# Killer Whale Predation on Sea Otters Linking Oceanic and Nearshore Ecosystems

J. A. Estes,* M. T. Tinker, T. M. Williams, D. F. Doak

After nearly a century of recovery from overhunting, sea otter populations are in abrupt decline over large areas of western Alaska. Increased killer whale predation is the likely cause of these declines. Elevated sea urchin density and the consequent deforestation of kelp beds in the nearshore community demonstrate that the otter's keystone role has been reduced or eliminated. This chain of interactions was probably initiated by anthropogenic changes in the offshore oceanic ecosystem.

Apex predators often initiate forces that cascade across successively lower trophic levels, sometimes reaching the base of the food web (*1*). Plant-herbivore interactions vary predictably with trophic complexity in such systems, being weak or strong when the number of trophic levels is odd or even, respectively (*2*). Sea otters (*Enhydra lutris*) and kelp forests provide a well-known example of this pattern (*3*). After being protected from overhunting, recovering otter populations transformed nearshore reefs from two- to three-trophic-level systems by limiting the distribution and abundance of herbivorous sea urchins, thereby promoting kelp forest development (*4*).

Sea otters abounded across the North Pacific rim until unregulated exploitation in the maritime fur trade reduced the species to near-extinction by the early 20th century (*5*). Population regrowth began when protection was afforded under the International Fur Seal Treaty. A geographically discordant recovery pattern ensued because of the fragmented distribution of surviving colonies, the discontinuous nature of their habitat, and the otter's limited dispersal ability (*5*, *6*). Consequently, by the 1970s otter populations had recovered to near maximum densities in some areas of their historic range, were growing rapidly in others, and remained absent from still others (*7*). The sea otter's predatory role in kelp forest ecosystems was discovered by contrasting inhabited with uninhabited areas (*8*) and by observing changes over time as the uninhabited areas were recolonized and their founding populations grew (*4*, *9*). In addition to showing the influence of sea otters on North Pacific kelp forests, this approach has demonstrated a breadth of indirect effects on coastal ecosystems (*10*). The sea otter's reputation as a keystone species (*11*) is based on these interactions and processes.

Recently, sea otter populations have declined precipitously and unexpectedly over large areas of western Alaska. We first detected this decline through population surveys at Adak Island in the central Aleutian archipelago, which indicated that the otter population decreased ~25% per year through the 1990s, resulting in nearly an order-of-magnitude overall reduction by 1997 (Fig. 1). Additional surveys of Little Kiska, Amchitka, and Kagalaska Islands all show population declines of similar timing and rate to that which occurred at Adak (Fig. 1). Aerial surveys of the Aleutian archipelago conducted by the U.S. Fish and Wildlife Service in 1965 and 1992 further indicate that these declines are occurring throughout the region (*12*). The concurrent and widespread nature of these declines strongly suggests a causal link with the oceanic environment.

Demographic explanations for the sea otter population declines are limited to reduced fertility, increased mortality, or redistribution. Of these, reduced fertility and redistribution can be excluded. Studies of radio-tagged sea otters at Amchitka Island in 1992–94 and Adak Island in 1995–96 show that birth rates of adult females and pup survival rates from birth to weaning were similar to those of stable populations. Redistribution is equally unlikely because the declines were synchronous over large areas—there have been no population buildups on some islands to account for the losses on others—and radio-tagged otters at Amchitka and Adak islands provided no indication of redistribution during the declines (*13*). From this we conclude that the sea otter population declines were caused by increased mortality.

Three lines of evidence point to increased predation by killer whales (*Orcinus orca*) as the reason for this mortality. First, although killer whales and sea otters have been observed in close proximity for decades, the first attack on a sea otter was seen in 1991. Subsequently, nine more attacks have been reported (*14*). We evaluated the likelihood that this cluster of recent observations was due to chance alone by summing the number of person-days spent in the Aleutian Islands by our research team before and after 1990 (3405 person-days before; 4005 after), estimating the attack rate from the post-1990 data (0.0015 attacks per day), and then calculating the probability of no attacks being seen before 1990 if the attack rate remained constant over the 27-year period. By modeling the expected number of observed attacks as a Poisson process, the probability of zero attacks being seen before 1990 is 0.006 (*15*).

