

# Quantification of the Relative Contribution of Major Histocompatibility Complex (MHC) and Non-MHC Genes to Human Immune Responses to Foreign Antigens

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**Understanding the extent to which genetic factors influence the immune response is important in the development of subunit vaccines. Associations with HLA gene polymorphisms appear insufficient to explain the range of variation in immune responses to vaccines and to infections by major pathogens. In this study of Gambian twins we report that regulation of the immune response to a variety of antigens from *Plasmodium falciparum* and *Mycobacterium tuberculosis* is controlled by factors which are encoded by genes that lie both within and outside the major histocompatibility complex (MHC). We define the relative contribution of these genes, which varies for different antigens. The cumulative genetic contribution of non-MHC genes to the total phenotypic variance exceeds that of the MHC-encoded genes.**

Genetic regulation of immune responses may have a major influence on susceptibility to numerous autoimmune and infectious diseases and on vaccine responsiveness. A vast literature of case control studies has addressed the role of HLA genes in determining variable susceptibility to disease, and many HLA associations are well defined (13, 30, 38). Studies of immune responses in rodents have identified an important role for non-major histocompatibility complex (MHC) as well as MHC genes in determining immune responses (18), but in humans the relative importance of MHC versus non-MHC genes in influencing antibody, and particularly cellular immune responses, has been little explored.

Methods for mapping and identifying genes contributing to complex phenotypes are increasingly available but remain labor-intensive. Thus, it is important to define the relative contributions of MHC genes and of those genes elsewhere in the genome that influence the human immune response to foreign (and to self) antigens. A recent study, for example, has provided evidence that interleukin-5 production by blood mononuclear cells in response to schistosomula sonicates is subject to control by a major codominant gene (29). Quantification of the effects of these genes and their subsequent identification and analysis may be important for understanding the pathogenesis of a large number of diseases and also for the development of novel subunit vaccines.

We have studied the genetic control of the humoral and cellular immune responses to antigens from the malarial parasite, *Plasmodium falciparum*, in a West African population where all individuals are repeatedly infected by this pathogen. Previous studies of immune responses to malarial antigens have identified substantial variation in the magnitude and type of immune responses made to this parasite (9, 24, 39), and such heterogeneity in response is seen as a potential obstacle to the

development of a successful malaria vaccine. Additionally, we have studied the cellular immune responses to the purified protein derivative (PPD), of *Mycobacterium tuberculosis*, another pathogen to which the great majority of this population will have been exposed.

A comparison between the immune responses of adult monozygous (MZ) and dizygous (DZ) twins who have shared the same environment is a powerful way of determining and dissecting the genetic contribution to the responses under study and of estimating the heritability,  $h^2$ , which may provide evidence of a genetic etiology. The value ranges from 0 to 1 (0 to 100%), with a low value indicative of a low genetic contribution to the trait and a high value suggestive of a larger genetic than environmental component (42). We undertook to study the cellular and humoral immune responses to a broad range of malarial antigens (including several malaria vaccine candidates) in a population of adult twins resident in The Gambia, West Africa: upon exposure to purified malarial antigens or synthetic peptides in vitro, activation of T cells from primed individuals may be demonstrated by proliferative responses and/or by the upregulation and secretion of a range of lymphokines, including gamma interferon and interleukin-4. Proliferative responses of individual Gambians to a single batch of soluble malarial antigen have shown consistency over time, although such antigen-specific responses may be suppressed in cells from acutely infected (clinical) malaria patients (23). Our findings demonstrate that the quantitative immune responses to a variety of antigens are subject to regulation by genetic factors and that the cumulative genetic contribution of non-MHC genes to the total phenotypic variance exceeds that of the MHC-encoded genes.

