receptors in the hippocampus (5). Long-term potentiation (LTP) in the hippocampus—a long-lasting form of neuronal plasticity and putative neurobiological substrate for learning in the mammalian brain (6)—produces a similar effect (7). Moreover, the induction of LTP before training increased the acquisition of the eyeblink response to a differential CR (8), and conditioning alone increased the amplitude of the monosynaptic granule cell population spike in response to perforant path stimulation (an increase resembling LTP) (9). Therefore, if these changes in AMPA receptor binding are functionally significant, stress should facilitate the acquisition of the conditioned eyeblink response, as reported here.

A wide range of evidence supports the notion that the cerebellum and its associated brain stem neuronal network form the essential circuitry for the basic CR (10). The hippocampus, however, plays a key modulatory role in eyeblink conditioning (11) and contributes a major source of afferents to the cerebellum by way of the retrosplenial cortex and pontine nuclei (12). Alternatively, stress effects could act more directly on the cerebellar circuit, for example, by way of the locus ceruleus (13).

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3. The EMG activity from the upper eyelid muscle was amplified and then electronically filtered to pass signals ranging from 300 Hz to 3 KHz to a window discriminator. The discriminator digitized activity that was greater than a threshold set just above the envelope of background activity. The digitized data were collected by computer and stored into 4-ms bins. CRs were determined trial by trial with the use of chi-square analysis with Yates's correction. A response was considered a CR if the number of counts recorded 100 ms before shock onset was greater than that predicted by a random distribution of counts recorded 100 ms before CS onset. In white noise–alone trials, analysis windows were extended to 200 ms. Trials with a significant difference between two contiguous 100-ms time windows in the time period before the CS were excluded. Unconditioned responses (URs) were not analyzed owing to the shock artifact associated with the US. Data were analyzed by ANOVA with repeated measures and planned comparisons with two between-group levels and four within-subject levels. To quantify EMG activity, we standardized the number of counts per bin relative to the period before the CS. The ANOVA was performed on the number of bins with significant Z scores (>1.96) during the period of the animal's response to the CS without presentation of the US for the four groups over 4 days.
4. As determined by the standard delay paradigm, rabbits typically acquire at least 80% CRs by the third day of training, whereas the results from rats are more variable. In the present study, rats within a cohort shipment were randomized before stress and training, and treatment was counterbalanced within and between training days. The relatively low number of CRs attained by both trained groups compared to other rat eyeblink conditioning studies [R. Skelton, *Behav. Neurosci.* **102**, 586 (1988); N. A. Schmajuk and B. A. Christiansen, *Physiol. Behav.* **48**, 755 (1990)] is therefore most likely attributable to cohort, strain, or methodological differences. For example, higher rates were obtained from the restrained Long-Evans rat [R. M. Adams, A. A. Zhang, D. Lavond, *Soc. Neurosci. Abstr.* **15**, 890 (1989)], notably a stressful condition. Under freely moving conditions, F1 hybrids between the Fischer 344 and Brown Norway strains exhibited more CRs than Fischer 344 rats [C. Weiss and R. F. Thompson, *Neurobiol. Aging* **13**, 319 (1992)].
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Establishment of Stable, Cell-Mediated Immunity That Makes "Susceptible" Mice Resistant to *Leishmania major*

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Cell-mediated, but not antibody-mediated, immune responses protect humans against certain pathogens that produce chronic diseases such as leishmaniasis. Effective vaccination against such pathogens must therefore produce an immunological "imprint" so that stable, cell-mediated immunity is induced in all individuals after natural infection. BALB/c mice "innately susceptible" to *Leishmania major* produce antibodies after substantial infection. In the present study, "susceptible" mice injected with a small number of parasites mounted a cell-mediated response and acquired resistance to a larger, normally pathogenic, challenge. This vaccination strategy may be applicable in diseases in which protection is dependent on cell-mediated immunity.