Second, we evaluated the impact of killer whales on sea otter populations at Adak Island by contrasting otter population trends and survival rates between Clam Lagoon, an area uniquely inaccessible to killer whales, and adjacent Kuluk Bay, an open coastal environment (Fig. 2). Sea otter numbers were stable from 1993 through 1997 in Clam Lagoon, whereas in Kuluk Bay they declined by 76%. In 1995, we marked 17 otters in Clam Lagoon and another 37 in Kuluk Bay with flipper tags and surgically implanted radio transmitters in order to compare their behavior and demography. There was virtually no movement of the marked animals between these areas. However, through year 1 of the study, the disappearance rate of sea otters in Kuluk Bay (65%) was greater than five times that of Clam Lagoon (12%), a trend that continued through year 2.

Finally, we estimated how many otters must have been eaten by killer whales to drive the decline rates, and then compared the actual number of observed attacks with the expected number of observed attacks based on this estimate. This analysis was done for the area between Kiska and Seguam Islands. Before the onset of the decline, an estimated 52,656 otters inhabited this area (*16*). Life table statistics (age-specific birth and death rates) were estimated from data collected during earlier field studies to construct a Leslie matrix for a stationary population. We then added an age-constant death rate (*17*) from killer whale predation sufficient to reduce the population by 78% over 6 years—the observed rate and magnitude of decline at Adak. The simulation was run by holding the number of individuals that died from killer whale predation constant over time, which produced a loss estimate of 6788 otters per year. The expected number of observed attacks produced by this approach is 5.05 for this 6-year period (*18*). This compares favorably with the 6 attacks that were seen.

Disease, toxins, and starvation, which are three other causes of elevated mortality in wildlife populations, can be dismissed as causes of the population declines. Any one of these should have produced substantial numbers of beach-cast carcasses, whereas very

J. A. Estes, Biological Resources Division, U.S. Geological Survey, A-316 Earth & Marine Sciences Building, University of California, Santa Cruz, CA 95064, USA. M. T. Tinker, Glenside Ecological Services, 79 High Street, Victoria, British Columbia, V8Z 5C8, Canada. T. M. Williams, Department of Biology, University of California, Santa Cruz, CA 95064, USA. D. F. Doak, Environmental Studies Board, University of California, Santa Cruz, CA 95064, USA.

*To whom correspondence should be addressed. E-mail: jestes@cats.ucsc.edu

few were found. Marked increases in sea urchin biomass during the population decline at Adak (Fig. 1) are further evidence against starvation, because sea urchins are the principal prey of sea otters in the Aleutian Islands (*19*). Although we looked specifically for signs of disease, none were found (*20*). Elevated contaminant concentrations have been reported in the Aleutian Islands (*21*), but subsequent analyses from 39 sites across the Aleutian archipelago have shown that these are restricted to a few small areas (*22*), which is inconsistent with the widespread declines in otter numbers.

The collective evidence thus leads us to conclude that increased killer whale predation has caused the otter declines. Although the population size and status of killer whales in the Aleutian Islands are unknown, these animals are commonly seen. From the energetic requirements of free-ranging killer whales and the caloric value of sea otters, we estimate that a single killer whale would consume 1825 otters per year and thus that the otter population decline could have been caused by as few as 3.7 whales (*23*).

Strikingly rapid changes in the kelp forest ecosystem have accompanied the sea otter population declines (Fig. 1). In 1987, when otters at Adak Island were near equilibrium density, the kelp forests were surveyed at 28 randomly selected sites (*4*). Otters were still numerous at Adak in 1991, when five of these sites were randomly chosen for the measurement of plant tissue loss to herbivory (*24*). Using similar procedures at the same sites in 1997, we resurveyed the kelp forest and repeated the measurements of plant tissue loss to herbivory. Over the 10-year interim, sea urchin size and density increased to produce an eight-fold increase in biomass, while kelp density declined by more than a factor of 12 (Fig. 1). The average rate of kelp tissue loss to herbivory increased from 1.1% per day in 1991 to 47.5% per day in 1997 (Fig.1). Observations made in August of 1997 revealed similar changes at Kiska, Amchitka, and Kagalaska Islands.

