T Helper Cell Subsets in Insulin-Dependent Diabetes

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Science, New Series, Volume 268, Issue 5214 (May 26, 1995), 1185-1188.

numbers are U24117 (chimp), U24118 (human), U24119 (gorilla), and U24120 (orang); alignments are available upon request.
9. See also L. C. Shimmin, H.-J. Chang, W.-H. Li, *Nature* **362**, 745 (1993). Our sequences for the human *ZFY* intron differ from that reported by Shimmin *et al.* at position 478, which represents the final T in a 12 T homopolymeric run. This may represent either a legitimate polymorphism in this region or more likely a sequencing discrepancy.
10. The topology of the most parsimonious tree was derived by exhaustive search with the PAUP package. The tree was rooted with the baboon sequence as an outgroup. The tree shown is 70 steps long [CI (consistency index) = 0.986, RI (retention index) = 0.923]; the next shortest tree, 72 steps long, results in a chimp-gorilla-human trichotomy.
11. This value was determined by using 14 My as the branching date for the orangutan lineage and 5 My as the age of the human/chimp-gorilla split, as proposed by M. Hasegawa, H. Kishino, and T. A. Yano [*J. Hum. Evol.* **18**, 461 (1989)]. The rate was derived by averaging over all pairwise comparisons.
12. M. Kreitman and R. R. Hudson, *Genetics* **127**, 565 (1991); R. R. Hudson, in *Mechanisms of Molecular Evolution*, N. Takahata and A. G. Clark, Eds. (Sinauer, Sunderland, MA, 1993, pp. 23–36.
13. The expected nucleotide diversity of Y chromosome loci may be reduced by the smaller effective population size of the Y chromosome (1/4 N_e relative to autosomal loci)—a given value of X thus leads to a fourfold reduction in predicted polymorphism relative to an autosomal locus. This effect, however, is offset by the apparently increased mutation rate for Y-linked sequences (9).
14. A. J. Berry, J. W. Ajioka, M. Kreitman, *Genetics* **129**, 1111 (1991).
15. We used a coalescence theory approach to estimate the probability of finding zero mutations in a current sample of 38 Y chromosomes as a function of the expected mutation rate, μ, and the deepest branching time, T. This probability is

$$p(0|T) = \prod_{i=1}^{37} (i/(i + \mu T))$$

where μ was estimated from the chimp, gorilla, and orangutan data as 0.135% per site per million years; given 729 sites, $\mu = 0.98$ per million years. We convert this to a Bayesian expectation for the time, given zero mutations, as

$$p(T|0) = p(0|T)p(T)/p(0) = p(0|T)dT/\int p(0|t)dt$$

and computed both an expected value for the time, T, as

$$\langle T \rangle = \int Tp(0|T)dT/\int p(0|t)dt$$

and a 95% maximum estimate for the time as T_m from

$$\int_0^{T_m} p(0|t)dt = 0.95\int_0^{\infty} p(0|t)dt$$

This yielded the values $\langle T \rangle$ = 270,000 years and T_m ~800,000 years. For a population of N Y chromosomes, $T = 2 \times$ generation time $\times N$. Thus for a 20-year generation time, the coalescence picture is one of an effective population of 7500 males. This is an exceedingly small population size for this entire 300,000 year period; it is far more likely that the coalescence model, which assumes worldwide uniform mixing and a constant effective population size, is not strictly applicable, and that humans recently fanned out around the world into groups which remained partially isolated after that time. That picture is more similar to the "star" phylogeny, each of the 38 chromosomal lineages remaining distinct after an original migration; such a star phylogeny corresponds to a $\langle T \rangle$ of 27,000 years for that original "migration."
16. C. B. Stringer and P. Andrews, *Science* **239**, 1263 (1988); M. H. Wolpoff, in *The Human Revolution, Behavioral and Biological Perspectives on the Origins of Modern Humans*, P. Mellars and C. Stringer, Eds. (Princeton Univ. Press, Princeton, NJ, 1989), pp. 62–108; D. W. Frayer, M. H. Wolpoff, A. G. Thorne, F. H. Smith, G. G. Pope, *Am. Anthropol.* **95**, 14 (1993); F. J. Ayala, A. Escalante, C. O'Huigin, J. Klein, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6786 (1994).
17. L. Vigilant, M. Stoneking, H. Harpending, K. Hawkes, A. C. Wilson, *Science* **253**, 1503 (1991); M. Hasegawa and S. Horai, *J. Mol. Evol.* **32**, 37 (1991).
18. M. Kimura, *J. Mol. Evol.* **16**, 111 (1980).
19. K. J. Matteson *et al.*, *Hum. Genet.* **69**, 263 (1985).
20. A. Chakravarti, K. H. Buetow, S. E. Antonarakis, C. D. Boehm, H. H. Kazazian, *Am. J. Hum. Genet.* **33**, 134a (1981).
21. D. Avramopoulos, A. Chakravarti, S. Antonarakis, *Genomics* **15**, 98 (1993).
22. A. Chakravarti, J. A. Phillips III, K. H. Mellits, K. H. Buetow, P. H. Seeburg, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6085 (1984).
23. S. Antonarakis *et al.*, *Hum. Genet.* **80**, 265 (1988).
24. A. Chakravarti, S. C. Elbein, M. A. Permutt, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1045 (1986).
25. W.-H. Li and L. A. Sadler, *Genetics* **129**, 513 (1991).
26. R. McCombie *et al.*, *Nat. Genet.* **1**, 348 (1992).
27. We thank D. Page for encouragement and C. Lowen for technical assistance. Supported by NIH grant GM 37997 to W.G. and by the Yale Science Development Fund.

