time of vertebrate myoglobin divergence were close.

Many eukaryotic genes that code for secretory proteins have introns between the signal peptide coding region and that of the mature protein (*22*). Neither the *Lumbricus* nor the *Chironomus* globin gene has an intron in this position. There is speculation that the absence of this intron in the *Chironomus* globin genes indicates that the total loss of all introns in *Chironomus* globins was caused by a single conversion event rather than by separate stochastic events (*23*). The absence of this intron in the *Lumbricus* chain *c* gene suggests that the lack may be a common feature shared by other genes of extracellular invertebrate hemoglobins.

Our results suggest that the third (central) intron of globin genes was lost at least 600 million years ago. A third intron should be sought in the genes for globins of lower invertebrates, particularly those of nematodes, flatworms, and protozoa (Fig. 1A). All these phyla have members with hemoglobins (*24*). It is possible that a third intron may not be found at all in the invertebrates, because comparison of animal and plant globins suggests a possible common ancestor 1500 million years ago (*4*).

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Oral *Salmonella typhimurium* Vaccine Expressing Circumsporozoite Protein Protects Against Malaria

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Immunization with radiation-attenuated malaria sporozoites induces potent cellular immune responses, but the target antigens are unknown and have not previously been elicited by subunit vaccines prepared from the circumsporozoite (CS) protein. A method is described here for inducing protective cell-mediated immunity to sporozoites by immunization with attenuated *Salmonella typhimurium* transformed with the *Plasmodium berghei* CS gene. These transformants constitutively express CS antigens and, when used to immunize mice orally, colonize the liver, induce antigen-specific cell-mediated immunity, and protect mice against sporozoite challenge in the absence of antisporozoite antibodies. These data indicate that the CS protein contains T cell epitopes capable of inducing protective cell-mediated immunity, and emphasize the importance of proper antigen presentation in generating this response. Analogous, orally administered vaccines against human malaria might be feasible.

ANIMALS AND HUMANS CAN BE PROtected against malaria by immunization with radiation-attenuated sporozoites. The basis of this immunity is complex and includes a humoral immune response to immunodominant repeat epitopes on the circumsporozoite (CS) protein, and cell-mediated immune responses to sporozoite or exoerythrocytic antigens (*1*). We previously reported that protective immunity induced by irradiated *Plasmodium berghei* sporozoites is mediated by antigen-specific T cells (*2*). These T cell populations may participate in cytotoxic or lymphokine-mediated killing of exoerythrocytic parasites in the liver (*3*). Unlike live sporozoites (*4*), killed sporozoites do not invade cells and are unable to induce protective immunity (*5*). Intracellular targeting of CS antigens may be necessary for induction of protective cell-mediated immune responses. We therefore investigated the use of attenuated *Salmonella* as vectors to present CS antigen.

Soon after oral inoculation, attenuated *Salmonella* vaccine strains are found in regional and systemic lymphatics where they are ingested by macrophages (*6*). Immunity induced by these strains is cell-mediated and its induction requires a period of intracellular survival by the organism (*7–9*). We used a plasmid containing the *P. berghei* CS protein gene to transform *S. typhimurium* WR4017, an avirulent strain with impaired ability to multiply within macrophages (*10*). This transformant vaccine constitutively expressed CS antigen, and induced antigen-specific cell-mediated immune responses in mice and protection from sporozoite challenge in the absence of measurable antibodies to CS protein. These studies identify the CS protein as a target antigen for protective cell-mediated immunity against sporozoites and demonstrate the feasibility of constructing analogous oral vaccines against human malarias.