## MATERIALS AND METHODS

**Study population.** The study was carried out in The Gambia between January 1992 and May 1993. An initial survey by field staff identified 272 complete twin pairs aged 14 years or above living in 222 rural villages; 271 of these pairs agreed to participate, and a 15-ml heparinized blood sample was drawn from each donor and processed within 4 h of collection. The study protocol was approved by the

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TABLE 1. References to specific malarial antigens cited in the text<sup>a</sup>

Antigen	Life cycle stage	Reference(s)
Th2R	Sporozoite	25
Th3R	Sporozoite	15
RLF	Sporozoite	4
PME-6	Merozoite (MSP-1)	12
PME-7	Merozoite (MSP-1)	12
190L	Merozoite (MSP-1)	10
190CST3	Merozoite (MSP-1)/sporozoite	35
3D7	Merozoite (MSP-2)	8
Fc27	Merozoite (MSP-2)	31
RESA	Erythrocytic	26, 39
(EENV) <sub>6</sub>	Erythrocytic	26, 39
(EENVEHDA) <sub>3</sub>	Erythrocytic	26, 39
F32	Soluble antigen	27
GLURP	Soluble antigen	6
Gametocyte lysate	Sexual stage	24

<sup>a</sup> This table provides information about the antigens used and, in certain cases, relevant experimental details and results of immunoepidemiological studies. It is not an exhaustive review of the literature.

Medical Research Council (MRC)/Gambia Government Joint Ethical Committee.

Unequivocal data were obtained from 267 twin pairs, who comprised 60 MZ (zygosity confirmed by minisatellite typing using five minisatellite probes [44]) and 207 DZ pairs aged between 14 and 92 years (median, 22 years). The majority of the twins were raised together: at the time of the study, 59 of the pairs (22%) were separated, residing in different (but nearby) villages; 23 pairs were more widely separated, and, as their cells could not be tested at the same time, they were excluded from analysis of the lymphoproliferative data. All of the twins were HLA class I typed by a standard microcytotoxicity assay, and class II typing was carried out by Southern blot hybridization using radiolabelled HLA-DRB and HLA-DQB probes.

**Lymphoproliferation assays.** Peripheral blood mononuclear cells ( $10^5$ /well) were cultured for 7 days in complete tissue culture medium by the methods described in reference 25. [<sup>3</sup>H]thymidine incorporation was measured by flat-bed liquid scintillation counting with a 1205 Betaplate counter (Wallac Oy, Turku, Finland). Each antigen or mitogen was tested in triplicate at a predetermined optimum concentration (to induce maximal secondary responses in malaria-exposed donors [data not shown]); up to nine unstimulated control wells were included for each donor, and specific vector control antigens were included (where available) for the recombinant proteins ( $\beta$ -galactosidase for PME-6, PME-7, and glutamate-rich protein [GLURP]; dihydrofolate reductase control for 190L). Details of the antigens used (which included components from all stages of the parasite life cycle) are included in references 4, 6, 8, 10, 12, 15, 25 to 27, 31, 35, and 39, detailed in Table 1. They were chosen on the basis of published immunoepidemiological data which indicated that several were potential malaria vaccine candidates. Phytohemagglutinin (Wellcome Diagnostics, Dartford, England) at 5  $\mu$ g/ml, PPD of *M. tuberculosis* (Evans Medical, Leatherhead, United Kingdom) at 50 U/ml, and *Candida albicans* extract (Hollister Stier, Spokane, Wash.) at a 1:1,000 dilution were used as system control antigens.

**ELISAs.** Plasma immunoglobulin G (IgG) antibody titers to all of the study antigens were measured by enzyme-linked immunosorbent assay (ELISA), and the optical density (OD) values were analyzed. The antigens used, coating concentrations, and sample dilutions are as in the references cited in Table 1.

**Statistical methods.** The geometric mean values for the test and control antigens were calculated; they were log transformed to normality and are expressed as log<sub>e</sub> (stimulation index) (1). For each antigen, the intrapair correlation (*r*) of the log<sub>e</sub> (stimulation index) was calculated for MZ and DZ twins separately by using the program TWINAN90 (43). Twice the difference between these values,  $2(r_{MZ} - r_{DZ})$ , yielded Falconer's estimate of heritability,  $h^2$ , the proportion of variance explained by additive genetic effects (7), although it should be noted that this is an upper bound estimate of the heritability. A maximum-likelihood path analysis was also carried out (22, 43) using the same program, to determine which combination of components of variance (additive genetic [A], dominant genetic [D], random environment [E], or environment common to cotwins [C]) most parsimoniously explained the observed data. In the event of a significant dominance effect, the broad-sense heritability was estimated from the model as the proportion of variance explained by additive variance plus dominant variance. For the antibody responses, the crude OD values were log transformed to normality, and these values were utilized by the TWINAN90 program to estimate the *r* values for each antigen.

