that reacts with all TCRαβ molecules and an antibody to clonotype that reacts with the transgenic TCR were used. The antibody to TCR and the Désiré-1 antibody to clonotype can simultaneously bind to the same TCR molecule. By double staining, the expression of the Des-TCR on anti-K^b–specific T cells and TCR expression on T cells of other specificities was compared. As shown in Fig. 3, only Des-TCR T cells down-regulated their receptors in the thymectomized animals; T cells with receptors of other specificities were unaffected. These experiments indicate that the rapid increase in K^b expression on the hepatocytes led to complete down-regulation of TCR on already tolerant cells. Four weeks after LPS injection, K^b levels on hepatocytes were low again and the level of Des-TCR was up-regulated, demonstrating the reversibility of the down-regulation.

Whereas in other studies the expression of small amounts of foreign antigen, including K^b, on pancreatic islet β cells was ignored by the immune system (6), expression of even lower amounts of K^b on hepatocytes in the uninduced mice rendered the T cells tolerant and partially down-regulated the TCR. It is possible that the different anatomical sites are responsible for this discrepancy. The use of thymus chimeras ruled out any contribution by the thymus. The rapid increase of K^b on hepatocytes was accompanied by a further down-regulation of TCR, possibly by deletion. These findings demonstrate not only that the dose of antigen influences the degree of tolerance on a single-cell level but also that tolerant T cells are not necessarily refractory to further contact with the tolerogen—they can still be driven into a deeper state of tolerance. Thus, tolerance may be the result of multiple interactions with antigen.

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19. LPS (10 mg) was injected intraperitoneally on days 1 and 3 (mRNA was isolated on day 4). The oligonucleotides used for amplification annealed to conserved H-2K allele sequences. After Southern (DNA) blot, PCR products derived from endogenous K^d (upper row) and transgenic K^b (lower row) were identified by specific oligonucleotides. Details of the PCR protocol have been published elsewhere (*3*). The CRP gene was within a 13-kb fragment containing 9 kb of the 5′ untranslated region. A 5.5-kb fragment containing the coding region of the K^b gene was inserted into the Eco RI site 5′ to the ATG of the CRP gene. This construct was used to establish CRP-K^b transgenic mice. To exclude elevated LPS-levels as a result of infection, we housed the CRP-K^b animals under specific pathogen-free conditions.
20. Hepatocytes were isolated after liver perfusion according to the method of Seglen (*10*). After staining with the biotinylated antibody to K^b (B8-24.3) (*11*) followed by fluoroscein isothiocyanate (FITC)–labeled streptavidin (Boehringer Mannheim, Germany), 10,000 events were collected in a FACScan Cytometer (Becton Dickinson).
21. For staining, the following antibodies were used: FITC-labeled anti-CD2 monoclonal antibody (mAb) RM2.-5 (*12*), phycoerythrin (PE)-conjugated anti-CD4 mAb GK1.5 (*13*), and biotinylated mAb H57-597 (*14*) to all β chains of TCRs. Cy-chrome–conjugated streptavidin (Pharmingen, San Diego) was used as a secondary reagent.
22. The FITC-conjugated mAb Désiré-1 (*15*) was used to detect the transgenic Des-TCR. Biotinylated mAb H57-597 (*14*) followed by Cy-chrome–conjugated streptavidin served as a pan-TCRαβ reagent, and the PE-conjugated mAb GK1.5 (*13*) was used for gating out $CD4^+$ cells.
23. Donor tail skin, including the epidermis and dermis, was transferred onto the tail of a recipient from which an appropriate piece of skin had been removed (*16*). If 75% of a graft was necrotic it was considered as rejected. To establish atxbm chimeras, we removed the thymuses of adult transgenic mice when the mice were 6- to 8-weeks-old under Ketanest-Rompun anesthesia using an adaptation of the method of Lo and Sprent (*17*). After 2 weeks the mice were grafted with two to four neonatal thymus lobes ($H\text{-}2^{dxk}$) under the kidney capsule followed 2 to 4 weeks later by lethal irradiation (9.5 Gy) and reconstitution with 5×10^6 T cell–depleted bone marrow cells as indicated [anti Thy-1.2 treatment with the specific antibody 30H12 (*18*) and complement-mediated lysis]. Eight weeks later the mice were skin grafted.