The transformant (WR4017/pMGB2) was constructed with an expression plasmid containing the entire *P. berghei* CS protein coding region (Fig. 1). Female BALB/c mice (6 to 8 weeks old) were immunized by subcutaneous (sc) injection of 10^4 bacteria or by oral administration of 10^9 or 10^{10}

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bacteria. *Salmonella* were cultured from liver homogenates for up to 3 weeks after sc or oral immunization of mice with WR4017/pMGB2 or WR4017. Mice immunized sc with WR4017/pMGB2 developed low levels of antibodies to CS protein that were detected at week 4 but not at week 5 (Table 1). Mice immunized orally with larger doses of WR4017/pMGB2 made no detectable antibodies (*11*). Mice immunized by either route were protected when challenged with sporozoites 4 to 9 weeks after immunization. Overall, 26 of 57 (46%) mice immunized with WR4017/pMGB2 became infected, compared with 32 of 35 (91%) mice immunized with WR4017 ($\chi^2 = 17.62$, $P = 0.0001$). Partial protection as reflected by delayed onset of parasitemia was seen in WR4017/pMGB2-immunized mice that became infected (mean prepatent period = 7.1 ± 0.29 days compared with 6.1 ± 0.11 days in WR4017 controls, $P < 0.005$, Student's t test). Although oral immunization was more efficacious than sc immunization (65% versus 37%, $\chi^2 = 4.97$, $P = 0.026$), it is clear that still greater vaccine efficacy will be needed. Vaccine failure in some mice may have resulted from plasmid instability or variable levels of CS expression in the transformants.

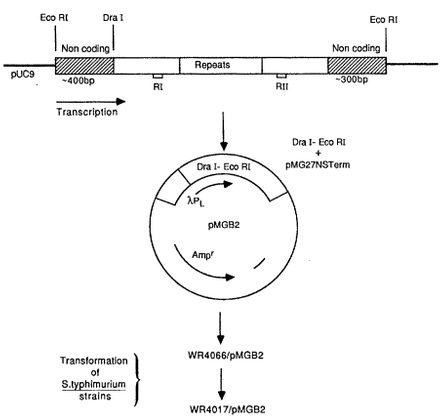

Fig. 1. Construction of WR4017/pMGB2. *P. berghei* DNA (*17*) was partially digested with Dra I and Eco R5, ligated to Eco RI linkers, and used to prepare a λgt10 genomic DNA library. A clone containing the full-length CS gene plus noncoding flanking sequences (crosshatched) was isolated as an Eco RI insert and subcloned into pUC9. RI and RII refer to regions conserved among species of *Plasmodium* (*18*). Plasmid pMGB2 was obtained by ligating a Dra I/Eco RI fragment into the Stu site of a λP_L-based plasmid expression vector, pMG27NSTerm, derived from pMG27N$^-$ (*19*). In *E. coli*, pMGB2 expresses full-length *P. berghei* CS protein with six additional amino acids at its amino terminus (Met-Asp-Pro-Trp-Arg-Lys). Plasmid pMGB2 was purified (*20*) and sequentially transformed into *S. typhimurium* WR4066 and WR4017 by a modification (*21*) of standard methods (*22*). Transformants expressing *P. berghei* CS repeat epitopes were identified by monoclonal antibody 3.28.1 (*17, 23*).

Protection in the absence of antibodies to sporozoites suggested the vaccine may have induced cell-mediated immunity. Studies with attenuated *S. typhimurium* have shown that specific cellular immunity against crude *Salmonella* lysates as well as O polysaccharide can be demonstrated by means of delayed-type hypersensitivity (DTH) skin tests (*6, 12*). Mice immunized orally with WR4017/pMGB2 developed DTH reactions to sporozoite lysates, as did sporozoite-immunized mice (Table 2), indicating that the vaccine induced a CS antigen–specific, cell-mediated immune response.

