function but would rather alter the intensity of part of the immune response.

In summary, with the use of mice lacking cytotoxic T cells we have generated data that support a dual role for $CD8^+$ T cells as effector and modulatory cells in the EAE model. The results indicate that the $CD8^+$ cells are not critical to, but play an adjunctive role in, disease induction and maintenance in the EAE model. This observation, however, may also reflect the bias on $CD4^+$ cells from exogenous antigen priming, and $CD8^+$ T cells may be more dominant in a spontaneous demyelinating disease, such as that from murine Theiler's virus infection or multiple sclerosis.

REFERENCES AND NOTES

1. R. Arnon, *Immunol. Rev.* **55**, 5 (1981).
2. S. S. Zamvil *et al.*, *Nature* **324**, 258 (1986).
3. H. Acha-Orbea, L. Steinman, H. McDevitt, *Annu. Rev. Immunol.* **7**, 371 (1989).
4. B. G. Arnason, B. D. Jankovic, B. H. Waksman, L. Wennerstein, *J. Exp. Med.* **116**, 177 (1962).
5. S. Sriram, L. Carrol, S. Fortin, S. Cooper, G. Ranges, *J. Immunol.* **141**, 464 (1988).
6. S. W. Brostoff and D. W. Mason, *ibid.* **133**, 1938 (1984).
7. C. B. Pettinelli and D. E. McFarlin, *ibid.* **127**, 1420 (1982).
8. S. Zamvil *et al.*, *Nature* **317**, 355 (1985).
9. D. G. Ando, J. Clayton, D. Kono, J. L. Urban, E. E. Sercarz, *Cell. Immunol.* **124**, 132 (1989).
10. J. M. Lemire and W. O. Weigle, *J. Immunol.* **137**, 3169 (1986).
11. D. M. Bitar and O. C. Whitacre, *Cell. Immunol.* **112**, 364 (1988).
12. J. D. Sedgwick *et al.*, *J. Immunol.* **145**, 2474 (1990).
13. J. D. Sedgwick, *Eur. J. Immunol.* **18**, 495 (1988).
14. S. Sriram and L. Carrol, *J. Neuroimmunol.* **17**, 147 (1988).
15. D. Sun, F. Qin, J. Chluba, T. J. Epplen, H. Wekerle, *Nature* **332**, 843 (1988).
16. O. Lider, L. M. B. Santos, C. Y. Lee, P. J. Higgins, H. L. Weiner, *J. Immunol.* **142**, 748 (1989).
17. P. J. Higgins and H. L. Weiner, *ibid.* **140**, 440 (1988).
18. H. Jiang *et al.*, *Science* **256**, 1213 (1992). In the accompanying report, the authors observed that mice depleted of $CD8^+$ T cells with antibodies after induction of primary EAE were susceptible to reinduction of disease, whereas untreated mice were resistant to or protected from further disease from reinduction.
19. P. W. Mathieson, K. J. Stapleton, D. B. G. Oliviera, C. M. Lockwood, *Eur. J. Immunol.* **21**, 2105 (1991).
20. W.-P. Fung-Leung *et al.*, *Cell* **65**, 443 (1991).
21. W.-P. Fung-Leung, T. M. Kundig, R. M. Zinkernagel, T.-W. Mak, *J. Exp. Med.* **174**, 1425 (1991).
22. W.-P. Fung-Leung *et al.*, unpublished data.
23. No statistical differences ($P < 0.05$) were found between the mutant and control mice with the Student's *t* test. The approximately twofold difference may not be significant but suggests that there may be a trend toward the mutant mice having less in vitro antigen-specific proliferation and IL-2 production compared with controls. The only other antigen responses known to date for the $CD8^{-/-}$ mutant are those toward alloantigens and viral antigens. Allo responses show no significant difference between mutants and controls in in vitro responses for $CD4^+$ MHC class II restricted T cells.
24. The amounts of MBP and adjuvant (pertussis toxin) used are about two to three times the amounts used in most protocols for inducing EAE in mice. Previous experiments have shown that a higher dose of MBP and adjuvant leads to a more severe and acute disease.
25. A. M. Brown, D. E. McFarlin, C. S. Raine, *Lab. Invest.* **46**, 171 (1982).
26. Histological analysis of brain and spinal cord sections of three mice that were moribund and killed showed typical demyelination and cellular infiltrates.
27. W. J. Karpus and R. H. Swanborg, *J. Immunol.* **143**, 3492 (1989).
28. ———, *ibid.* **146**, 1163 (1991).
29. T. R. Mossman and R. L. Coffman, *Adv. Immunol.* **46**, 111 (1989).
30. M. B. Powell *et al.*, *Int. Immunol.* **2**, 539 (1990).
31. K. Sakai *et al.*, *J. Immunol.* **137**, 1527 (1988).
32. D. Mason, *Immunol. Today* **12**, 57 (1991).
33. K. E. Ellerman, J. M. Powers, S. W. Brostoff, *Nature* **331**, 265 (1988).
34. A. Miller *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 421 (1992).
35. M. K. Racke *et al.*, *J. Immunol.* **146**, 3012 (1991).
36. S. R. Sambhara and R. G. Miller, *Science* **252**, 1424 (1991).

