Brief Definitive Report

GENETIC CONTROL OF THE IMMUNE RESPONSE IN MICE TO A *PLASMODIUM FALCIPARUM* SPOROZOITE VACCINE

Widespread Nonresponsiveness to Single Malaria T Epitope in Highly Repetitive Vaccine

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Much effort has been devoted to developing vaccines against different stages in the life cycle of the malarial parasite. Infection is initiated by injection of sporozoites by mosquitoes. If this stage in the life cycle can be blocked, then infection can be prevented. Vaccines against sporozoites are based on the following observations. A family of analogous surface proteins (circumsporozoite [CS] proteins) are found on all malarial sporozoites, and contain an immunodominant repeating epitope, the central third of the CS protein. Antibodies to this repeating epitope block infection (1–4).

One antisporozoite vaccine against *Plasmodium falciparum*, currently being tested in humans, has been produced as a fusion protein between the repeat sequence of the malaria parasite [MDP$(NANP)_{15}$NVDP$(NANP)_{15}$NVDP] (one-letter amino acid code) and 32 amino acids encoded by a tetracycline-resistance gene read out of frame (tet_{32}) (5). If the goal of vaccination is a high antibody titer over a limited period of time, then T cell help can be supplied in the vaccine from a carrier unrelated to the sporozoite protein itself. If boosting is desirable from natural infection, or if cellular immunity also kills the parasites in the liver, then T cell epitopes must derive from the parasite protein. We determined whether the repeating peptide of parasite origin or the tet_{32} peptide contained the T cell epitope(s). This report shows that T cell epitopes are contained within both the repeating peptide and tet_{32}. The immune response to the T epitopes was under *Ir* gene control, mapping to the I-A and I-E regions of the mouse H-2 complex, but only mice bearing the $I\text{-}A^b$ gene responded to the T cell epitope from the repeat. If an analogous situation applies in humans, boosting by natural exposure to sporozoites, through mosquito bites, may require addition to the

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vaccine of another epitope for T cell recognition from elsewhere in the CS protein.

Materials and Methods

Mice. H-2-congenic mice on the B10 background were bred at our breeding facility or were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. The following were used: R32 tet_{32}, $MDP(NANP)_{15}NVDP(NANP)_{15}NVDP$ Tet 1–32, where Tet 1–32 refers to the first 32 amino acids encoded by a tetracycline resistance gene, read out of frame; R32LR, $MDP(NANP)_{15}NVDP(NANP)_{15}NVDPLR$; 24-mer, $NP(NANP)_5NA = (NPNA)_6$; 7-mer, PNANPNA; 11-mer, $P(NANP)_2NA$; 16-mer, $NP(NANP)_3NA = (NPNA)_4$; 40-mer, $(NANP)_{10}G$; 60-mer, $(NANP)_{15}G$.

Immunization. For antibody production, mice were given a primary intraperitoneal injection of 80 μg of antigen emulsified in H37Ra CFA (Difco Laboratories, Detroit, MI) on day 0, followed by secondary and tertiary intraperitoneal injections of 20 μg of antigen (aqueous) on days 21 and 31. Sera from a bleed on day 41 were used.

For T cell proliferation studies (6), 100 μg of R32 tet_{32} emulsified in CFA was injected in the base of the tail of mice on day 0. On day 10, draining nodes were removed. Cells were seeded at 4×10^5 cells/well (flat-bottom microtiter plates, 3596; Costar, Cambridge, MA) in 0.2 ml of RPMI containing 10% FCS, 5×10^{-5} M 2-ME, penicillin, streptomycin, and antigen. On day 5, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to quadruplicate cultures, 16 h later, cells were harvested, and incorporated ^{3}H was assessed by β emission spectroscopy.

Estimation of Specific IgG. Specific IgG was estimated by ELISA. Briefly, 60-mer (2 μg/ml, 50 μl) was added to polyvinyl wells for 16 h. Wells were then blocked, sera added, and a goat anti-mouse IgG coupled to alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added. Finally substrate (104–105; Sigma Chemical Co., St. Louis, MO) was added, and absorbance was read at 405 nm. Sera were tested at various dilutions and compared with a control serum defined to contain 1,000 U of specific IgG per milliliter.