Many nonreplicating antigens can induce either delayed-type hypersensitivity (DTH) or antibody-mediated responses, depending on quantitative variables such as antigen dose (1). A concentration of antigen that is subimmunogenic for the induction of antibody can induce DTH (1). Chronic administration of such low doses results in "low-zone paralysis" (2, 3), in which animals do not produce as strong an antibody response to subsequent challenge as do untreated animals. This state of unresponsiveness for the induction of antibody is associated with the expression of DTH to the antigen (4) and is therefore more appropriately referred to as "low-zone immune deviation." Low-zone immune deviation is probably associated with the induction of antigen-specific

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$CD8^+$ T cells that suppress the induction of the humoral response (5). Thus, it may be possible to produce an "imprint" on the immune system by giving concentrations of antigen that are subimmunogenic for the induction of antibody so that the immune response to the particular antigen is locked into a cell-mediated mode.

We tested our "imprinting" hypothesis in an animal model that corresponds to infection in humans where infection by a given pathogen produces either a stable and effective cell-mediated response or an ineffective antibody-mediated response (6, 7). BALB/c mice appear to be "innately susceptible" to *Leishmania major*, a protozoan parasite that causes cutaneous leishmaniasis. These mice mount an antibody response to the parasite after infection. Resistant mouse strains, in contrast, respond to infection with a stable, cell-mediated response (7).

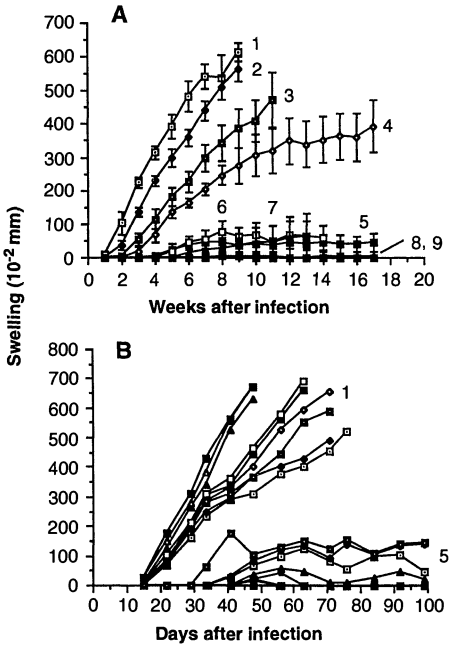

Fig. 1. The kinetics of increase in foot size of "susceptible" BALB/c mice after subcutaneous inoculation with different numbers of *Leishmania major* promastigotes. There were ten mice per group. Parasites from strain MHOM/IL/80/Friedelin were grown as described (*24*). Feet were measured as described (*25*). Mice were obtained from either the Animal Facility (Department of Microbiology) or the Animal Resource Center of the University of Saskatchewan. Care of animals was according to institutional guidelines. (**A**) Kinetics of the mean (± SE) increase in foot size after subcutaneous infection with different numbers of parasites. (**B**) Increase in foot size of individual mice (belonging to groups 1 and 5) after subcutaneous infection with 10^4 or 10^6 parasites. Similar experiments were performed three times with similar results. Number of parasites given: Group 1, 1×10^6; 2, 3.3×10^5; 3, 1×10^5; 4, 3.3×10^4; 5, 1×10^4; 6, 3.3×10^3; 7, 1×10^3; 8, 3.3×10^2; and 9, 1×10^2.

We attempted to generate an "imprint" in "susceptible" BALB/c mice by infecting them with small numbers of parasites so that their subsequent response to a normally pathogenic challenge with *L. major* would be locked into a cell-mediated mode and hence modulated to become similar to the response of resistant strains.

