

Evidence of Genetic Susceptibility to *Chlamydia trachomatis*-Induced Pelvic Inflammatory Disease in the Pig-Tailed Macaque

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The macaque model of chlamydial pelvic inflammatory disease (PID) demonstrates individual variability in the time of onset of intrapelvic adhesions. Some animals develop adhesions rapidly, within 2 weeks after a single tubal inoculation with *Chlamydia trachomatis*, while in others, adhesions are not observed until 2 weeks after a second tubal inoculation. To test whether this variability correlates with major histocompatibility complex (MHC) class I haplotype, we used macaque alloantisera and mouse anti-HLA monoclonal antibodies to determine the MHC class I haplotypes of 44 *C. trachomatis*-infected macaques (*Macaca nemestrina*). Macaques developing gross tubal adhesions after the first chlamydial inoculation were classified as susceptible ($n = 29$), while those not developing adhesions until after the second chlamydial inoculation were classified as relatively resistant ($n = 15$), to adhesion formation. Three antibody specificities correlated with susceptibility (odds ratio [OR] 5.2, $P < 0.01$; OR 6.1 and 4.3, $P < 0.05$), and two correlated with relative resistance to adhesions (OR 0.1, $P < 0.05$; OR 0.2, $P < 0.01$). Because several of these antibodies are cross-reactive, as many as five different MHC class I alleles (three increasing and two decreasing ORs) or as few as two different MHC class I alleles (one increasing and one decreasing OR) could be correlated with risk of adhesion formation. We conclude that in macaques, susceptibility or relative resistance to rapid formation of tubal adhesions is correlated with expression of MHC class I alleles, consistent with reports of MHC class I restriction of chlamydial immunopathology in humans.

The hypothesis that genetic factors influence susceptibility to the inflammatory complications of *Chlamydia trachomatis* infection has been strongly supported by epidemiologic studies of both ocular and genital *C. trachomatis* infections in humans (8, 26). For example, the major histocompatibility complex (MHC) class I allele HLA B27 is a well-documented risk factor for Reiter's syndrome, a form of polyarthritis that can occur as a sequela of *C. trachomatis* infection (18). More recently, the occurrence of *C. trachomatis*-induced pelvic inflammatory disease (PID), as opposed to infection limited to the cervix, has been associated with expression of HLA A31, C2, and C3 (7). Similarly, antibody response to a chlamydial antigen, the 57-kDa chlamydial heat-shock protein (cHSP), was correlated with expression of the MHC class II alleles DR and DQ in ocular trachoma patients (15). Although chlamydial replication takes place within intracellular membrane-bound inclusion bodies, indicating that MHC class II molecules present chlamydial antigen, CD8 T lymphocytes appear to be activated during chlamydial salpingitis in the macaque model, suggesting that MHC class I molecules also present chlamydial antigens (10, 27).

A mouse model has also been used to investigate genetic susceptibility to complications of *C. trachomatis* infection. Using this well-characterized model, susceptibility to chlamydial PID varied by mouse genotype (25), and both increased and decreased immune responses to cHSPs were associated with expression of specific MHC (*H-2*) alleles (29). Although the pig-tailed macaque MHC has not been as well characterized as

that of the mouse, the macaque has proven to be a valuable model of human chlamydial salpingitis and PID (14). With this model, a relatively low inoculum of *C. trachomatis* reliably establishes infection (13) and eventually induces adhesions similar to those seen in human PID (11, 12). Some animals develop adhesions rapidly, within 2 weeks after a single tubal inoculation with *C. trachomatis*, while in others, adhesions are not observed until 2 weeks after a second tubal inoculation. This model thus offers an opportunity for testing the relationship between MHC haplotype and the rapidity of progression of immunopathology in an animal which has immunologic responses to chlamydia similar to those of humans (10, 16). To accomplish this, we used two similar detection methods (one using macaque alloantisera [MnLA] and one using mouse anti-HLA monoclonal antibodies [MAbs] [2, 3]) to define MHC class I haplotypes in *C. trachomatis*-infected macaques. Antibody reactions to MHC class I molecules (specificities) were then compared between animals that developed adhesions rapidly and those that developed lesions slowly. Our purpose in this investigation was to establish whether evidence of genetic susceptibility to PID could be observed in the macaque and, if so, to develop hypotheses regarding the role of MHC class I alleles in susceptibility to PID that could be tested by using the macaque model.