Results

The influence of *Ir* genes on the antibody response to 24-mer (administered without carrier) was examined initially. Different H-2-congenic mice were immunized, and anti-NANP specific IgG was determined (Table I). Of the mice examined, only those carrying the *I-A*^{b} gene produced specific antibody. These results indicated that the 24-mer contained a T epitope capable of being seen by mice expressing an I-A^b molecule, as well as a B epitope. As the B10 mice express only an I-A^b molecule and no I-E molecule, the response maps to *I-A*^{b}. It has previously (7) been shown that $(NANP)_{n \geq 2}$ contains the B epitope, since $(NANP)_2$ can inhibit the interaction of antisporozoite mAb and sporozoites.

To determine the minimum size of this T epitope, lymph node proliferation assays were performed. B10, B10.A (5R), and B10.D2 mice were immunized with R32 tet_{32}. Lymph node cells were then restimulated in vitro with different antigens (Fig. 1). As shown, R32 tet_{32} efficiently stimulated cells from all strains. B10.D2 cells, as expected, could not be stimulated with any $(NANP)_n$ constructs, even as large as the 60-mer ($n = 15$). B10 and B10.A(5R) cells were capable of optimal restimulation with 16-mer, moderate restimulation with 11-mer, but could not be restimulated with 7-mer. The minimal effective T epitope is thus between the 11-mer and 16-mer.

Similarly, the *Ir* gene control of the response to R32 tet_{32} was mapped (Table II). Mice (carrying the I-A^b gene) that responded to 24-mer also responded to

TABLE I

Ir Gene Control of Response to 24-mer

Strain	H-2 alleles								Anti-NANP IgG (U/ml)	
	K	A	B	J	E	C	S	D	Exp. 1	Exp. 2
B10.S	s	s	s	s	s	s	s	s	<1, <1, <1, <1, <1	ND
B10.BR	k	k	k	k	k	k	k	k	<1, <1, <1, <1, <1	<1, <1, <1, <1, <1
B10.HTT	s	s	s	s	k	k	k	d	<1, <1, <1, <1, <1	ND
B10.A(5R)	b	b	b	k	k	d	d	d	105, 10, <1, 164, 159	27, 10, 114, 75, 2
B10	b	b	b	b	b	b	b	b	80, <1, 24, 10, 252	588, 292, 751, 1789
B10.D2	d	d	d	d	d	d	d	d	<1, 3, <1, <1, <1	ND
B10.MBR	b	k	k	k	k	k	k	q	ND	<1, <1, <1, <1, <1
B10.A(4R)	k	k	b	b	b	b	b	b	ND	<1, <1, <1, <1, <1

Each value represents serum from one mouse.