26 October 1994; accepted 16 March 1995

T Helper Cell Subsets in Insulin-Dependent Diabetes

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It has been proposed that the development of insulin-dependent diabetes is controlled by the T helper 1 (T_H1) versus T_H2 phenotype of autoreactive T_H cells: T_H1 cells would promote diabetes, whereas T_H2 cells would actually protect from disease. This proposition was tested by establishing cultures of T_H1 and T_H2 cells that express an identical diabetogenic T cell receptor and comparing their ability to initiate disease in neonatal nonobese diabetic mice. T_H1-like cells actively promoted diabetes; T_H2-like cells invaded the islets but did not provoke disease—neither did they provide substantial protection.

Insulin-dependent diabetes mellitus is an autoimmune disease characterized by infiltration of leukocytes into the islets of Langerhans of the pancreas and breakdown of glucose homeostasis as a result of destruction of insulin-producing beta cells (*1*). The leukocyte infiltrate, termed insulitis, is a heterogeneous population, composed of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, macrophages, and dendritic cells (*2*). T lymphocytes play a primary role (*3*), but the relative contribution of $CD4^+$ and $CD8^+$ cells is uncertain, in particular the importance of each subset in provoking or perpetuating disease and whether each has a unique function (*4*). Nonetheless, there have been several reports of $CD4^+$ T cells, by themselves, instigating diabetes (*5–7*).

$CD4^+$ T lymphocytes fall into two major classes: T helper 1 (T_H1) cells, which secrete interferon γ (IFN-γ) and are primarily associated with cellular immunity; and T_H2 cells, which produce interleukin-4 (IL-4) and IL-10 and are mainly involved in humoral immunity (*8*). Several studies have correlated diabetes with T_H phenotype, leading to the idea that T_H1 cells promote disease whereas T_H2 cells protect from it, dampening the activity of T_H1 effectors (*9*). The most substantive arguments in support of this notion derive from studies in mice. Artificial introduction of lymphokines or antibodies to lymphokines that favor T_H1 or disfavor T_H2 cell development generally promotes diabetes, whereas the converse inhibits disease (*10*). Along similar lines, C57Bl mice are more likely to develop a T_H1 and less likely to develop a T_H2 response than are BALB/c mice (*11*), and transgenic animals that express a T cell receptor (TCR) reactive to an islet cell neoantigen develop diabetes on the former but not the latter genetic background (*12*). Finally, there appears to be a correlation between the amount and type of lymphokines secreted by invading lymphocytes and how aggressively the lymphocytes attack an islet graft, high IFN-γ and low IL-4 levels being associated with destruction (*13*).

However, the above set of arguments appears to be inconclusive for three major reasons. First, conflicting data do exist. For example, pancreatic expression of IL-10, a T_H2 lymphokine, actually promotes disease in nonobese diabetic (NOD) mice (*14*). Second, many of the points cited above rely on effects provoked by manipulating the levels of particular lymphokines; however, lymphokines are highly pleiotropic and control many phenomena besides T_H1 and T_H2 phenotype: the differentiation of $CD8^+$ T cells, B cell differentiation and effector diversification, natural killer cell activity, antigen-presenting cell function, and lymphocyte circulation. In addition, beta islet cells appear

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to be exquisitely sensitive to the cytotoxic effects of lymphokines (*15*). Finally, it is becoming increasingly clear that the T_H1-T_H2 split is an oversimplification. There are now several examples of antigen-experienced $CD4^+$ T cells that secrete a pattern of lymphokines distinct from the classical T_H1 or T_H2 pattern (*16*). There is also mounting evidence that αβ $CD8^+$ and γδ T cells can exhibit a T_H1-T_H2–like dichotomy in the lymphokines they secrete (*17*) and thus may be able to similarly, or even more effectively, drive the cellular or humoral arms of an immune response.