(Portions of this work were published previously [12].)

MATERIALS AND METHODS

Animals. Forty-four sexually mature pig-tailed macaques (*Macaca nemestrina*) were enrolled in this study. All monkeys were housed at the University of Washington's Regional Primate Research Center.

Inoculation. Serovar D (P0124), a human endometrial isolate of *C. trachomatis*, was divided into aliquots of 6×10^6 inclusion-forming units per ml in sucrose-phosphate glutamate buffer and frozen at -70°C until used. At laparot-

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omy, the fallopian tubes were inoculated directly through the fimbrial os with 0.2 ml of chlamydias per tube, and the cervix was inoculated with 0.1 ml. The fallopian tubes and cervix were inoculated two times at 2-week intervals ($n = 18$ monkeys) or three times at 2-week intervals ($n = 26$ monkeys) to establish chronic chlamydial infection (11, 12). Data for the reported observations were collected at the time of the first (baseline observation) and second inoculations only. During this time period, all animals received identical treatments.

Visual assessment. At the time of the first and second inoculations, progression of disease was scored visually and was also monitored by video recording of the upper reproductive tract. Direct observations of gross tubal pathology and adhesions were also made at laparotomy following experimental treatment after the second inoculation, at laparoscopy following the final inoculation, and at hysterectomy but are not reported here (11, 12). The following scoring index (gross adhesion score) was used to evaluate tubal damage pre- and posttreatment: 0, normal; 1, dilatation (edema) of fallopian tubes and erythema; 2, dilatation plus mild adhesions (peritubal); 3, dilatation plus moderate adhesions (peritubal and adnexal); and 4, dilatation plus severe adhesions (peritubal, periadnexal, and peritoneal).

Animals developing grossly evident adhesions (gross adhesion score of ≥ 2) following the first inoculation with *C. trachomatis* (viewed at the time of the second intratubal inoculation) were classified as susceptible, while those that did not develop lesions until after the second inoculation (viewed at laparoscopy or at hysterectomy) were considered relatively resistant. At the infectious dose of *C. trachomatis* used, all experimental macaques eventually develop adhesions (11, 12). The surgical team responsible for scoring adhesions was blinded as to the MHC status of all animals.

Specimen collection. To detect chlamydial infection, swabs were obtained from the cervix for cell culture and ligase chain reaction and from the fimbrial os of each tube for ligase chain reaction and cell culture every 2 weeks throughout the experiment and at hysterectomy (11, 12).

MHC class I typing. (i) MnLA. To identify individual macaque MHC class I haplotypes, peripheral blood leukocytes (PBL) were isolated from blood samples drawn from each animal at the beginning of the study. Standard two-step microcytotoxicity assays using 47 alloantisera previously identified in *M. nemestrina* were used to type MHC class I molecules on the PBL. Briefly, these macaque alloantisera were developed by using skin allografts and/or subcutaneous injections of peripheral blood lymphocytes from full sibling or parental donor *M. nemestrina* (2). The alloantisera were then screened for reactivity with donor lymphocytes from unrelated macaques, and conventional immunogenetic methods were applied for population and family data analysis (6, 9, 17). The 47 alloantisera were characterized as reacting with MHC antigens encoded by A-like, B-like, or undefined macaque loci (Table 1). These alloantisera were cross-reactive, reacting either with similar antigenic sites (epitopes) shared by more than one MHC molecule (public epitopes) or with more than one epitope (multispecific) (2).

(ii) Mouse anti-HLA MAb. Macaque PBL were also assessed by using a panel of 22 mouse MAbs to human class I HLA epitopes (Table 2) in standard two-step microcytotoxicity tests. Mouse MAbs were obtained from the American Type Culture Collection, Duke University, Genetic Systems Corporation, or Stanford University (3). Both methods (MnLA and MAb) were used in order to more sensitively identify the MHC class I haplotype.

Microcytotoxicity tests. Microcytotoxicity tests were performed by the technique of Terasaki and McLelland (23), modified for use in the macaque (1). Briefly, macaque PBL were isolated over a Ficoll-Hypaque density gradient (Pharmacia Fine Chemicals, Piscataway, N.J.) and then suspended in a balanced salt solution to which complement had been added. The cell suspensions were added to antiserum (MnLA or MAb) microdroplet preparations, incubated at room temperature with a stain indicating nonviable cells, and read by light microscopy.

