sup; daf-2(e1370) hermaphrodites at the nonpermissive temperature and examining cross progeny for dauer formation. The descendants of the cross progeny were also examined to ensure that the mutations were not unlinked, noncomplementing mutations. We also mapped many of the mutations by testing for linkage with *unc-29,* which maps near *daf-16,* or else with *daf-16*–linked restriction fragment length polymorphisms using PCR (*32*).
15. One such gene may be *daf-18.* This gene has been identified by a single mutation that suppresses both dauer formation and the life-span extension of *daf-2* mutants (*7*, *10*, *28*). However, many *daf-18* individuals show severe morphological abnormalities, suggesting that this gene has other, possibly essential, functions (*28*). The fact that we did not find any *daf-18* alleles supports this hypothesis. In addition, we note that because we screened F_2 progeny of mutagenized animals, we would have missed mutants that were maternally rescued.
16. We first attempted to clone *daf-16* by positional mapping but found that the gene was located in a gap in the physical map between cosmids AE7 and ZK39. To isolate *daf-16::Tc1* insertion mutants, we screened *daf-2(sa189); mut-6* animals for spontaneous mutants that did not become dauers when cultured at 20°C. One mutant, *mu147,* also suppressed dauer formation at 25°C. This mutation failed to complement *daf-16(m26)* and was closely linked to *unc-29*, which maps near *daf-16*. *mu147* was subsequently crossed to either *unc-29(e1072); daf-2(e1370)*; *him-5(e1490)* or *daf-2(e1370)*; *him-5(e1490)* mutants, and homozygous Daf-16(−) and Daf-16(+) recombinants were obtained. Genomic DNA was prepared from these recombinants and analyzed by Southern blot hybridization with the 1.6-kb Tc1 sequence as probe. A 6.1-kb Tc1-hybridizing fragment was detected in the Xba I–digested genomic DNA, which was present in 20 of 20 *daf-16*(−) recombinants but absent in 15 of 15 *daf-16*(+) recombinants and also absent in the wild-type strain (N2). DNA from the corresponding region was then extracted from agarose gels and circularized by self-ligation. An inverse PCR strategy was used to identify a Tc1-containing fragment with the expected size of 5.1 kb. The Tc1-specific primers used for inverse PCR were 5′-CCTTGTTCGAAGCCAGCTACAATGGC-3′ and 5′-TGATCGACTCGATGCCACGTCGTTGT-3′. The 5.1-kb PCR product was cloned into the pGEM-T vector (Promega, Madison, WI), and the 0.6-kb flanking Tc1 sequence was removed by digestion with Eco RV. The remaining sequence was then used as a probe in subsequent experiments.
17. R. H. A. Plasterk and H. G. A. M. van Luenen, in *C. elegans II,* D. R. Riddle, T. Blumenthal, B. Meyer, J. Priess, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997), pp. 97–116.
18. J. Sulston *et al., Nature* **356**, 37 (1992); R. Wilson *et al., ibid.* **368**, 32 (1994).
19. RT-PCR was performed with an SL1 primer to obtain the 5′ end of the gene and with Q_T, Q_O, and Q_I to obtain the 3′ end by rapid amplification of cDNA ends (RACE), as well as with several internal primers (positioned as shown in Fig. 2B). In addition, blast searches with sequences contained on R13H8 identified three cDNA clones (yk13f11, yk31f10, and yk32f8) in a *C. elegans* EST database**.** Complete sequences of clones yk13f11 and yk31f10 were then obtained, and both contained the longer form of the transcripts, whereas the majority of the RT-PCR products (with mixed-stage RNA preparations) contained the shorter form (see Fig. 2). In addition, the longer spliced form was also detected by RT-PCR.
20. E. Kaufmann and W. Knochel, *Mech. Dev.* **57**, 3 (1996).
21. K. Lin, J. Dorman, A. Rodan, C. Kenyon, data not shown.
22. X. Chen *et al., Nature* **389**, 85 (1997).
23. P. Flakoll, M. G. Carlson, A. Cherrington, in *Diabetes Mellitus,* D. LeRoith, S. I. Taylor, J. M. Olefsky, Eds. (Lippincott-Raven, Philadelphia, PA, 1996), pp. 121–131.
24. R. M. O'Brien and D. K. Granner, in *ibid*., pp. 234–241.
25. R. M. O'Brien *et al., Mol. Cell. Biol.* **15**, 1747 (1995).
26. D. B. Friedman and T. E. Johnson, *Genetics* **118**, 75 (1988).
27. J. Z. Morris, H. A. Tissenbaum, G. Ruvkun, *Nature* **382**, 536 (1996).
28. J. B. Dorman, B. Albinder, T. Shroyer, C. Kenyon, *Genetics* **141**, 1399 (1995).
29. S. Murakami and T. E. Johnson, *ibid.* **143**, 1207 (1996).
30. Neither gain of function (*n1046)* nor dominant negative (*sy100)* mutations in the *C. elegans* Ras homolog *let-60* affected *C. elegans* life-span (because these mutants cannot lay eggs, their gonads were ablated to prevent premature death from internal hatching). In addition, *let-60(n1046gf)* did not suppress the dauer-constitutive phenotype of *daf-2(e1370)* (J. Apfeld and C. Kenyon, unpublished data).
31. C. E. Finch, *Longevity, Senescence, and the Genome* (Univ. of Chicago Press, Chicago, IL, 1990).
32. B. D. Williams *et al., Genetics* **131,** 609 (1992).
33. M. Krause and D. Hirsh, *Cell* **49**, 753 (1987).
34. M. A. Frohman, *Methods Enzymol.* **28**, 341 (1993).
35. K. L. Clark, E. D. Halay, E. Lai, S. K. Burley, *Nature* **364**, 412 (1993).
36. S. Ogg *et al.*, *ibid.* **389**, 994 (1997).
37. We thank N. Ahmada for technical assistance with positional mapping (*16*); B. Albinder, M. Macrae, J. Reiter, T. Wang, D. Eisenstadt, I. Reichardt, and J. Blumstein for helping to isolate and characterize trimethylpsoralen- and Tc1-induced *daf-16* mutants; J. Apfeld in our laboratory for investigating the role of Ras in the *daf-2* signaling pathways (*30*); members of the Kenyon lab for discussions and comments on the manuscript; and Y. Kohara for sending us EST clones of *daf-16***.** Supported by NIH grant AG11816. C.K. is the Herbert Boyer Professor of Biochemistry and Biophysics.

