1 antibody together with the prevention of EAE by transfer of PLP-specific Th2 clones is consistent with a direct effect of costimulatory molecules on cytokine secretion. Alternatively, the polarization of Th cell responses by the addition of anti-B7 antibodies, observed in this study, may be an indirect effect of the antibodies themselves. A number of cell types, including T cells, macrophages, and dendritic cells, constitutively express B7-2 and can up-regulate expression of B7-1 upon activation. Cross-linking costimulatory molecules on the surface of these cells by antibody may lead to the production of specific cytokines and change the cytokine milieu in the microenvironment, thereby polarizing Th cell responses. If the latter hypothesis is correct, then cross-linking B7-1 versus B7-2 would lead to the production of different cytokines, such as IL-12, that may promote or inhibit generation of Th1 versus Th2 cells. The identification of intracellular signals that are generated by interaction of B7-1 and B7-2 with the same counterreceptors (CD28 and CTLA-4) on a Thp cell may provide insight into the molecular mechanisms responsible for Th cell differentiation, allowing selective manipulation of the immune response in disease.

Experimental Procedures

Mice

Female SJL/J mice 4 to 8 weeks of age (Jackson Laboratory, Bar Harbor, ME) were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, as stated in Department of Health and Human Services publication 85-23 (revised in 1985).

Transgenic B10.PL mice expressing TCRs for MBP have been described previously (Goverman et al., 1993) and express Vα2.3- and Vβ8.2-containing TCR genes from a B10.PL-derived T cell hybrid specific for MBP Ac(1–11). Separate TCR α and β chain transgenic lines were established and the lines bred together to generate mice expressing a complete transgenic TCR. Expression of transgenes was evaluated by genotyping tail DNA and by flow cytometry of peripheral blood T cells utilizing MAbs specific for TCR Vα2 and Vβ8.2 regions. An additional MBP TCR transgenic line expressing Vα4 and Vβ8.2 that recognizes MBP Ac(1–17) was also utilized (Lafaille et al., 1994) (gift of Dr. C. Janeway, Yale University, New Haven, CT).

Antigens

PLP(139–151) (HSLGKWLGHPDKF), PLP(190–209) (SKTSASIGSLCADARMYYGVL), and MBP Ac(1–11) (AcASQKRPSQRHG) peptides were prepared according to published sequences (Laursen et al., 1984; Newman et al., 1987) except that serine was always substituted for cysteine at position 140 in the peptide PLP(139–151) (Tuohy et al., 1989). Peptides were synthesized in the laboratory of Dr. R. Laursen (Department of Chemistry, Boston University, Boston, MA) on a Milligen model 9050 synthesizer using Fmoc chemistry or by H. Freisheim (Medical College of Ohio, Toledo, OH) on an Applied Biosystems model 430A synthesizer using Milligen PAL resins or RINK amide resins.

Antibodies

Anti-B7-1 (1G10) and anti-B7-2 (2D10) have been described previously (Nabavi et al., 1992; Chen et al., 1994) and were derived from the same somatic cell fusion. The MAbs were purified from ascitic fluid on protein G columns. The anti-B7-1 and B7-2 antibodies are of the IgG2a and IgG2b isotypes, respectively. A second anti-B7-2 antibody (GL-1) of rat IgG2a isotype was obtained from ATCC. Hybridoma cells were grown as ascites and purified over a protein G column. Purified rat IgG2a and IgG2b antibodies (Pharmingen, San Diego, CA) were used as isotype controls. A rat anti-Vβ7 (TR-310) antibody (rat IgG2a) was purified from ascitic fluid on a protein G column and used as an additional control. The rat MAb to mouse IL-4 was the gift of Dr. W. E.

Paul (National Institutes of Health, Bethesda, MD), and the anti-Vβ3 antibody KJ25a, used as a control, was the gift of Dr. J. Kappler (National Jewish Hospital and Research Center, Denver, CO).

Induction of EAE

SJL mice were injected subcutaneously in the flank with 25–100 μg of peptide PLP(139–151) and 400 μg of Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI) in an emulsion consisting of equal volumes of PBS and CFA. Each mouse was also injected intravenously on day 0 and day 2 or 3 with 10^9 heat-killed Bordetella pertussis bacilli (Massachusetts Public Health Biological Laboratories, Boston, MA). To test the efficacy of the antibodies in the induction and inhibition of EAE in vivo, we injected mice with anti-B7 or control antibodies (100 μg/mouse) intraperitoneally every other day following immunization, according to various protocols as described in Results.

Clinical assessment was carried out daily according to the following criteria: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony, moderately clumsy gait, poor righting ability, or some combination of these symptoms; 3, limb weakness; 4, limb paralysis; 5, moribund state. Animals were sacrificed at the termination of the experiment or at the peak of the disease. Mice that showed no clinical signs were sacrificed at the conclusion of the experiment. Brains and spinal cords were removed and fixed in 10% formalin, and paraffin-embedded sections were stained with Luxol fast blue–hematoxylin and eosin for light microscopy. Histological disease was quantified by counting inflammatory foci in meninges and parenchyma (Sobel et al., 1989).

