The Influence of the Swine Major Histocompatibility Genes on Antibody and Cell-mediated Immune Responses to Immunization with an Aromatic-dependent Mutant of *Salmonella typhimurium*

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ABSTRACT

Eighty-two major histocompatability complex (MHC) swine leukocyte antigen (SLA) defined miniature pigs from 16 litters were examined for serum agglutinating antibody titer and O-polysaccharide (O-ps) specific peripheral blood lymphocyte blastogenesis following two parenteral vaccinations with 1×10^8 aromatic-dependent (aroA) *Salmonella typhimurium* and following oral challenge with 1×10^{12} virulent parent *S. typhimurium*. Least mean squares analysis allowed separate determinations of the effects of MHC genotype, dam, sire and litter. In most cases only litter significantly influenced both lymphocyte blastogenesis and antibody titer before and after vaccination and following challenge. However, pig SLA haplotype significantly influenced the degree of O-ps specific lymphocyte proliferation six days after the second vaccination ($p < 0.004$). Lymphocyte proliferation and serum agglutinating antibody response six days after primary vaccination were negatively correlated ($r^2 = -0.68$, $p < 0.001$). In most cases, "dd" and "gg" homozygous and "dg" heterozygous pigs, having the same MHC class II region, behaved immunologically as a group distinct from the other genotypes.

RÉSUMÉ

Quatre-vingt-deux porcs nains, ayant des complexes majeurs d'histocompatibilité (CMH) connus, provenant de 16 portées différentes ont été examinés afin de vérifier les titres sériques et l'effet blastogénique du polysaccharide-O sur des lymphocytes circulants suite à deux vaccinations parentérales avec 1×10^8 colonies d'un mutant aro A de *Salmonella typhimurium* suivi d'une provocation orale avec 1×10^{12} colonies de souche sauvage de *S. typhimurium*. L'analyse des carrés moyens a permis de séparer les effets du génotype de CMH, de la mère, du père et de la portée. Dans la plupart des cas, seulement l'effet de la portée a influencé les tests précédents avant et après la vaccination ainsi qu'après provocation. Cependant, les animaux haplotype ont significativement ($p < 0.0004$) influencé la prolifération lymphocytaire six jours après la deuxième vaccination. La prolifération des lymphocytes et les titres sériques six jours après la première vaccination étaient toutefois corrélés négativement ($r^2 = 0.68$, $p < 0.0001$). Dans la plupart des cas, les homozygotes « dd » et « gg » et les hétérozygotes « dg » ayant le CMH de classe 2 identique, se comportaient immunologiquement de façon différente par rapport aux autres génotypes. (*Traduit par Dr Pascal Dubreuil*).

INTRODUCTION

As in mice and man, the pig major histocompatibility complex (MHC) swine leukocyte antigen (SLA), is associated with graft rejection, production of lymphocytotoxic serum antibodies, immune response (Ir) genes and stimulation of mixed lymphocyte reactions (1). The SLA genotype has been associated with differences in the magnitude of the antibody response to a *Bordetella bronchiseptica* vaccine (2) and a pseudorabies vaccine (3) in commercial pigs and with decreased incidence of encysted *Trichinella spiralis* larvae in miniature pigs (4). The SLA genotype was associated with antibody response to hen egg white lysozyme (HEWL) and the synthetic peptide (T,G)-A--L in miniature swine (5) and antibody response to HEWL in commercial pigs (6). As well, SLA genotype was also shown to significantly influence serum immunoglobulin (IgG) concentration (7), antibody response to HEWL and sheep red blood cells (srbc) and cutaneous delayed type hypersensitivity (DTH) to dinitrochlorobenzene (DNCB) and tuberculin purified protein derivative (PPD) in miniature pigs (8). The SLA genotype also influenced uptake and killing of *Salmonella typhimurium* and *Staphylococcus aureus* by peripheral blood monocytes (9).

The purpose of this study was to determine the influence of SLA genotype on antibody and cell-mediated immune response to parenteral vaccination with an aromatic-dependent mutant of *Salmonella typhimurium* and oral challenge with its virulent parent organism.