Killer whales and sea otters have co-inhabited the west-central Aleutian archipelago for much of the past half century, and probably for millennia before. Thus, it is necessary to ex-

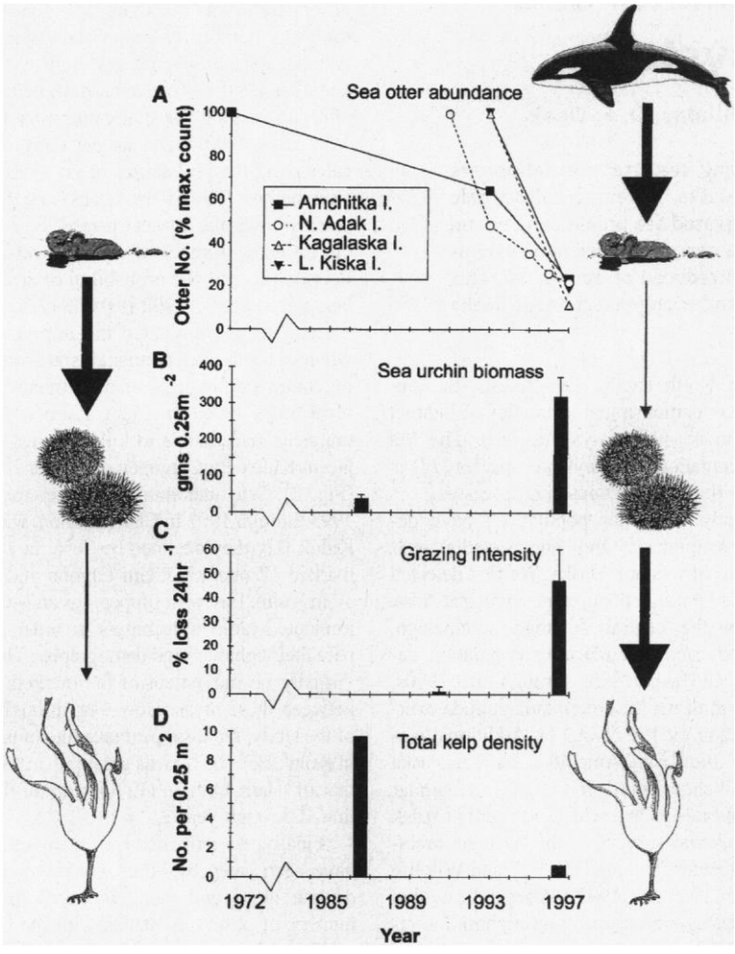


**Fig. 1.** (**A**) Changes in sea otter abundance over time at several islands in the Aleutian archipelago and concurrent changes in (**B**) sea urchin biomass, (**C**) grazing intensity, and (**D**) kelp density measured from kelp forests at Adak Island. Error bars in (B) and (C) indicate 1 SE. The proposed mechanisms of change are portrayed in the marginal cartoons—the one on the left shows how the kelp forest ecosystem was organized before the sea otter's decline and the one on the right shows how this ecosystem changed with the addition of killer whales as an apex predator. Heavy arrows represent strong trophic interactions; light arrows represent weak interactions.