37. We thank M. Schilham and A. Rahemtulla for helpful comments and constructive criticisms. Also thanks to M. Schilham for technical assistance and I. Ng for secretarial help. Supported by the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Multiple Sclerosis Society (United States).

21 January 1992; accepted 2 April 1992

Role of $CD8^+$ T Cells in Murine Experimental Allergic Encephalomyelitis

Hong Jiang, Sheng-le Zhang, Benvenuto Pernis

The course of experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis, is affected by immunoregulatory T lymphocytes. When animals are immunized with encephalitogenic peptide of myelin basic protein and recover from the first episode of EAE, they become resistant to a second induction of this disease. Animals depleted of $CD8^+$ T cells by antibody-mediated clearance were used to examine the role of $CD8^+$ T cells in EAE. These cells were found to be major participants in the resistance to a second induction of EAE but were not essential for spontaneous recovery from the first episode of the disease.

We have studied EAE, induced in B10.PL ($H\text{-}2^u$) mice by immunizing (*1*) the mice with 1-9Nac, the encephalitogenic NH_2-terminal–acetylated peptide of the first nine amino acids of mouse myelin basic protein (MBP) (*2*, *3*). This disease was reproducible: in 102 mice, 94% of the animals developed EAE, with mean severity of 3.5 and maximum severity of 5.0; the mean onset day was ~20 days after the first immunization; the disease lasted ~13 days; and recovery was spontaneous. The first episode induced resistance; after a second immunization, most animals did not show any symptoms, and in those that did (only 28% of the total animals) the disease was less severe and of shorter duration (mean severity was 0.4, maximum severity was 2.5, and the duration was ~9 days). Spontaneous relapses were rare in our experiments: only 3 of 96 mice receiving the first immunization relapsed (2 weeks after recovery).

We then depleted the $CD8^+$ T cells from B10.PL mice with monoclonal antibodies (MAbs) to mouse CD8 (*4*, *5*). We tested two MAbs to CD8: HO-2.2 [murine immunoglobulin M (IgM)] and 53-6.72 (rat IgG2a). Only the latter completely depleted $CD8^+$ T cells (Fig. 1 and Table 1). This depletion was evident on day 3 after the MAb injection and lasted for ~2 weeks, which suggests that the $CD8^+$ T cells were eliminated and not merely prevented from being detected with the same labeled MAb by the unlabeled MAb that was injected in vivo. In fact, an overnight culture of spleen cells from day 10 of 53-6.72–injected mice still showed almost a complete absence of $CD8^+$ T cells. The $CD8^+$ T cells were probably eliminated by antibody-dependent cytotoxicity (ADCC), because MAb 53-6.72 could bind to the Fc receptors of mouse monocytes. This $CD8^+$ T cell depletion was followed by the slow appearance of new $CD8^+$ T cells.