These results imply that epitopes capable of inducing cell-mediated immunity are contained within the CS protein and that such immunity can be induced by incorporation of this gene into a bacterial host. Class II major histocompatibility complex (MHC)–restricted T cell epitopes have been identified on the *P. falciparum* CS protein that provide help for antibody production against CS repeats (*13*). However, other epitopes will be needed if CS vaccines are to induce and expand class I–restricted T cells with cytotoxic or lymphokine-mediated activities against exoerythrocytic stages. Although exogenously presented protein antigens are generally processed by endosomal recycling pathways and associate preferentially with class II MHC antigens (*14*), the introduction of such proteins into the cytoplasm of cells can induce class I–restricted

Table 1. Protective immunity induced by *S. typhimurium* (WR4017) transformed with a plasmid expressing the *P. berghei* CS protein (WR4017/pMGB2). Mice were immunized at time 0 with a single subcutaneous (sc) or oral (po) dose. One experiment included normal mice and mice immunized with two intravenous (iv) doses of irradiated sporozoites, Antibodies to sporozoites were measured by immunofluorescence (IFA) and enzyme-linked immunosorbent assay (ELISA) (*11*). Mice were challenged intravenously with 1500 *P. berghei* salivary gland sporozoites and blood samples were examined for parasites daily from days 6 to 12 after challenge.

Immunogen	Route	Dose	IFA*	ELISA†	No. infected / No. challenged	Efficacy (%)‡
		Week 4 after immunization				
WR4017	sc	10^4	0	0.034	5/5	
WR4017/pMGB2	sc	10^4	1+	0.147	5/9	44
		Week 5 after immunization				
WR4017	sc	10^4	0	0.057	10/10	
WR4017/pMGB2	sc	10^4	0	0.073	12/18	33
WR4017	po	10^{10}	0	0.060	5/5	
WR4017/pMGB2	po	10^{10}	0	0.059	4/10	60
WR4017	po	10^9	0	0.063	5/5	
WR4017/pMGB2	po	10^9	0	0.058	3/10	70
		Week 9 after immunization				
WR4017	po	10^9	ND§	ND	7/10	
WR4017/pMGB2	po	10^9	ND	ND	2/10	71
None			0	0.035	7/8	
γ-SPZ	iv	75,000 and 20,000	4+	1.477	0/5	100

*Immunofluoresence of air-dried *P. berghei* sporozoites reacted with serum diluted 1:10. †Optical density for sera diluted 1:100. The mean + 2 SD for seven control sera was 0.089. ‡Efficacy calculated as 100 × [1− (% WR4017/pMGB2 infected/% WR4017 infected)]. §ND, not done.

Table 2. Delayed-type hypersensitivity responses (DTH) in mice immunized with irradiated *P. berghei* sporozoites (γ-SPZ), *S. typhimurium* (WR4017), or *S. typhimurium* transformed with a plasmid expressing the *P. berghei* CS protein (WR4017/pMGB2). Mice were immunized with a single oral dose of 10^9 bacteria or with two doses (75,000 and 20,000) of irradiated sporozoites. DTH responses were determined 8 or 9 weeks later in groups of ten mice by measuring footpad swelling 24 hours after injecting phosphate-buffered saline (PBS) or a sporozoite lysate (*24*). Results are expressed as the mean ± standard error of footpad swelling (in millimeters). Statistical significance was determined by analysis of variance (ANOVA) and Newman-Keuls test for multiple comparisons.

Group	Experiment 1		Experiment 2	
	PBS	Sporozoite lysate	PBS	Sporozoite lysate
WR4017/pMGB2	0.00 ± 0.00	0.12 ± 0.036*	0.00 ± 0.00	0.08 ± 0.029†
WR4017	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
γ-SPZ	0.01 ± 0.01	0.11 ± 0.028*	0.00 ± 0.00	0.09 ± 0.038†
Normal	ND‡	ND	0.00 ± 0.00	0.00 ± 0.00

*Significantly different ($P < 0.01$) from PBS controls; ANOVA $F = 9.631$ ($P < 0.0001$). †Significantly different ($P < 0.05$) from PBS controls; ANOVA $F = 4.657$ ($P < 0.0003$). ‡ND, not done.

cytotoxic T cells (*15*). We speculate that intracellular targeting of WR4017/pMGB2 led to expression of CS antigen on the cell surface in association with class I MHC molecules and the induction of a cell-mediated immune response. This technique should be useful for precise mapping of critical T cell epitopes, and for construction of vaccines against other diseases in which cell-mediated immunity is important, such as leprosy, leishmaniasis, schistosomiasis, and infection with rickettsia or the human immunodeficiency virus.