The variance explained by additive genetic effects can be decomposed into that explained by the HLA class II haplotype and that explained by all other genes, assuming that there is no interaction between these two classes of genes. By considering those DZ twin pairs who share the same HLA class II haplo-

types (haplo-identical DZ twins) but who, unlike MZ twins, may differ at any other locus, we were able to decompose Falconer's estimate of heritability into these two components:  $h^2 = 2(r_{MZ} - r_{DZ}) = 2(r_{MZ} - r_{\text{haplo-identical DZ}}) + 2(r_{\text{haplo-identical DZ}} - r_{DZ}) = h^{n2} + h^{i2}$ , where  $h^{n2}$  is the proportion of the phenotypic variance explained by non-HLA class II genes and  $h^{i2}$  is that explained by the class II haplotype. The contribution of HLA class II-encoded genes to the additive genetic variance is therefore the ratio  $h^{i2}/h^2$ , and that of the non-class II-encoded genes is  $h^{n2}/h^2$ . The 95% confidence interval (CI) estimates for  $h^2$ ,  $h^{i2}$ , and  $h^{n2}$  were calculated based upon reference 42.

## RESULTS

**Genetic regulation of lymphoproliferative responses.** The quantitative responses of 60 pairs of MZ twins were compared with those of 207 DZ pairs, although due to differences in the

TABLE 2. Heritability of cell-mediated immune responses to malarial antigens based upon lymphocyte stimulation indices in adult twin pairs

Stage and antigen (kDa) <sup>a</sup>	MZ		DZ		$h^{2b}$	<i>P</i> <sup>c</sup>	95% CI	Model <sup>d</sup>
	<i>n</i>	<i>r</i> <sup>e</sup>	<i>n</i>	<i>r</i>				
<b>Sporozoite</b>								
Th2R (2.1)	59	0.49	197	0.18	0.62	0.009	0.15, 1.0	AE
Th3R (2.6)	59	0.58	195	0.25	0.66	0.003 <sup>f</sup>	0.23, 1.0	AE
RLF (30)	23	0.39	79	0.37	0.04	0.45	0.0, 0.84	CE
<b>Blood stage</b>								
<b>MSP-1</b>								
PME-6 (21)	59	0.59	189	0.55	0.08	0.33	0.0, 0.46	CE
PME-7 (15)	59	0.37	192	0.34	0.06	0.38	0.0, 0.31	CE
190L (25)	36	0.57	126	0.29	0.56	0.03	0.24, 0.88	AE
190CST3 (27)	35	0.38	126	0.26	0.24	0.23	0.0, 0.57	CE
<b>MSP-2</b>								
MSP-2 (3D7) (50)	59	0.27	197	0.11	0.32	0.13	0.0, 0.88	CE
MSP-2 (Fc27) (50)	56	0.47	193	0.24	0.46	0.04	0.0, 0.92	AE
<b>RESA</b>								
RESA (116)	58	0.61	197	0.40	0.42	0.03	0.02, 0.82	AE
(EENV) <sub>6</sub> (2.8)	58	0.47	189	0.33	0.28	0.12	0.0, 0.76	CE
(EENVEHDA) <sub>3</sub> (2.8)	58	0.55	189	0.16	0.80*	0.001 <sup>f</sup>	0.34, 1.0	ADE
<b>Soluble antigen component</b>								
F32	40	0.64	136	0.48	0.32	0.09	0.0, 0.65	CE
GLURP (80)	56	0.54	181	0.43	0.22	0.16	0.0, 0.72	CE
<b>Sexual stage: gametocyte lysate</b>								
	40	0.74	135	0.35	0.78	0.001 <sup>f</sup>	0.27, 1.0	AE
<b>System control antigens</b>								
Phytohemagglutinin	60	0.62	199	0.52	0.20	0.16	0.0, 0.57	CE
PPD	33	0.57	124	0.19	0.76	0.01	0.19, 1.0	AE
Candida	50	0.57	157	0.38	0.38	0.06	0.0, 0.84	CE

<sup>a</sup> Approximate molecular mass, excluding expression constructs.