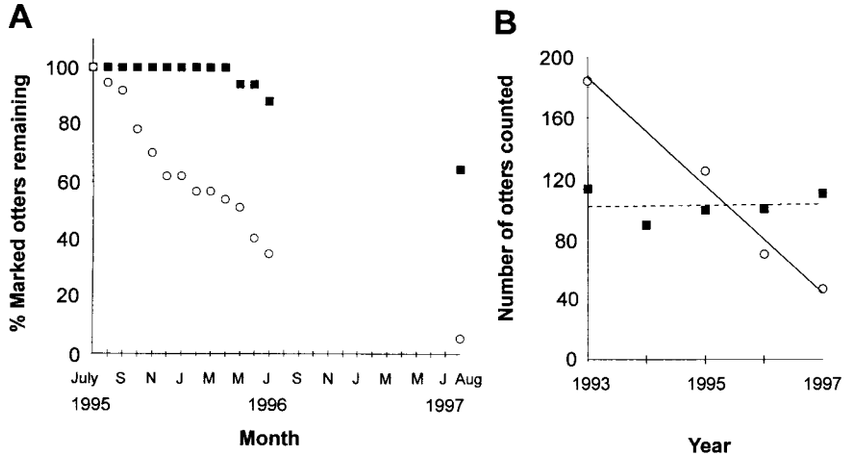


**Fig. 2.** Population trends and survival rates of sea otters in Clam Lagoon (solid squares) and adjacent Kuluk Bay (open circles), Adak Island, Alaska. (**A**) The rate of population change $r$, calculated as the slope of the linear best fit to the natural log of the number of otters counted versus year, for Kuluk Bay between 1993 and 1997 was −0.345 (SE = 0.058), which is significantly different from 0 ($R^2$ = 0.946, $P$ = 0.027). In Clam Lagoon, the rate of change over this same period was 0.006 (SE = 0.034), which is not significantly different from 0 [$R^2$ = 0.011, $P$ = 0.867; statistical power to detect $r \geq 0.1$ = 0.9]. The measured rates in Kuluk Bay and Clam Lagoon differed significantly ($\chi^2$ = 27.26, 1 df, $P < 0.001$). (**B**) Survival rates of marked sea otters differed significantly between Clam Lagoon (0.88 year$^{-1}$) and Kuluk Bay (0.35 year$^{-1}$; $\chi^2$ = 13.52, 1 df, $P < 0.001$).

plain why the behavior of killer whales toward sea otters has recently changed. The most likely explanation is a shift in the prey resource base for killer whales. Some killer whale groups or individuals feed on marine mammals (*25*), including Steller sea lions and harbor seals, and populations of both these species recently have collapsed across the western North Pacific. Sea lion populations began to decline in the late 1970s, and their numbers had reached minimum levels in the Aleutian islands by the late 1980s (*26*), a time that coincides with the onset of otter declines. Although the exact cause of the pinniped decline is uncertain (*27*), it probably relates to reduced abundance and altered species composition of their prey (*28*). Recent population declines of piscivorous marine birds are consistent with this explanation (*29*). Why forage fish stocks have shifted is not well understood, although the change was likely caused by some combination of effects from the region's burgeoning fisheries, increased ocean temperature, and depletion of baleen whales (*30*).

Regardless of the ultimate cause, sea otter population declines and the consequent collapse of kelp forest ecosystems almost certainly have been driven by events in the offshore oceanic realm. Our proposed explanation involves a chain of ecological interactions, beginning with reduced or altered forage fish stocks in the oceanic environment, which in turn sent pinniped populations into decline. Pinniped numbers eventually became so reduced that some of the killer whales who once fed on them expanded their diet to include sea otters. This shift in killer whale foraging behavior created a linkage between oceanic and coastal ecosystems and in so doing transformed coastal kelp forests from three- to four-trophic-level systems, thereby releasing sea urchins from the limiting influence of sea otter predation. Unregulated urchin populations increased rapidly and overgrazed the kelp forests, thus setting into motion a host of effects in the coastal ecosystem.

Parts of this scenario are well documented, others are more speculative, and still others have yet to be evaluated. Nonetheless, the data are sufficient to make several points of broader ecological significance. First, our findings afford evidence of the often underappreciated importance that uncommon and transient species can have in controlling community structure, demonstrating further that such species can link interactions across ecosystems. Although intersystem linkages are becoming increasingly well known (*31*), this example is unusual because the linkage is formed through the activities of a top-level carnivore. Additionally, our results are relevant to understanding food web dynamics, because they demonstrate that adding another apex predator to a system under top-down control has predictable effects on plant populations at the base of the food chain. Finally, results from this long-term study have implications for both the approach to and scale of other ecological field studies. The events reported here could not have been chronicled or even detected in a short-term study, were unanticipated, and thus seem poorly suited for analysis by a priori hypothesis testing. These points emphasize the potential significance of large-scale ecological events and the consequent need for large-scale approaches in ecological research.