We have addressed more directly the question of whether the T_H phenotype of $CD4^+$ T cells influences the development of diabetes. T_H1 and T_H2 cells that express an identical, diabetogenic TCR were produced and their ability to provoke disease in neonatal NOD mice compared. This strategy allows evaluation of the two subsets as isolated entities and permits their comparison independently of TCR specificity. The latter point is critical given that the conditions required for T_H1 versus T_H2 cell growth could favor expansion of specificities directed at different islet cell antigens, variable in their diabetogenic potential, or specificities directed against the same antigen but with different affinities, a situation already suggested in other systems (*18*).

To generate $CD4^+$ T cells with identical TCRs but different effector phenotypes, we made use of the BDC2.5 transgenic mouse strain (6). These animals carry rearranged TCR α and β chain genes from a $CD4^+$ T cell clone, BDC2.5 (*19*), which was isolated from a diabetic NOD mouse, is specific for an islet cell antigen, and is capable of initiating disease in neonatal NOD recipients. Most T cells in this strain display the BDC2.5 TCR; allelic exclusion of the β chain is complete but of the α chain only partial (6, 7). We have now rendered the αβ T cell repertoire of the BDC2.5 strain monospecific by eliminating any contribution by endogenous TCR α genes, which we accomplished by breeding in a null mutation of the TCR α locus (Cα°) (*20*). Spleens from BDC2.5/Cα° mice contain only $CD4^+$, not $CD8^+$, T cells (Fig. 1A). These uniformly express the transgene-encoded $V_β4$ chain and most appear to be naïve, displaying a $CD25^-CD44^{low}CD62L^{high}$ phenotype (Fig. 1A); in addition, they express no CD69 and high levels of CD45RB (7). Thus, splenocytes from BDC2.5/Cα° mice could be used as a source of naïve T cells that express a single, potentially diabetogenic receptor.

Naïve $CD4^+$ T cells can differentiate into either T_H1 or T_H2 effectors, depending on the lymphokine milieu present at the time of initial activation (*21*, *22*). The T_H phenotype is stable for several months when

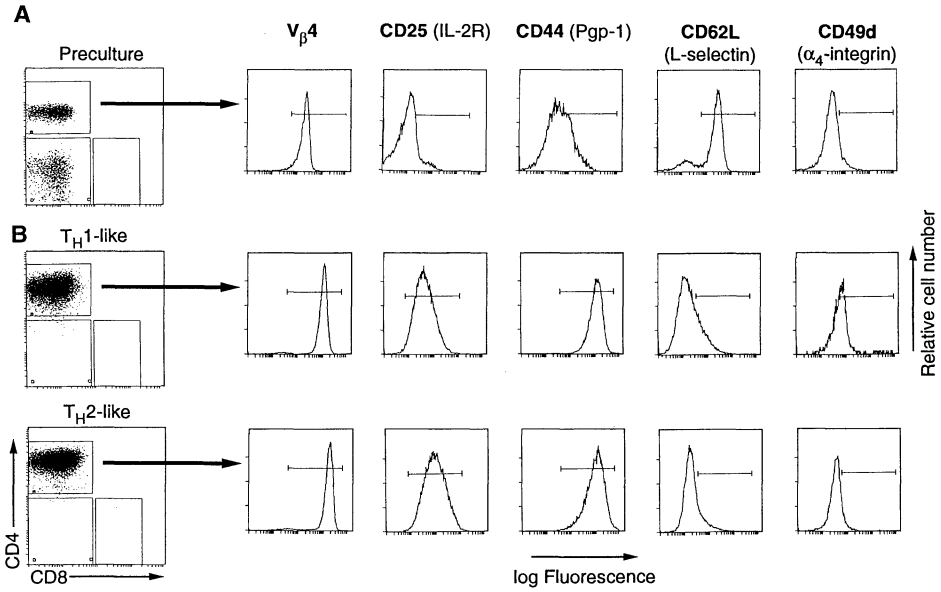

Fig. 1. Cell surface phenotype of splenocytes from BDC2.5/Cα° mice. CD4-CD8 profiles from the input splenocytes (**A**) and cultured cells (**B**) at the left; staining (*24*) profiles for $V_β4$, CD25, CD44, CD49d, and CD62L of gated $CD4^+$ T cells at the right. Preculture splenocytes contained 45 to 55% $CD4^+$ T cells and lacked $CD8^+$ T cells. After culture that favors the generation of either T_H1- or T_H2-like cells (*25*), the population was ≥95% $CD4^+V_β4^+$ T cells. Fluorescence above backround control is indicated by horizontal bar in each histogram.

