link the cytoskeleton with the signal transduction pathways.

REFERENCES AND NOTES

1. K. Burridge, K. Fath, T. Kelly, T., G. Nuckolls, C. Turner, *Annu. Rev. Cell Biol.* **4**, 487 (1988).
2. B. Herman and W. J. Pledger, *J. Cell Biol.* **100**, 1031 (1985); M. Schliwa, T. Nakamura, K. R. Porter, V. Euteneuer, *ibid.* **99**, 1045 (1984); S. Kellie, T. C. Holme, M. J. Bissel, *Exp. Cell Res.* **160**, 259 (1985); T. David-Pfeuty and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6687 (1980).
3. J. A. Wilkins and S. Lin, *Cell* **28**, 83 (1982); K. Burridge and J. R. Feramisco, *Cold Spring Harbor Symp. Quant. Biol.* **46**, 587 (1982); B. M. Jockusch and G. Isenberg, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3005 (1981).
4. J. A. Wilkins and S. Lin, *J. Cell Biol.* **102**, 1085 (1986); R. R. Evans, R. M. Robson, M. H. Stromer, *J. Biol. Chem.* **259**, 3916 (1984); J. J. Otto, *Cell Motil. Cytoskeleton* **6**, 48 (1986).
5. J. A. Wilkins, M. A. Risinger, S. Lin, *J. Cell Biol.* **103**, 1483 (1986).

5a. J. A. Butler and S. Lin, unpublished data.

6. J. A. Wilkins, M. A. Risinger, E. Coffey, S. Lin, *J. Cell Biol.* **106**, 130a (1987).
7. S. Lin, M. A. Risinger, J. A. Butler, in *Cytoskeletal and Extracellular Proteins,* U. Aebi and J. Engel, Eds. (Springer Series in Biophysics, Springer-Verlag, Berlin, 1989), vol. 3, pp. 341–344.
8. M. A. Risinger and S. Lin, *J. Cell Biol.* **107**, 256a (1988).
9. J. W. Tamkun *et al.*, *Cell* **46**, 271 (1986).
10. T. Pawson, *Oncogene* **3**, 491 (1988); C. A. Koch, D. Anderson, M. F. Moran, C. Ellis, T. Pawson, *Science* **252**, 669 (1991).
11. I. Sadowski, J. C. Stone, T. Pawson, *Mol. Cell. Biol.* **6**, 4396 (1986).
12. B. J. Mayer *et al.*, *Nature* **332**, 272 (1988).
13. M. L. Stahl, C. R. Ferenz, K. L. Kelleher, R. W. Kriz, J. Knopf, *ibid.*, p. 269.
14. U. S. Vogel *et al.*, *ibid.* **335**, 90 (1988).
15. H.-C. R. Wang and T. Parsons, *J. Virol.* **63**, 291 (1989).
16. H. Hirai and H. E. Varmus, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8592 (1990).
17. C. Ellis *et al.*, *Nature* **343**, 377 (1990).
18. M. L. Wahl *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1568 (1989); B. Margolis *et al.*, *Cell* **57**, 1101 (1989); J. Meisenhelder, P.-G. Suh, S. G. Rhee, T. Hunter, *ibid.*, p. 1109.
19. C. A. Koch, M. Moran, I. Sadowski, T. Pawson. *Mol. Cell. Biol.* **9**, 4131 (1989).
20. M. Matsuda *et al.*, *Science* **248**, 1537 (1990).
21. A. B. Reynolds, S. B. Kanner, H.-C. R. Wang, J. T. Parson, *Mol. Cell Biol.* **9**, 3951 (1989).
22. M. Moran *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8622 (1990).
23. B. J. Mayer, P. Jackson, D. Baltimore, *ibid.* **88**, 627 (1991).
24. D. Anderson *et al.*, *Science* **250**, 979 (1990).
25. B. J. Druker, H. J. Mamon, T. M. Roberts, *N. Engl. J. Med.* **321**, 1383 (1989).
26. D. G. Drubin *et al.*, *Nature* **343**, 288 (1990).
27. V.-P. Lehto, V.-M. Wasenius, P. Salven, M. Saraste, *ibid.* **334**, 388 (1988).
28. D. L. Rimm and T. D. Pollard, *Gene* **75**, 323 (1989).
29. F. A. O. Marston, in *DNA Cloning: A Practical Approach*, D. M. Glover, Ed. (IRL Press, Oxford, Washington, DC, 1987), vol. 3, pp. 59–88. The peptide (AANPGGRPKEPHLHSYKEAC; residues 150 to 168) was made on a 430A Peptide Synthesizer (Applied Biosystems), with the use of solid-phase synthesis. Peptide (5 mg) was conjugated to keyhole limpet hemacyanin (5 mg) with MBS (*m*-maleimidobezoyl-*N*-hydroxysulfosuccinimide ester) [T. Kitagawa and T. Aikawa, *J. Biochem.* (*Tokyo*) **79**, 233 (1976)]. Both fusion protein (as inclusion bodies) and peptide antigens were mixed with adjuvant before immunization. Proteins (5 mg) from inclusion bodies solubilized in urea were conjugated to CNBr-activated Sepharose 4B (Pharmacia). Peptides (100 μg) were coupled to aminoethyl BioGel P-150 (Bio-Rad) beads with MBS. After binding to the respective columns, antibodies (from 0.5 to 1.0 ml of primary antiserum) were eluted at low pH in a volume of 1.5 to 3 ml and immediately neutralized. Resulting antibodies were diluted 1:10 for incubation with blots.
30. A Hinc II restriction fragment of tensin (530 bp from 1547 to 2076) was subcloned into pGEX-3X (*31*). The transformed cells (DH5α) were grown overnight, induced with isopropyl β-D-thiogalactopyranoside (0.1 mM) for 2 hours, washed in PBS, and sonicated. After centrifugation (15,000*g* for 10 min) the supernatant was incubated with beaded agarose (4%) conjugated with glutathione (Sigma) for purification of the fusion protein between glutathione transferase and SH2 domain of tensin. Confluent untransformed or RSV-transformed rat fibroblasts grown in Dulbecco's modified Eagle's medium supplemented with calf serum (10%) (iron-supplemented, HyClone) were lysed in RIPA buffer (2 ml of 1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM tris, pH 8.0) supplemented with 2 mM diisopropylfluorophosphate, 2 mM phenylmethylsulfonyl fluoride, pepstatin (5 μg/ml), leupeptin (10 μg/ml), 2.5 mM EDTA, and 100 μM sodium vanadate on ice for 20 min and clarified by centrifugation at 16,000*g*.
31. D. B. Smith and L. M. Corcoran, in *Current Protocols in Molecular Biology,* F. A. Ausubel *et al.*, Eds. (Greene and Wiley-Interscience, New York, 1990), pp. 16.7.1–16.7.8.