MATERIALS AND METHODS

EXPERIMENTAL PROTOCOL

All pigs were held in an isolation unit for at least three days prior to the commencement of the experiment. Pigs were vaccinated with live aroA

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Salmonella typhimurium (1×10^8) in 1 mL normal saline injected intramuscularly on day 1. A second, identical immunization was performed two weeks later. One week later all of the pigs were challenged orally with 1×10^{12} *S. typhimurium* 3860C, the parent of the mutant vaccine strain, in 10 mL normal saline. Blood samples for serum and lymphocytes were collected from the retro-orbital sinus of each pig five times during the protocol. On day 0, one day before vaccination, day 6 and day 13 after the first vaccination, day 20, six days after the second vaccination, and day 27, six days after challenge.

BACTERIA

Salmonella typhimurium 3860, a nalidixic acid resistant strain, was originally associated with salmonellosis in boars (10). After recovery from an experimentally infected calf it was designated *S. typhimurium* 3860C and an aroA mutant was produced by transposon deletion(11). The parent and aroA strains were grown in L-broth (10 mL) overnight at 37°C with shaking and then cultured on L-agar in a Roux flask with 50 μg/mL nalidixic acid (nal) (Sigma Chemical Co., St. Louis, Missouri). Flasks were incubated at 37°C for 24 hours. Bacterial suspensions were pooled and washed twice in normal saline with centrifugation at 6000 *g* for 15 minutes and resuspended in 1 mL aliquots in lyophilization medium (5% w/v dextran, MW 70,000; 10% w/v sucrose and 1% w/v sodium glutamate in ddH_2O) for storage at −70°C.

ANIMALS

Miniature pigs were originally obtained from the National Institutes of Health, Bethesda, Maryland (1) and are the result of an original cross between a miniature boar (Vita Veterinary Laboratory, Ames, Iowa) and a "Pigmee" sow (Hormel Institute, Massachusetts). Pigs from three SLA inbred lines ($SLA^{a/a}$, $SLA^{c/c}$ and $SLA^{d/d}$) homozygous for a distinct set of MHC haplotypes were examined. Pigs from a recombinant line ($SLA^{g/g}$) were also examined that had the MHC I region of the $SLA^{c/c}$ and the MHC II region of $SLA^{d/d}$. The breeding herd was housed conventionally at the Eramosa Research Station of the Ontario Veterinary College. The study utilized 82 pigs representing the genotypes, $SLA^{a/a}$, $SLA^{a/c}$, $SLA^{c/c}$, $SLA^{c/d}$, $SLA^{d/d}$, $SLA^{d/g}$ and $SLA^{g/g}$ with 14, 12, 11, 7, 25, 7 and 6 animals of each genotype used, respectively. A total of 16 litters of pigs were utilized with the pigs of genotypes $SLA^{a/c}$ and $SLA^{g/g}$ resulting from breedings with two different sires while the pigs of the other genotypes were the products of three different sires.

During experiments pigs were housed in the Veterinary Microbiology and Immunology Isolation Facility at the University of Guelph. Pigs were fed twice daily and given water *ad libitum*. Miniature pigs were brought to isolation at 45 days of age and housed until euthanized by intravenous (IV) barbituate (Euthanyl Forte, M.T.C. Pharmaceuticals, Cambridge, Ontario).

All experiments involving animals followed the guidelines of the Guide to the Care and Use of Experimental Animals.

O-POLYSACCHARIDE PREPARATION

Salmonella typhimurium 3860C grown in five L-agar Roux flasks + nal was washed twice in normal saline with centrifugation (6000 *g* for 15 minutes), resuspended in 1% v/v formalin, incubated overnight at 4°C prior to washing and lyophilization. Lipopolysaccharide (LPS) was isolated (12), and resuspended in distilled, deionized water (ddH_2O) at 1 mg/mL O-polysaccharide (O-ps) was purified from LPS by weak acetic acid hydrolysis (13). After chloroform extraction (5 ×), the aqueous product was pooled, dialyzed against ddH_2O and lyophilized. The resulting O-ps was resuspended to 1 mg/mL saline and stored at −20°C.