the differentiated cells are transferred into mice (*22*). Following such protocols, we divided spleen cells from BDC2.5 transgenics into two parallel cultures—one activated in the presence of IL-2 and IFN-γ plus IL-4 monoclonal antibody (mAb) (T_H1-like), the other activated in the presence of IL-4 plus IFN-γ mAb (T_H2-like). After 4 days of culture in either condition, the input T cells expanded two- to fourfold and exhibited a blast-like morphology (7). Both final populations were over 95% $CD4^+V_β4^+$ T cells and were activated, as shown by their expression of CD25, upregulation of CD44, and down-modulation of CD62L [Fig. 1, compare (A) with (B)]. Though subject to some interexperimental variation, the cultured cells were largely $CD45RB^{-/low}$ and between 15 to 40% $CD69^+$ (7).

Portions of cells from each culture were then assayed for function. To test susceptibility to restimulation, we incubated them in microtiter plates coated with mAbs against $V_β4$ or CD3 and, after 36 hours, quantitated [^{3}H]thymidine incorporation (Fig. 2A). Cells from the two cultures proliferated similarly upon restimulation by anti-$V_β4$, in a dose-dependent manner; parallel results were obtained with anti-CD3 (7). Supernatants from restimulated cultures were tested for production of IL-2, IL-4, and IFN-γ (Fig. 2B). The initial culture conditions clearly generated discrete phenotypes: One condition led to cells secreting high concentrations of IFN-γ and substantial IL-2, but little or no IL-4; the other generated cells making high concentrations of IL-4 and little IL-2 or IFN-γ. Hence, we could generate cell lines with a T_H1- or T_H2-like phenotype that display the same potentially diabetogenic TCR.

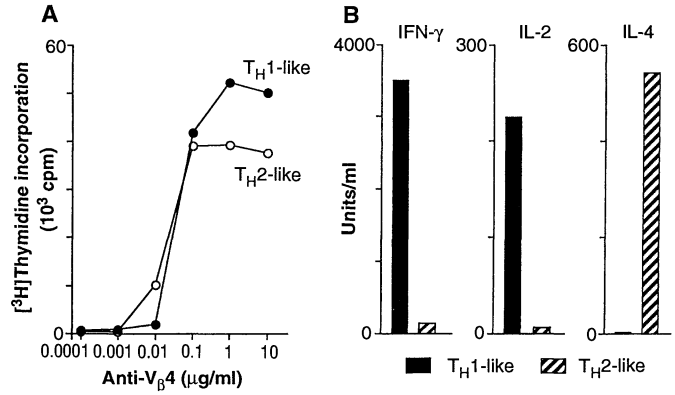

Fig. 2. Functional properties of T_H1- and T_H2-like cells. (**A**) Proliferation after restimulation. T cells were restimulated by $V_β4$ mAb (*26*). Values are displayed as the mean of triplicates; standard deviations were <15% of the mean and are not shown. (**B**) Lymphokine profiles. T cells were restimulated on anti-$V_β4$–precoated plates and supernatants removed and tested for IL-4 and IFN-γ by ELISA or IL-2 by bioassay (*27*).