Mutants of *S. typhi* have been used safely in humans as oral vaccines and elicit cellular immunity against *S. typhi* antigens (*9, 16*). Preliminary data indicate that galE (Ty21A) or aroA (541Ty) mutants of *S. typhi*, and *S. typhimurium* strain WR4017, express *P. falciparum* antigens when transformed with plasmids containing the *P. falciparum* CS gene. Thus it may be possible to develop an orally administered vaccine for the prevention of malaria.

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Increased Attention Enhances Both Behavioral and Neuronal Performance

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Single cells were recorded from cortical area V4 of two rhesus monkeys (*Macaca mulatta*) trained on a visual discrimination task with two levels of difficulty. Behavioral evidence indicated that the monkeys' discriminative abilities improved when the task was made more difficult. Correspondingly, neuronal responses to stimuli became larger and more selective in the difficult task. A control experiment demonstrated that changes in general arousal could not account for the effects of task difficulty on neuronal responses. It is concluded that increasing the amount of attention directed toward a stimulus can enhance the responsiveness and selectivity of the neurons that process it.

OUR PERCEPTUAL SYSTEMS DO NOT always work at their peak (*1*). As an extreme example, perceptions may seem to dull, then disappear, as we drift off to sleep. Likewise, within the visual system, neurons in the cerebral cortex of sleeping cats give weakened sensory responses that do not distinguish among incoming stimuli as well as neuronal responses in the awake animal (*2*). Moreover, neurons in certain cortical areas of awake monkeys show different degrees of responsiveness, depending on whether the monkey is idle, engaged in a detection task, or engaged in a discrimination task (*3*). It has not been clear from these physiological studies, however, whether neuronal responsiveness varies with changes in state, level of arousal, the specific task required of the animal, or the amount of attention devoted to the stimuli. To test specifically whether the amount of attention, or cognitive "effort," devoted to a stimulus affects how it is coded within the visual system, we studied the responses of visual neurons to stimuli presented within the same perceptual task at different levels of difficulty.

Neurons were studied in area V4 within the extrastriate cortex of monkeys. Area V4 is an intermediate station along the pathway from the primary visual cortex into the temporal lobe, a pathway critically involved in object recognition (*4*). It appears to be the first cortical area along this pathway in which neuronal responses are gated by spatially directed selective attention. Previously, it was found that when a monkey attends to a stimulus within the receptive field of a V4 neuron, the neuron's responses to nearby, but ignored, stimuli within the receptive field are greatly attenuated (*5*), possibly explaining why unattended stimuli are not normally perceived. Our goal in the present study was to determine if even the neuronal responses to attended stimuli are affected by "how much" attention, or effort, is devoted to them. To test this, we trained two rhesus monkeys to maintain fixation on a small spot and to discriminate the orientation or color of a stimulus presented within the receptive field of a neuron in V4. Every cell was tested with stimuli presented within the context of the same discrimination task at two levels of difficulty.

The task used was a modified version of matching-to-sample. While the monkey held a bar and gazed at a fixation spot, a sample stimulus appeared for 200 msec, and 400 to 600 msec later a test stimulus appeared for 200 msec at the same location (*6*). When the test stimulus was identical to the preceding sample (a "matching" trial), the animal was rewarded with a drop of water if it released the bar immediately; when the test stimulus differed from the sample (a "nonmatching" trial), the animal was rewarded only if it delayed release for 700 msec. Half the trials were matching and half nonmatching. The

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