32. We thank R. O. Hynes for the CEF cDNA libraries; T. D. Pollard for the expression plasmids; T. Bestor, B. Geiger, H. Hanafusa, S. Hata, M. Hemler, M. Krangel, and A. Neyfakh for discussions; and C. S. Lin for preparing figures and editorial assistance. Supported by grants from the National Institutes of Health (GM 38318 to L.B.C., GM 22289 to S.L., and postdoctoral fellowships to S.D. and M.L.L.) and the Muscular Dystrophy Association (to M.L.L.). B.J.D. is a recipient of the NIH Clinical Investigator Award.

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Protection Against Malaria by Vaccination with Sporozoite Surface Protein 2 Plus CS Protein

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The circumsporozoite (CS) protein has been the target for development of malaria sporozoite vaccines for a decade. However, immunization with subunit vaccines based on the CS protein has never given the complete protection found after immunization with irradiated sporozoites. BALB/c mice immunized with irradiated *Plasmodium yoelii* sporozoites produced antibodies and cytotoxic T cells against a 140-kilodalton protein, sporozoite surface protein 2 (SSP2). Mice immunized with P815 cells that had been transfected with either SSP2 or CS genes were partially protected, and those immunized with a mixture of SSP2 and CS transfectants were completely protected against malaria. These studies emphasize the importance of vaccine delivery systems in achieving protection and define a multi-antigen sporozoite vaccine.

IMMUNIZATION WITH RADIATION-ATTENUATED sporozoites protects animals and humans against malaria (*1, 2*). Antibodies and cytotoxic T lymphocytes (CTLs) against the CS protein are thought to be primary in mediating this immunity, and immunization of mice with peptides from the *P. berghei* CS protein (*3*) or with *Salmonella typhimurium* transformed with the *P. berghei* CS protein gene (*4*) partially protects against moderate sporozoite challenge. However, no CS subunit vaccine has produced protection against *P. berghei* comparable to that induced by irradiated sporozoites or provided any protection against highly infectious 17XNL *P. yoelii* sporozoites (*5, 6*). This observation suggested that other sporozoite or liver stage antigens contribute to this $CD8^+$ T cell–dependent immunity (7).