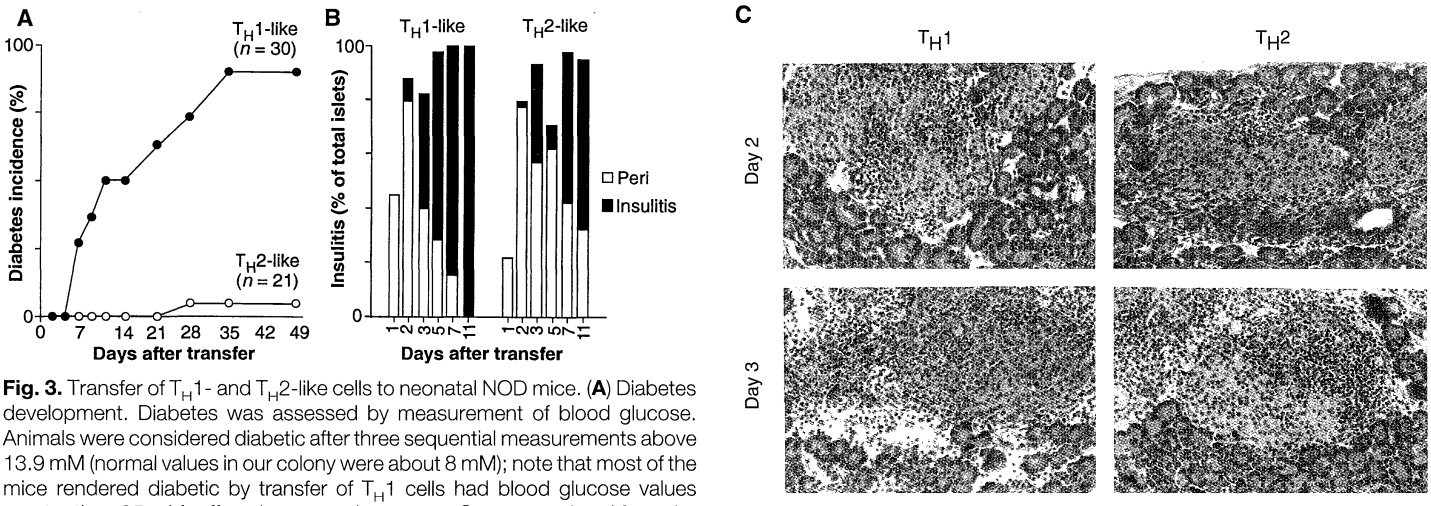

Fig. 3. Transfer of T_H1- and T_H2-like cells to neonatal NOD mice. (**A**) Diabetes development. Diabetes was assessed by measurement of blood glucose. Animals were considered diabetic after three sequential measurements above 13.9 mM (normal values in our colony were about 8 mM); note that most of the mice rendered diabetic by transfer of T_H1 cells had blood glucose values greater than 25 mM, off-scale on our glucometer. Onset was dated from the first of the sequential diabetic measurements. (**B**) Insulitis time course. Multiple hematoxylin-eosin–stained pancreatic sections were scored for peri-insulitis and insulitis. Approximately 50 islets were scored per pancreas, and several pancreata (two to four) were examined at each time point. Insulitis is shown as the percentage of infiltrated islets per total islets scored for each group and time point. (**C**) Representative islets, showing light infiltration 2 days after transfer of T_H1 or T_H2 cells, and more prominent infiltrates after 2 days.

Diabetes is rapidly induced in neonatal NOD mice after transfer of splenocytes from prediabetic BDC2.5 transgenics, but only if they are first activated in vitro (*7*). Newborn NOD mice were segregated into three groups: One received 1×10^7 to 2×10^7 T_H1-like BDC2.5/Cα° cells, another got the same number of T_H2-like cells, and the remaining group was left unmanipulated. The recipients were monitored for diabetes by measuring nonfasting blood glucose levels from the fifth or seventh day after transfer (Fig. 3A). The T_H1-like cells rapidly precipitated diabetes in almost all recipients: By day 14 after transfer, 50% (of 30) showed sustained severe hyperglycemia; by day 35, 90% were diabetic. In contrast, only 1 of the 21 recipients of T_H2-like cells developed hyperglycemia. All of the unmanipulated controls remained normoglycemic.

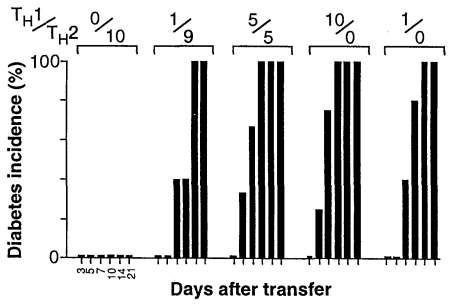

Fig. 4. Transfer of mixtures of T_H1- and T_H2-like cells. Neonatal NOD mice each received 1×10^7 total T cells, as T_H1-like cells, T_H2-like cells, or mixtures of the two, as shown. One control group received only 1×10^6 T_H1-like T cells (far right). At the indicated day, mice were tested for diabetes as described in Fig. 3. The results represent a composite of three experiments, performed with T_H1- and T_H2-like cells from BDC2.5 mice.

There are two possible explanations for the absence of diabetes in recipients of T_H2-like BDC2.5/Cα° cells. Either the T_H2-like cells could not home to the pancreatic islets or, once there, they could not initiate or sustain an anti–islet cell attack. To distinguish between these possibilities, we compared the rate and magnitude of insulitis in recipients of T_H1- and T_H2-like cells (Fig. 3B). Both T_H types were efficient invaders for the first few days, but they differed thereafter. The attack by T_H1 cells progressed so that by day 11 all of the islets exhibited insulitis, usually an overwhelming one; in contrast, the attack by T_H2-like cells did not progress much beyond the initial stage. Representative pancreas sections are shown in Fig. 3C, where the two T_H types have penetrated by day 2, but by day 3 the progression of T_H1-like cells was already more aggressive. In both cases, the infiltrates consisted largely of $CD4^+V_\beta4^+$ T cells, but also included a substantial number of surface immunoglobulin M^+ (IgM^+) B cells (*7*). Immunohistology further revealed that the IgG1 versus IgG2a phenotype of isotype-switched B cells in the islets correlated with the known influence (*23*) of T_H1 and T_H2 lymphokines on switching: IFN-γ promotes switching to IgG2a, and recipients of T_H1-like cells had an exaggerated ratio of $IgG2a^+/IgG1^+$ B cells in pancreas and spleen 7 days after transfer; IL-4 favors switching to IgG1, and mice that received T_H2-like cells had predominantly $IgG1^+$ B cells in pancreas and spleen (*7*). These data differentiate between the two possible scenarios: The lack of diabetes in recipients of T_H2-like cells was not due to an inability to home to the pancreas, but rather to an incapacity to initiate or sustain an autoimmune attack.