In the initial experiments, we injected BALB/c mice in the footpad with different numbers (10^2 to 10^6) of parasites in the hope that the administration of a small number of parasites would result in a stable, cell-mediated DTH response. We monitored lesion development and disease progression by measuring the increase in size of the injected foot. The mean increase in foot size varied with the time after infection for mice injected with different numbers of parasites (Fig. 1A). The increase in foot size of individual mice that had been subcutaneously inoculated revealed four patterns: (i) the foot size of most mice that received between 10^2 and 10^3 parasites did not increase, confirming similar studies by

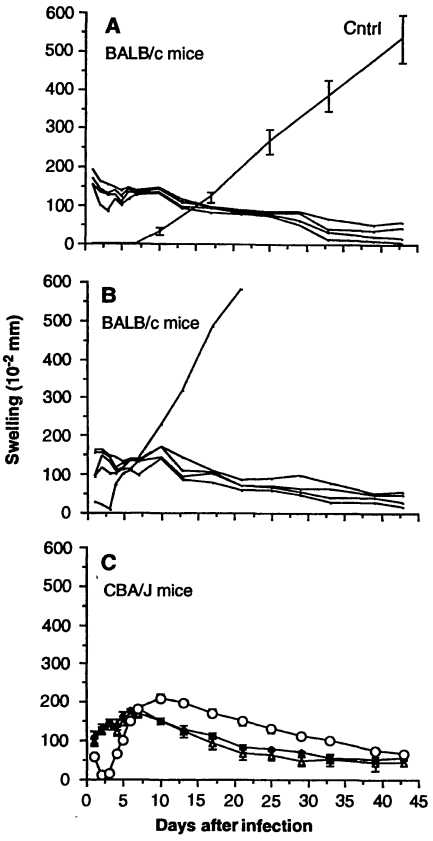

Fig. 2. Exposure to a low dose of parasites renders "susceptible" BALB/c mice resistant and increases the resistance of "resistant" CBA/J mice groups (four or five mice) of both "susceptible" and "resistant" strains were injected subcutaneously in the right foot with 10^2 or 10^3 parasites and challenged 105 days later in the contralateral foot with 10^7 parasites. (**A**) Kinetics of foot size of individual BALB/c mice made resistant by exposure to 10^3 parasites after challenge, and the mean (± SE) increase in foot size of ten normal mice exposed to the challenge alone (Cntrl). (**B**) Kinetics of foot size of individual BALB/c mice exposed to 10^2 parasites and challenged with 10^7 parasites. (**C**) Mean (± SE) increase in foot size of ten CBA/J mice pre-exposed to either 0 (circles), 10^2 (squares), or 10^3 (triangles) parasites and challenged with 10^7 parasites.

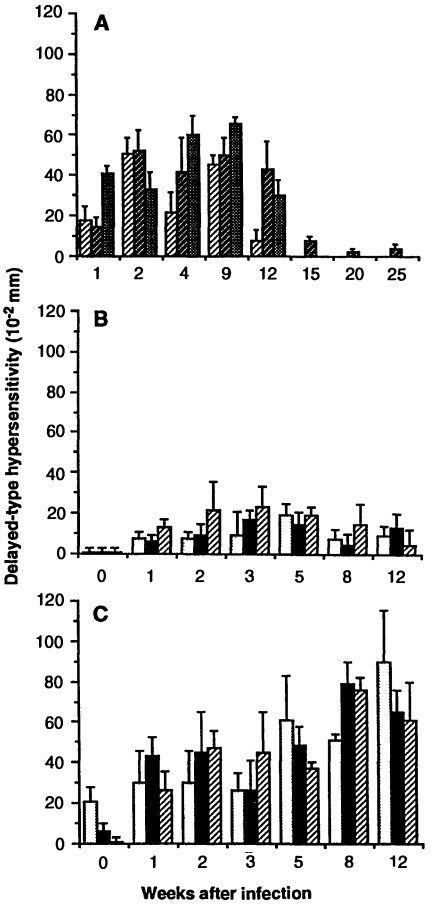

Fig. 3. Kinetics of parasite-specific DTH expressed by normal BALB/c mice receiving different doses of parasites and by BALB/c mice made resistant and challenged with a high dose of parasites. Normal mice, or mice rendered resistant, were injected subcutaneously in the foot with the low dose (3.3×10^2) (**A**) or high dose (10^6) (**B**) of parasites. Individual mice were killed at the indicated times after primary or secondary infection, and their spleen cells harvested (*25*). Parasite-specific DTH was estimated by measuring the 24-hour, antigen-dependent swelling caused by the transfer of 10^7 splenic white cells, with and without antigen, to the footpads of normal syngeneic recipients (five recipients with and five without antigen). Each bar represents the measured DTH from one mouse. Error bars: SE. Parasite antigen (*26*) was given at approximately 25 μg per footpad, an amount that by itself did not cause swelling. (**C**) Mice were challenged with the high dose (10^6) of parasites 120 days after exposure to the low dose (3.3×10^2) of parasites.