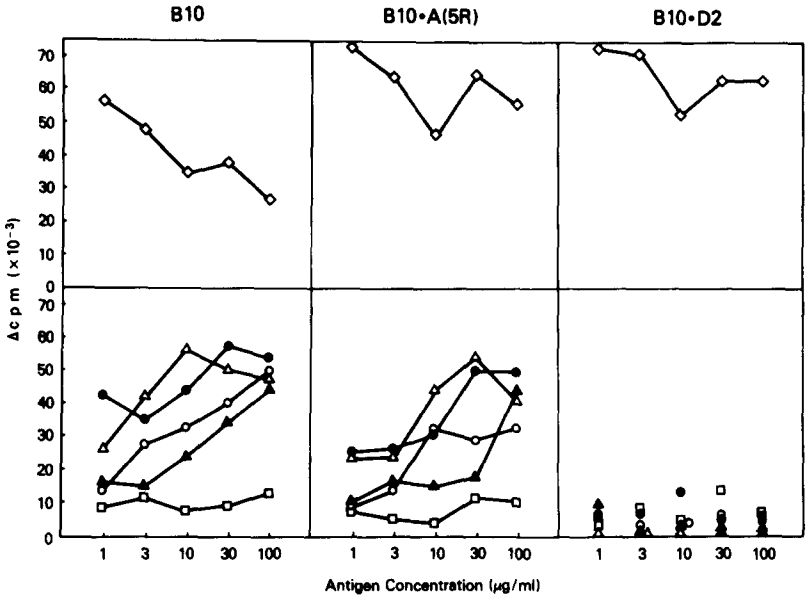

FIGURE 1. B10, B10.A(5R), and B10.D2 mice were immunized in the base of the tail with R32tet$_{32}$, as described. 10 d later, draining lymph node cells were challenged in vitro with different concentrations of either R32tet$_{32}$ (◇), 60-mer (○), 40-mer (●), 16-mer (△), 11-mer (▲), or 7-mer (□), and proliferation was assessed by incorporation of [^{3}H]thymidine 4 d later. Background cpm (in the absence of antigen) was 17,654 for B10 cells, 15,817 for B10.A(5R) cells, and 23,067 for B10.D2 cells. Δcpm given in the figure are (cpm with antigen) − (cpm without antigen).

R32 tet$_{32}$. This was expected since R32 tet$_{32}$ contains two blocks of (NANP)$_{15}$. However, certain strains that did not respond to 24-mer did respond to R32 tet$_{32}$ (B10.HTT, B10.D2), whereas other strains (B10.S, B10.BR, B10.GD) responded poorly. While B10.D2 cells contain two possible class II restriction molecules (I-A^d, I-E^d), B10.GD cells contain only I-A^d. The differential response between B10.D2 and B10.GD, then, suggests that R32 tet$_{32}$ contains a T epitope(s) recognized in association with I-E^d not I-A^d. Fig. 1 shows that the T epitope seen by B10.D2 cells is not (NANP)$_{n>6}$, since these cells did not respond to any construct of (NANP)$_n$ up to $n = 15$ (also see Table III, below).

The differential response of B10.HTT to 24-mer and R32 tet$_{32}$ shows that

TABLE II

Ir Gene Control of Response to R32 tet_{32}

Strain	H-2 alleles								Response to 24-mer*	Anti-NANP IgG (U/ml)	
	K	A	B	J	E	C	S	D		Exp. 1	Exp. 2
B10.S	s	s	s	s	s	s	s	s	−	<1, <1, <1, <1, <1	<1, <1, <1, <1
B10.BR	k	k	k	k	k	k	k	k	−	<1, 3, <1, <1, <1	ND
B10.HTT	s	s	s	s	k	k	k	d	−	686, 91, 809, 155, 107	ND
B10.A(5R)	b	b	b	k	k	d	d	d	+	451, 809, <1, 107, <1	ND
B10	b	b	b	b	b	b	b	b	+	1,180, 461, 275, 431	ND
B10.D2	d	d	d	d	d	d	d	d	−	97, 40, 53, 78	105, 68, 101, 89, 93
B10.GD	d	d	b	b	b	b	b	b	−	ND	43, 4, <1, <1, <1

* See results from Table I.

TABLE III

Definition of Non–24-mer T Epitope(s) by Immunization with Various Peptides

Immunogen	Anti-NANP IgG (U/ml)	
	B10.D2	B10.HTT
R32 tet_{32}	70, 57, 161, 153, 127	123, 379, 244, <1, 162
24-mer	<1, <1, <1, <1, <1	<1, <1, <1, <1, <1
40-mer	<1, <1, <1, <1, <1	<1, <1, <1, <1, <1
60-mer	<1, <1, <1, <1, <1	<1, <1, <1, <1, <1
R32LR	<1, <1, <1, <1, <1	<1, <1, <1, <1, <1

$E_\alpha^k E_\beta^s$ can also be a restriction element for a T epitope(s) within R32 tet_{32}, but distinct from the 24-mer. This *Ir* gene control maps to $E_\alpha^k E_\beta^s$ in the B10.HTT, since B10.S, which expresses the same I-A^s molecule as B10.HTT but expresses no I-E molecule, does not respond. The results of Table II do not indicate whether B10 mice are responding to a T epitope within R32 tet_{32} in addition to that contained within 24-mer. The B10.A(5R) response to R32 tet_{32} probably maps to the I-A^b molecule shared with the B10 strain, which does not express an I-E molecule. However, an additional role of the $E_\alpha^k E_\beta^b$ molecule also expressed by the B10.A(5R) mice cannot be ruled out with available recombinant strains.

