Bovine Leukocyte Antigen Major Histocompatibility Complex Class II *DRB3**2703 and *DRB3**1501 Alleles Are Associated with Variation in Levels of Protection against *Theileria parva* Challenge following Immunization with the Sporozoite p67 Antigen

Keith T. Ballingall,^{1*} Anthony Luyai,^{1†} G. John Rowlands,¹ Jill Sales,² Anthony J. Musoke,¹ Subash P. Morzaria,^{1‡} and Declan J. McKeever^{1§}

International Livestock Research Institute, Nairobi, Kenya,¹ and BioSS, Edinburgh EH9 3JZ, Scotland, United Kingdom²

Received 4 December 2003/Returned for modification 10 January 2004/Accepted 9 February 2004

Initial laboratory trials of an experimental subunit vaccine against *Theileria parva* based on the 67-kDa major sporozoite surface antigen revealed a range of responses to challenge. We have analyzed convergence in seven sets of monozygotic twins which suggests that genetic factors may have an influence in determining the degree of protection provided by p67 immunization. In addition, we have examined whether allelic diversity at major histocompatibility complex class II loci influences protection. Analysis of bovine leukocyte antigen *DRB3* diversity in 201 animals identified significant associations with vaccine success (*DRB32703; $P = 0.027$) and vaccine failure (*DRB3**1501; $P = 0.013$). Furthermore, *DRB3**2703 was associated with the likelihood of immunized animals showing little to no clinical signs of disease following challenge. We discuss the acquired and innate immune mechanisms that may be behind the associations described here.**

Theileria parva is a tick-borne parasite of cattle in sub-Saharan Africa that causes a fatal lymphoproliferative disease known as East Coast fever (ECF). The life cycle of the parasite in the mammalian host in many respects resembles that of *Plasmodium falciparum*, the causative agent of human malaria (9). Infective sporozoites of *T. parva* rapidly invade host lymphocytes after inoculation by an infected tick. Their subsequent differentiation to multinucleated schizonts is associated with transformation of the infected cell to a state of uncontrolled proliferation (10). By associating with the cellular spindle apparatus, the parasite divides in synchrony with the infected cell, allowing rapid expansion of aggressively invasive infected lymphoblasts (8). Susceptible animals rarely survive beyond 3 weeks of infection. Animals that recover from infection are solidly immune to homologous challenge, and numerous studies have established that protection is primarily mediated by major histocompatibility complex (MHC) class I-restricted parasite-specific cytotoxic T lymphocytes (reviewed in reference 9). However, cattle immunized with sporozoite lysate or those naturally exposed to repeated challenge develop neutralizing antibodies against a 67-kDa molecule (p67) found on the surface of the sporozoite (4, 12). These observations prompted an evaluation of the plausibility of a p67-based neutralizing vaccine against *T. parva*. In preliminary trials of an

Escherichia coli recombinant p67 product, a range of ECF reactions were observed following 70% lethal dose challenge, despite the observation of high titers of specific antibody in all immunized animals (11). While a third of the immunized cattle showed no protection against challenge, the remaining animals were all protected to some degree. Of these, a third developed no clinical signs of infection and two-thirds showed mild or moderate symptoms. No association was evident between the outcome of challenge and antibody titer in this and subsequent trials (11, 14). Indeed, no immunological parameter has been identified that correlates well with the variable outcomes of challenge in p67-immunized cattle.

The MHC genetic region encodes cell surface molecules that are central to the induction and regulation of adaptive immunity (6, 19). Numerous associations have been identified between the highly polymorphic MHC class I and class II loci and autoimmune disease, infectious disease, and responses to immunization (2, 5, 7, 17, 18, 23).

The primary aim of this study was to establish if allelic diversity at the extensively polymorphic and principal expressed MHC class II *DRB3* locus plays a role in determining the variation in protection against *T. parva* challenge following immunization with the sporozoite p67 antigen.

MATERIALS AND METHODS

Animals. Monozygotic twin cattle were derived by splitting Boran (*Bos indicus*) or Boran/European *Bos taurus* embryos and implanting them in surrogate dams. All other cattle used in the study were 5- to 8-month-old Boran animals obtained from the ECF-free International Livestock Research Institute ranch. All animals were seronegative for *T. parva* at the outset of the study.

