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Requirement for CD8$^+$ Cells in T Cell Receptor Peptide–Induced Clonal Unresponsiveness

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T cell receptor (TCR) vaccination in rats prevents the development of experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis. The mechanism of this potential immunotherapy was examined by vaccinating mice with an immunogenic peptide fragment of the variable region of the TCR V_β8.2 gene. Another immunogen that usually induces an immune response mediated by V_β8.2$^+$ T cells was subsequently inhibited because specific clonal unresponsiveness (anergy) had been induced. Depletion of CD8$^+$ cells before TCR peptide vaccination blocked such inhibition. Thus, the clonal anergy was dependent on CD8$^+$ T cells, and such immunoregulatory T cells may participate in the normal course of EAE.

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease induced in animals by immunization with myelin basic protein (MBP) and is considered to be a model for the human demyelinating disease multiple sclerosis (*1*). It has served as an important animal model in the development of strategies for immunotherapy. The studies reported here are based on the model developed by Vandenbark and co-workers and Howell and co-workers (*2*, *3*) in which TCR peptide vaccination prevented the subsequent development of EAE in rats after immunization with MBP. Because the mechanism of such TCR peptide–based vaccination has not been elucidated, we transferred this TCR peptide vaccination into a mouse model to study mechanisms of induced unresponsiveness. We found that T cell clonal anergy developed in T cells bearing the receptor against which such peptide-based vaccination was carried out. Furthermore, CD8$^+$ regulatory cells were involved in the induction of such anergy.

To test the immunogenicity of TCR peptide vaccination in mice, we immunized groups of DBA/2 and (PL/J × SJL)F_1 mice with several different peptides that corresponded to several different regions of the TCR β and α chains, including a peptide composed of amino acids 39 through 61 of the complementarity determining region 2 (CDR2) of V_β8.2 (V_β8.2 CDR2). Both DBA/2 and (PL/J × SJL)F_1 mice mounted a brisk proliferative response to the V_β8.2 CDR2 peptide but not to other tested peptides (*4*). We then investigated whether prior vaccination with the V_β8.2 CDR2 peptide could influence the immune response of DBA/2 mice to a synthetic peptide, the T cell response against which is predominantly mediated by V_β8.2$^+$ CD4$^+$ T cells (*5*). DBA/2 mice were vaccinated with the V_β8.2 CDR2 peptide 10 days before immunization with a synthetic peptide that consisted of amino acids 110

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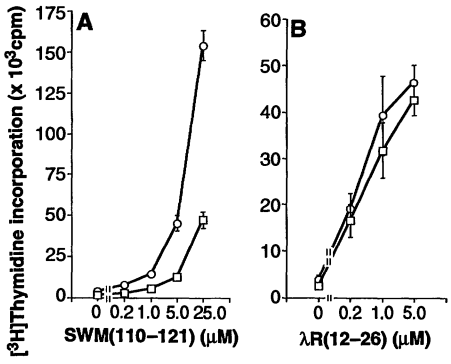

Fig. 1. Effect of TCR V_β8.2 CDR2 vaccination on the response of DBA/2 mice to (**A**) SWM(110–121) or (**B**) C1 λR(12–26). Groups of four DBA/2 mice were vaccinated at the base of the tail with 100 μg of the TCR V_β8.2 CDR2 peptide (DTGHGLRLIHYSYGAGSTEKGDI) (open squares) or a control peptide from TCR V_α1(46–59) (ALGMSISDGFKEEG) emulsified in CFA (open circles) (*18*). Control groups were vaccinated with control peptides or CFA alone. Ten to 12 days later, the mice were immunized in the flanks or foot pads with 100 μg each of SWM(110–121) or C1 λR(12–26) emulsified in CFA. Eight days after this immunization, single-cell suspensions were prepared from the inguinal lymph nodes. Lymph node cells (0.5×10^6) pooled from four mice were cultured for 4 days with different amounts of either SWM(110–121) or C1 λR(12–26). We determined the incorporation of [^{3}H]thymidine added (1 μCi per well) for the last 18 hours of culture by counting the harvested cells in a scintillation counter. Means of triplicate cultures ± SEM at each dose are shown; the data are representative of three separate experiments. The response to mycobacterial purified protein derivative in mice immunized with SWM(110–121) was comparable to that of the groups vaccinated with the V_β8.2 CDR2 or control peptides (290,660 ± 12,453 cpm for the group vaccinated with the V_β8.2 CDR2 peptide versus 321,622 ± 835 cpm for the group vaccinated with the control peptide).