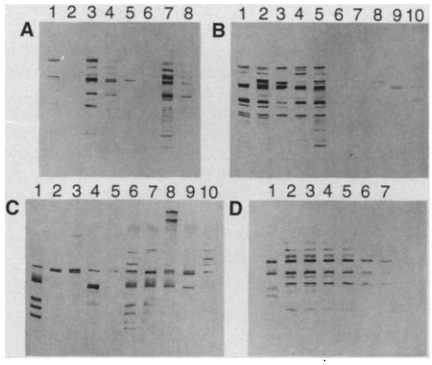

Fig. 4. Protein immunoblot analysis of the parasite-specific IgG1 and IgG2a antibodies from infected BALB/c mice. (**A**) Analysis of pooled sera from five mice infected with different numbers of parasites at different times after infection. Lanes 1 and 2, IgG1 and IgG2a antibodies, respectively, from the sera of mice infected with 10^5 parasites collected 1 month after infection; lanes 3 and 4, IgG1 and IgG2a antibodies, respectively, from mice infected with 10^7 parasites 1 month after infection; lanes 5 and 6, IgG1 and IgG2a antibodies, respectively, from mice infected with 10^3 parasites 2 months after infection; and lanes 7 and 8, IgG1 and IgG2a antibodies, respectively, from mice infected with 10^5 parasites 2 months after infection. (**B**) Antibodies from five individual mice, collected 1 month after infection with 10^6 parasites. Lanes 1 to 5, IgG1 antibodies; lanes 6 to 10, IgG2a antibodies. (**C**) Antibodies from five mice exposed to 3.3×10^2 parasites and challenged 120 days later with 10^6 parasites. Sera were collected 1 month after challenge. Lanes 1 to 5, IgG1 antibodies; lanes 6 to 10, Ig2a antibodies. (**D**) Estimation by protein immunoblot of the relative IgG1 parasite-specific antibody in normal mice (B) and resistant mice (C) 1 month after challenge with 10^6 parasites. Lane 1, IgG1 antibodies in the pooled sera of resistant mice at a dilution of 1:100; lanes 2 to 7, antibodies of normal mice detected at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200, respectively. *Leishmania major* promasigotes (10^9) were harvested and washed three times in phosphate-buffered saline. The pellet was resuspended in 700 μl of Laemmli buffer (*27*) and heated at 100°C for 3 min. Fifty microliters of the extract per gel were run on SDS–polyacrylamide gel electrophoresis (10%) with the use of a mini-PROTEAN II Electrophoresis Cell (Bio-Rad). The separated proteins were electroblotted onto nitrocellulose membranes (Bio-Rad) at 15 V for 15 min with the use of a Trans-Blot Semi-dry Transfer Cell (Bio-Rad), and the membrane was blocked with bovine serum albumin (3%) in tris-buffered saline (TBS) for 30 min at room temperature (RT). The membrane was incubated with sera at a dilution of 1:100 in TBS containing 0.05% Tween-20 (TTBS) for 60 min at RT. After three washes in TTBS, the membrane was incubated with horseradish peroxidase–conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Inc.) at a dilution of 1:1000 in TTBS for 60 min at RT. After three washes with TTBS and one with TBS, the membrane was developed at RT in the dark for 10 min, and the reaction was stopped by immersion of the membrane in distilled water.

others (*7*); (ii) the feet of some mice, given relatively few parasites, showed a small swelling that subsequently disappeared (Fig. 1B); (iii) the injection of ~10^6 parasites led to a progressive increase in foot size (Fig. 1B); and (iv) the foot size of some of the mice that had been given an intermediate number (~10^4) of parasites increased substantially and reached a steady or gently undulating state (Fig. 1B). These mice probably had a substantial number of parasites whose rapid and further proliferation was contained (*8*).