Genetic analysis. Genomic DNA of individual animals was obtained from 20 ml of peripheral blood using standard procedures. All cattle were typed at the highly polymorphic MHC class II *DRB3* locus by PCR-restriction fragment length polymorphism (RFLP) analysis according to the method of van Eijk et al. (20) using a combination of RsaI, BstYI, and HaeIII restriction enzyme patterns.

* Corresponding author. Present address: Department of Veterinary Clinical Studies, The University of Edinburgh, Easterbush Veterinary Centre, Easterbush, Roslin, Midlothian, Scotland, United Kingdom. Phone: 44 (0)131 650 6273. Fax: 44 (0)131 650 6588. E-mail: k.ballingall@ed.ac.uk.

† Present address: Department of Animal Science, Oklahoma State University, Stillwater, Okla.

‡ Present address: SAO, Bangkok, Thailand.

§ Present address: The Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, Scotland, United Kingdom.

TABLE 1. ECF reaction index scores for monozygotic twin pairs

Pair and animal no.	Index score
Immune	
Bp208	1.57
Bp209	0.28
1	
Bp388	6.17
Bp389	8.85
2	
Br66	9.09
Br67	9.55
3	
Br37	0.49
Br38	0.45
4	
Bs23	7.52
Bs24	8.32
5	
Bs26	5.30
Bs27	8.87
6	
Bs28	9.22
Bs29	8.54
7	
Bs30	7.32
Bs31	4.78

Alleles were designated according to recognized nomenclature guidelines (www.projects.roslin.ac.uk/bola/dr3pcr.html).

Cloning and nucleotide sequence analysis. Exon 2 fragments from bovine leukocyte antigen (BoLA) *DRB3* alleles were amplified by PCR and cloned into the pGEM T-easy vector (Promega). Nucleotide sequence was determined from three independent clones using the fmol sequencing kit (Promega) and analyzed using the DNASIS MAX package (Hitachi Software, San Bruno, Calif.).

Immunization and monitoring of cattle. Cattle were immunized intramuscularly by inoculation of *E. coli* recombinant p67 products in a proprietary adjuvant formulation as described by Musoke et al. (11). All cattle were challenged with a previously determined 70% lethal dose of cryopreserved *T. parva* sporozoites (Muguga stock) inoculated subcutaneously over a parotid lymph node. Clinical, parasitological, and hematological parameters were monitored after challenge as described elsewhere (16). Briefly, rectal temperatures were recorded daily after challenge, schizont parasitosis in the draining lymph node was evaluated from day 5 by analysis of Giemsa-stained smears of needle aspirates, and corresponding analysis of a distant node was conducted daily after parasites were first observed. Similarly, daily blood samples were analyzed for total white blood cell count and the presence of tick-infective piroplasms in erythrocytes. Data were collected from 201 immunized Boran cattle in the course of 13 laboratory challenge experiments exploring various regimens for the delivery of the p67 sporozoite antigen.

Statistically derived index of reactions to challenge. Traditional classification of ECF reactions as nonreactor, mild, moderate, or severe (1) precluded a rigorous statistical analysis of challenge data. A statistically derived reaction index (16) was therefore used to provide an objective assessment of the severity of the ECF reaction in each animal. Based on principal component analysis of 13 parasitological, clinical and hematological parameters recorded daily after challenge, a reaction index score of between 0 and 10 was calculated for each animal. Scores correspond roughly to the traditional classification as follows: 0 to 0.99, nonreactor; 1 to 3.49, mild reactor; 3.5 to 5.99, mild or moderate reactor; 6 to 7.99, moderate or severe reactor; 8 to 10, severe reactor.

Statistical analysis of reaction index data. The relationship between reaction index score and possession of particular BoLA *DRB3* alleles was investigated using a multiple regression model. Only animals that had at least one allele identified were included in the analysis. None of the animals were twins or full siblings. Each of the alleles that occurred in any of the animals was included in the model as a dummy variable. The ECF scores were obtained from 13 different

experiments exploring various regimens for the delivery of the p67 sporozoite antigen. As such, the experimental protocol was fitted as a random factor in the model. Estimates of the model parameters were obtained using the REML directive in the Genstat software package (6th ed.). The scores from seven pairs of twins were also analyzed separately using a one-way analysis of variance.