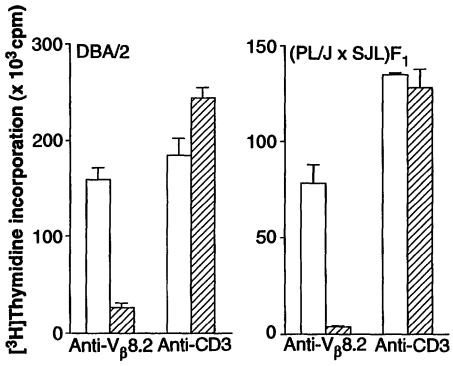

Fig. 2. TCR antibody–induced (receptor cross-linking) proliferation of lymph node cells from either DBA/2 or (PL/J × SJL)F_1 mice vaccinated with either a control peptide (open bars) or with the V_β8.2 CDR2 peptide (hatched bars) as described (Fig. 1). Ten to 15 days later, TCR antibody cross-linking assays were performed with the use of antibodies to V_β8.2 (F23.2) (anti-V_β8.2) (*19*) and CD3 (145.2.C11) (anti-CD3) (*20*). Fifty microliters of a solution of antibodies (5 μg/ml) specific to the β chain of the heterodimeric TCR or the CD3 complex were coated on a 96-well plate. After 18 to 24 hours of incubation at 37°C, the plates were washed with phosphate-buffered saline, and 0.2×10^6 lymph node cells pooled from three to four mice in each group were dispensed into each well. Two days later, 1 μCi of [^{3}H]thymidine was added to each well, and the cells were harvested 18 hours later. The extent of proliferation was determined by scintillation counting. Mean counts per minute of triplicate cultures ± SEM are shown after subtraction of background counts (in the absence of antibody). The background counts for the groups vaccinated with the control or V_β8.2 CDR2 peptides were 1719 ± 373 and 465 ± 291 cpm, respectively (DBA/2 mice) and 1449 ± 133 and 456 ± 55 cpm, respectively [(PL/J × SJL)F_1 mice]. Data are representative of eight separate experiments.

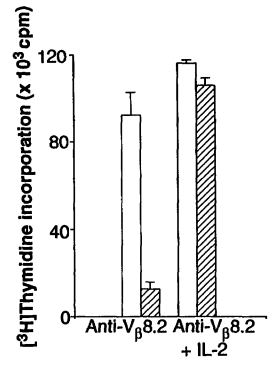

Fig. 3. V_β8.2 CDR2 peptide–induced unresponsiveness is overcome by the addition of exogenous IL-2. (PL/J × SJL)F_1 mice were immunized with the V_β8.2 CDR2 peptide (hatched bars) or a control peptide (open bars). After 10 days, we assayed inguinal lymph node cells for proliferation, in the absence or presence of exogenously added IL-2, by cross-linking V_β8.2 TCRs with immobilized anti-V_β8.2 as described (Fig. 2). Data shown has had background counts subtracted (without IL-2, 885 ± 96 and 703 ± 50 cpm for cells from control peptide– and V_β8.2 peptide–vaccinated mice, respectively; with IL-2, 19,000 ± 1,353 and 26,939 ± 1,774, respectively). Similar results were obtained with DBA/2 mice.