To identify more precisely the T epitope(s) in R32 tet_{32} that is recognized by B10.D2 and B10.HTT mice, these mice were immunized with R32 tet_{32}, R32LR (which lacks the tet_{32} peptide), and different constructs of $(NANP)_n$, where n = 6, 10, or 15 (Table III). Neither strain of mouse produced antibody in response to $(NANP)_n$ as large as the 60-mer, indicating that the failure to respond to 24-mer (Table I) was not due to the small size of the 24-mer. Neither of the strains that responded to R32 tet_{32} responded to R32LR, indicating that tet_{32} contained the functional T epitope(s) for those two strains, and that the variant repeats, NVDP, contained within R32LR, and which were absent from the 60-mer, were not sufficient to elicit T cell help. Lymph node cell transformation studies confirmed this finding, showing that while the B10, B10.HTT, and B10.D2 mice, which had been immunized with R32 tet_{32} could all respond to R32 tet_{32}, only the B10 mice could respond to R32LR (data not shown). Thus, of all strains tested, only those mice carrying the *I-A^b* gene could respond to the malaria-specific sequence present in R32 tet_{32}, which is contained within $NP(NANP)_3NA$.

Discussion

Immunity to sporozoites requires constant high levels of specific antibody and possibly non–antibody dependent cellular mechanisms of immunity (8). To maintain high levels of antibody after vaccination, a vaccine must have certain properties: (*a*) it must contain an appropriate B epitope(s); (*b*) it must contain T epitopes, covalently linked to the B epitopes, that are capable of being recognized by all who receive the vaccine; (*c*) for natural boosting to occur, T epitopes must derive from the organism. Because of *Ir* gene control (9, 10) only a subset of individuals will respond to any given epitope.

Although human trials with R32 tet_{32} have only just commenced, the results presented here from murine studies suggest that some of the above criteria may be absent for this vaccine. Firstly, R32 tet_{32} is capable of stimulating significant levels of antibody in only four of seven inbred strains of mice. These mice are homozygous, however, at each H-2 locus, and the response to R32 tet_{32} in an outbred population could be expected to be much better. Disappointingly, however, more strains respond to T epitopes present in Tet 32 than in the malaria-encoded sequence, R32. Only two of nine strains of mice (representing one of eight different possible I-A and I-E restriction molecules, including hybrid molecules) could respond to the malaria sequence, $(NANP)_6$. If an analogous situation occurs in humans, many people may not recognize the malaria T epitope, even though they may recognize the tet_{32} T epitope(s), and could thus not mount an anamnestic response to sporozoite inoculation. Although not directly addressed here, malaria-specific T epitopes are crucial for a vaccine reliant on high levels of cellular immunity. Studies (8) in murine malaria suggest an important role for cellular immune mechanisms in sporozoite clearance.

The results of this study have broader implications for peptide vaccine development in general. Peptide vaccines must contain all the relevant immunologic information in a single sequence of amino acids. Invariant B epitopes recognized by protective antibodies must be covalently linked to invariant T epitopes, at least one of which must be recognized by T cells of all who receive the vaccine. The T and B epitopes present in the peptide must also be on the same molecule in the organism for boosting to occur (11, 12). Should the molecule in the organism that contains the required B epitope contain few or no T epitopes, then a vaccine will need to contain artificial T epitopes, and thus require repetitive administration to boost antibody levels. This may be the case with R32 tet_{32}.

Summary

Different H-2 congenic strains of mice were immunized with a *P. falciparum* sporozoite vaccine currently being tested in humans, or with different segments of the vaccine molecule. Specific IgG production or lymph node cell proliferation in response to different antigens was then determined. Only four of seven strains (representing three of eight possible different class II restriction molecules) responded to the vaccine. Of those restriction molecules, only one, $I\text{-}A^b$, was associated with a response to a malaria-encoded T epitope [contained within $NP(NANP)_3NA$], while the other two molecules ($E_\alpha{}^dE_\beta{}^d$ and $E_\alpha{}^kE_\beta{}^s$) were associated with a T cell response to a nonmalarial epitope(s) carboxyterminal to the

malaria sequence and encoded by a tetracycline resistance gene, read out of frame. If an analogous situation applies in humans, natural boosting by sporozoites will be very restricted. This has serious implications for the effectiveness of the vaccine, since constant high levels of antisporozoite antibodies and possibly antibody-independent T cell effector functions are required for immunity.

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