Statistics. Incidence of MHC class I epitopes was defined as the number of susceptible animals expressing an epitope/29, or the number of resistant animals expressing an epitope/15, and was expressed as a percentage. The incidence difference (absolute value of percent susceptible animals minus percent resistant animals) was determined for each antibody specificity tested. Risk of early adhesion formation in macaques expressing, versus those not expressing, a given epitope was calculated as an odds ratio (OR):

$$OR = (h \cdot K) / (H \cdot k)$$

$$y = \log_e OR$$

$$V = (1/h) + (1/k) + (1/H) + (1/K)$$

$$\text{chi-square value } (\chi^2_{\text{inf}}) = (1/V)y^2$$

where h is adhesions by 2 weeks, reactive to antibody; K is no adhesions by 2 weeks, not reactive to antibody; H is no adhesions by 2 weeks, reactive to antibody; k is adhesions by 2 weeks, not reactive to antibody; and V is variance.

Because the OR is undefined in the presence of values of zero, where appropriate, Haldane's modification of Woolf's formula,

$$OR = [(2h + 1)(2K + 1)] / [(2H + 1)(2k + 1)]$$

TABLE 1. Association of specificities of MnLA with risk of adhesion formation following a single exposure to *C. trachomatis*

MnLA ^a	Specificity incidence		OR	P value	HLA class I specificity ^b
	No. (% of 29) susceptible to early adhesions	No. (% of 15) resistant to early adhesions			
1	17 (59)	12 (80)	0.4	NS ^c	U
2	4 (14)	1 (7)	2.2	NS	A
3	19 (66)	4 (27)	5.2	<0.01	B
4	9 (31)	9 (60)	0.3	<0.1	A
6	13 (45)	9 (60)	0.5	NS	B
7	19 (66)	9 (60)	1.3	NS	A
8	8 (28)	3 (20)	1.5	NS	B
9	9 (31)	2 (13)	2.9	NS	A
10	3 (10)	2 (13)	0.8	NS	A
12	3 (10)	0	4.1	NS	A
13	3 (10)	2 (13)	0.8	NS	B
16	1 (3)	0	1.6	NS	U
17	4 (14)	3 (20)	0.6	NS	B
20	4 (14)	2 (13)	1.0	NS	B
21	1 (3)	0	1.6	NS	U
25	4 (14)	4 (27)	0.4	NS	B
26	0	2 (13)	0.1	<0.05	U
31	5 (17)	2 (13)	1.4	NS	B
32	18 (62)	11 (73)	0.6	NS	A
34	2 (7)	1 (7)	1.0	NS	U
35	1 (3)	2 (13)	0.2	NS	U
36	5 (17)	0	7.0	<0.1	B
37	5 (17)	3 (20)	0.8	NS	B
38	4 (14)	1 (7)	2.2	NS	B
41	18 (62)	6 (40)	2.5	NS	U
42	12 (41)	8 (53)	0.6	NS	A
45	10 (35)	8 (53)	0.5	NS	A
46	10 (35)	8 (53)	0.5	NS	B
53	6 (21)	2 (13)	1.7	NS	U
54	4 (14)	0	5.5	NS	U
57	13 (45)	7 (47)	0.9	NS	U
58	15 (52)	9 (60)	0.7	NS	U
59	21 (72)	10 (67)	1.3	NS	U
61	8 (28)	3 (20)	1.5	NS	U
67	6 (21)	0	8.6	<0.1	U
69	7 (24)	2 (13)	2.1	NS	U
70	2 (7)	1 (7)	1	NS	U
72	1 (3)	1 (7)	0.5	NS	U
73	5 (17)	3 (20)	0.8	NS	U
74	3 (10)	3 (20)	0.5	NS	U
75	8 (28)	3 (20)	1.5	NS	U
76	5 (17)	8 (53)	0.2	<0.01	U
77	7 (24)	6 (40)	0.5	NS	U
79	20 (69)	11 (73)	0.8	NS	U
80	6 (21)	2 (13)	1.7	NS	U
81	4 (14)	1 (7)	2.2	NS	U

^a One MnLA did not react with any macaque PBL tested and is not reported.
^b A, encoded by an A-like locus in the macaque; B, encoded by a B-like locus; U, encoded by an unidentified locus.
^c NS, not significant.

$$y = \log_e OR$$

$$V = [1/(h + 1)] + [1/(k + 1)] + [1/(H + 1)] + [1/(K + 1)]$$

$$\text{Chi-square value } (\chi^2_{\text{inf}}) = (1/V)y^2$$

was used to augment the values for h , H , k , and K (28, 24). This formula is used in the study of HLA antigens. Resultant P values of less than 0.05 were considered significant.