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Natural Vertical Transmission of Western Equine Encephalomyelitis Virus in Mosquitoes

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The mechanism by which western equine encephalomyelitis (WEE) virus and other mosquito-borne alphaviruses (Togaviridae) survive during periods of vector inactivity is unknown. Recently, three strains of WEE virus were isolated from adult *Aedes dorsalis* collected as larvae from a salt marsh in a coastal region of California. This provides evidence of vertical transmission of WEE virus in mosquitoes in nature. Vertical transmission in *Ae. dorsalis* and closely related mosquito species may be an important mechanism for the maintenance of WEE virus in temperate regions in North America where horizontal transmission of the virus is seasonal.

Western equine encephalomyelitis virus (Togaviridae: *Alphavirus*) has been recognized as a cause of summertime epidemics and epizootics of encephalitis in humans and horses, respectively, in the western United States and Canada since the 1930s (*1*, *2*). The summertime transmission cycle of WEE virus in rural agricultural areas of western North America principally involves *Culex tarsalis* mosquitoes as vectors and passerine birds as amplifying hosts (*1*). In temperate climates, annual transmission of the virus ends when vector mosquitoes cease blood-feeding in autumn, which coincides with a decrease in ambient temperature and daylight. There is evidence that WEE virus persists in endemic areas between transmission seasons (*1*, *2*). Howev-

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Table 1. Results of cross-neutralization tests with five western equine encephalomyelitis (WEE) serocomplex viruses, one eastern equine encephalomyelitis (EEE) serocomplex virus, and three unidentified viruses (indicated by question marks) isolated from *Aedes dorsalis* collected in California during 1991–1992. Antibody preparations were as described (*11*).

Virus	Strain	Antibody to						
		WEE Fleming	WEE BFS-1703	DAV-3340	Highlands J	Fort Morgan	Sindbis	EEE
WEE	Fleming	5,120	2,560	5,120	<20	<20	80	<20
WEE	BFS-1703	5,120	2,560	10,240	<20	40	160	<20
?	DAV-3340	5,120	1,280	10,240	<20	<20	80	<20
?	DAV-5875	5,120	2,560	20,480	<20	<20	160	<20
?	DAV-5880	2,560	2,560	20,480	<20	<20	80	<20
Highlands J	B-230	320	160	1,280	160	20	160	<20
Fort Morgan	CM4-146	80	20	40	<20	1,280	40	<20
Sindbis	EgAr 339	80	40	320	<20	<20	2,560	<20
EEE*	NJ/60	<20	<20	<20	<20	<20	<20	5,120

*EEE virus is the representative of the EEE serocomplex.

er, despite intensive study it is not known how the virus overwinters locally and is introduced into a subsequent summertime transmission cycle. Postulated mechanisms include viral persistence in hibernating or diapausing adult female Cx. *tarsalis*, vertical transmission of virus to progeny of Cx. *tarsalis* or other vector species, and persistence of virus in chronically infected vertebrate hosts (*1*). We report here evidence of vertical transmission of WEE virus in mosquitoes in nature.

In 1991 and 1992, *Aedes dorsalis* mosquitoes were collected from a salt marsh at Morro Bay in San Luis Obispo County, California, as part of an intensive field investigation of the ecology of a newly recognized California serogroup virus (Bunyaviridae: *Bunyavirus*) (*3*). Larvae and pupae were collected by dipping; adults were collected by sweep netting or in light traps that had been baited with dry ice. The mosquitoes were transported alive to the Department of Entomology at the University of California, Davis, where larvae and pupae were mass-reared to the adult stage at room temperature. Trapped, netted, and reared adults were segregated by species and gender into pools of up to 50 mosquitoes each and stored at −80°C. These mosquito pools were transported on dry ice to the School of Public Health, University of California, Berkeley, where they were tested for virus by plaque assay in Vero cell cultures (*4*). Viral isolates were identified by plaque reduction neutralization (Nt) tests with the use of hyperimmune mouse ascitic fluids prepared to each of six alphaviruses, including five members of the WEE serocomplex and one member of the eastern equine encephalomyelitis (EEE) serocomplex (*5*).