In Vitro Proliferative Response and Cytokine Production of Antigen-Primed LNCs

Groups of mice immunized subcutaneously with peptide PLP(139–151) in CFA for the induction of EAE were sacrificed at various timepoints during the course of disease. Inguinal, prescapular, and para-aortic lymph nodes were removed and dissociated in Dulbecco's minimum essential medium (DMEM). LNCs from these mice were used for in vitro proliferative assays and production of cytokines following in vitro stimulation.

Proliferative Assays

For in vitro proliferation, LNCs were cultured for 72 hr in flat-bottomed 96-well plates in the presence of various concentrations of peptide and then pulsed with 1 μCi of [^{3}H]thymidine for the last 16–18 hr of incubation, and mean thymidine incorporation in triplicate wells was calculated. The data of the proliferative assays are presented as stimulation indices for easy comparison of the proliferative responses: mean cycles per minute in the test wells divided by mean counts per minute in the control wells (with medium).

Cytokine ELISA

The amount of cytokines produced by LNCs was determined by ELISA. LNCs obtained from mice immunized with PLP(139–151) in CFA and injected with the various antibodies were activated with PLP(139–151) control peptide in vitro, and culture supernatants were collected from separate wells on day 1 and day 2. In brief, 96-well plates were coated overnight with primary anti-cytokine capture antibody (1 μg/ml) specific for a particular cytokine. The plates were washed twice and, after a 2 hr incubation with PBS plus 15% blocking reagent (Kirkegaard and Perry Laboratories, Gaithersburg, MD), the supernatants from the activated clones and the standards were added. After overnight incubation at 4°C, the plates were washed, and diluted biotinylated anti-cytokine-detecting MAb (1 μg/ml) was added and incubated at room temperature for 1 hr. The plates were washed and further developed by adding avidin–peroxidase and its substrate, and absorbance was measured on a 450 nm wavelength spectrophotometer. The amount of cytokine in each supernatant was extrapolated from the standard curve. The antibody pairs used were as follows, listed by capture/biotinylated detection: IL-2, JES6-1A12/JE5H4; IL-4, BVD4-1D11/BVD6-24G2; IL-10, JES5-2A5/SXC-1; IFNγ, R4-6A2/XMG1.2 (all from Pharmingen, San Diego, CA). The standards were recombinant cytokine curves generated in 1:2 dilutions from 39 to 2500 pg/ml for IL-2, IL-4, and IL-10 and from 78 to 5000 pg/ml for IFNγ with detectable readings at the lowest dilutions.

In Vitro Differentiation and Phenotype of Transgenic T Cells

Spleen cells (5 × 10^6/ml) obtained from a naive MBP TCR transgenic mouse (Goverman et al., 1993) were equally divided into four aliquots and cultured in a 24-well plate with one of the following antibody reagents at 20 μg/ml: anti-B7-1 (1G10), anti-B7-2 (2D10), purified rat immunoglobulin, or PBS, with or without addition of the antigen MBP Ac(1–11) (10 μg/ml). Culture supernatants were harvested 2 days after activation and tested for cytokine production by ELISA. The cultures were further propagated for an additional 21 days by addition of media containing 5% human IL-2. The live T cells were purified, resuspended at 5 × 10^6 cells/ml, and activated in the presence of the corresponding antibody reagent and syngeneic spleen cells, such as APCs (10^7/ml), with or without addition of MBP Ac(1–11) (10 μg/ml). The culture supernatants were harvested 48 hr after activation, and cytokine content was determined by ELISA.

Derivation and Maintenance of T Cell Clones

SJL mice were injected subcutaneously in each flank with 100 μg of PLP(139–151) in CFA plus 400 μg of M. tuberculosis H37RA and then injected with anti-B7-1 antibody (100 μg intravenously on days 0 and 2) (Razi-Wolf et al., 1992) or control hamster IgG (Cappel, Durham, NC). LNCs were removed from mice 10 days after immunization and cultured for 5 days with PLP(139–151) peptide (20 μg/ml) in DMEM containing 1% autologous serum. Live cells were isolated on a Ficoll–Hypaque gradient and grown in 5% IL-2 medium (T-STIM, Collaborative Biomedical Products, Bedford, MA) for 14 days. The cells were activated once again in the presence of PLP(139–151) and SJL spleen cells as APCs. The T cells were cloned by limiting dilution 3 days after the last activation and individual T cell clones expanded to obtain sufficient numbers. The cloned cells were activated with PLP(139–151), and the cytokine profile of each T cell clone was established by cytokine ELISAs.

Adoptive Transfer of Clones

T cell clones were activated with APCs and peptide PLP(139–151) (20 μg/ml) for 3 days, after which live cells were isolated by Ficoll–Hypaque density gradient centrifugation. Clones (Th1 or Th2; 5 × 10^6/mouse) were injected intravenously into mice that were injected subcutaneously with 50 μg of PLP(139–151) in CFA at the time of transfer. A control line secreting IL-4 and IL-10 specific for hen–egg white lysozyme (HEL) was also transferred into PLP peptide-immunized mice. A Th2 clone (1E3, producing IL-4 and IL-10) was also transferred into mice after the first sign of disease (limp tail). The disease course was followed for 25–30 days.

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