Depletion of $CD8^+$ T cells had no effect on the induction of EAE in B10.PL mice, as measured by the incidence and the severity of the disease (Table 2). The spontaneous recovery was similar in $CD8^+$ T cell–depleted mice and in control mice; however, $CD8^+$ T cells began to reappear ~2 weeks after depletion, and it is possible that these $CD8^+$ T cells contributed to the final stage of the spontaneous recovery in $CD8^+$ T cell–depleted mice. Furthermore, $CD8^+$ T cell depletion performed after recovery in 15 mice did not by itself induce any relapses. But $CD8^+$ T cell depletion did affect the resistance to a second induction of EAE. In three separate experiments, each with controls, $CD8^+$ T cell depletion eliminated the resistance to a second induction of the disease in mice that had recovered from the first episode of EAE; there was also a significant effect on the severity

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032.

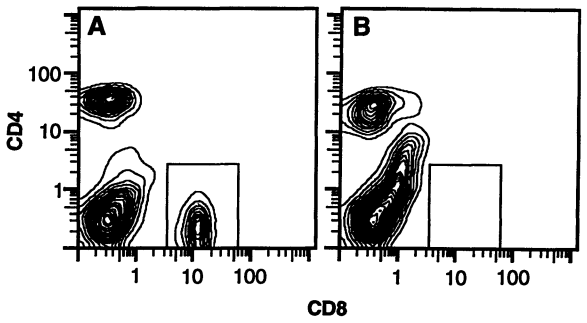

Fig. 1. CD8+ T cell depletion in B10.PL mice. Mice were injected intraperitoneally with a 40% ammonium sulfate–precipitated preparation of ascitis of a rat MAb to mouse CD8 (53-6.72; American Type Tissue Culture Collection) at 0.3 mg per mouse on two consecutive days; the effect of this MAb on both CD8+ and CD4+ T cells was monitored by checking the number of CD8+ and CD4+ T cells in the peripheral blood of injected mice every 7 days from the third day of the injection. Mononuclear cells were purified from 75 μl of peripheral blood drawn from the tail vein of the mice on a lymphocyte separation medium gradient (Organon Teknicka) and were labeled with fluorescein isothiocyanate (FITC)–conjugated MAb 53-6.72 and phycoerythrin-conjugated MAb GK1.5 (anti-CD4). Two-color fluorescence analysis was done on a FACSTAR Plus Dual Laser cell sorter and immunofluorescence microscope. (**A**) The profile of CD8+ and CD4+ T cells from normal mouse. (**B**) Analysis of mouse peripheral blood on day 3 after anti-CD8 MAb injection, in which CD8+ T cells are 0.06% and CD4+ T cells are 21% of total mononuclear cells of peripheral blood.

of the second episode (Table 3). Our data show that CD8+ T cells participate in the resistance to EAE that normally follows an acute episode of the disease in intact animals. In contrast, a previous study noted no effect of CD8+ T cell depletion on the resistance to a second induction of the disease in rats (6). The difference is probably a result of different experimental conditions. It is also possible that in rats, a species in which activated T cells can express class II major histocompatibility complex (MHC) antigens, CD4+ T suppressor cells are also involved in the immunoregulation of EAE (7, 8); double negative (CD4− CD8−) T cells may be involved as well in the regulation of EAE in this species (9).

In B10.PL mice, CD8+ T cells that were induced in response to the activated CD4+ T cells that produced the first episode of EAE were the primary cause of resistance to a second episode. The mechanism for this effect is unknown, but rats that have recovered from EAE mediated by an encephalitogenic CD4+ T cell line (S1) contain CD8+ T cells capable of lysing the encephalitogenic S1 T cells (10). Thus, the mechanism operating in our system might be one of direct lysis or suppression of the CD4+ anti-MBP peptide T cells by complementary, specific CD8+ T lymphocytes. Our depletion experiments also eliminate the possibility that, in our mice, the resistance to a second induction of EAE was due to antibodies to the 1-9Nac peptide of MBP; examination of the serum of our mice for such antibodies by enzyme-linked immunosorbent assay (ELISA) yielded only negative results.

We have no clear evidence for participation of CD8+ T cells in the spontaneous recovery from EAE (Table 2), in agreement with the observation that recovery from EAE occurs normally in T cell–deficient animals in which the disease was induced by passive administration of polyclonal encephalitogenic T cells (11, 12).