Table 1. Percentage of CD4$^+$ cells that were V_β8.2$^+$. DBA/2 or (PL/J × SJL)F_1 mice were vaccinated with either V_β8.2 CDR2 or a control peptide. Ten or 20 days later, on the day of the TCR cross-linking assay, flow cytometric analysis was performed on peripheral blood lymphocytes with the use of anti-V_β8.2(F23.2) (*19*) and anti-CD4 (GK 1.5) (*22*). Data on three animals in each group are shown.

Strain	V_β8.2$^+$ CD4$^+$ cells (%)	
	Control-vaccinated	V_β8.2 CDR2–vaccinated
DBA/2	14.7	14.7
	13.4	14.7
	14.1	12.6
(PL/J × SJL)F_1	8.3	9.9
	8.5	8.6
	8.8	8.6

through 121, which corresponds to an immunogenic determinant of sperm whale myoglobin [SWM(110–121)]. The T cell proliferative response of these mice was diminished in comparison to that of similarly immunized mice that had received a control TCR peptide vaccination (Fig. 1). This reduction in the response of V_β8.2$^+$ T cells to SWM(110–121) was specific: in comparison to this response, DBA/2 mice vaccinated with the TCR peptide were unaffected in their response to a determinant consisting of amino acids 12 through 26 from the C1 protein of λ repressor [λR(12–26)], which does not predominantly use V_β8.2$^+$ T cells (6, 7) (Fig. 1).

We then investigated whether vaccination with the V_β8.2 CDR2 peptide diminished the immune response in an antigen-specific way or whether it led to inactivation of most (or all) T cells expressing the V_β8.2 receptor. Using monoclonal antibodies (MAbs) to selected TCR β chains or to CD3, we assayed proliferation induced by receptor cross-linking in lymphocytes from DBA/2 and (PL/J × SJL)F_1 mice that had been vaccinated 10 to 15 days before with either the V_β8.2 CDR2 peptide or with another TCR-based peptide as a control. In eight separate experiments, lymphocytes from groups of three or more mice vaccinated with the V_β8.2 CDR2 peptide had decreased proliferation in response to receptor cross-linking with a MAb specific for the TCR V_β8.2 (F23.2) in comparison to the response of mice vaccinated with an irrelevant control peptide or complete Freund's adjuvant (CFA) alone (Fig. 2). Some variation in the amount of unresponsiveness induced by the V_β8.2 CDR2 peptide vaccination among individual mice was observed. However, most mice in each experiment and all pooled cells tested gave data similar to that presented in Fig. 2. Proliferation induced by antibodies directed at CD3 or antibodies directed at other TCRs remained unaffected in all groups of mice. This diminished response of V_β8.2 CDR2 peptide–vaccinated mice to receptor cross-linking (Fig. 2) was overcome by the addition of exogenous interleukin-2 (IL-2) to the cultures. Proliferation increased to control activity in all tested cultures to which recombinant IL-2 was added (Fig. 3).