19 August 1997; accepted 15 October 1997

TECHNICAL COMMENTS

Immune Response and Myoblasts That Express Fas Ligand

Henry T. Lau *et al.* (*1*) report that syngeneic myoblasts that expressed Fas ligand (FasL, CD95L) protected allogeneic islets of Langerhans from immune rejection when cotransplanted under the kidney capsule. The presumed immune privilege conferred by exogenous expression of FasL in this system appeared to be similar to the naturally occurring immune protection described in the anterior chamber of the eye (*2*), in the rodent testis (*3*), and in malignant melanoma (*4*), all of which express endogenous FasL. These studies generated considerable interest in the scientific community because they suggested a method for generating gene- or tissue-specific tolerance with broad applications to organ transplantation. In contrast with these results, others have found that exogenous FasL expression, either by tumor cells or by islets, targeted the cells for rapid destruction by neutrophils (*5*, *6*). Moreover, although a recent report showed a FasL-mediated inhibition of antibody production, it also stated that an inflammatory response was observed (*7*).

To investigate potential variables leading to these divergent findings, we carried out experiments designed to replicate closely those described by Lau *et al.* (*1*). Primary skeletal myoblasts were isolated from C3H mice and transduced with a retroviral vector that directs murine FasL expression from the LTR promoter. Functional FasL expression was confirmed by cytotoxicity to Fas-expressing Jurkat cells (*8*). Unexpectedly, transduced myoblasts underwent rapid apoptosis during differentiation, which suggests that skeletal myoblasts express Fas, in contrast with the findings of Lau *et al.* (*1*). Although shown earlier to occur in postnatal cardiac and skeletal muscle tissues (*9*), Fas expression had, to our knowledge, not been examined in cultured cells. With the use of an antibody to mouse Fas, Jo2 (*10*), we confirmed Fas expression in myoblasts of C3H (Fig. 1) and C57BL/6 strains (*11*).