The encephalitogenic CD4+ T cells, when fully activated, cause the disease, but they also directly or indirectly suppress themselves so that EAE, in our mice, tends to be a self-limiting event. Simultaneously, during the allergic episode, the activated CD4+ T cells may induce a secondary immunization process that produces complementary suppressor CD8+ T cells. These CD8+ T cells are generated during the latter days of the allergic episode, persist after the recovery, and are responsible for the resistance to the induction of the second episode of EAE for an extended period.

We assume that the MAb to CD8 injected after recovery eliminated the CD8+ T cells that had been primed by the CD4+ T cell proliferation burst responsible for the first episode of encephalomyelitis. Thus, susceptibility to the disease was reinstated in spite of the progressive reappearance of new CD8+ T lymphocytes.

In the accompanying paper by Koh *et al.* (13), results from a different approach are reported that fit well with our data and demonstrate a role of CD8+ T cells in the immunoregulation of EAE. However, we had a much lower rate of spontaneous relapse and found no evidence that the CD8+ T cells contribute to the pathogenesis of the disease. These differences are probably a result of whole MBP being used as the immunogen by Koh *et al.* instead of the 1-9Nac MBP peptide that we used. If the 1-9Nac MBP peptide does not bind to the H-2^u class I MHC molecules, it would be unable to provide a target for pathogenic CD8+ T cells; other peptides derived from the processing of whole MBP, however, may do so.

Table 1. CD8+ T cell depletion in B10.PL mice. The percentage (mean ± SD) of total peripheral mononuclear cells that were CD8+ or CD4+ from 12 mice before and after injection of 53-6.72 (as described in Fig. 1) was determined. This MAb had no effect on CD4+ T cells; the slight increase in the proportion of CD4+ T cells corresponds to what would be expected if CD8+ T cells were eliminated.

Day*	CD4+ T cells	CD8+ T cells
0	19.3 ± 2.8	15.0 ± 1.5
3	19.8 ± 3.2	0.0
10	19.6 ± 1.5	0.4 ± 0.7
17	21.5 ± 4.1	2.8 ± 1.5
24	21.9 ± 1.0	3.4 ± 1.9
31	20.2 ± 0.8	3.1 ± 0.7
48	27.0 ± 5.1	4.3 ± 1.6

*After first injection of antibody.

Table 2. The effect of CD8+ T cell depletion on the onset of EAE in B10.PL mice. EAE induction and CD8+ T cell depletion were done as described in (1) and Fig. 1; CD8+ T cell depletion was started simultaneously with EAE induction. In the control group, there was no CD8+ T cell depletion, and an ammonium sulfate–precipitated preparation of normal rat serum was used instead of MAb 53-6.72.

Group	Incidence	Severity		Mean onset day
		Mean	Max	
Control	18/20	3.2	5.0	23.2
Depleted	15/16	3.1	5.0	21.6

Table 3. EAE after second challenge in CD8+ T cell–depleted, EAE-recovered mice. EAE induction and CD8+ T cell depletion were done as described in (1) and Fig. 1; EAE reinduction was done 2 to 4 weeks after the mice recovered from the first induction of EAE with simultaneous CD8+ T cell depletion. In the control group, EAE was reinduced with 1-9Nac MBP in EAE-recovered mice, and the mice were injected with 0.3 mg of ammonium sulfate–precipitated preparation of normal rat serum on day 1 and day 2 to serve as a control for rat anti-mouse CD8 antibody 53-6.72.

Group	Incidence	Severity		Mean onset day
		Mean	Max	
Experiment 1				
Control	1/5	0.2	1.0	25
Treated*	4/4	2.5	4.0	21
Experiment 2				
Control	2/5	0.7	2.5	22
Treated*	6/6	2.6	3.5	20
Experiment 3				
Control	2/7	0.3	1.5	19
Treated*	8/9	2.1	3.5	14
Summary				
Control	5/17 (29%)	0.4	2.5	22
Treated*	18/19 (95%)†	2.4‡	4.0	17

*Reinduced and CD8-depleted. †Chi-square test of the significance of the difference in incidence between the EAE-reinduced group and the EAE-reinduced and CD8+ T cell–depleted group; $P < 0.001$. ‡Student's t test of the significance of the difference in the mean severity between the EAE-reinduced group and the EAE-reinduced and CD8+ T cell–depleted group; $P < 0.001$.