To see whether administration of the

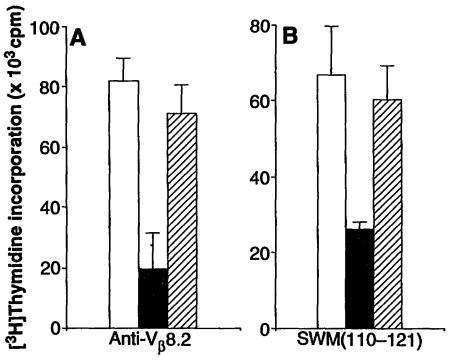

Fig. 4. Effect of $CD8^+$ depletion on $V_\beta 8.2$ CDR2 peptide–induced unresponsiveness. (**A**) Proliferation of T cells from a group of mice depleted of $CD8^+$ cells and vaccinated with the $V_\beta 8.2$ CDR2 peptide (hatched bar), a group only vaccinated with the $V_\beta 8.2$ CDR2 peptide (closed bar), and a group vaccinated only with a control peptide (open bar). DBA/2 mice were treated intraperitoneally for 3 days with antibodies to CD8 (53.6.7) (100 μg per day) (*21*). Upon maximum depletion (>90%) as checked by flow cytometry, mice were vaccinated subcutaneously at the base of the tail with $V_\beta 8.2$ CDR2 in CFA. Two groups not depleted of $CD8^+$ cells were included in the experiment, one vaccinated with the same $V_\beta 8.2$ CDR2 peptide and the other vaccinated with a control peptide. Ten days after vaccination, we performed a receptor cross-linking assay of lymph node cells using immobilized anti-$V_\beta 8.2$ as described (Fig. 2). Results are shown ± SEM of triplicate cultures after subtraction of background (no antibody) counts, which were 24,796 ± 3,477, 11,666 ± 1,435, and 13,485 ± 3,751 cpm, respectively, for the three groups. (**B**) In a separate set of experiments, the same groups of mice as in (A) were immunized with SWM(110–121) 10 days after vaccination with control peptide or $V_\beta 8.2$ CDR2 with or without CD8 depletion. Eight days later, draining lymph node cells were used in a SWM(110–121)–specific T cell proliferation assay as described (Fig. 1). Shading of bars is as in (A). Mean counts per minute ± SEM of triplicate cultures with 5 μM SWM (110–121) are shown after subtraction of background [cultures in the absence of SWM(110–121)] counts, which were 13,686 ± 858, 16,187 ± 1,115, and 30,509 ± 1,415 cpm for the respective groups. These experiments were repeated twice.

$V_\beta 8.2$ CDR2 peptide led to a decrease in circulating $V_\beta 8.2^+$ T cells, we assayed peripheral lymph nodes and blood lymphocytes of DBA/2 and (PL/J × SJL)F_1 mice for $V_\beta 8.2^+$ T cells 10 to 20 days after they were vaccinated with the $V_\beta 8.2$ CDR2 peptide. There was no demonstrable decrease in the number of $V_\beta 8.2^+$ T cells in either strain in comparison to normal mice (Table 1). Antibodies that bind to T cells that have the $V_\beta 8.2$ TCR are generated in rats after $V_\beta 8.2$ CDR2 peptide vaccination (*8*). However, using the enzyme-linked immunosorbent assay and cloned $V_\beta 8.2^+$ T cells or $V_\beta 8.2^+$ T cell hybridomas, we could not detect in these vaccinated DBA/2 mice any antibodies that could recognize $V_\beta 8.2^+$ T cells, although we found peptide-specific antibodies. These results are similar to the results of others (*9*). Additionally, we could not detect antibodies to $V_\beta 8.2$ TCRs bound to the $V_\beta 8.2^+$ T cells of the vaccinated animals (*4*).

We used vaccination with TCR peptides because vaccination with attenuated whole T cells effectively generates antiidiotypic T cells with regulatory functions (*10, 11*). This suggested that TCRs might be recognized directly by immune cells or that TCR proteins might undergo intracellular processing and be presented by major histocompatibility complex (MHC) class I molecules on the T cell surface where they could serve as targets for regulatory cells. Although there is no direct evidence to support this hypothesis, a significant proportion of TCR chains are degraded intracellularly and do not reach the cell surface (*12, 13*). Also, T cells recycle their surface class I MHC molecules (*14*). TCR β chains and MHC class I molecules can be histochemically detected in the same subcellular compartment (*15*). Thus, these two products can possibly associate within the cell and be presented on the T cell surface as the processed TCR chain in the context of an MHC class I molecule as a target of regulation. The original studies on $V_\beta 8.2$ CDR2 vaccination in rats suggested a possible role for a $CD8^+$ regulatory cell (*2, 3*). More recent studies demonstrate two points. (i) $CD8^+$ T cells are involved as regulators of murine EAE, although they may not be involved in the regulation of primary episodes of demyelination but in the regulation that leads to resistance to a second induction of EAE (*16*). (ii) CD8 knock-out mice have more chronic EAE and a higher frequency of relapse (*17*). These studies suggest that $CD8^+$ T lymphocytes might participate as immune regulators in animal models of EAE.