LYMPHOCYTE BLASTOGENESIS ASSAY

Peripheral blood lymphocytes (PBL) were collected, purified and blastogenesis was performed as described previously (14) in 96-well microtiter plates (A/S Nunc, Roskilde, Denmark). Treatments for each pig were: four wells with PBL and medium only, six with PBL and O-ps as the stimulating antigen, and two with PBL and Concanavalin (Con-A) (Sigma). Each well received 1×10^5 PBL in 100 μL of RPMI + penicillin G (100 IU) and streptomycin (40 μg/mL) (ps). The O-ps and Con-A were added at 10 μg/mL in 100 μL aliquots. Each plate had six blank wells and six containing only RPMI + ps. Plates were incubated (37°C, 5% CO_2 for 24 hours) prior to pulsing all wells with 0.5 μCi [^{3}H]-methyl thymidine (Dupont, Mississauga, Ontario) in 20 μL of RPMI. After eighteen hours, the cells were harvested (PHD, Cambridge Technology Inc., Cambridge, Massachusetts) and the radioactivity (cpm) detected in a scintallation counter (Model 460, Packard, Downer's Grove, Illinois). For each pig, the highest and lowest cpm for cells stimulated with O-ps were discarded and the remaining counts averaged. The average cpm for the unstimulated cells for the individual was then subtracted and the value expressed as the change in cpm.

BACTERIAL MICROAGGLUTINATION

Salmonella typhimurium 3860C from an L-agar + nal Roux flask, incubated overnight at 37°C were harvested, washed and killed with formalin as described previously (14) and resuspended for storage in 0.1% v/v formalinized saline at 4°C. The bacterial suspension was diluted to an OD of 1.0 at 550 nm for use in the assay. As controls, known positive and negative anti-*Salmonella typhimurium* sera plus bacteria and bacteria plus diluent only were included. The plate was incubated at room temperature overnight prior to reading the agglutinating titer.

ANTIBODY-MEDIATED LYMPHOCYTOTOXICITY

The SLA typing sera were made by cross immunizations with lymphocytes between pigs of disparate haplotypes. The SLA typing was performed by a modification of a standard antibody-mediated lymphocytotoxicity test (15).

STATISTICAL ANALYSIS

Analysis was by least squares using the SAS general linear model proce-

TABLE I. Analysis of variance[a] for O-ps specific lymphocyte blastogenic response and serum agglutinating antibody response to *Salmonella typhimurium* 3860C

Dependent variable	R^2 %	Model	SLA	Sire	Sex	Litter
Blastogenesis						
Day 0	74.9	0.0001[b,c]	0.667	0.916	0.190	0.0001
Day 6	49.4	0.0045	0.790	0.762	0.433	0.011
Day 13	65.6	0.0001	0.750	0.045	0.760	0.724
Day 20	51.5	0.0023	0.004	0.409	0.270	0.026
Day 27	74.6	0.0001	0.430	0.719	0.113	0.0001
Agglutination						
Day 0	70.3	0.0001	0.630	0.910	0.960	0.0001
Day 6	55.8	0.0004	0.909	0.278	0.889	0.026
Day 13	64.2	0.0001	0.432	0.432	0.746	0.0016
Day 20	35.4	0.1679	0.935	0.630	0.612	0.368
Day 27	62.2	0.0001	0.324	0.804	0.757	0.0052

[a]Analysis of variance computed using Statistical Analysis System (SAS)
[b]Probability > F
[c]p values ≤ 0.05 are considered significant

TABLE II. Least square means (LSM) and their standard error (SE) for effect of SLA genotype on lymphocyte proliferation to *Salmonella typhimurium* 3860C O-ps six days after the second vaccination (Day 20)