The demonstration of the existence of regulatory CD8+ T cells in mice that recovered from the first induction of EAE will lead to the study of the specificity of these CD8+ T cells, with regard to both their cellular and molecular targets, as well as to their mechanism of function.

REFERENCES AND NOTES

1. EAE was induced in B10.PL mice (8 to 10 weeks old, Jackson Laboratory) by subcutaneously injecting 100 μg of purified 1-9Nac MBP (Ac-Ala-Ser-Gln-Arg-Lys-Pro-Ser-Gln-Arg; Ac = acetyl) emulsified with complete Freund's adjuvant (CFA). Pertussis toxin (0.1 μg, List Biological) was injected intravenously 24 and 72 hours later. Symptoms of EAE were evaluated daily [S. Zamvil *et al.*, *J. Exp. Med.* **162**, 2107 (1985)]: 0, no signs; 1, weakness of tail; 2, mild paresis of hind limbs (paraparesis); 3, severe paraparesis; 4, complete paralysis of hind limbs (paraplegia) or one side of limbs (hemiplegia); and 5, death.
2. S. Zamvil *et al.*, *Nature* **324**, 258 (1986).
3. D. Ando, J. Clayton, D. Kono, J. Urban, E. Sercarz, *Cell. Immunol.* **124**, 132 (1989).
4. C. Leclerc, M.-P. Schutze, E. Deriaud, G. Przewlocki, *J. Immunol.* **145**, 1343 (1990).
5. M. Eichelberger, W. Allan, M. Zijlstra, R. Jaenisch, P. C. Doherty, *J. Exp. Med.* **174**, 857 (1991).
6. J. D. Sedgwick, *Eur. J. Immunol.* **18**, 495 (1988).
7. W. J. Karpus and R. H. Swanborg, *J. Immunol.* **143**, 3942 (1989).
8. K. Ellerman, J. Powers, S. Brostoff, *Nature* **331**, 265 (1988).
9. D. Sun, H. Wekerle, K. Raper, D. Gold, *Cell. Immunol.* **137**, 292 (1991).
10. D. Sun, Y. Qin, J. Chluba, J. Epplen, H. Wekerle, *Nature* **332**, 843 (1988).
11. D. O. Willenborg, P. Sjollenic, G. Dante, *J. Immunol.* **136**, 1676 (1986).
12. K. Sakai, T. Namikawa, T. Kunishita, K. Yamanouchi, T. Tabira, *ibid.* **137**, 1527 (1986).
13. D.-R. Koh, W.-P. Fung-Leung, A. Ho, D. Gray, T.-W. Mak, *Science* **256**, 1210 (1992).

14. We thank D. Nitecki of Cetus Corporation for providing purified 1-9Nac peptide of MBP. Supported by a grant from Lifegroup Incorporated, Padova, Italy.

21 January 1992; accepted 7 April 1992

G Protein Activation of a Hormone-Stimulated Phosphatase in Human Tumor Cells

Ming Gui Pan, Tullio Florio, Philip J. S. Stork*

The growth-inhibiting peptide hormone somatostatin stimulates phosphotyrosine phosphatase activity in the human pancreatic cell line MIA PaCa-2. This hormonal activation was mediated by a pertussis toxin–sensitive guanosine 5′-triphosphate–binding protein (G protein) in the membranes of these cells. Activation of this G protein by somatostatin stimulated the dephosphorylation of exogenous epidermal growth factor receptor prepared from A-431 cells in vitro. This pathway may mediate the antineoplastic action of somatostatin in these cells and in human tumors and could represent a general mechanism of G protein coupling that is utilized by normal cells in the hormonal control of cell growth.