<sup>b</sup>  $h^2$  is the Falconer estimate of heritability (7), defined as  $2(r_{MZ} - r_{DZ})$  except for the result marked with an asterisk, where there was a significant dominance effect, as shown by the path model. In this case, Falconer's  $h^2$  would be an overestimate, and so the broad sense heritability was estimated.

<sup>c</sup> Significance level for the test of the existence of a genetic effect on the proliferative response to the antigen: due to the multiplicity of significance tests, these values should be taken only as indicative of their magnitude.

<sup>d</sup> For an explanation of the model, reference should be made to "Statistical methods" in Materials and Methods.

<sup>e</sup> Intraclass correlations for MZ and DZ twin pairs.

<sup>f</sup> Result remains significant after correction for multiple comparisons.

TABLE 3. Correlation between antigens that showed significantly heritable responses in proliferation assays

Antigen	Correlation with:						
	Th2R	Th3R	190L	Fc27	RESA	(EENVEHDA) <sub>3</sub>	Gam
Th3R	0.5						
190L	0.19	0.22					
Fc27	0.52	0.40	0.20				
RESA	0.53	0.36	-0.01	0.48			
(EENVEHDA) <sub>3</sub>	0.37	0.44	0.10	0.40	0.40		
Gam	0.30	0.25	0.15	0.20	0.29	0.07	
PPD	0.42	0.23	0.00	0.30	0.53	0.35	0.22

number of cells obtained from each of the donors, it was not possible to test every donor against every antigen. As shown in Table 2, responses to almost 50% of the antigens show evidence of a significant heritable component, generally consistent with an additive model of individual allele effects. In the case of the synthetic peptide (EENVEHDA)<sub>3</sub>, however, the path model provided a better fit that was consistent with a dominance component. Calculation of the intra-person correlation between the phenotypes showing the highest heritabilities (based upon differences from the mean of each pair) indicates a high degree of correlation between several of the antigens (Table 3), indicating the likely existence of several common genetic pathways.

For those responses that showed a significant heritable component (i.e., >50%) the contribution of the shared MHC class II genes ( $h^2$ ) could be estimated by comparing the  $r$  values of the HLA DRB-DQB haplo-identical DZ twin pairs with the whole of the DZ cohort (Table 4). It is evident that, apart from the responses to 190L, ring-infected erythrocyte surface antigen (RESA), and PPD, the  $r$  values of the class II haplo-identical DZ pairs are no greater than the values for the DZ pairs as a whole, suggesting that the contribution of the HLA class II region to the total phenotypic variance of these responses is small. The contribution of genes lying outside the HLA class II region ( $h^2$ ) was estimated by comparing the  $r$  values of the class II haplo-identical DZ pairs with those of the MZ pairs (Table 5): it is apparent that such genes are, for many of the responses elicited, cumulatively the major genetic de-

TABLE 4. Effect of shared HLA class II haplotype: intraclass correlations ( $r$ ) of lymphoproliferative responses in haplo-identical DZ twin pairs compared with the whole DZ twin cohort

Antigen	Haplo-identical DZ pairs		All DZ twin pairs		$h^{2a}$	$P^b$	95% CI
	$n$	$r$	$n$	$r$			
	Th2R	43	0.09	197			
Th3R	43	0.15	195	0.25	0.00	0.30	0.00, 0.64
190L	26	0.55	126	0.29	0.52	0.07	0.00, 1.00
MSP-2 (Fc27)	41	0.16	193	0.24	0.00	0.35	0.00, 0.65
RESA	43	0.42	197	0.40	0.04	0.43	0.00, 0.59
(EENVEHDA) <sub>3</sub>	39	-0.02	189	0.16	0.00	0.18	0.00, 0.69
Gam	28	0.35	135	0.35	0.00	0.48	0.00, 0.72
PPD	26	0.30	124	0.19	0.22	0.28	0.00, 1.00

<sup>a</sup> Heritability values range from 0 to 1; values <0 have been entered as 0.

<sup>b</sup>  $P$  values refer to a test of the haplo-identical DZ twins (two haplotypes shared) versus all other DZ twins, since the haplo-identical group is a subset of the whole DZ cohort.