RESULTS

Evaluation of ECF reactions in monozygotic twins. ECF reaction index scores for individual animals from the seven pairs of naïve monozygotic twins are presented in Table 1. (This table also includes an additional pair of immune twins which have not been included in the present analysis.) With the exception of one pair, the immunized twins all displayed symptoms of ECF, with reaction index values ranging from 4.79 to 9.22. Neither of the remaining pair of twins (Br37 and Br38) showed pyrexia, schizont parasitosis, or hematological variation from day 0 values (data not shown). Both animals scored less than 1.0 on the reaction index. Analysis of variance revealed that the within-pair variability of index score was considerably less than the between-pair variability score ($P = 0.005$). This similarity of response within monozygotic twin pairs is likely to reflect the influence of genetic rather than environmental factors in determining the final outcome of challenge following vaccination.

BoLA MHC class II *DRB3* allelic frequencies in p67-immunized cattle. The BoLA *DRB3* alleles were identified by PCR-RFLP analysis in 201 Boran cattle subjected to immunization and challenge. The distribution of these alleles across the 201 animals is presented in Table 2, subgrouped with respect to the

TABLE 2. Table showing the number (frequency) of BoLA *DRB3* alleles occurring in animals that develop low or high ECF following p67 vaccination^a

BoLA <i>DRB3</i> allele	No. (frequency) in animals that develop:	
	Low ECF	High ECF
Unknown	9 (0.039)	7 (0.040)
02	16 (0.070)	14 (0.081)
03	9 (0.039)	7 (0.040)
06	10 (0.044)	2 (0.011)
11	16 (0.070)	11 (0.063)
12	5 (0.022)	4 (0.023)
13	1 (0.004)	1 (0.006)
15	14 (0.061)	6 (0.034)
16	3 (0.013)	8 (0.046)
18	7 (0.031)	2 (0.011)
20	3 (0.013)	2 (0.011)
22	13 (0.057)	10 (0.058)
23	10 (0.044)	2 (0.011)
27	14 (0.061)	12 (0.069)
31	57 (0.250)	51 (0.293)
32	1 (0.004)	1 (0.006)
33	11 (0.048)	8 (0.046)
34	13 (0.057)	12 (0.069)
35	4 (0.018)	3 (0.017)
37	1 (0.004)	1 (0.006)
44	1 (0.004)	1 (0.006)
49	9 (0.039)	9 (0.052)
54	1 (0.004)	0 (0.000)
Total	228	174

^a Note that in Table 2 each animal is included twice. Unknown corresponds to BoLA *DRB3* alleles that could not be accurately assigned using the methods described here. Low ECF, ECF index <6; high ECF, ECF index ≥6.

FIG. 1. Range of distribution of ECF reaction scores in 201 vaccinated and challenged animals.

ECF index scores. A total of 22 alleles were identified in the sample, though the frequency of eight of these was less than 2%. The distribution of frequencies of the ECF reaction scores is plotted in Fig. 1. The scores are clearly not normally distributed, as distinct peaks are observed towards both ends of the distribution. This suggests that the population is not homogeneous, incorporating subpopulations with markedly lower or higher mean scores than the average.

To determine whether there is an association between the presence of a particular MHC allele and ECF reaction index values, a multiple regression model was fitted to the ECF score data, with each allele included as a dummy variable. Only three alleles, 06, 16, and 23, appeared to influence the value of the reaction score. Output from the reduced regression model with these three alleles included as explanatory variables is shown in Table 3. There is evidence of an effect for both of alleles 16 and 23 (P values of 0.013 and 0.027, respectively) and to a lesser extent for allele 06 ($P = 0.040$). Animals that were heterozygous or homozygous for allele 16 tended to have higher-than-average scores (2.5 above the mean), and those with alleles 06 or 23 tended to have lower reaction scores (1.9 and 2.2 below the mean, respectively).