We then examined whether "susceptible" BALB/c or resistant CBA/J mice, exposed to small numbers of parasites, would acquire resistance to a dose that normally leads to progressive lesion development in BALB/c mice. Both BALB/c (Fig. 2, A and B) and "resistant" CBA/J mice (Fig. 2C), when given a low dose of parasites, either acquired systemic resistance (all BALB/c mice, with the exception of a mouse exposed to 10^2 promastigotes) or became more resistant (CBA/J mice) to a large challenge of parasites given about 2 months after primary exposure. This increased resistance of resistant mice after exposure to low doses of parasites confirms earlier work (*9*). We thus defined a standard protocol of vaccination that renders "susceptible" BALB/c mice resistant and resistant CBA/J mice more resistant to a challenge with parasites that is normally pathogenic in BALB/c mice. Resistance in BALB/c mice takes longer than 1 month to become reliably established and can be transferred to normal, irradiated mice by the standard assay for detecting protective cells (*10*). Such passive protection requires the transfer of $CD4^+$ T cells and is long lasting (potent protective cells are present in the spleen 10 months after exposure to a low dose of parasites) (*11*). The establishment of protection in BALB/c mice by exposure to small numbers of parasites, as assessed either by in situ challenge or by the ability of spleen cells from exposed mice to confer protection on normal mice, has been observed in 15 out of 15 experiments.

Our approach for the establishment of resistance in "susceptible" BALB/c mice rests on two suppositions: (i) primary immunization with high and low doses of parasites results in the long-term induction of antibody and DTH, respectively, and (ii) challenge with a high dose of parasites that results in the long-term induction of only an immunoglobulin G (IgG) antibody response in normal BALB/c mice will result in the induction of DTH in mice made resistant after exposure to a low dose of parasites. The observations of Figs. 3 and 4 support the validity of these suppositions. Figure 3 shows the kinetics of parasite-specific DTH expressed by normal BALB/c mice after injection with a small number (3.3×10^2) or large number (10^6) of parasites and by BALB/c mice made resistant with the low dose of parasites and challenged with the high dose. Figure 4 shows the IgG1 and IgG2a antibodies to parasites induced by different doses of parasites in normal BALB/c mice and the corresponding antibodies induced in resistant BALB/c mice after challenge with a large, normally pathogenic dose of parasites.

These observations support our hypothesis in several respects. (i) Injection with high doses (10^5 and 10^6) of parasites induced the production of antibodies that were detectable 2 months after challenge, whereas the injection of 10^3 parasites, which rendered mice resistant, induced much smaller amounts of antibodies that were barely detectable (Fig. 4A). (ii) A small number of parasites induced a larger and more prolonged DTH response than a large number (Figs. 3, A and B). There was a small and transient DTH response after injection with a high dose (Fig. 3B), confirming classical studies in this system (*7*). (iii) Mice made resistant after exposure to a low dose of parasites expressed a prolonged DTH response after a normally pathogenic challenge, and their production of parasite-specific IgG1 decreased by about eightfold (Fig. 4D). Production of the corresponding IgG2a antibody increased substantially in some cases.

These findings are similar to those of the murine immune response to *Trichinella spiralis*. Susceptible mice mount a T_H2-like response and produce IgG1 antibody, whereas resistant mice mount a T_H1-like response with the production of IgG2a antibody (*12*). Such a change in isotype production is expected because interferon-γ (IFN-γ), produced by T_H1-like cells, both suppresses the induction of IgG1 and enhances the production of IgG2a antibody (*13, 14*). We have found, as expected (*15*), that parasite-specific $CD4^+$ T cells in resistant, but not in normal, BALB/c mice produce large amounts of IFN-γ after exposure to a normally pathogenic challenge of parasites (*16*).

Standard vaccination procedures for humans should be effective in a genetically diverse population of individuals that produce different immune responses after natural infection. The importance of giving a sufficiently low dose (<3.3×10^4 *L. major* promastigotes in the case of mice) is demonstrated by this report. Such a low dose not only protects "susceptible" mice but makes resistant mice more resistant (Fig. 2), and the administration of such a low dose thus provides universal protection.