A total of 29,841 *Ae. dorsalis* adults (14,038 collected as immatures and 15,803 collected as adults) was assayed for virus in 666 pools. Three viral isolations were made: DAV-3340, from a pool of males collected as larvae on 14 August 1991; DAV-5875, from a pool of females collected as larvae on 25 August 1992; and DAV-5880, from a pool of males collected as larvae on 25 August 1992. These isolations can be explained only by vertical transmission because each isolation was made at a time when stock WEE virus was not being used in the laboratory and because DAV-3340 was reisolated from the original mosquito pool (no attempt was made to reisolate DAV-5875 or DAV-5880). In one-way Nt tests, the three field isolates were indistinguishable from each other (Table 1). In two-way Nt tests, isolate DAV-3340 was indistinguishable from the Fleming and BFS-1703 strains of WEE virus. Thus, we concluded that the three field isolates from *Ae. dorsalis* are strains of WEE virus.

The isolations of WEE virus from *Ae. dorsalis* are evidence that the virus is transmitted vertically in mosquitoes in nature. In addition, these viral isolations augment the limited experimental and field evidence for vertical transmission of alphaviruses in mosquitoes. Low levels of vertical transmission of Ross River virus in *Aedes vigilax* (*6*) and Sindbis virus in *Aedes australis* (*7*) have been demonstrated in the laboratory; EEE virus has been isolated on one occasion from field-collected *Culiseta melanura* larvae (*8*); and there has been one report each of the isolation of EEE and Semliki Forest (SF) viruses from field-caught male mosquitoes (*9*). However, the isolation of EEE and SF viruses from males could be attributed to contamination of pools with parts of infected female mosquitoes or to venereal transmission.

The importance of vertical transmission of WEE virus in *Ae. dorsalis* to the maintenance of the virus in temperate areas of North America is unknown. As stated previously, summertime transmission of WEE virus in North America principally involves Cx. *tarsalis* and passerine birds. However, the results of serological and virological surveys suggest that mammalophilic *Aedes* mosquitoes and wild small mammals are sometimes involved in the transmission of WEE virus (*2*, *10*). Because *Aedes* mosquitoes overwinter as diapausing eggs or larvae in temperate areas of North America, it is possible that WEE virus survives winter conditions in the immature stages of these mosquitoes and that the bird–Cx. *tarsalis* cycle is initiated from virus amplified during the spring in a mammal-*Aedes* cycle. For example, a summertime transmission cycle involving *Aedes melanimon* (a member of the *Ae. dorsalis* complex) and the blacktail jackrabbit (*Lepus californicus*) has been documented in parallel with the bird–Cx. *tarsalis* cycle in the Sacramento Valley of California (*10*). Because Cx. *tarsalis* feeds on rabbits (*1*) and jackrabbits develop viremias sufficient to infect Cx. *tarsalis* (*1*, *10*), it is possible that WEE virus was transferred from the jackrabbit–*Ae. melanimon* cycle to the bird–Cx. *tarsalis* cycle. In this situation, vertically infected female *Ae. melanimon* may have initiated the transmission of WEE virus in the springtime. However, laboratory studies to demonstrate vertical transmission of WEE virus in a field population of *Ae. melanimon* were negative (*1*). Similarly, tests on over 1475 field-collected male *Ae. melanimon* and 46,267 adult *Ae. melanimon* reared from field-collected larvae and pupae failed to provide evidence for vertical transmission of WEE virus (*1*).

In California, almost all documented human cases of WEE have occurred in residents of the inland rural agricultural valleys (*1*). The apparent lack of WEE viral activity in coastal areas of California, including San Luis Obispo County, may be related to a limited number of Cx. *tarsalis* mosquitoes, the principal endemic and epidemic vector of WEE virus, or to the relatively cool ambient temperatures in these areas. One or both of these factors may impede amplification of WEE virus and, consequently, the occurrence of human cases of WEE in coastal California.