To avoid Fas/FasL-mediated self-destruction of myoblasts, we generated primary myoblasts from Fas-deficient C57BL/6 lpr mice, the mouse counterpart to human autoimmune lymphoproliferative syndrome (ALPS). When transduced with the FasL vector, lpr myoblasts did not self-destruct on differentiation in vitro. Nontransduced or FasL-transduced lpr myoblasts were injected under the kidney capsule of congenic C57BL/6 mice. Mice were killed 1, 3, 7, 14, and 26 days after transplantation, and their kidneys were removed for histological examination (Fig. 2). Kidneys transplanted with nontransduced myoblasts appeared normal at all time points (Fig. 2, A, C, and E). In contrast, each kidney transplanted with FasL-expressing myoblasts had a prominent white abscess that was abundant in neutrophils; these abscesses appeared by day 1, were pronounced by day 3, and disappeared by day 26 (Fig. 2, B, D, and F, respectively). Moreover, in contrast with the findings of Lau *et al.* (*1*), co-implantation of allogeneic C3H islets of Langerhans with congenic FasL-expressing myoblasts led to accelerated destruction of the islets (Fig. 2G). Untransduced myoblasts differentiated and persisted for at least 26 days, but FasL myoblasts were destroyed by the granulocytic infiltrate (Fig. 2, H and I). It is therefore unclear how Lau *et al.* (*1*) were

able to generate FasL-expressing myoblasts that persisted as differentiated multinucleated myotubes for more than 80 days in vivo. Moreover, our data show that islet destruction is not prevented but accelerated by FasL-expressing myoblasts, presumably through a bystander effect mediated by infiltrating neutrophils.

Our findings are in direct conflict with those reported by Lau *et al.* (*1*). Subtle technical differences can perhaps be invoked; however, it is unclear from our studies how myoblasts that express both Fas and FasL can avoid apoptosis while differentiating or be available to induce apoptosis of invading lymphocytes, as proposed by Lau *et al.* Even if clones of non-Fas–expressing myoblasts were selected by Lau *et al.* (*1*) and were therefore spared from apoptosis, it remains an enigma how such FasL-expressing myoblasts escaped the granulocytic response that we observed and which resulted in premature elimination of both Fas-deficient myoblasts (lpr) and islets. It has been suggested that the exact quantity of FasL expressed may be critical in determining whether immunoprotection or immunodestruction occurs (*12*). However, we and others have found that, although low amounts of FasL expression do not result in granulocytic infiltration, these amounts also do not protect against T cell–mediated allograft rejection (*6*, *13*). FasL-specific activity is known to vary as a result of polymorphisms (*14*); however, both Lau *et al.* (*1*) and we used the C57BL/6 form of FasL known to have reduced cytotoxic potential. Taken together, these findings suggest that, although FasL may have a role along with other factors in the immune privilege of the eye and testis, expression of endogenous FasL alone is unlikely to suffice.

In support of this conclusion, most cell types and tissues that have been genetically engineered to express FasL have been shown to undergo destruction by neutrophils (*5*, *6*). Thus, FasL expression has complex consequences (*15*), and further investigation of the effects of dosage, cell context, and microenvironment are warranted. Our observations, although discouraging for transplant purposes, suggest other applications for FasL and new approaches for defining the molecular determinants requisite for immune protection.