A monoclonal antibody (MAb), Navy Yoelii Sporozoite 4 (NYS4), produced by immunization of BALB/c mice with irradiated *P. yoelii* sporozoites, recognizes the surface of *P. yoelii* sporozoites and a 140-kD protein in extracts of sporozoites (*8*); this 140-kD protein is designated SSP2 (*9*). The *P. yoelii* SSP2 gene encodes a protein of 826 amino acids. It has a sequence distinct from that of the CS protein, but, like the CS protein, it has a sequence of six amino acids tandemly repeated multiple times [QGPGAP for the CS protein; NPNEPS for SSP2 (*10*)] and an area with significant similarity to the conserved region II of CS proteins (*9*).

To determine if mice immunized with *P. yoelii* sporozoites produced CTLs against SSP2, we transfected a 1.5-kb fragment of the SSP2 gene into P815 mastocytoma cells ($H\text{-}2^d$) (*11*). This fragment encodes 477 amino acids, including two regions of short, tandemly repeated peptide sequences, PNN and NPNEPS, and the region of similarity to CS region II (*9*). The fragment does not include any sequence similar to the only known CTL epitope on the *P. yoelii* CS protein (*12*). We derived nine different

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Table 1. Protection against sporozoite challenge after immunization with SSP2 clone 3.9, CS clone 1.5, or both. Four- to six-week-old BALB/c mice were immunized five times at 2-week intervals by ip injection of 2×10^8 irradiated (100 Gy ^{137}Cs) cells of SSP2 clone 3.9, CS clone 1.5, or of each. Control mice were immunized with either pSV2neo-transfected P815 or nontransfected P815 cells. Two weeks after the final dose, sera were tested for antibodies to *P. yoelii* sporozoites by an indirect fluorescent antibody test (*8*) so that the maximum titer could be determined, and mice were challenged by iv injection of 200 *P. yoelii* (17XNL, clone 1.1) sporozoites. Naive control BALB/c mice were injected with fivefold dilutions of 200 sporozoites. Six mice were challenged in each group. We assessed protection for 14 days after challenge by examining blood smears.

Immunogen	Antibody titer	Dose of sporozoites	No. infected	Protection (%)
P815 cells	0	200	6	0
pSV2neo-P815	0	200	6	0
SSP2 clone 3.9	1:3,200	200	3	50
CS clone 1.5	1:12,800	200	2	67
SSP2 3.9 + CS 1.5	1:12,800	200	0	100
		200	6	
		40	5	
		8	6	
		1.6	2	

clones by limiting dilution. We used these clones to generate effectors and as targets in a standard chromium-release CTL assay. To produce effectors, we used each clone to stimulate in vitro spleen cells from BALB/c mice (H-2^d) that had been immunized with irradiated *P. yoelii* sporozoites. All the transfectant clones were shown to express SSP2 by an indirect fluorescent antibody test and by immunoblot (*14*), but only one of the nine clones (SSP2 3.9) could be lysed by effectors produced by the stimulation of immune spleen cells with the homologous clone (*14*). The CTLs were only present in cultures of spleen cells from immunized mice (Fig. 1A) and were antigen-specific; they did not lyse P815 cells transfected with the gene encoding the CS protein (*15*) (Fig. 1B). All cytotoxic activity was eliminated by in vitro depletion of CD8$^+$ T cells but was unaffected by depletion of CD4$^+$ T cells (*16*) (Fig. 1C). Mice immunized with irradiated *P. yoelii* sporozoites produced CTLs against not only the CS protein (*12*) but also against SSP2.