TABLE 5. Effect of non-MHC class II genetic factors: intraclass correlations ( $r$ ) of lymphoproliferative responses of haplo-identical pairs of DZ twins compared with MZ pairs

Antigen	MZ		Haplo-identical DZ		$h^2$	$P$	95% CI
	$n$	$r$	$n$	$r$			
	Th2R	59	0.49	43			
Th3R	59	0.58	43	0.15	0.86	0.007	0.18, 1.00
190L	36	0.57	26	0.55	0.04	0.47	0.00, 0.73
MSP-2 (Fc27)	56	0.47	41	0.16	0.62	0.05	0.00, 1.00
RESA	58	0.61	43	0.42	0.38	0.10	0.00, 0.97
(EENVEHDA) <sub>3</sub>	58	0.55	39	-0.02	0.57	0.001	0.00, 1.00
Gam	40	0.74	28	0.35	0.78	0.01	0.08, 1.00
PPD	33	0.57	26	0.30	0.54	0.11	0.00, 1.00

terminants of the magnitude of the proliferative immune responses to those antigens.

**Genetic regulation of humoral responses.** The quantitative IgG responses (based upon the OD values) to three of the malarial antigens (the 83- and 42-kDa components of merozoite surface protein 1 [MSP-1] and the Fc27 allelic variant of MSP-2) show evidence of a significant heritable effect (Table 6). The contribution of the HLA class II region to the total  $h^2$  is greater than was noted for the lymphoproliferative responses (Table 7), with a rather smaller contribution from the non-HLA class II genes (Table 8,  $h^2$ ).

## DISCUSSION

This study demonstrates that heritable factors contribute significantly to the proliferative responses of peripheral blood mononuclear cells to a variety of malarial antigens and also to PPD. While the influence of environmental factors, such as exposure, may be a greater determinant of overall responsiveness to several of the antigens, it is apparent that for those antigen responses that demonstrate a significant heritable component (many of which include known T-cell epitopes), the contribution of HLA class II-encoded genes to the genetic variance is generally much less than the cumulative effect of genes that lie outside this region. Furthermore, it is likely that these non-class II-encoded genes lie outside the entire 4-Mb MHC region, for the estimated recombination rate across this region is approximately 2% (based upon data from reference 19); 98% of the DZ twin pairs with shared class II haplotypes will therefore share the rest of their MHC genes.

Previous studies of immune responses to malarial antigens among populations living in areas where malaria is endemic have failed to demonstrate clear evidence of HLA class II-mediated genetic restriction of cell-mediated immune re-

TABLE 6. Heritability of IgG antibody levels (OD values) from analysis of plasma samples from adult twin pairs by ELISA<sup>a</sup>

Antigen	MZ		DZ		$h^2$	$P$	95% CI <sup>b</sup>	Model
	$n$	$r$	$n$	$r$				
MSP-1 (83 kDa)	60	0.70	205	0.49	0.42	0.01	0.09, 0.75	AE
MSP-1 (42 kDa)	54	0.64	188	0.38	0.52	0.009	0.0, 0.92	AE
MSP-2 (3D7)	59	0.54	204	0.43	0.22	0.17	0.0, 0.66	CE
MSP-2 (Fc27)	59	0.59	203	0.37	0.44	0.03	0.02, 0.86	AE
(EENVEHDA) <sub>3</sub>	60	0.47	207	0.28	0.30	0.06	0.0, 0.77	CE

<sup>a</sup> Annotations as for Table 2.

<sup>b</sup> As  $h^2$  values range between 0 and 1, values <0 are entered as 0.