To investigate whether a $CD8^+$ subpopulation of cells might be involved in inducing specific T cell unresponsiveness in mice vaccinated with the $V_\beta 8.2$ CDR2 peptide, we depleted $CD8^+$ cells from DBA/2 mice and (PL/J × SJL)F_1 mice before vaccination. Three days after depletion of $CD8^+$ cells, DBA/2 and (PL/J × SJL)F_1 mice were vaccinated with 100 μg of the $V_\beta 8.2$ CDR2 peptide in CFA. Two weeks after this vaccination, T cells from these mice were assayed for receptor-mediated cross-linking activation. The induced unresponsiveness seen in DBA/2 and (PL/J × SJL)F_1 mice that were vaccinated with only the $V_\beta 8.2$ TCR peptide was prevented by this depletion of $CD8^+$ cells with monoclonal antibody (Fig. 4A). A second group of DBA/2 mice that were depleted of $CD8^+$ cells and vaccinated with the $V_\beta 8.2$ CDR2 peptide was then immunized with SWM(110–121) in CFA. Their responsiveness was compared to (i) control animals that were not depleted of $CD8^+$ cells but immunized with only a control peptide and then subsequently immunized with the SWM peptide or (ii) animals who received $V_\beta 8.2$ CDR2 peptide vaccination and SWM(110–121) immunization without $CD8^+$ cell depletion (Fig. 4B). DBA/2 mice depleted of $CD8^+$ cells at the time of $V_\beta 8.2$ CDR2 peptide vaccination were responsive to subsequent immunization with SWM(110–121), whereas the experimental group that received only $V_\beta 8.2$ CDR2 peptide vaccination without $CD8^+$ depletion had the previously demonstrated (Fig. 1) diminished response to SWM(110–121).

Thus, $V_\beta 8.2$ CDR2 peptide–induced unresponsiveness is mediated by a $CD8^+$ cell subpopulation in vaccinated animals. The $CD8^+$ cell subpopulation regulates the $V_\beta 8.2^+$ T cell population by inducing unresponsiveness, not deletion. These data, in support of previous work on the role of $CD8^+$ T cells in murine EAE, suggest the possibility that $CD8^+$ T cells regulate the severity, or block the second induction, of EAE. This may be achieved by a regulatory circuit in which $CD8^+$ T cells have, as targets of regulation, the $CD4^+$ $V_\beta 8.2^+$ T cells necessary for the induction or perpetuation of EAE. The potential application of TCR peptide vaccination as immunotherapy in a mouse model of EAE remains to be tested.

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Treatment and Prevention of Rat Glioblastoma by Immunogenic C6 Cells Expressing Antisense Insulin-Like Growth Factor I RNA

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Rat C6 glioma cells express insulin-like growth factor I (IGF-I) and form rapidly growing tumors in syngeneic animals. When transfected with an episome-based vector encoding antisense IGF-I complementary DNA, these cells lost tumorigenicity. Subcutaneous injection of IGF-I antisense-transfected C6 cells into rats prevented formation of both subcutaneous tumors and brain tumors induced by nontransfected C6 cells. The antisense-transfected cells also caused regression of established brain glioblastomas when injected at a point distal to the tumor. These antitumor effects result from a glioma-specific immune response involving CD8$^+$ lymphocytes. Antisense blocking of IGF-I expression may reverse a phenotype that allows C6 glioma cells to evade the immune system.