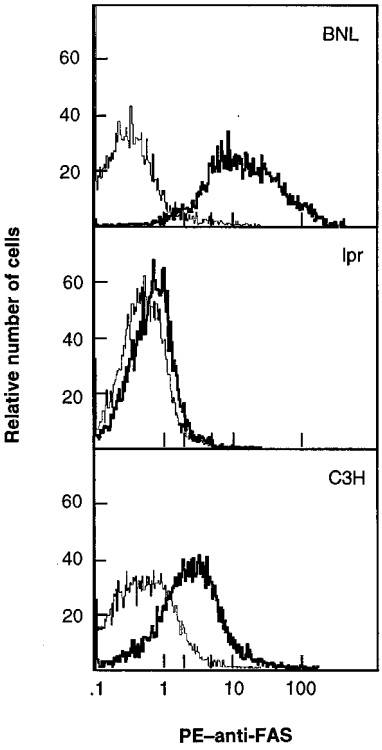

Fig. 1. Expression of Fas as analyzed by FACS. Cell populations were analyzed by FACS with the use of antibodies against murine Fas combined with phycoerythrin (PE). Thin and thick lines show FACS plots for control cells and antibody-treated cells, respectively. BNLc12 cells, derived from liver, exhibit a shift in fluorescence indicative of Fas expression and serve as a positive control (top panel). lpr primary myoblasts, which are deficient in Fas, exhibit no such shift and serve as a negative control (middle panel). C3H primary myoblasts (bottom panel) exhibit a shift in fluorescence not as great as that seen with BNL cells, but significantly higher than controls, confirming primary myoblast expression of Fas. C57BL/6 myoblasts gave FACS results indistinguishable from those of C3H myoblasts (data not shown). Methods: Cells were rinsed with phosphate-buffered saline (PBS) and incubated for 2 to 20 min in PBS containing 1 mM EDTA in order to detach the cells from the dish, and the cell suspension (2×10^5 cells) was then rinsed twice in PBS and incubated for 20 min on ice with the mouse-Fas–specific antibody, PE-labeled Jo2 antibody at 0.5 μg/ml (Pharmingen, San Diego, California) in medium containing deficient RPMI, 4% fetal bovine serume, and 10 mM Hepes. Cells were then rinsed with FACS buffer and analyzed for Fas expression by FACS for PE.