The number of animals possessing at least one of these alleles in each of the reactor groups is shown in Table 4. Allele 06 was present in 12 of the cattle, while 11 animals possessed allele 16. One animal was heterozygous for alleles 06 and 16, and scored 7.69 on the reaction index. Eleven animals possessed allele 23, and interestingly, the proportion of these animals in the nonreactor category (reaction index score < 1.0) was 0.55, compared with 0.22 for the population as a whole. Conversely, no animals possessing allele 16 yielded reaction index scores of less than 1.

TABLE 3. Estimated effects on ECF index of possessing certain alleles compared with not possessing these alleles ($n = 194$)

Allele	Frequency	Mean	SE	95% confidence interval	P
Other	0.91	4.86	0.36		
06	0.03	-1.91	0.93	[-3.74, -0.08]	0.040
16	0.03	2.46	0.99	[0.51, 4.40]	0.013
23	0.03	-2.17	0.98	[-4.10, -0.24]	0.027

TABLE 4. Number of animals (proportion) in each of the reactor groups with at least one of the significant alleles^a

Allele	No. (proportion) of animals in group ^b :		
	Nonreactor	Mild or moderate reactor	Severe reactor
06	4 (0.33)	7 (0.58)	1 (0.08)
16	0 (0)	6 (0.55)	5 (0.45)
23	6 (0.55)	4 (0.36)	1 (0.09)
Other	35 (0.22)	85 (0.53)	41 (0.25)

^a Note that one animal with alleles 06 and 16 has been included twice and that animals with two unknown alleles have been omitted ($n = 194$).

^b Nonreactor, < 1 ; mild or moderate reactor, 1 to 8; severe reactor, > 8 .

BoLA *DRB3* nucleotide sequence determination. Second exon nucleotide sequence of the PCR-RFLP analysis-defined alleles 06, 16, and 23 was determined for at least three different animals expressing each allele. In accordance with existing BoLA class II classification (www.projects.roslin.ac.uk/bola/dr3pcr.html) the alleles were found to correspond to BoLA *DRB3**2201, *DRB3**1501, and *DRB3**2703, respectively.

DISCUSSION

Analysis of convergence in monozygotic twins during the course of this study has provided evidence that genetic factors have an influence in determining the final outcome of p67 immunization and challenge. Initially the purpose of this study was to evaluate the influence of MHC class II genotype on the outcome of immunization of cattle with a prototype subunit vaccine against *T. parva*. We wished to test the hypothesis that the outcome of immunization was dependent on the magnitude and specificity of the antibody response, which in turn was under the control of the MHC class II genotype. However, it became apparent that the immune response of all vaccinated cattle was characterized by high antibody titers (of the order of 10^4 to 10^5), dominated by immunoglobulin G isotypes (11, 13). As reported previously (13, 14), no association was apparent in any of the immunization experiments between the outcome of challenge and either the magnitude or the linear specificity of the antibody response. In addition, none of the immunized animals demonstrated consistent CD4⁺-T-cell responses (11, 13). We have subsequently observed that sera from nonreactor and severe-reactor animals also show similar titers of antibody that binds the sporozoite surface as assessed by flow cytometry (our unpublished observations), suggesting that these groups do not differ significantly in the recognition of conformational epitopes. In light of these observations, it is not surprising that we were unable to demonstrate associations between the magnitude or specificity of the antibody response and the presence of individual BoLA *DRB3* alleles. Rather, our results indicate that presentation of p67 epitopes was similarly effective in all animals irrespective of the MHC class II genotype.