We examined the reliability of this form of vaccination in "susceptible" BALB/c mice. All 21 mice exposed to 3.3×10^2 promastigotes and all 12 mice exposed to 10^3 promastigotes were rendered resistant, as shown by their response to a subsequent challenge with a normally pathogenic dose. Only 12 out of 16 mice were made resistant after exposure to 10^2 promastigotes. Thus, all 33 BALB/c mice previously exposed to between 3.3×10^2 and 10^3 promastigotes were protected against a normally pathogenic challenge of the parasite.

"Susceptible" individuals or mice have the capacity to mount a protective response but do not do so for regulatory reasons. Patients with cutaneous and visceral leishmaniasis recover after drug treatment and gain resistance to reinfection as the antibody titer decreases and cell-mediated immunity is expressed, as assessed by skin reactivity to parasite antigens (6). BALB/c mice given either irradiation (*17*), cyclosporin A (*18*), or a monoclonal antibody that results in the depletion of $CD4^+$ T cells (*19*) near the time of infection mount a stable and protective cell-mediated response. The implication—that susceptible humans and animals have the intrinsic ability to mount protective responses—underlies attempts to develop universally effective vaccines.

The approach used here should be applicable in the development of vaccines to other pathogens that cause chronic disease and are preferentially susceptible to a specific cell-mediated attack. The dependence of the type of immunity induced on the dose of antigen administered is likely to reflect the existence of different thresholds for different inductive events (*20*). *Mycobacterium tuberculosis* (*21*), *Mycobacterium leprae* (*21*), *Schistosoma mansoni* (*22*), and human immunodeficiency virus type–1 (*23*) are probably preferentially susceptible to cell-mediated attack. We suggest that the strategy of vaccination described here for *L. major* will also apply to these human pathogens.

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Planar Induction of Anteroposterior Pattern in the Developing Central Nervous System of *Xenopus laevis*

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It has long been thought that anteroposterior (A-P) pattern in the vertebrate central nervous system is induced in the embryo's dorsal ectoderm exclusively by signals passing vertically from underlying, patterned dorsal mesoderm. Explants from early gastrulae of the frog *Xenopus laevis* were prepared in which vertical contact between dorsal ectoderm and mesoderm was prevented but planar contact was maintained. In these, four position-specific neural markers (*engrailed-2, Krox-20, XlHbox 1*, and *XlHbox 6*) were expressed in the ectoderm in the same A-P order as in the embryo. Thus, planar signals alone, following a path available in the normal embryo, can induce A-P neural pattern.

Spemann proposed that dorsal mesoderm can induce neural development in the ectoderm of the amphibian embryo in two ways: by vertical induction, in which signals pass from the involuted dorsal mesoderm to overlying ectoderm, or by planar induction, in which signals pass from the dorsal mesoderm to the ectoderm around the dorsal lip (*1*). However, planar induction appeared to be ruled out when Holtfreter found that urodele exogastrulae lack any obvious neural differentiation (*2*). In these abnormal embryos, mesoderm evaginates during gastrulation, instead of invaginating, and fails to make vertical contact with ectoderm, while presumably retaining planar contact. In other studies vertical signals from dorsal mesoderm were found to induce neural development and A-P neural pattern (*3*). Thus, it has been assumed that neural differentiation and patterning are normally induced by vertical signals alone.

Recent results with *Xenopus laevis*, however, provide evidence for planar neural induction. Exogastrulae express two panneural genes, the neural cell adhesion molecule (N-CAM) and the neurofilament-like protein (NF3) (*4*, *5*), as well as the anterior-specific *xhox-3* (6). However, the possibility of vertical induction cannot be fully excluded because it is difficult to observe the internal movements of mesoderm during exogastrulation. To avoid this problem, studies of planar induction have been carried out in planar explants of mesoderm and ectoderm ["Keller" explants (*7*)], in which the absence of vertical contacts has been well documented (*8*). These explants ex-

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