In BALB/c mice, protection induced by immunization with irradiated *P. yoelii* sporozoites was eliminated by in vivo treatment of immune mice with antibodies to CD8$^+$ T cells (anti-CD8) (7), indicating that protection is dependent on CTLs. CTLs against the CS protein adoptively transfer protection (*17*). However, the presence of CTLs and antibodies (*5*, *6*, *12*, *18–20*) to the CS protein does not indicate that mice or humans will be protected; this observation suggests that immune responses to other sporozoite or liver stage antigens contribute to irradiated sporozoite-induced protection. To determine if immunity to SSP2 is protective, we injected BALB/c mice intraperitoneally (ip) five times at 2-week intervals with 2×10^8 irradiated (100 Gy ^{137}Cs) cells of a line of P815 cells that had been transfected with the SSP2 gene and challenged with 200 *P. yoelii* sporozoites 2 weeks after the last dose. The mice produced antibodies to SSP2 and sporozoites and CTLs against SSP2 (*14*), and two of six mice did not become infected. Controls immunized with pSV2neo-transfected P815 cells or nontransfected P815 cells all became infected.

This protection was induced by immunization with mastocytoma cells expressing SSP2. In previous experiments, immunization with vaccinia (*6*), *S. typhimurium* (*5*), or pseudo-rabies virus (*18*) transformed with the *P. yoelii* CS protein gene failed to protect mice against malaria, despite induction of antibodies and specific CTLs. To determine if the lack of protection with the CS protein vaccines was a function of the expression and delivery systems, we immunized mice with irradiated P815 cells transfected with the *P. yoelii* CS protein gene (*15*). Two of four clones (CS 20.8 and CS 1.5) provided partial protection (one of six and four of six mice were protected, respectively). Having achieved only partial protection with SSP2 or CS immunization, we studied the combination. Complete protection was demonstrated after immunization with a mixture of SSP2 clone 3.9 and CS clone 1.5 (Table 1). Furthermore, this regimen induced high levels of antibodies to sporozoites (Table 1) and antigen-specific, CD8$^+$ T cell–dependent CTLs (Fig. 2).

To determine if this protection, like that after immunization of BALB/c mice with irradiated sporozoites, was dependent on CD8$^+$ T cells, we immunized mice with either SSP2 3.9, CS 1.5, or both and then treated the mice with anti-CD8$^+$ (*6*, 7). The partial immunity induced by SSP2 or the CS protein and the complete protection induced by the combination were completely reversed by depletion of CD8$^+$ T cells (Table 2). All the control mice immunized with either pSV2neo-transfected P815 cells or nontransfected P815 cells became infected.

It generally takes two to eight *P. yoelii* sporozoites to infect 50% of mice (ID_{50}) (Table 1). Therefore, 100% protection of