Despite the lack of an apparent association between MHC phenotype and the immune response to vaccination, significant associations (P values = 0.027 and 0.013, respectively) were observed between two of the alleles (*DRB3**2703 and *DRB3**1501) and the reaction of vaccinated animals to challenge. Such associations could have arisen through various direct or indirect mechanisms. Direct mechanisms focus on the major functional role of MHC class II molecules in presenta-

tion of antigenic peptides to specific CD4⁺ T cells. In addition to provision of help for the observed strong B-cell response, priming of p67-specific T cells may have direct T-cell-mediated effects on recently infected lymphocytes, either through cytotoxicity or cytokine-mediated inhibition of parasite development. Although p67 is not expressed by the parasite after invasion, it is shed by the sporozoite as it enters the host cell and remains associated with the lymphocyte membrane (21). It is possible that this shed antigen is endocytosed and processed for presentation by MHC class II, which is expressed by activated bovine lymphocytes (15). Overlapping-peptide analysis of cattle immunized with native p67 formulated in complete Freund's adjuvant suggests that T-cell epitopes are distributed throughout the molecule (13). Under circumstances where such mechanisms were affecting parasite development, it is possible that MHC class II phenotype would influence their efficacy, despite having no detectable effect on the antibody responses.

An alternative indirect explanation for our observations is that the *DRB3* locus is simply a genetic marker for a linked gene or genes that are involved in controlling the infection. In this regard, a number of studies have already linked the *DRB3*2703* allele with resistance to disease, including severe clinical mastitis in Holstein cattle (17) and persistent lymphocytosis associated with bovine leukemia virus (22, 24). Although the identity and function of the linked element remains to be determined, its potential role in resistance to protozoal as well as bacterial and viral infection is of substantial interest.

The fact that no association was observed between the magnitude, specificity or neutralizing capacity of the antibody response and reaction to challenge does not exclude a role for antibody in the outcome of these trials. It is possible that all animals neutralized the challenge infection equally effectively and that the range of observed reactions reflected variation in additional innate responses that addressed the reduced breakthrough infection with variable efficacy. Evidence supporting this possibility emerges from previous studies that established the dose dependence of disease severity following *T. parva* challenge. A threshold dilution was observed below which all animals react severely, but above which a range of reactions were recorded (3).

In the light of these observations, a plausible interpretation of the outcome of the p67 vaccine trials is that the variation among the vaccinated animals in reaction to challenge arose from intrinsic differences in innate immune capacity. Our evidence that genetic factors influence the outcome of challenge and our identification of significant associations between the severity of reaction and MHC class II genotype suggest that the relevant innate mechanisms may be controlled by genetic elements linked to the MHC.

ACKNOWLEDGMENTS

This work was funded by the Consultative Group on International Agricultural Research (CGIAR). K. Ballingall is currently supported by the Wellcome Trust (grant 065442).

REFERENCES

1. Anonymous. 1989. Theileriosis in eastern, central and southern Africa, p. 187–188. In T. T. Dolan (ed.), *Proceedings of a workshop on East Coast fever immunisation*. ILRAD, Nairobi, Kenya.