Glioblastoma is the most frequent brain tumor in man and is usually fatal. Both human and rat glioma cells express high amounts of IGF-I. We previously reported that transfection of rat C6 glioma cells (*1*) with an antisense IGF-I cDNA transcriptional cassette driven by the mouse metallothionein-I promoter abrogated their tumorigenicity in syngeneic rats (*2*). This antisense expression vector was episomal and included the Epstein-Barr virus origin of replication and the gene encoding nuclear antigen 1, which together drive extrachromosomal replication (*2*, *3*). Southern blot analysis indicated that the episome was stably maintained for at least 6 months in this cell line (*4*). Although the transfected cells differed morphologically from parental (nontransfected) cells, their viability was unaffected by activation of the transgene in vitro (*2*, *4*). Rats injected with these transfected glioma cells have remained tumor-free for 13 months. In contrast, rats injected with parental C6 cells consistently developed large tumors within 3 weeks after injection.

We noted an accumulation of large mononuclear infiltrates, consisting predominantly of lymphocytes, at the sites of injection of the transfected glioma cells before the lesions disappeared (*2*). This observation suggested the possibility that antisense-mediated inhibition of IGF-I rendered the glioma cells more immunogenic, and hence that their loss of tumorigenicity might have an immune basis.

To determine whether glioma cells transfected with the antisense IGF-I transcriptional cassette elicit a host immune response against parental glioma cells in vivo, we subcutaneously injected 10^7 parental cells into each of ten syngeneic BDX rats (*1*) at a site above the left hind leg. After 4 to 6 days, when the resulting tumors were less than 1 cm in diameter, we injected 10^7 IGF-I antisense-transfected glioma cells at a site above the right hind leg. In all ten rats the tumors regressed within 3 weeks and the animals have remained tumor-free for 13 months (Table 1). In control animals, including rats secondarily injected with glioma cells transfected with the vector devoid of the IGF-I sequence, tumors persisted and after 1 to 2 months grew to a size that necessitated sacrifice of the animals (Table 1).

We next investigated the effect of the transfected glioma cells on rats from which large, established glioblastomas (2 to 3 cm in diameter, usually observed 3 to 4 weeks after injection) had been surgically excised. Twelve such rats were divided into two groups. Six animals received no further treatment, and, of these, five had a local recurrence of tumor after approximately 3 weeks, reflecting the incomplete excision of the primary tumor. The other group of six animals received an injection of 10^7 IGF-I antisense-transfected glioma cells immediately after surgery, and none of them showed tumor recurrence at the excision site or elsewhere over a 12-month period (Table 1).

To establish whether treatment by IGF-I antisense-transfected cells was effective against glioblastomas established in the brain, we injected a group of rats intracranially with 1×10^6 to 2×10^6 parental or transfected C6 cells. After 1 week several rats were sacrificed and their brain tissue prepared for histopathological examination. Of the remaining rats, six of six that had been injected with IGF-I antisense-transfected cells have survived 4 months with no evidence of tumor development, whereas five of five injected with parental cells either died after 3 to 4 weeks or were sacrificed when tumor growth was evident at the point of injection. A second group of six rats was injected intracranially with 1×10^6 to 2×10^6 parental cells and, 2 to 3 days later, injected subcutaneously above the right hind leg with 10^7 transfected cells. These rats have all remained tumor-free for 4 months (Table 1).

During tumor involution in each of the above experiments, we noted abundant infiltration of mononuclear cells accompanied by neovascularization at sites proximal to persisting glial elements (Fig. 1, A and B). In late stages of tumor involution (2 weeks after injection of the transfected cells), 70 to 80% of the infiltrating cells were CD8$^+$ lymphocytes (Fig. 1C), as deduced by staining with an antibody to CD8. The remaining infiltrating cells were macrophages and CD8$^-$ lymphocytes. Sections of normal rat spleen stained with the same antibody showed the expected proportion of CD8$^+$ cells (~10%).

To determine whether IGF-I antisense-transfected glioma cells could prevent glioblastoma development, we subcutaneously injected 11 rats above the left hind leg with 10^7 parental glioma cells and above the

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