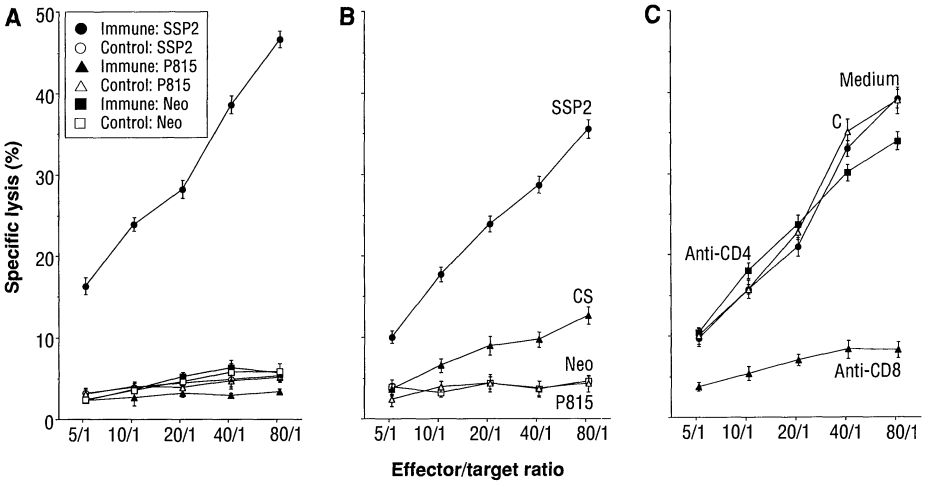

Fig. 1. Cytotoxicity for SSP2-expressing targets of spleen cells (from mice immunized with irradiated *P. yoelii* sporozoites). (**A**) Sporozoite immunization requirement for cytotoxicity. Immune (●, ▲, ■) and control (○, △, □) spleen cells stimulated in vitro with SSP2 clone 3.9 were incubated with ^{51}Cr-labeled targets: SSP2 clone 3.9 (●, ○), pSV2neo-transfected P815 cells (■, □), and P815 cells (▲, △). (**B**) Antigen specificity of cytotoxicity. Immune cells stimulated by SSP2 clone 3.9 were incubated with ^{51}Cr-labeled targets: SSP2 clone 3.9 (●), CS clone 1.5 (▲), pSV2neo-transfected cells (□), and P815 cells (△) (See Fig. 2 for data demonstrating that CS clone 1.5 is a target for CS-specific CTLs). (**C**) CD8$^+$ T cell–dependent cytotoxicity. Immune cells stimulated by SSP2 clone 3.9 treated with anti-CD8 (*24*) + C (▲), anti-CD4 (*16*) + C (■), C (△), or medium (●) were incubated with ^{51}Cr-labeled SSP2 clone 3.9. Error bars represent SEM of triplicate assays, except in (A), which represents SEM of three experiments done in triplicate.

18 mice against 200 *P. yoelii* sporozoites by the combination vaccine in two experiments (Tables 1 and 2) represents significant immunity. To further assess this immunity, we challenged six mice that had been immunized with both CS 1.5 and SSP2 3.9 with 5×10^3 sporozoites. After this challenge, at least 5×10^2 ID_{50}, four of the six were completely protected.

Table 2. Depletion of $CD8^+$ T cells eliminates vaccine-induced protection. BALB/c mice were immunized and evaluated as in Table 1, except that mice immunized with only SSP2 clone 3.9 or CS clone 1.5 received 4×10^8 cells per dose, whereas recipients of both transfectants received 2×10^8 cells of each clone. On days 10 to 13, 16, 19, 22, and 25 after last immunization, mice were injected ip with 0.5 mg of MAb 19/178, a mouse IgG2a to CD8 antigen (*24*), or an unrelated mouse IgG2a control, MAb 1B3. On day 14, six mice were challenged with 200 *P. yoelii* sporozoites for each treatment. Analysis of spleen cells from separate groups of immunized mice demonstrated that on day 14, >99% of splenic $CD8^+$ T cells were eliminated by this regimen. A total of 6/6 naive mice injected with 200 sporozoites and 6/6 injected with 40 became infected.

Immunogen	MAb	Number infected	Protection (%)
SSP2 3.9		2	67
SSP2 3.9	Control	3	50
SSP2 3.9	Anti-CD8	6	0
CS 1.5		2	67
CS 1.5	Control	1	83
CS 1.5	Anti-CD8	6	0
SSP2 3.9 + CS 1.5		0	100
SSP2 3.9 + CS 1.5	Control	0	100
SSP2 3.9 + CS 1.5	Anti-CD8	6	0
pSV2neo-P815		6	0
P815 cells		6	0

The target of SSP2-induced CTLs has not been established. We expect that, as with irradiated sporozoite (*12, 21*) and CS protein (*18*) vaccine-induced CTLs, these CTLs eliminate malaria-infected hepatocytes. Development of malaria preerythrocytic vaccines has been based on the observation that immunization of animals (*1*) and humans (*2*) with irradiated sporozoites protects against malaria. The demonstration that a MAb to the CS protein protected mice against malaria (*22*) led researchers to consider the CS protein for the target of virtually all development work on preerythrocytic stage malaria vaccines. Despite efforts to develop CS protein vaccines against rodent and human malarias, no such vaccine has provided protective immunity approaching that provided by irradiated sporozoites. Our findings provide an explanation for these observations and a method for overcoming this deficiency. Mice immunized with irradiated sporozoites induce both humoral and T cell responses, not only against the CS protein, but also against SSP2, and immunization with the CS protein and SSP2 provides additive protection against sporozoite challenge. These studies identify the components and provide a compelling rationale for the development of a multi-antigen human sporozoite vaccine.