## References and Notes


1. S. R. Carpenter and J. F. Kitchell, *The Trophic Cascades in Lakes* (Cambridge Univ. Press, Cambridge, 1993).
2. N. G. Hairston *et al.*, *Am. Nat.* **94**, 421 (1960); S. D. Fretwell, *Oikos* **50**, 291 (1987); M. E. Power, *Science* **250**, 811 (1990).
3. P. D. Steinberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8145 (1995).
4. J. A. Estes and D. O. Duggins, *Ecol. Monogr.* **65**, 75 (1995).
5. K. W. Kenyon, *North American Fauna* **68**, 1 (1969).
6. M. L. Riedman and J. A. Estes, *Biol. Rep. U.S. Fish Wildl. Serv.* **90** (**14**), 1 (1990).
7. L. M. Rotterman and T. Simon-Jackson, in *Selected Marine Mammals of Alaska*, J. W. Lentfer, Ed. (PB88-17462, National Technical Information Service, Springfield, VA, 1988), pp. 237–275.
8. J. A. Estes and J. F. Palmisano, *Science* **185**, 1058 (1974).
9. G. R. Van Blaricom and J. A. Estes, Eds., *The Community Ecology of Sea Otters* (Ecological Studies No. 65, Springer-Verlag, NY, 1988).
10. D. O. Duggins *et al.*, *Science* **245**, 170 (1989); P. K. Dayton, *Fish. Bull.* **73**, 230 (1975); D. O. Duggins, *Ecology* **61**, 447 (1980); D. C. Reed and M. S. Foster, *ibid.* **65**, 937 (1984); J. A. Estes, in *Aquatic Predators and Their Prey*, S. P. R. Greenstreet and M. L. Tasker, Eds. (Fishing News Books, Oxford, 1996), pp. 65–72.
11. M. E. Power *et al.*, *Bioscience* **46**, 609 (1996).
12. By 1965, otter populations had recovered to pre-exploitation levels at most of the Aleutian islands, from Kiska in the west to Adak in the east (*5*). Of the 21 islands in this region that were surveyed in both 1965 and 1992, sea otter counts decreased at all but one, for an average reduction of 58%. The 1965 data are from (*5*); the 1992 data are from T. J. Evans *et al.*, *Technical Report MMM 97-5* (U.S. Fish and Wildlife Service, Anchorage, AK, 1997).
13. Among resightings of radio-tagged otters at Adak (1635 resightings of 52 otters) and Amchitka (3711 resightings of 98 otters), the maximum distances moved were 4.31 and 6.95 km, respectively. From 1992 to 1997, most of the marked animals that were lost from these populations disappeared suddenly and without a trace, after being seen regularly in predictable locations through months of study.
14. B. B. Hatfield *et al.*, *Mar. Mamm. Sci.*, in press.
15. This probability was calculated from the Poisson probability density function $f(x) = e^{-\mu}\mu^x/x!$, for $\mu = 5.1$ (the expected number of attacks seen) and $x = 0$ (the number of attacks actually seen).
16. This number was obtained from counts made during a 1965 aerial survey (*5*) and adjusted upward by a factor of 5.62 to account for the proportion of animals that were not seen. The adjustment factor was calculated from a 1972 estimate of sea otter abundance at Amchitka Island [estimate, 6432; from J. A. Estes, in *The Environment of Amchitka Island*, M. L. Merritt and R. G. Fuller, Eds. (TID-26712, U.S. Energy Research and Development Administration, Springfield, VA, 1977), pp. 511–526] divided by the number of otters counted at Amchitka in the 1965 aerial survey (1144).
17. The age-constant death rate was inferred from the age-constant rates of otter disappearance seen in our field studies of marked sea otters at Adak Island.
18. The expected number of observed attacks was calculated as $N(t/T)(a/A)$, where $N = 40{,}728$ otters, which is the estimated number eaten by killer whales between 1991 and 1997; $t = 21{,}677$ hours, which is the number of person-hours of field time spent by our research team during this period; $T = 52{,}560$ hours (that is, 6 years); $a = 1$ km, which is the observer's sighting window [that is, two times the maximum distance from observers that attacks have been seen (*14*)]; and $A = 3327$ km, which is the area's coastal length.