			Probability of significant difference						
Haplotype	LSM (cpm)	SE LSM	aa	ac	cc	cd	dd	dg	gg
aa	2471.0	821.7	—	ns[a]	ns	ns	0.02	ns	0.10
ac	1521.8	696.1		—	ns	ns	0.005	ns	ns
cc	2343.4	658.8			—	ns	0.02	ns	0.07
cd	1491.1	1182.7				—	0.02	ns	ns
dd	6783.7	1526.9					—	0.0003	0.0007
dg	1004.0	921.0						—	ns
gg	867.9	730.9							—

[a]Not significant

TABLE III. Least square means (LSM) and standard errors (SE) for effect of SLA genotype on serum agglutinating antibody response to *Salmonella typhimurium* 3860C six days after challenge (Day 27)

			Probability of significant difference						
Haplotype	LSM ($1/\log_2$)	SE LSM	aa	ac	cc	cd	dd	dg	gg
aa	7.15	0.591	—	ns[a]	0.03	0.15	0.05	0.003	0.0001
ac	6.94	0.510		—	0.03	ns	0.06	0.003	0.0001
cc	5.74	0.482			—	ns	ns	0.07	0.01
cd	5.32	0.873				—	ns	ns	ns
dd	4.45	1.127					—	ns	ns
dg	4.19	0.680						—	ns
gg	4.21	0.539							—

[a]Not significant

dure (16). Agglutinating antibody response and lymphocyte blastogenic response were analyzed on data from 82 miniature pigs from 16 litters using the following model:

$$Y_{ijklm} = \mu + SLA_i + SIRE_j + LITTER(SIRE)_{jk} + SEX_l + ERROR_{ijklm}$$

where

Y_{ijklm} = an observed value for serum agglutinating antibody response to *S. typhimurium* 3860C, or an observed value for lymphocyte blastogenic response to *S. typhimurium* 3860C O-ps;

μ = the population mean for that trait;

SLA_i = a fixed effect due to piglet SLA genotype;

$SIRE_j$ = a random effect due to sire;

$LITTER(SIRE)_{jk}$ = a random effect due to litter nested within sire;

SEX_l = a fixed effect due to sex of pig;

$ERROR_{ijklm}$ = a random residual error term.

Using the type III sums of squares, this model tested the hypotheses that SLA genotypes, sires, litter and sexes did not differ for the traits monitored. Sire effects were tested with the litter mean square as the error term. Genotype, litter and sex effects were tested with the error mean square. Least square means were calculated to compare the response between SLA genotypes for each trait.

RESULTS

LYMPHOCYTE BLASTOGENESIS

The SLA genotypes dd, dg and particularly gg had lower innate (prevaccination) lymphocyte stimulation than aa, ac, cc and cd pigs. Six days after primary vaccination the same pattern was evident with dd, dg and gg lower than aa, ac, cc and cd. Genotypes dd and dg had negative estimated response values. However, thirteen days after primary vaccination, SLA dd, dg and gg pigs were now higher responders than aa, ac and cc pigs. After secondary vaccination only dd pigs were still significantly higher responders than any of the other genotypes. After challenge, cc and cd pigs had the highest lymphocyte proliferative values and aa pigs the lowest (Fig. 1).

Litter ($p < 0.0001$) significantly influenced the innate lymphocyte proliferative response to O-ps, but SLA, sire and sex did not. Only litter ($p < 0.011$) significantly affected the primary response six days after vaccination, while only sire ($p < 0.045$) significantly influenced the primary response at day 13 (Table I). The SLA ($p < 0.004$), (Tables I and II) and litter ($p < 0.026$) significantly affected the secondary lymphocyte proliferative response to O-ps on day 20. Only litter ($p < 0.0001$) affected the response after challenge (Table I).

BACTERIAL MICROAGGLUTINATION

Regardless of SLA genotype, serum antibody at six days rose significantly from prevaccination but did not decline significantly by day 13. Secondary vaccination and challenge produced variable changes in antibody titer (Fig. 2).