REFERENCES AND NOTES

1. R. S. Nussenzweig, J. Vanderberg, H. Most, C. Orton, *Nature* **216**, 160 (1969).
2. D. F. Clyde, H. Most, V. C. McCarthy, J. P. Vanderberg, *Am. J. Med. Sci.* **266**, 160 (1973); K. H. Rieckmann, R. L. Beaudoin, J. S. Cassells, D. W. Sell, *Bull. WHO* **57** (suppl. 1), 261 (1979).
3. J. E. Egan *et al.*, *Science* **236**, 453 (1987); F. Zavala *et al.*, *J. Exp. Med.* **166**, 1591 (1987); J. P. Tam *et al.*, *ibid.* **171**, 299 (1990).
4. J. C. Sadoff *et al.*, *Science* **240**, 336 (1988); A. Aggarwal *et al.*, *J. Exp. Med.* **172**, 1083 (1990).
5. A. A. Lal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8647 (1987); J. Flynn *et al.*, *Mol. Microbiol.* **4**, 2111 (1990); Y. Charoenvit *et al.*, *J. Immunol.* **146**, 1020 (1991); M. Sedegah *et al.*, *Bull. WHO* **68** (suppl.), 109 (1990).
6. M. Sedegah *et al.*, *in Technological Advances in Vaccine Development*, L. Lasky, Ed. (Liss, New York, 1988), pp. 295–309.
7. W. R. Weiss *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 573 (1988).
8. Y. Charoenvit, M. F. Leef, L. F. Yuan, M. Sedegah, R. L. Beaudoin, *Infect. Immun.* **55**, 604 (1987).
9. R. Hedstrom *et al.*, *Bull. WHO* **68** (suppl.), 152 (1990); W. O. Rogers, personal communication.
10. Abbreviations for the amino acids are as follows: A, Ala; E, Glu; G, Gly; N, Asn; P, Pro; Q, Gln; and S, Ser.
11. A 1.5-kb fragment of the gene encoding the *P. yoelii* SSP2 protein (*9*) was cloned into a Sma I site of the expression vector pcEXV3 [J. Miller and R. N. Germain, *J. Exp. Med.* **164**, 1478 (1986)]. P815 mouse tumor cells ($H\text{-}2^d$ mastocytoma) (American Type Culture Collection) were cotransfected with purified plasmid DNA and pSV2neo DNA by calcium phosphate precipitation [M. Wigler *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1373 (1979); (*23*)]. Transfectants were also prepared with pSV2neo DNA alone. To obtain stable cell lines, the day after transfection we cultured SSP2-transfected P815 cells for 1 week in the presence of G418 (Geneticin, Gibco) at 2 mg/ml and then 200 μg/ml to maintain cell stability. We derived single-cell colonies of the transfected cell lines by limiting dilution and then screened them by immunofluorescence with MAb NYS4 (*8*) to detect SSP2.
12. W. R. Weiss *et al.*, *J. Exp. Med.* **171**, 763 (1990).
13. BALB/c ($H\text{-}2^d$) mice were immunized intravenously (iv) at 2-week intervals with three doses (5×10^4, 3×10^4, and then 3×10^4) of irradiated (100 Gy ^{137}Cs) 17XNL, clone 1.1, *P. yoelii* sporozoites prepared as described (*6*). Control mice were not