19. J. A. Estes *et al.*, in *Worldwide Furbearer Conference Proceedings*, J. A. Chapman and D. Pursley, Eds. (Univ. of Maryland Press, Frostburg, MD, 1981), pp. 606–641.
20. Gross observation and hematological analyses of 66 sea otters captured at Adak, Amchitka, Kiska, and Kanaga Islands during the summer of 1997 failed to provide any known sign of disease. All of these animals appeared to be in excellent health (D. Jessup, Senior Wildlife Veterinarian, California Department of Fish and Game, Santa Cruz, CA, personal communication).
21. J. A. Estes *et al.*, *Mar. Poll. Bull.* **34**, 486 (1997).
22. S. Reese, thesis, University of California, Santa Cruz (1998).
23. We have estimated that 40,000 sea otters would have to have been eaten to drive the observed decline. The minimal number of killer whales necessary to consume this number of otters was determined by measuring the caloric value of sea otters; estimating the field metabolic rate of killer whales, discounted for assimilation efficiency; and then equating these values to estimate the number of sea otters needed to fuel a wild killer whale. The caloric content of adult sea otters, determined by adiabatic bomb calorimetry of homogenized carcasses, averaged 1.81 ± 0.04 kcal $gm^{-1}$ of wet weight. Field metabolic rate (FMR) was 7934 watts (W) for female and 11,800 W for male killer whales (51 to 59 kcal $kg^{-1}$ of killer whale per day). Values for FMR were based on field metabolic rates of odontocetes (D. P. Costa and T. M. Williams, unpublished data) and their basal metabolism [B. Kriete, thesis, Univ. of British Columbia (1995)]. Our estimate of killer whale FMR compares with the 30 to 62 kcal $kg^{-1}$ $day^{-1}$ reported by L. G. Barrett-Lennard *et al.* [*Report for the North Pacific Universities Marine Mammal Consortium* (Univ. of British Columbia, Vancouver, BC, Canada, 1994)], R. W. Baird [thesis, Simon Frasier University, Vancouver, BC, Canada, (1994)], and B. Kriete [thesis, Univ. of British Columbia, Vancouver, BC, Canada, (1995)]. The caloric value of sea otters compares with a range of 0.78 to 3.55 kcal $gm^{-1}$ of wet weight for fish and other marine mammals that make up the killer whale diet. An adult male sea otter weighing 34 kg provides 61,540 kcal (34,000 gm × 1.81 kcal $gm^{-1}$ of wet weight); a 23-kg adult female otter provides 41,630 kcal. From this, we calculated that an adult female killer whale feeding exclusively on sea otters would need three male or five female sea otters per day, and an adult male would require five male or seven female otters per day. The average consumption rate (five otters per whale per day) was divided into the sea otter loss estimate to determine how many killer whales would be needed to account for the losses. Based on this approach, 3.7 killer whales feeding exclusively on sea otters would be sufficient to drive the population decline.
24. These measurements of plant tissue loss were obtained by placing preweighed pieces of tissues from blades of the four most common kelp species—*Alaria fistulosa*, *Laminaria groenlandica*, *Agarum cribrosum*, and *Thalassiophyllum clathrus*—on the seafloor and recording their change in mass over 24 hours relative to that of adjacent caged controls. Five replicates were done for each species at each site.
25. J. R. Heimlich-Boran, *Can. J. Zool.* **66**, 565 (1988); J. K. B. Ford *et al.*, *Killer Whales: The Natural History and Genealogy of* Orcinus orca *in British Columbia and Washington State* (Univ. of British Columbia Press, Vancouver, BC, 1994).
26. A. E. York *et al.*, in *Metapopulations and Wildlife Conservation*, D. R. McCullough, Ed. (Island Press, Washington, DC, 1996), pp. 259–292.
27. M. A. Pascual and M. D. Adkison, *Ecol. Appl.* **4**, 393 (1994).
28. R. L. Merrick *et al.*, *Can. J. Fish. Aquat. Sci.* **54**, 1342