To determine whether T_H2 cells can inhibit T_H1 effectors, we transferred both cell types together, mixed at different ratios (Fig. 4). The recipients of a 1:1 mix developed hyperglycemia at a rate and penetrance identical to that of the recipients of T_H1-like cells alone, indicating that at this ratio the T_H2-like cells were not protective. We then reduced the number of T_H1-like cells to one-tenth the initial concentration to see whether a large excess of T_H2-like cells would protect. All the animals that received reduced numbers of T_H1-like cells alone developed diabetes, albeit at a slower rate than those that got the full complement of T_H1-like cells. When the reduced number of T_H1-like cells was combined with a ninefold excess of T_H2-like cells, all the recipients also developed diabetes, at a rate that was further reduced, but only negligibly.

Our data provide direct evidence both in support of, and contradictory to, conventional views on the role of T_H cell subsets in the development of diabetes. An important role for the T_H1 subset is substantiated by the rapid onset of disease in NOD neonates after transfer of this cell type alone. This result does not mean that no other effectors are involved, but these can now be identified systematically by transfer of T_H1-like cells into mice that lack defined leukocyte subsets. Likewise, the role of individual lymphokines and other effector molecules produced by donor or host cells can be dissected with the appropriate mutant animals. The transfer results do not advocate a role for T_H2 cells in precipitating diabetes nor, surprisingly, do they support the concept that T_H2 cells afford disease protection. T_H1-like cells could aggressively invade the islets and destroy β cells in the presence of a ninefold excess of

T_H2-like cells. These results concern only the effector phase of the disease and do not bear on the role of T_H1-T_H2 cells in earlier events. However, they are in accord with the observation that artificial expression by islet cells of IL-10, an immunosuppressive lymphokine with documented effects on T_H1 cells, actually promotes insulitis and diabetes rather than inhibits them (*14*). Some of the major challenges remaining are to prove the role of T_H1 cells in spontaneous diabetes in rodents and humans, to understand the role of accessory cells or molecules in regulating the T_H1-T_H2 balance, and to find the least interventional means to divert T_H1 cells to a T_H2 phenotype in ongoing disease.