There were no significant differences between pigs by SLA genotype in prevaccination antibody and all inverse $\log_2$ agglutination titers were less than 1.0. There were no remark-

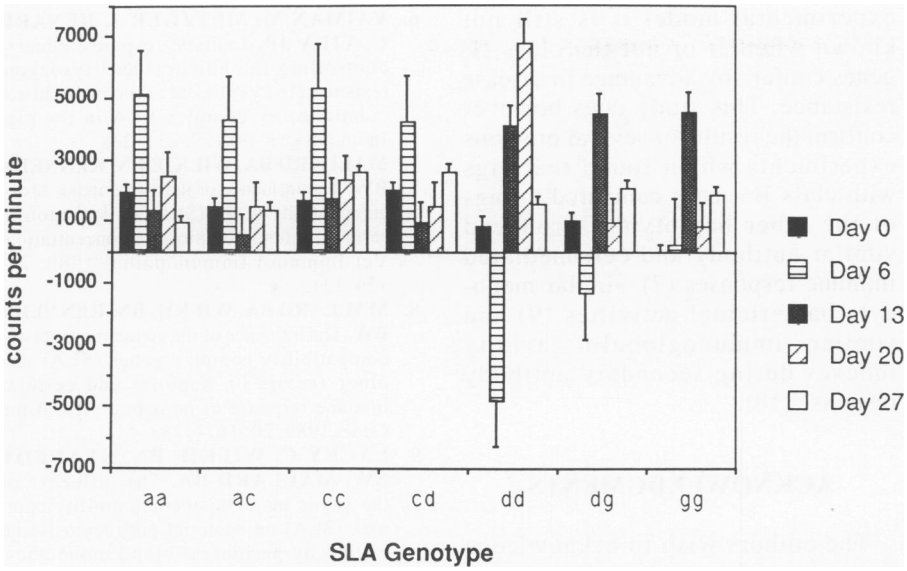

Fig. 1. Least square means for the effect of SLA on lymphocyte proliferation to *Salmonella typhimurium* 3860C O-ps before vaccination (Day 0, solid bar), following primary parenteral vaccination with 1 × 10^8 aroA *S. typhimurium* (Day 6, dashed bar; Day 13, stippled bar), following an identical secondary parenteral vaccination (Day 20, diagonally stripped bar) and following oral challenge with 1 × 10^{12} virulent *S. typhimurium* 3860C (Day 27, open bar).