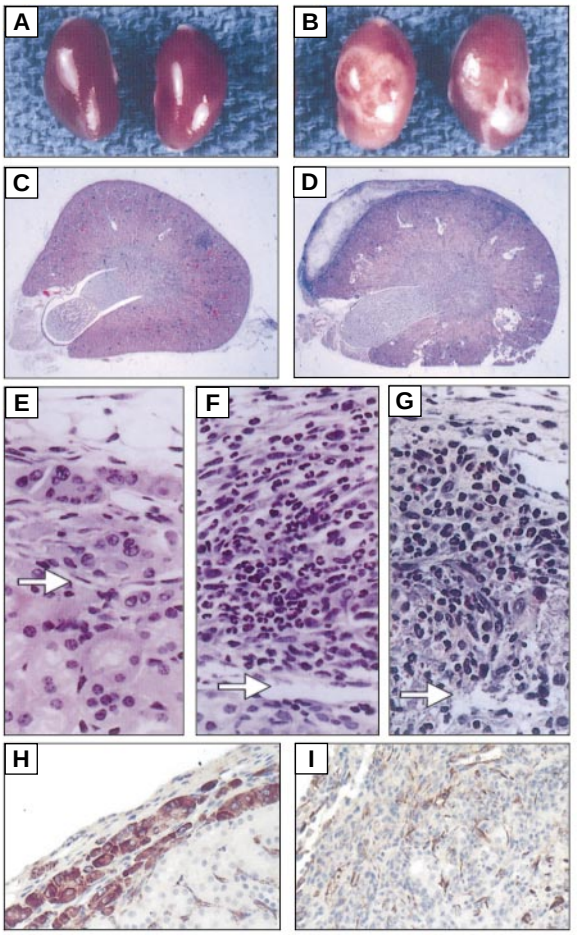

Fig. 2. Implantation into kidney. Nontransduced or FasL-transduced lpr myoblasts were injected under the kidney capsule of congenic C57BL/6 mice (2×10^6 myoblasts per kidney, 10 mice per group). (**A**, **C**, and **E**) Kidneys transplanted with nontransduced myoblasts appear normal at all time points as visualized by hematoxylin/eosin (HE) staining of sections. (**B**, **D**, and **F**) In contrast, each kidney transplanted with FasL-expressing myoblasts has a raised white lesion revealed by histochemical analysis to be abundant in neutrophils as identified by nuclear morphology. A requirement for host Fas expression for the granulocytic response was shown by the persistence over time of FasL-transduced myoblasts transplanted into Fas-deficient lpr hosts (data not shown). (**G**) Allogeneic islets of Langerhans (600-800/transplant) from C3H mice are rejected in 3 ± 1 days when co-transplanted with congenic FasL-transduced lpr myoblasts (2×10^6 per transplant) into streptozocin-induced diabetic C57BL/6 mice ($n = 3$), as determined by serum glucose measurements. By comparison, C3H islets transplanted alone are rejected in 10 ± 1 days ($P = 0.001$, Student's t test). Histology at 7 days of co-transplanted kidneys is identical to that of FasL-expressing myoblasts injected alone, and islets are no longer identifiable. Junction between normal renal tissue and the subcapsular grafts is indicated by arrows. (**H**) Immunohistochemical staining with antibodies against desmin (brown) reveals that implantation of untransduced myoblasts led to the formation of differentiated multinucleate myotubes, which were detected for at least 26 days (data not shown). (**I**) By contrast, FasL-transduced myoblasts provoke an intense granulocytic infiltrate and are destroyed within 7 days. (A and B) show grafts 3 days after transplantation; ×4 magnification; (C and D) 3 days after, ×8; (E to G) 7 days after, ×400; and (H and I) 7 days after, ×200.

Sang-Mo Kang
Department of Surgery, University of California, San Francisco, CA 94143, USA
Andreas Hofmann*
Department of Molecular Pharmacology, School of Medicine, Stanford University, Stanford, CA 94305–5332, USA
David Le
Department of Surgery, University of California
Matthew L. Springer
Department of Molecular Pharmacology, School of Medicine, Stanford University
Peter G. Stock
Department of Surgery, University of California
Helen M. Blau
Department of Molecular Pharmacology, School of Medicine, Stanford University
E-mail: hblau@cmgm.stanford.edu

*Present address: Institut für Biochemie I, Medizinische Fakultät der Universität zu Köln, 50931 Köln, Germany.

REFERENCES AND NOTES

1. H. T. Lau, M. Yu, A. Fontana, C. J. Stoeckert Jr., *Science* **273**, 109 (1996).
2. T. S. Griffith, T. Brunner, S. M. Fletcher, D. R. Green, T. A. Ferguson, *ibid.* **270**, 1189 (1995).
3. D. Bellgrau *et al.*, *Nature* **377**, 630 (1995).
4. M. Hahne *et al.*, *Science* **274**, 1363 (1996).
5. K. Seino, N. Kayagaki, K. Okumura, H. Yagita, *Nature Med.* **3**, 165 (1997).
6. J. Allison, H. M. Georgiou, A. Strasser, D. L. Vaux, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3943 (1997); S.-M. Kang *et al.*, *Nature Med.* **3**, 738 (1997).
7. H. Arai, S. Y. Chan, D. K. Bishop, G. J. Nabel, *Nature Med.* **3**, 843 (1997).
8. A. Hofmann and H. M. Blau, *Somat. Cell Mol. Genet.*, in press.
9. H. Yamada, M. Nakagawa, I. Higuchi, R. Ohkubo, M. Osame, *J. Neurol. Sci.* **134**, 115 (1995); R. Watanabe-Fukunaga *et al.*, *J. Immunol.* **148**, 1274 (1992).
10. J. Ogasawara *et al.*, *Nature* **364**, 806 (1993).
11. S.-M. Kang *et al.*, data not shown.
12. H. T. Lau and C. J. Stoeckert, *Nature Med.* **3**, 727 (1997).
13. D. Vaux, personal communication.
14. N. Kayagaki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3914 (1997).
15. D. R. Green and C. F. Ware, *ibid.*, p. 5986.
16. We thank S. Nagata for supplying cDNA for Southern blotting probes; C. Goodnow for supplying lpr mice; Z. Lin, P. Kraft, and N. Bracey for technical assistance; and N. Asher, A. Estellés, and D. Vaux for helpful discussion. Supported by a Howard Hughes Medical Institute Physician Research Fellowship to S.M.K., a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft to A.H., an NSRA (F32 HL08991) to M.L.S., and grants from NIH (R01-CA59717, R01-HD18179, and MERIT award R37-AG09521) to H.M.B.