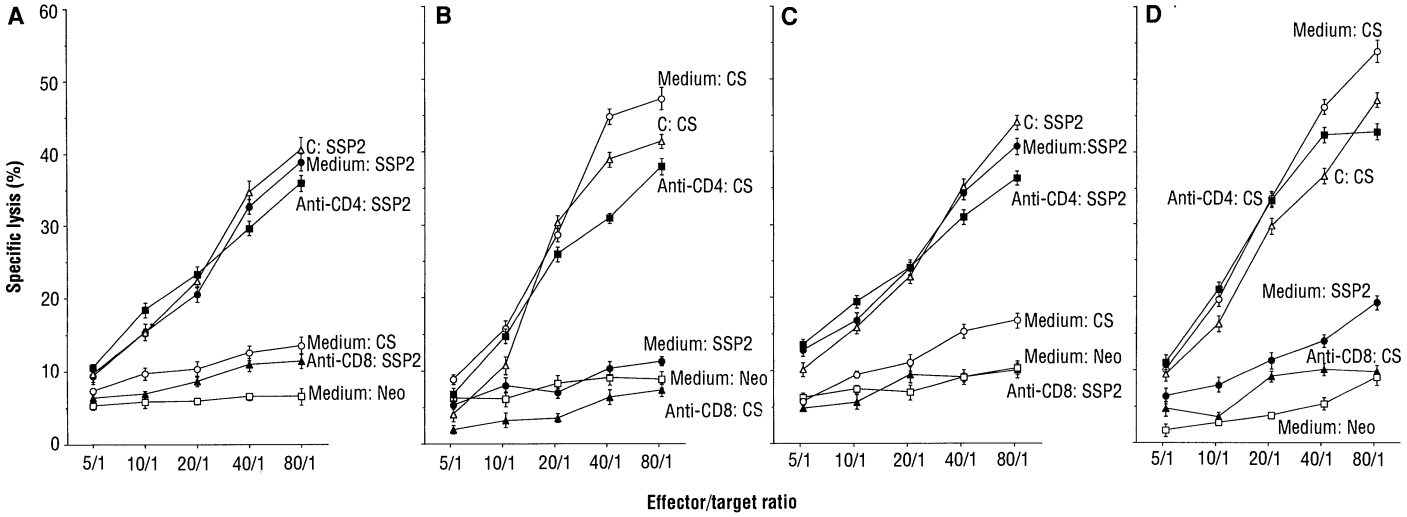

Fig. 2. Cytotoxicity of T cells from mice immunized with SSP2 clone 3.9, CS clone 1.5, or both, for homologous targets. Spleen cells from BALB/c mice immunized with five doses of SSP2 clone 3.9 (**A**), CS clone 1.5 (**B**), or the mixture of SSP2 clone 3.9 and CS clone 1.5 (**C** and **D**) and protected against a challenge of 200 sporozoites (Table 1) were stimulated in vitro with SSP2 clone 3.9 (A and C) or CS clone 1.5 (B and D). Cytotoxicity against SSP2 clone 3.9 (●), CS clone 1.5 (○), and pSV2neo-transfected P815 (□) cells was determined. In each experiment, effectors incubated with homologous targets were depleted of $CD8^+$ (▲) or $CD4^+$ (■) T cells or treated with complement alone (△) as in Fig. 1C. Error bars represent SEM of triplicate assays.

immunized. Two weeks later, 5×10^6 immune or control spleen cells were stimulated in vitro at 37°C with 2×10^5 cells of SSP2 clone 3.9 or CS clone 1.5 (*15*) that had been treated with mitomycin C (50 μg/ml, 30 min) in 2 ml of Dulbecco's modified Eagle's medium complete medium (*23*). After 6 days, cells were harvested, counted, and incubated for 6 hours at 37°C at various effector/target ratios with 5×10^3 ^{51}Cr-labeled cells of SSP2 clone 3.9, CS clone 1.5, pSV2neo-transfected P815 cells, or nontransfected P815 cells. Percent specific lysis was determined as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). The spontaneous release values (medium control counts per minute/detergent-release counts per minute) for all targets of all experiments reported in this paper were similar (mean ± SEM, 10.34 ± 1.02%).

14. S. Khusmith *et al.*, unpublished data.
15. The entire coding sequence of the CS gene of the 17XNL strain of *P. yoelii* [A. A. Lal *et al.*, *J. Biol. Chem.* **262**, 2937 (1987)] was cloned into the Pst I–Eco RI restriction site of the expression vector pcDL-SR-α-296 [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)]. This vector contains an SR-α-promotor, a splicing site for the SV40 late gene, and a polyadenylation signal from the SV40 late region. We cotransfected mouse P815 cells (H-2^d) with plasmid DNA and pSV2neo DNA by electroporation using a Gene Pulser (Bio-Rad) and standard conditions [G. Chu, H. Mayakawa, P. Berg, *Nucleic Acids Res.* **15**, 1311 (1987)]. Stable cell clones were derived as above (*11*) and screened with MAb NYS1 (*8*) to detect CS protein.
16. We achieved depletion of either CD4$^+$ or CD8$^+$ T cells by incubating cells that had been stimulated in vitro for 6 days with SSP2 clone 3.9 with either anti-CD8 MAb 19/178 (*24*) or an antibody to CD4 (anti-CD4), MAb RL172 [R. Ceredig, J. W. Lowenthal, M. Nabhoiz, H. R. Macdonald, *Nature* **314**, 98 (1985)], and complement (C).
17. P. Romero *et al.*, *ibid.* **341**, 323 (1989).
18. M. Sedegah *et al.*, unpublished data.
19. S. L. Hoffman *et al.*, *Science* **237**, 639 (1987).
20. A. Malik, J. E. Egan, R. A. Houghten, J. C. Saduff, S. L. Hoffman, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3300 (1991).
21. S. L. Hoffman *et al.*, *Science* **244**, 1078 (1989).
22. P. Potocnjak, N. Yoshida, R. S. Nussenzweig, V. Nussenzweig, *J. Exp. Med.* **151**, 1504 (1980).
23. S. Kumar *et al.*, *Nature* **334**, 258 (1988).
24. G. Hammerling, U. Hammerling, L. Flaherty, *J. Exp. Med.* **150**, 108 (1979).
25. We note with sadness that R. L. Beaudoin, our friend and colleague, passed away on 22 May 1990. We thank R. Hedstrom for providing the 1.5-kb SSP2 gene fragment, W. Rogers for advice and manuscript review, L. H. Miller for manuscript review, and S. Matheny for providing mosquitoes for the production of *P. yoelii* sporozoites. Supported by the Naval Medical Research and Development Command work unit nos. 3M161102.BS13.AK.111 and 3M162770A870.AN.121. Experiments reported here were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services, Publ. NIH 86-23, 1985).