RESULTS

At the time of laparotomy, 29 of 44 animals had developed adhesions and were classified as susceptible, while 15 of 44 had not yet developed adhesions and so were classified as resistant.

TABLE 2. Association of specificities of mouse anti-HLA MABs with risk of adhesion formation following a single exposure to *C. trachomatis*

MAB clone ^a	Specificity incidence		OR	P value	HLA class I specificity
	No. (% of 29) susceptible to early adhesions	No. (% of 15) resistant to early adhesions			
5.4	11 (37.9)	3 (20)	2.4	NS ^b	HLA A2,3,28,29,30,31,68,69
8.1	0	1 (7)	0.2	NS	HLA B8
16.1	2 (6.9)	1 (7)	1.0	NS	
25.1	27 (93.1)	15 (100)	0.4	NS	HLA A,B,C
25.2	15 (51.7)	8 (53)	0.9	NS	HLA A,B,C
34.1	10 (34.5)	6 (40)	0.8	NS	HLA A29
41.1	14 (48.3)	2 (13)	6.1	<0.05	Broadly polymorphic
41.2	21 (72.4)	10 (67)	1.3	NS	HLA A,B,C
41.3	15 (51.7)	3 (20)	4.3	<0.05	Broadly polymorphic
55.2	4 (13.8)	3 (20)	0.6	NS	HLA A3
65.2	3 (10.3)	1 (7)	1.6	NS	Broadly polymorphic
77.1	25 (86.2)	14 (93)	0.4	NS	HLA Bw6
77.2	23 (79.3)	12 (80)	1.0	NS	HLA A,B,C
84.1	4 (13.8)	1 (7)	2.2	NS	HLA A25,32
84.2	2 (6.9)	0	2.8	NS	HLA B14,18,39,59
85.1	9 (31.0)	8 (53)	0.4	NS	HLA A23,24,25,32,Bw-short 27,38,49,52,53,57,59,63
88.1	0	1 (7)	0.2	NS	HLA A,B,C

^a Four MABs did not react with any macaque PBL tested and are not reported.

^b NS, not significant.

The MnLA and MABs used were highly reactive in the animals studied. The incidence of specificities detected with MnLA ranged from 0 to 71% (Table 1), and those with mouse MABs ranged from 0 to 96% (Table 2). The incidence difference between susceptible and relatively resistant animals for each specificity ranged from 0 to 39% for MnLA and from 0 to 35% for MABs. Three specificities (MnLA3 [Table 1], 41.1, and 41.3 [Table 2]) were associated with susceptibility to early formation of adhesions (ORs, 5.2, 6.1, and 4.3, respectively). These specificities were detected in 52, 36, and 41%, respectively, of the 44 macaques tested. MnLA3 is associated with a B-like allele in the macaque, but both 41.1 and 41.3 are broadly polymorphic in humans (Tables 1 and 2). Two specificities, MnLA26 and MnLA76 (Table 1), were associated with relative resistance (ORs, 0.1 and 0.2, respectively) to early formation of adhesions. Although MnLA26 was detected in only 5% of the population tested, MnLA76 was detected in 30%, indicating that a common allele in this population is associated with relative resistance to adhesions.

In a small number of animals ($n = 8$), both alleles associated with susceptibility and alleles associated with relative resistance to early formation of adhesions were detected. Five animals were susceptible to early adhesions; three were resistant.