REFERENCES AND NOTES

1. J.-F. Bach, *Endocr. Rev.* **15**, 516 (1994); M. A. Atkinson and N. K. MacLaren, *N. Engl. J. Med.* **331**, 1428 (1994).
2. L. A. O'Reilly *et al.*, *Eur. J. Immunol.* **21**, 1171 (1991).
3. A. Miyazaki *et al.*, *Clin. Exp. Immunol.* **6**, 622 (1985); M. Ogawa *et al.*, *Biomed. Res.* **6**, 103 (1985); S. Makino, M. Harada, Y. Kishimoto, Y. Hayashi, *Exp. Anim.* **35**, 495 (1986); M. Harada and S. Makino, *ibid.* **35**, 501 (1986); Y. Mori *et al.*, *Diabetologia* **29**, 244 (1986); T. Koike *et al.*, *Diabetes* **36**, 539 (1987); J. A. Shizuru, C. Taylor-Edwards, B. A. Banks, A. K. Gregory, C. G. Fathman, *Science* **240**, 659 (1988); M. Dardenne, F. Lepault, A. Bendelac, J.-F. Bach, *Eur. J. Immunol.* **19**, 889 (1989); P. Sempé *et al.*, *ibid.* **21**, 1663 (1991).
4. A. Bendelac, C. Carnaud, C. Boitard, J.-F. Bach, *J. Exp. Med.* **166**, 823 (1987); B. J. Miller, M. C. Appel, J. O'Neil, L. S. Wicker, *J. Immunol.* **140**, 52 (1988); Y. Wang, O. Pontesilli, R. G. Gill, F. G. La Rosa, K. J. Lafferty, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 527 (1991); B. J. Bradley, K. Haskins, F. G. La Rosa, K. J. Lafferty, *Diabetes* **41**, 1603 (1992).
5. E.-P. Reich, R. S. Sherwin, O. Kanagawa, C. A. Janeway Jr., *Nature* **341**, 326 (1989); K. Haskins and M. McDuffie, *Science* **249**, 1433 (1990); N. Nakano, H. Kikutani, H. Nishimoto, T. Kishimoto, *J. Exp. Med.* **173**, 1091 (1991); S. W. Christianson, L. D. Schultz, E. H. Leiter, *Diabetes* **42**, 44 (1993).
6. J. Katz, B. Wang, K. Haskins, C. Benoist, D. Mathis, *Cell* **74**, 1089 (1993).
7. J. Katz, unpublished results.
8. P. Scott, *Curr. Opin. Immunol.* **5**, 391 (1993); W. E. Paul and R. A. Seder, *Cell* **76**, 241 (1994).
9. R. S. Liblau, S. M. Singer, H. O. McDevitt, *Immunol. Today* **16**, 34 (1995).
10. N. Sarvetnick *et al.*, *Nature* **346**, 844 (1990); M. Debray *et al.*, *J. Autoimmun.* **4**, 237 (1991); I. L. Campbell, T. W. Kay, L. Oxbrow, L. C. Harrison, *J. Clin. Invest.* **87**, 739 (1991); M. J. Rapoport *et al.*, *J. Exp. Med.* **178**, 87 (1993); K. J. Pennline, E. Roque-Gaffney, M. Monahan, *Clin. Immunol. Immunopathol.* **71**, 169 (1994); S. Trembleau *et al.*, *J. Exp. Med.* **181**, 817 (1995).
11. J. P. Sypek *et al.*, *J. Exp. Med.* **177**, 1797 (1993); F. P. Heinzel, D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, M. K. Gately, *ibid.*, p. 1505; L. M. C. C. Leal, D. W. Moss, R. Kuhn, W. Müller, F. Y. Liew, *Eur. J. Immunol.* **23**, 566 (1993); L. C. C. Afonso *et al.*, *Science* **263**, 235 (1994).
12. B. Scott *et al.*, *Immunity* **1**, 73 (1994).
13. N. N. Shehadeh, F. La Rosa, K. J. Lafferty, *J. Autoimmun.* **6**, 291 (1993).
14. L. Wogensen, M.-S. Lee, N. Sarvetnick, *J. Exp. Med.* **179**, 1379 (1994); M. S. Lee, L. Wogensen, J. Shizuru, M. B. Oldstone, N. Sarvetnick, *J. Clin. Invest.* **93**, 1332 (1994); M. Moritani *et al.*, *Int. Immunol.* **6**, 1927 (1994).
15. K. Bendtzen *et al.*, *Science* **232**, 1545 (1986); C. Pukel, H. Baquerizo, A. Rabinovitch, *Diabetes* **37**, 133 (1988).
16. M. Alzona, H.-M. Jäck, R. I. Fischer, T. M. Ellis, *J. Immunol.* **153**, 2861 (1994); S. Vollmer, A. Menssen, P. Trommler, D. Schendel, J. C. Prinz, *Eur. J. Immunol.* **24**, 2377 (1994); N. K. Nanda, E. E. Sercarz, D.-H. Hsu, M. Kronenberg, *Int. Immunol.* **6**, 731 (1994).
17. A. S. Lagoo *et al.*, *Eur. J. Immunol.* **24**, 3087 (1994); D. A. Ferrick *et al.*, *Nature* **373**, 255 (1995); R. A. Seder and G. G. Le Gros, *J. Exp. Med.* **181**, 5 (1995).
18. C. Pfeifer *et al.*, *J. Exp. Med.* **181**, 1569 (1995).
19. K. Haskins *et al.*, *ibid.* **160**, 452 (1984); M. Hattori *et al.*, *Science* **231**, 733 (1986).
20. K. L. Philpott *et al.*, *Science* **256**, 1448 (1992).
21. R. A. Seder, W. E. Paul, M. M. Davis, B. Fazekas de St. Groth, *J. Exp. Med.* **176**, 1091 (1992); M. Croft, D. D. Duncan, S. L. Swain, *ibid.*, p. 1431; C.-S. Hsieh, S. E. Macatonia, A. O'Garra, K. M. Murphy, *Int. Immunol.* **5**, 371 (1993); D. D. Duncan and S. L. Swain, *Eur. J. Immunol.* **24**, 2506 (1994); E. Schmitt, P. Hoehn, T. Germann, E. Rüde, *ibid.*, p. 343.
22. S. L. Swain, *Immunity* **1**, 543 (1994).
23. C. Esser and A. Radbruch, *Annu. Rev. Immunol.* **8**, 717 (1990); F. D. Finkelman and J. Holmes, *ibid.*, p. 303.
24. Analysis by three-color flow cytometry was performed as described (*6*), after staining with either KT4-10 (anti-$V_\beta4$), 7D4 (anti-CD25), IM7 (anti-CD44), or MEL-14 (anti-CD62L), in conjunction with anti-CD4 and anti-CD8.
25. Cell suspensions were prepared from spleens of 3- to 5-week-old BDC2.5/Cα^o mice. Red cells were lysed in 0.87% ammonium chloride. Remaining cells were washed and resuspended at 5×10^5 cells/ml in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 1× nonessential amino acids, 1 mM glutamine, penicillin and streptomycin, and 50 μM 2-mercaptoethanol, divided in half, and put into culture. T_H1-like cultures: concanavalin A (5 μg/ml), recombinant murine IL-2 (r-mulL-2, 100 U/ml), r-mulFN-γ (1000 U/ml), and mulL-4 mAb (11B11, 15 μg/ml). T_H2-like cultures: concanavalin A (5 μg/ml), r-mulL-4 (500 U/ml), and mulFN-γ mAb (AN-18.17.24 and R4-6A2, 15 μg/ml). Cells were used after culture for 4 days at 37°C and 10% CO_2.
26. For anti-$V_\beta4$ stimulation, enzyme-linked immunosorbent assay (ELISA)–grade 96-well plates were coated with 10-fold serial dilutions of affinity-purified mAb KT4-10, starting from 10 μg/ml, overnight at 4°C. After washing, 5×10^4 cultured cells were added to each well. The cultures were incubated for 48 hours at 37°C, 10% CO_2 including a 12-hour pulse with 1 μCi per well of [^{3}H]thymidine before harvesting.
27. IFN-γ ELISA was performed with paired mAb AN 18.17.24 and biotin-conjugated R4-6A2, and paired IL-4 mAbs (BVD4-1D11 and biotin-conjugated BVD6-24G2 were purchased from PharMingen). ELISAs were performed as described by the manufacturer's protocol. The concentration of lymphokines was determined by comparison with a standard curve of recombinant cytokine. IL-2 concentration was determined by bioassay with the IL-2 indicator line CTLL-2.