2. Bodmer, W. F. 1995. Evolution and function of the HLA region. *Cancer Surv.* 22:5–16.
3. Cunningham, M. P., C. G. Brown, M. J. Burridge, A. J. Musoke, R. E. Purnell, D. E. Radley, and C. Sempabwa. 1974. East Coast fever: titration in cattle of suspensions of *Theileria parva* derived from ticks. *Br. Vet. J.* 130:336–345.
4. Dobbelaere, D. A., S. Z. Shapiro, and P. Webster. 1985. Identification of a surface antigen on *Theileria parva* sporozoites by monoclonal antibody. *Proc. Natl. Acad. Sci. USA* 82:1771–1775.
5. Gelder, C. M., R. Lambkin, K. W. Hart, D. Fleming, O. M. Williams, M. Bunce, K. I. Welsh, S. E. Marshall, and J. Oxford. 2002. Associations between human leukocyte antigens and non-responsiveness to influenza vaccine. *J. Infect. Dis.* 85:114–117.
6. Germain, R. N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* 76:287–299.
7. Hill, A. V., C. E. Allsopp, D. Kwiatkowski, N. M. Anstey, P. Twumasi, P. A. Rowe, S. Bennett, D. Brewster, A. J. McMichael, and B. M. Greenwood. 1991. Common West African HLA antigens are associated with protection from severe malaria. *Nature* 352:595–600.
8. Hulliger, L., K. H. Wilde, C. G. Brown, and L. Turner. 1964. Mode of multiplication of *Theileria* in cultures of bovine lymphocytic cells. *Nature* 203:728–730.
9. McKeever, D. J., E. L. Taracha, W. I. Morrison, A. J. Musoke, and S. P. Morzaria. 1999. Protective immune mechanisms against *Theileria parva*: evolution of vaccine development strategies. *Parasitol. Today* 15:263–267.
10. Morrison, W. I., B. M. Goddeeris, W. C. Brown, C. L. Baldwin, and A. J. Teale. 1989. *Theileria parva* in cattle: characterization of infected lymphocytes and the immune responses they provoke. *Vet. Immunol. Immunopathol.* 20:213–237.
11. Musoke, A. J., S. Morzaria, C. Nkonge, E. Jones, and V. Nene. 1992. A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle. *Proc. Natl. Acad. Sci. USA* 89:514–518.
12. Musoke, A. J., V. M. Nantulya, F. R. Rurangirwa, and G. Buscher. 1984. Evidence for a common protective antigenic determinant on sporozoites of several *Theileria parva* strains. *Immunology* 52:231–238.
13. Musoke, A. J., V. Nene, and D. J. McKeever. 1995. Epitope specificity of bovine immune responses to the major surface antigen of *Theileria parva* sporozoites, p. 57–61. In R. M. Chanock, F. Brown, H. S. Ginsberg, and E. Norrby (ed.), *Vaccines 95*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
14. Nene, V., E. Gobright, R. Bishop, S. Morzaria, and A. Musoke. 1999. Linear peptide specificity of bovine antibody responses to p67 of *Theileria parva* and sequence diversity of sporozoite-neutralizing epitopes: implications for a vaccine. *Infect. Immun.* 67:1261–1266.
15. Quade, M. J., and J. A. Roth. 1999. Dual-colour flow cytometric analysis of phenotype, activation marker expression, and proliferation of mitogen-stimulated bovine lymphocyte subsets. *Vet. Immunol. Immunopathol.* 67:33–45.
16. Rowlands, G. J., A. J. Musoke, S. P. Morzaria, S. M. Nagda, K. T. Ballingall, and D. J. McKeever. 2000. A statistically derived index for classifying East Coast fever reactions in cattle challenged with *Theileria parva* under experimental conditions. *Parasitology* 120:371–381.
17. Sharif, S., B. A. Mallard, B. N. Wilkie, J. M. Sargeant, H. M. Scott, J. C. Dekkers, and K. E. Leslie. 1998. Associations of the bovine major histocompatibility complex DRB3 (*BoLA-DRB3*) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. *Anim. Genet.* 29:185–193.
18. Tomlinson, I. P., and W. F. Bodmer. 1995. The HLA system and the analysis of multifactorial genetic disease. *Trends Genet.* 11:493–498.
19. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601–624.
20. van Eijk, M. J., J. A. Stewart-Haynes, and H. A. Lewin. 1992. Extensive polymorphism of the *BoLA-DRB3* gene distinguished by PCR-RFLP. *Anim. Genet.* 23:483–496.
21. Webster, P., D. A. Dobbelaere, and D. W. Fawcett. 1985. The entry of sporozoites of *Theileria parva* into bovine lymphocytes in vitro. *Immunoelectron microscopic observations*. *Eur. J. Cell Biol.* 36:157–162.
22. Xu, A., M. J. van Eijk, C. Park, and H. A. Lewin. 1993. Polymorphism in *BoLA-DRB3* exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. *J. Immunol.* 151:6977–6985.
23. Yoshikawa, T., Y. Suzuki, A. Nomoto, T. Sata, T. Kurata, and S. Tamura. 2002. Antibody responses and protection against influenza virus infection in different congenic strains of mice immunized intranasally with adjuvant-combined A/Beijing/262/95 (H1N1) virus hemagglutinin or neuraminidase. *Vaccine* 21:60–66.
24. Zanotti, M., G. Poli, W. Ponti, M. Polli, M. Rocchi, E. Bolzani, M. Longeri, S. Russo, H. A. Lewin, and M. J. van Eijk. 1996. Association of *BoLA* class II haplotypes with subclinical progression of bovine leukaemia virus infection in Holstein-Friesian cattle. *Anim. Genet.* 27:337–341.