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Biohybrid Artificial Pancreas: Long-Term Implantation Studies in Diabetic, Pancreatectomized Dogs

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Diabetic complications such as neuropathy, retinopathy, and renal and cardiovascular disease continue to pose major health risks for diabetic patients. Consequently, much effort has focused on approaches that could replace conventional insulin therapy and provide more precise regulation of blood glucose levels. The biohybrid perfused artificial pancreas was designed to incorporate islet tissue and a selectively permeable membrane that isolates this tissue from the immune system of the recipient. Biohybrid pancreas devices containing canine islet allografts were implanted in ten pancreatectomized dogs requiring 18 to 32 units of injected insulin daily. These implants resulted in good control of fasting glucose levels in six of these animals without further exogenous insulin for periods of up to 5 months.

THE USE OF CONVENTIONAL INSULIN therapy for control of glucose levels in diabetic patients is associated with a number of drawbacks. These include the need for daily injections and a high degree of patient compliance with a strict regimen of diet and exercise. Furthermore, normoglycemia may still be difficult to achieve, particularly in Type I diabetics. Although controversial, data suggest that the long-term complications associated with diabetes may result from this lack of optimal glycemic control (*1*).

Studies of diabetic patients with pancreatic transplants indicate that normoglycemia can be achieved without administration of exogenous insulin (*2*). However, many potential problems are associated with the use of whole or segmental pancreatic transplants. These include the limited availability of donor organs, the need to provide drainage for exocrine systems, and the requirement for generalized immunosuppression. Transplantation of isolated human islet allografts offers a partial solution to some of these problems, but still involves the use of immunosuppression and the difficulties in obtaining significant numbers of purified human islets. A number of studies have focused on approaches designed to decrease the immune response to transplanted islet tissue. These include modification of the donor islet tissue by ultraviolet radiation, prolonged tissue culture or use of antisera against immunocytes (*3*), treatment of the recipient with antisera targeted at subpopulations of immunocytes (*4*), and the use of immunologically privileged transplantation sites such as the brain, testicle, or thymus (*5*). Although these approaches have shown varying degrees of success when used alone and in combination in diabetic laboratory rodents, convincing proof of long-term efficacy in larger animals is generally lacking.

The concept of the biohybrid artificial implantable pancreas was developed so that these problems associated with conventional pancreas or islet transplantation could be overcome. The device utilizes a selectively permeable membrane with a nominal molecular mass cut-off of 50 kD (*6*). The tubular membrane is coiled inside a protective housing that provides a compartment for the islet cells. The membrane is connected at each end to a standard (6 mm) arterial polytetrafluoroethylene (PTFE) graft that extends beyond the housing and is used to connect the device to the vascular system as an arteriovenous shunt. Blood flow through the device from the graft and tubular membrane results in exchange of glucose, insulin, and factors necessary for sustained islet viability across the membrane between the circulating blood and the cell compartment. Antibodies and lymphocytes responsible for immune rejection are, however, excluded from the cell compartment (*7*). This immunoisolation facilitates the use of xenogeneic islets, which can be isolated in large numbers.

Various approaches to the biohybrid pancreas have been described (*8*). To establish the potential of this type of device as a viable therapy for diabetic patients, several critical elements must be demonstrated. These include (i) the ability to support long-term islet viability and function; (ii) long-term patency of the vascular shunt and biocompatibility with respect to thrombosis; (iii) protection of islet allografts or xenografts from immune rejection; (iv) elimination of the need for exogenous insulin therapy in

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