The discovery of vertical transmission of WEE virus in *Ae. dorsalis* is sufficient cause to reexamine the role of vertical transmission in the maintenance of this virus in temperate areas of North America. Particular attention should be given to North American members of the *Ae. dorsalis* complex (*Ae. dorsalis*, *Ae. melanimon*, and *Ae. campestris*) because WEE virus has been isolated from each member of the complex, and members of the complex occur throughout the geographical distribution of WEE virus in North America. Viral tests should be done on large numbers of *Aedes*

mosquitoes collected as immatures from known areas of WEE viral activity in order to provide further evidence for vertical transmission of WEE virus in nature. Finally, consideration should be given to factors that may influence the efficiency of vertical transmission of WEE virus in mosquitoes. For example, the temperature and salinity of the aquatic environment in which immature mosquitoes develop may influence the efficiency of vertical transmission.

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Lack of Acidification in *Mycobacterium* Phagosomes Produced by Exclusion of the Vesicular Proton-ATPase

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The success of *Mycobacterium* species as pathogens depends on their ability to maintain an infection inside the phagocytic vacuole of the macrophage. Although the bacteria are reported to modulate maturation of their intracellular vacuoles, the nature of such modifications is unknown. In this study, vacuoles formed around *Mycobacterium avium* failed to acidify below pH 6.3 to 6.5. Immunoelectron microscopy of infected macrophages and immunoblotting of isolated phagosomes showed that *Mycobacterium* vacuoles acquire the lysosomal membrane protein LAMP-1, but not the vesicular proton–adenosine triphosphatase (ATPase) responsible for phagosomal acidification. This suggests either a selective inhibition of fusion with proton-ATPase–containing vesicles or a rapid removal of the complex from *Mycobacterium* phagosomes.

Mycobacterium spp. are the causative agents of a spectrum of human diseases. Both *Mycobacterium tuberculosis* and *M. avium* have attracted attention through their increasing prevalence, particularly among immunocompromised individuals, and their resistance to current chemotherapeutic regimens (*1*, *2*). The key to *Mycobacterium*'s success lies in its interaction with the host macrophage and its strategies for survival inside this potentially hostile cell. Much attention has been directed toward the nature of *Mycobacterium*-containing vacuoles, and although the literature concerning the accessibility of these compartments to freshly endocytosed material is contradictory (*3*), a significant number of laboratories have reported failure of electron-dense colloids to enter previously formed *Mycobacterium* vacuoles (*4*). In addition, recent analysis of *M. avium*–containing vacuoles revealed a correlation between inhibition of lysosomal fusion and survival of bacteria in macrophages from bcg^r and bcg^s mice (*5*). The restricted fusigenicity of the mycobacterial vacuole may extend beyond limiting access of lysosomal hydrolases to the bacilli, and both Gordon (*6*), indirectly through the use of acridine orange, and Crowle (*7*), using the weak base DAMP, had reported that the bacterial vacuole was relatively less acidic than neighboring lysosomes.

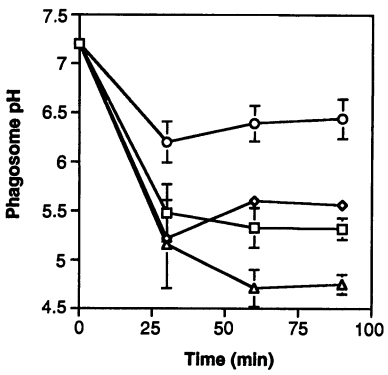

Fig. 1. Measurement of phagosomal pH. Graph illustrates the pH of phagosomes formed around human IgG–coated latex beads, *L. mexicana* tissue-culture amastigotes, zymosan, and *M. avium* (*9*). The pH of IgG-beads (◇), zymosan (△), and *Leishmania* phagosomes (□) rapidly decreased below 5.5 within the first 30 min after uptake. In contrast, the pH of *M. avium* phagosomes (○) decreased to about 6.3 before equilibrating to 6.5. The pH of the phagosomes was measured by spectrofluorimetry of NHS-carboxyfluorescein–labeled particles (*9*).

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