(1997); R. L. Merrick and D. G. Calkins, *U.S. Department of Commerce, NOAA Tech. Rep. NMFS* **126**, 153 (1996).
29. A. Springer, *Alaska Sea Grant Report* **93-01**, 14 (Univ. of Alaska, Fairbanks, AK, 1993).
30. National Research Council, *The Bering Sea Ecosystem* (National Academy Press, Washington, DC, 1996).
31. G. A. Polis and D. R. Strong, *Am. Nat.* **147**, 813 (1996); G. A. Polis *et al.*, *Annu. Rev. Ecol. Syst.* **28**, 289 (1997).
32. Supported by grants from NSF and the Office of Naval Research, and by a contract from the U.S. Navy. The Alaska Maritime National Wildlife Refuge provided logistic support for work in the Aleutian Islands. We thank C. Dominick, B. Konar, J. Meehan, K. Miles, and J. Stewart for field assistance and K. Clifton, D. Croll, E. Danner, L. Fox, B. Lyon, R. Ostfeld, M. Power, and A. Springer for comments on the manuscript.

27 May 1998; accepted 20 July 1998

# Induction of Antigen-Specific Cytotoxic T Lymphocytes in Humans by a Malaria DNA Vaccine

**Ruobing Wang,* Denise L. Doolan,* Thong P. Le,† Richard C. Hedstrom, Kevin M. Coonan, Yupin Charoenvit, Trevor R. Jones, Peter Hobart, Michal Margalith, Jennifer Ng, Walter R. Weiss, Martha Sedegah, Charles de Taisne, Jon A. Norman, Stephen L. Hoffman‡**

CD8$^+$ cytotoxic T lymphocytes (CTLs) are critical for protection against intracellular pathogens but often have been difficult to induce by subunit vaccines in animals. DNA vaccines elicit protective CD8$^+$ T cell responses. Malaria-naïve volunteers who were vaccinated with plasmid DNA encoding a malaria protein developed antigen-specific, genetically restricted, CD8$^+$ T cell–dependent CTLs. Responses were directed against all 10 peptides tested and were restricted by six human lymphocyte antigen (HLA) class I alleles. This first demonstration in healthy naïve humans of the induction of CD8$^+$ CTLs by DNA vaccines, including CTLs that were restricted by multiple HLA alleles in the same individual, provides a foundation for further human testing of this potentially revolutionary vaccine technology.

During 1990–1994, the administration of "naked" plasmid DNA encoding a specific protein antigen was shown to induce expression of the protein in mouse myocytes (*1*), to elicit antibodies against the protein (*2*), and to manifest protection against influenza (*3*) and malaria (*4*) that was dependent on CD8$^+$ T cell responses against the expressed protein. Hundreds of publications have now reported the efficacy of DNA vaccines in small and large animal models of infectious diseases, cancer, and autoimmune diseases (*5*).

DNA vaccines elicit antibodies and CD4$^+$ T cell responses in animals, but their major advantage at the immunological level has been their capacity to induce antigen-specific CD8$^+$ T cell responses, including CTLs, which is a major mechanism of protection against intracellular pathogens. Important to our method of developing a malaria vaccine is the induction of CD8$^+$ T cell responses against *Plasmodium falciparum* –infected hepatocytes (*6*). The lysis of cells in a standard chromium release assay was used as a surrogate for antihepatocyte responses, because it has been established that CD8$^+$ CTLs, which recognize peptide-pulsed target cells, also recognize and eliminate parasite-infected hepatocytes (*6*). On the basis of our work with rodents (*4*, *7*) and our work and that of others with rhesus monkeys (*8*, *9*), we have developed a plan for manufacturing and testing the efficacy of a multigene *P. falciparum* liver-stage DNA vaccine in humans (*10*). This has been contingent on establishing that DNA vaccination of humans is safe and induces antigen-specific, genetically restricted, CD8$^+$ T cell–dependent CTLs. Recently, the presence of CTL responses in human immunodeficiency virus (HIV)–infected individuals after vaccination with plasmid DNA encoding the *nef*, *rev*, or *tat* genes or the *env* and *rev* genes of HIV was reported (*11*). Interpreting these results is difficult because of the concurrent HIV infection, which has been demonstrated to prime individuals for a CTL response that is independent of immunization.