TABLE 7. Effect of shared HLA class II haplotype: intraclass correlations ( $r$ ) of IgG (OD) responses in haplo-identical DZ twin pairs compared with the whole DZ twin cohort

Antigen	Haplo-identical DZ pairs		All DZ twin pairs		$h^2$	$P$	95% CI
	$n$	$r$	$n$	$r$			
	MSP-1 (83 kDa)	46	0.51	205			
MSP-1 (42 kDa)	43	0.47	188	0.38	0.18	0.23	0.00, 0.71
MSP-2 (Fc27)	44	0.58	203	0.37	0.42	0.05	0.00, 0.88

sponses to defined malarial antigens (28, 40), and multiple regression analysis of data from 496 Gambian subjects indicated that HLA-related variation accounted for no more than 14% of the total variation in the measured immune responses (28). Nevertheless, a significant MHC effect was apparent in the response to 190L from a conserved region of MSP-1. This region has been shown to contain a variety of T-cell epitopes that are recognized in association with HLA-DR, DQ, or DP molecules (10); it is also possible that other restriction elements mapping to the MHC account for our findings.

Interactions between peptides and MHC molecules are permissive (20), and the specificity of the immune response is maintained by the specificity of the recognition by the T cell itself (33, 34). This latter effect may account, at least in part, for the increased correlation of MZ pairs over class II haplo-identical DZ pairs, as factors outside the MHC class II region have been demonstrated to influence T-cell receptor V $\beta$  gene expression (5). Genetic variation in antigen processing or in T-cell activation pathways may also account for our results, and a small Swedish study has provided evidence that differences at the level of the antigen-presenting cell may account for some of the non-MHC-related genetic differences in antigen-induced T-cell proliferation (41); based upon the observed correlations between the antigens in our study, it is likely that several common pathways are involved. A number of polymorphic genes that may affect the activity of a wide variety of immune response cells have been identified, although their functional status and role in human disease susceptibility remain to be defined (13). Three non-MHC polymorphic genes that have been demonstrated to influence human infectious disease susceptibility include mannose binding protein (37), CD32 (3), and the secretor (*FUT2*) blood group locus (2).

Our results also demonstrate evidence of genetic regulation over the detected level of IgG antibody to certain of the malarial antigens, notably to the 42- and 83-kDa fragments of MSP-1 (levels of which were also noted to be highly heritable in a cohort of Gambian twin children [12a]). Previous twin studies have reported genetic regulation over antibody re-

sponses to diphtheria toxoid and to measles (11), pneumococcal capsular polysaccharide (14), malarial antigens (36), and *Helicobacter pylori* (17), although in none of these studies was quantification of the MHC contribution defined.

The contribution of the class II genes to the total genetic variance for each of the antibody responses is clearly quite different and may reflect the presence of a T-cell epitope(s) required to provide help for antibody production to that antigen. It is therefore of interest that both the antibody and proliferative responses to the Fc27 allelic variant of MSP-2 demonstrated significant genetic variance, for more T-cell epitopes have been recognized in this variant than in the 3D7 strain (32).

Animal studies using strains of genetically selected high- and low-antibody-producing Biozzi mice have defined several regions of the mouse genome that show strong linkage to antibody responsiveness (21): the two strongest linkages were to markers that mapped to the *Igh* locus (on chromosome 12) and the *H-2* region on chromosome 17 (high- and low-antibody-producing mice have been shown to display different allotype determinants [16]). The authors postulate that VH gene polymorphism could influence antigen recognition, while CH genes could modulate antibody effector functions. Additional linkage was found to the proximal part of mouse chromosome 6, which includes several candidate genes: the *Cd8* locus, *Tcrb*, and *Igk*. Such genes may well play a role in the regulation of human immune responses, and genetic linkage studies are in progress to address this issue.

In summary, therefore, our data highlight the role of genetic factors in the acquired immune response of humans to defined parasite antigens and imply that significant polymorphisms exist in a wider range of immune response genes than hitherto recognized. Identification of these genes offers the potential for novel disease prevention and intervention strategies in the future; furthermore, their functional significance in relation to the design and efficacy of malarial (and other) vaccines needs clarification.

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TABLE 8. Effect of non-MHC genetic factors: intraclass correlations ( $r$ ) of OD values of haplo-identical pairs of DZ twins compared with MZ pairs

Antigen	MZ		Haplo-identical DZ		$h^2$	$P$	95% CI
	$n$	$r$	$n$	$r$			
	MSP-1 (83 kDa)	60	0.70	46			
MSP-1 (42 kDa)	54	0.64	43	0.47	0.34	0.13	0.00, 0.90
MSP-2 (Fc27)	59	0.59	44	0.58	0.02	0.48	0.00, 0.53

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