Pertussis toxin–sensitive G proteins mediate a wide range of functions within the cell, including the control of cell growth (*1*, *2*). Constitutively active mutants of these proteins have been implicated in the pathogenesis of some human neoplasms (*3*). However, the effectors that G protein pathways activate to mediate growth regulation have not been fully identified; these effectors may include phosphotyrosine phosphatases (PTPs). Inhibition of endogenous PTP activity in nontransformed cells in culture can induce cellular transformation (*4*), and the injection of exogenous PTP into *Xenopus* oocytes can reverse the mitogenic effect of insulin-stimulated tyrosine kinase activity (*5*). These findings suggest that PTP activity may counteract the actions of tyrosine kinases to promote normal cell growth, and that interfering with this activity may lead to malignant transformation.

In vitro, the enzymatic activity of PTPs far exceeds that of tyrosine kinases (*6*, *7*). Therefore, PTPs are probably regulated in vivo to maintain a balance between phosphorylation and dephosphorylation. Cellular responses to increased tyrosine kinase activity may generate rapid stimulation of specific PTPs followed by a return to resting levels. G proteins provide hormone receptors with the ability to generate rapid stimulation and termination of effector pathways.

To explore the possibility that G proteins regulate PTP activity, we examined the effects of somatostatin on the undifferentiated human pancreatic cancer cell line MIA PaCa-2 (*8*). This cell line expresses somatostatin receptors and displays hormonally regulated PTP activity (*9*). To characterize the regulation of this PTP activity, we evaluated the action of somatostatin on cell membranes and on whole cells. We measured phosphatase activity with the synthetic substrate *p*-nitrophenyl phosphate (*p*-Npp) (*10*), which releases a spectrophotometrically detectable cleavage product after incubation with phosphatases.

The MIA PaCa-2 cell may contain membrane-associated and cytoplasmic PTPs as well as serine-threonine phosphatase activity. To ensure that the assay was specific for membrane-associated PTP activity, we incubated MIA PaCa-2 membranes with the substrate *p*-Npp and the phosphatase inhibitors microcystin–leucine-arginine (M-LR) and $ZnCl_2$. M-LR is a specific inhibitor of the major serine-threonine protein phosphatases 1 and 2A (*11*). At micromolar concentrations, $ZnCl_2$ inhibits cytosolic PTPs (*12*) more effectively than membrane PTPs. These inhibitors only slightly reduced the basal phosphatase activity (less than 10%), which suggests that a low amount of serine-threonine phosphatase activity was present in our membrane preparations (*13*). The phosphatase activity that remained after inhibition by M-LR was unable to dephosphorylate specific substrates of serine-threonine phosphatases (*14*) and was completely blocked by vanadate (50 μM), an inhibitor of PTP (*15*) (Fig. 1). Therefore, only PTPs were active under the conditions of our assay.

Under conditions of substrate excess, the phosphatase activity from unstimulated membranes was proportional to the length of time and the quantity of membrane proteins assayed (*13*). Inclusion of somatostatin-14 (1 μM) doubled this activity at all times during a 1-hour assay. Vanadate completely blocked both the stimulated and the basal phosphatase activity (Fig. 1). Somatostatin was active over a wide range of concentrations; half-maximal stimulation of phosphatase activity occurred with 2 nM somatostatin. This is similar to the somatostatin concentration that produces a half-maximal effect on growth in this cell line (*16*).

We evaluated phosphatase activity in membranes incubated in the presence of guanosine 5′-triphosphate (GTP) (200 μM) and 5′-guanylylimidodiphosphate (GppNHp, 200 μM), with and without somatostatin-14 (1 μM). GppNHp and GTP (*17*) increased phosphatase activity of MIA PaCa-2 membranes (Fig. 2); the addition of somatostatin further increased this activity (Fig. 2). However, when we incubated cell membranes with the nonhydrolyzable guanine nucleotide analog guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S, 200 μM), somatostatin stimulation of phosphatase activity was completely blocked (Fig. 2). This demonstrates that the effect

M. G. Pan and P. J. S. Stork, The Vollum Institute for Advanced Biomedical Research and Department of Cell Biology and Anatomy, Oregon Health Sciences University, L474, 3181 SW Sam Jackson Park Road, Portland, OR 97201.
T. Florio, The Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, L474, 3181 SW Sam Jackson Park Road, Portland, OR 97201.

*To whom correspondence should be addressed.