28. We thank M. Owen and A. Hayday for the Cα^o line; A. Livingstone, J. Langhorne, and R. Ceredig for antibodies; C. Waltzinger and C. Ebel for assistance in flow cytometric analyses; the staff of the Centre de Service des Animaux de Laboratoire/Centre National de la Recherche Scientifique (CNRS), J. Hergueux, P. Michel, N. Zinck, and W. Magnant for help in maintaining the mice; P. Gerber for technical help; and T. Ding for assistance with histology. Supported by institute funds from the Institute National de la Santé et de la Recherche Medicale, CNRS, and the Centre Hospitalo–Universitaire Regional and by grants to D.M. and C.B. from the Association pour la Recherche sur le Cancer and the Juvenile Diabetes Foundation International (JDFI). J.D.K. was supported by postdoctoral fellowships from the JDFI and the Foundation pour la Recherche Médicale.

8 February 1995; accepted 25 April 1995

Requirement for Phosphatidylinositol Transfer Protein in Epidermal Growth Factor Signaling

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Stimulation of phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis is a widespread mechanism for receptor-mediated signaling in eukaryotes. Cytosolic phosphatidylinositol transfer protein (PITP) is necessary for guanosine triphosphate (GTP)–dependent hydrolysis of PIP_2 by phospholipase C–β (PLC-β), but the role of PITP is unclear. Stimulation of phospholipase C–γ (PLC-γ) in A431 human epidermoid carcinoma cells treated with epidermal growth factor (EGF) required PITP. Stimulation of PI-4 kinase in cells treated with EGF also required PITP. Coprecipitation studies revealed an EGF-dependent association of PITP with the EGF receptor, with PI-4 kinase, and with PLC-γ.

The regulation of PLC activity by tyrosine kinases occurs by a signaling mechanism distinct from that of GTP-dependent pathways (*1*). Cytosolic PLC-γ associates with specific phosphotyrosine residues on activated receptor tyrosine kinases at the plasma membrane, including phosphotyrosine residues at the COOH-terminus of the human EGF receptor (*2*, *3*). Association in vitro is mediated by the Src homology 2 domains of PLC-γ and results in tyrosine phosphorylation of PLC-γ (*3*, *4*), but these events are insufficient to stimulate phosphoinositidase activity in intact cell membranes (*5*), and substrate presentation appears to be an important criterion (*6*). It is

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