Accordingly, 20 healthy, malaria-naïve adults were recruited and randomized into four dosage groups of five individuals. Three injections of 20, 100, 500, or 2500 μg of plasmid DNA encoding the *P. falciparum* circumsporozoite protein (PfCSP) (*12*) were administered at 4-week intervals in alternate deltoids (*13*). The details of recruitment, safety, and tolerability were reported elsewhere (*14*). To assess CTL responses, we collected peripheral blood mononuclear cells (PBMCs) from each volunteer before vaccination, 2 weeks after the second immunization, and 2 and 6 weeks after the third immunization. These cells were either assayed while fresh for recall antigen-specific CTL responses (*15*) or were frozen (*16*) for subsequent study. In parallel, CTL assays were carried out with PBMCs from nonimmunized control volunteers. Cytolytic activity was assessed after both primary and secondary in vitro restimulation against HLA-matched and HLA-mismatched PfCSP-specific and control targets. The percent lysis and the percent specific lysis were determined as described (*15*). The most sensitive and specific method (*17*) for demonstrating the presence of CTLs was with effector cells that were expanded in vitro by exposure to cells infected with canary pox (ALVAC) expressing the PfCSP (*18*) and with target cells that were sensitized with PfCSP-derived synthetic peptides (*19*). There was no apparent difference between the primary and secondary assays (*20*) or between the fresh and frozen specimens (*21*).

For logistical reasons, fresh PBMCs were studied only before vaccination and after the second immunization in the 20- and 100-μg-dosage groups but were studied before vaccination and after all immunizations in the 500- and 2500-μg-dosage groups, with the exception of one individual (*13*). For 14 individuals, adequate amounts of frozen PBMCs were available for further analysis. A typical pattern of CTL responses is presented in Fig. 1A. These responses were peptide-specific and genetically restricted because there was little or no recognition of autologous targets that were incubated with the control peptide or of HLA class I–mismatched targets that were incubated with the specific peptide. This activity was shown to be CD8$^+$ T cell–dependent by restimulating

R. Wang, Malaria Program, Naval Medical Research Institute, Bethesda, MD 20889–5607, USA, and Henry M. Jackson Foundation, Rockville, MD 20852, USA. D. L. Doolan, Malaria Program, Naval Medical Research Institute, Bethesda, MD 20889–5607, USA, and Pan American Health Organization, Regional Office of the World Health Organization, Washington, DC 20037, USA. T. P. Le, R. C. Hedstrom, Y. Charoenvit, T. R. Jones, W. R. Weiss, M. Sedegah, S. L. Hoffman, Malaria Program, Naval Medical Research Institute, Bethesda, MD 20889–5607, USA. K. M. Coonan, Medical Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21701, USA. P. Hobart, M. Margalith, J. A. Norman, Vical, San Diego, CA 92121, USA. J. Ng, C. W. Bill Young Department of Defense Bone Marrow Donor Program, Naval Medical Research Institute, Bethesda, MD 20889–5607, USA. C. de Taisne, Pasteur-Merieux Connaught-France, 69007 Lyon, France.

*These authors contributed equally to this work.
†Present address: Clinical Research, Pasteur-Merieux Connaught-USA, Swiftwater, PA 18370, USA.
‡To whom correspondence should be addressed. E-mail: hoffmans@nmripo.nmri.nnmc.navy.mil