22 August 1997; accepted 22 October 1997

Response: Kang *et al.* raise several issues with regard to engineered expression of FasL on myoblast as a means of giving immunoprotection to islet allografts. In our initial studies, we observed the expression of FasL on myoblasts from C57BL/6 mice cultured over a long term; when differentiated in vitro, these cells did not undergo apoptosis and continued to express functional FasL. In contrast, Kang *et al.* report that FasL expression of C3H myoblasts results in apoptosis after differentiation. It is unclear why FasL expression differs among different myoblast populations; perhaps apoptosis resistance is acquired during multiple passage, or susceptibility to FasL-induced apoptosis may be strain dependent. We have transfected nonobese diabetic (NOD) mice myoblasts and observed spontaneous cell death; however, when myoblasts were preselected with prolonged culture with soluble FasL, we obtained apoptosis-resistant cells that permitted subsequent functional expression of FasL. Preliminary co-transplantation experiments with allogeneic islets with these NOD FasL$^+$ myoblasts have not resulted in prolonged survival. This cell mortality may be a result of the greater complexity in the killing of primed T cells that infiltrate and destroy the islets, as one would expect to find in the diabetic NOD recipient.

With regard to the issue of neutrophilic infiltration, we have reexamined the histology of the composite grafts from our initial study on day 3 after transplantation and have observed local inflammation, but islets and myoblast were present. Histology at day 7 revealed local pockets of neutrophilic infiltration, but again, islets and myoblast were identified. By the fifth week after transplantation [as we originally observed (*1*)], there was resolution of the inflammation, and fused muscle cells were seen on histology. The prolongation of islet allograft survival we observed appears to be a bystander effect of local expression of FasL in which there is no specificity in the killing of infiltrating T cells. Thus, there may be a race between the muscle cells and islets to survive the initial inflammation and still effect apoptosis of infiltrating activated T cells directed against the allogeneic islets. Under such circumstances, transplantation of borderline numbers of islets required for correction of hyperglycemia would not result in long-term correction of the diabetic state, because the initial inflammation would result in some attrition of islets. In this regard, as noted in our initial studies (*1*), we observed fluctuation in glucose in diabetic mice receiving the highest numbers of FasL expressing myoblast (2×10^6). In retrospect, this may have represented the initial inflammation, which subsided 3 weeks after transplantation, as reflected in stabilization of glucose in the blood (*1*). The amount of FasL expression may be critical in this regard in that there may be a balance between FasL inducted local inflammation and immunoprotection (*2*).

We agree that immune privilege is more than the expression of FasL and that there are other factors at work, especially in light of our inability to extend these findings to the NOD mouse model of spontaneous diabetes. However, our initial studies showing that even a bystander effect can prolong islet allograft survival (*1*) suggest that expression of FasL in the context of alloantigen or autoantigen (as in the case of islets) may enable specific killing of T cells that are activated toward such coexpressed antigens. Although muscle cell expression of FasL in a local fashion may not be applicable across all stains or species (because of self apoptosis or destruction by neutrophils in a confined space), ectopic FasL expression (in the context of an alloantigen or autoantigen on engineered cells such as muscle) administered systemically may effect specific attenuation of an immune response. This attenuation has been demonstrated by Arai *et al.* with the use of an allogeneic tumor cell engineered to express FasL (*3*). These recent findings may help define a role for the use of engineered FasL expression in the modulation of immune response.

Henry T. Lau
Department of Pediatric Surgery, Johns Hopkins Hospital, Baltimore, MD 21287–3716, USA
C. J. Stoeckert
Department of Hematology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

REFERENCES

1. H. T. Lau, M. Yu, A. Fontana, C. J. Stoeckert Jr., *Science* **273**, 109 (1996).
2. H. T. Lau and C. J. Stoeckert, *Nature Med.* **3**, 727 (1997).
3. H. Arai, S. Y. Chan, D. K. Bishop, G. J. Nabel, *ibid.*, p. 843.

19 September 1997; accepted 22 October 1997

Immune Response and Myoblasts That Express Fas Ligand

Sang-Mo Kang, Andreas Hofmann, David Le, Matthew L. Springer, Peter G. Stock and Helen M. Blau

Science **278** (5341), 1322-1324.
DOI: 10.1126/science.278.5341.1322