DISCUSSION

Previous studies using the mouse model indicated that more than one mechanism may contribute to genetic susceptibility to *C. trachomatis* infection. Variability in susceptibility to chlamydial infection was seen among mouse strains with the same MHC (*H-2*) haplotype, indicating that susceptibility was not entirely due to expression of the *H-2* gene complex (25). However, MHC class I restriction of the immune response to *C. trachomatis* is supported by several lines of evidence. MHC class I restriction of immunopathology following chlamydial infection is plausible, because chlamydial antigen may be presented to CD8 cells in the context of MHC class I molecules. This possibility is supported by evidence that mouse immune responses to two chlamydial heat shock proteins, cHSP60 and cHSP70, vary by *H-2* haplotype (29). Chlamydial infection in the macaque pocket salpingitis model resulted in detection of

both CD4 and CD8 lymphocyte responses and a Th1 pattern of cytokine production (27), indicating that MHC class I antigen presentation likely occurred in this model. The role of MHC class I in chlamydial antigen presentation has also been confirmed in a mouse model, where the dominant epitope recognized by a *C. trachomatis*-specific CD8 cell line was presented on the murine MHC class I L^d molecule (22). Similarly, the HLA B27 molecule appears to present a chlamydial peptide to CD8 T lymphocytes (19).

Although the mouse model offers the advantage of a well-defined genotype, the macaque model, due to the genetic similarity of macaques and humans, may be more appropriate for investigating human genetic susceptibility to chlamydial disease. Extensive similarity between macaque and human MHC class I and II has been reported (2–5, 20, 21). MHC class I genetic identity between pig-tailed macaques and humans has been determined to be 78% for all HLA epitopes, 84% for public epitopes, and 97% for private epitopes, using mouse anti-HLA MABs (3). In this population of macaques, evidence was found for alleles correlated with relative resistance to rapid adhesion formation. This finding may be unique to the macaque, because in humans, HLA A31, B27, and C2 are associated with susceptibility to chlamydial pathogenesis (7, 18).

If the antibody specificities detected distinct MHC class I alleles, as many as five different alleles may influence the macaque model response to chlamydia. However, the MAB results do not indicate whether single or multiple antigens, or their HLA homologies, were detected due to their broad polymorphism. Although MnLA3 is associated with a B-like allele in the macaque, MnLA26 and MnLA76 are associated with unidentified loci. Because antibody specificities were associated with both increased and decreased ORs of early adhesion formation, there may be at least two macaque alleles that affect the response to chlamydia. These may be macaque homologs of HLA A31, B27, or C2, which predispose humans to increased pathology following *C. trachomatis* infection (7, 18). Alternatively, MHC class I antigens unique to the macaque may act to increase or decrease susceptibility to PID.

In a small number of animals, both alleles associated with susceptibility and alleles associated with relative resistance to

early formation of adhesions were detected. Five animals were susceptible to early adhesions; three were resistant. Therefore, no conclusions can be drawn in regard to a greater effect of alleles associated with relative susceptibility or resistance. A small number of animals with coexpression of alleles might be expected by chance.

For several reasons, it is premature to infer a direct association between the findings in this study and the well-established correlations between human MHC class I haplotype and disease. The current study used a broad approach, detecting species-specific as well as cross-specific patterns of immunoreactivity to MHC class I antigens. Since macaque PBL have also been shown to cross-react with HLA class I-specific MAbs (3), both MnLA and mouse anti-HLA MAbs were used for class I analysis in this study. The large number of specificities tested is warranted, considering the relative lack of information regarding this species. The appropriateness of this approach is evident, considering the high detection rate of specificities in assays using both MnLA and mouse anti-HLA MAbs. However, the locus assignments of pig-tailed macaque MHC class I, defined by using MnLA (2), may not correspond to those of HLA class I loci. Because the purpose of this study was to identify whether MHC class I antigens are associated with risk of adhesion formation in the macaque model, direct correlations between HLA and MnLA were not attempted in this study. Molecular methods, such as DNA typing using MnLA and HLA class I sequence-specific oligonucleotide probes or restriction fragment length polymorphism, are necessary to identify correlations between haplotypes in the human and macaque (21).

These results suggest that rapid development of upper genital tract adhesions is influenced by MHC class I alleles in the macaque model and provide a rationale for continued study in this area. Future investigations should use fewer alloantisera or MAbs, should employ molecular methods, and should evaluate a larger population of animals. Use of this model offers important advantages over studies of human clinical populations, in which multiple chlamydial exposures at uncertain time points and with unknown strains have generally occurred prior to development of PID. Larger trials with direct identification of alleles will more clearly define the pig-tailed macaque as a model of genetic susceptibility to *C. trachomatis*-induced PID.

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