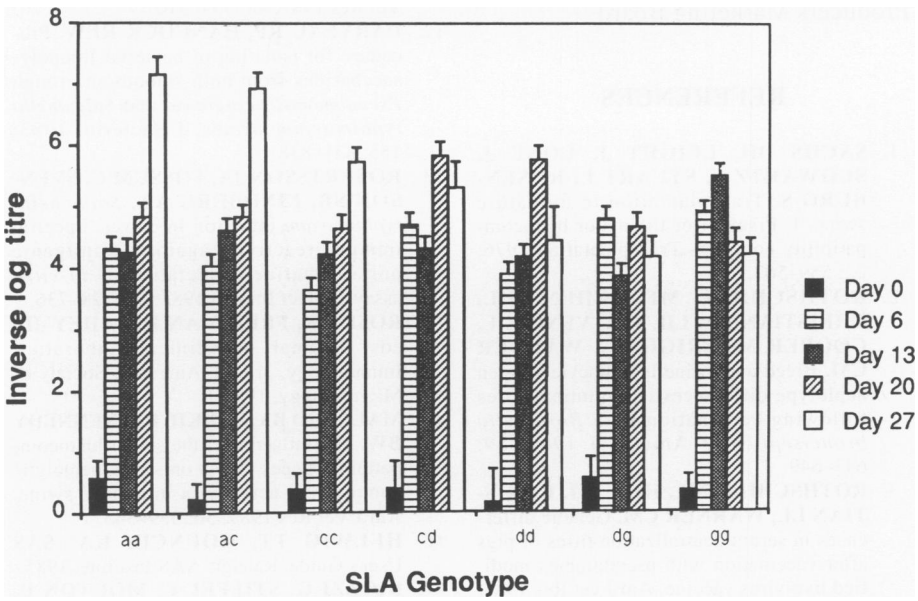

Fig. 2. Least square means for the effect of SLA on serum agglutinating antibody titer to *Salmonella typhimurium* 3860C before vaccination (Day 0, solid bar), following primary parenteral vaccination with 1 ×10^8 aroA *S. typhimurium* (Day 6, dashed bar; Day 13, stippled bar), following an identical secondary parenteral vaccination (Day 20, diagonally stripped bar) and following oral challenge with 1 × 10^{12} virulent *S. typhimurium* 3860C (Day 27, open bar).

able patterns of SLA influence on serum antibody response at 6 or 13 days after primary vaccination or six days after secondary vaccination (not shown). After challenge however dd, dg and gg pigs were lower responders than either aa or cc with haplotypes cc and cd moderate responders (Table III, Fig. 2).

(17). Experiments in this laboratory with SLA defined miniature pigs have indicated that sire, litter and dam more significantly affected antibody and cell-mediated response to a variety of antigens than did SLA related genes (8). Genetic effects besides SLA were also found to significantly influence monocyte function (9)

Litter ($p < 0.0001$, $p < 0.026$, $p < 0.0016$) significantly influenced innate serum antibody titers and the primary antibody response to vaccination at day 6 and 13 respectively. None of the variables had a significant effect on secondary antibody response and only litter ($p < 0.0052$) significantly affected antibody response after challenge (Table I).

At most sampling times the statistical model reasonably accounted for the phenotypic variation in lymphocyte blastogenic response (49.4% to 74.6%) and serum agglutinating antibody titer (35.4% to 70.3%) (Table I).

Pearson correlation coefficients revealed a negative correlation between lymphocyte proliferation on day 6 following primary vaccination and serum agglutinating antibody titer on day 6 ($r^2 = -0.68$, $p < 0.001$) and day 13 ($r^2 = -0.58$, $p < 0.001$) following primary vaccination.

DISCUSSION

This study addressed the influence of SLA on lymphocyte blastogenic response to O-ps and serum agglutinating antibody response following primary and secondary parenteral vaccination with an aroA *Salmonella typhimurium* and following oral challenge with the parent *S. typhimurium* 3860C. The SLA significantly influenced blastogenic response six days after secondary vaccination and notably affected the agglutinating antibody response six days after oral challenge. Litter significantly affected antibody and CMI responses at almost all sampling times while sire influenced the blastogenic response 13 days after primary vaccination. The effect of sex of the pig was not significant at any time. There were also large environmental effects not explained by the statistical model.

The MHC genes are not the only genes that influence immunological responses. Mouse H-2 genes were found to account for 20% of the interline difference between high and low responder mice selected for antibody response to srbc, and 25% of the differences between lines in peak agglutinin response to secondary immunization with somatic antigens of *S. typhimurium* was due to MHC loci

serum immunoglobulin concentrations (7) and antibody avidity maturation (18).

There was some evidence of epistatic gene effects between SLA class I and II loci since the gg genotype (SLA II of d, I of c) had higher antibody titers than either dd or cc pigs when their responses were averaged over time (not shown). Lymphocytes of gg pigs also responded less than either the dd or cc pig lymphocytes averaged over time, indicating that having these particular class I and II alleles in combination had a negative effect on lymphocyte proliferation to O-ps. An epistatic effect of haplotype g over either d or c has also been observed for monocyte killing of *Salmonella typhimurium* (9). There was no evidence in this study that heterozygosity *per se* influenced antibody or cell-mediated responses.

The kinetics of response differed by SLA genotypes. The dd, dg and gg groups of pigs had a delayed primary blastogenic response in comparison with the aa, ac, cc and cd groups with the degree of peak response similar for both groups. An SLA related difference in immune response kinetics has been observed previously by Mallard *et al* (8) for antibody response to srbc, HEWL and (T,G)-A--L.

Pearson correlation coefficients indicated a moderate negative correlation between lymphocyte proliferation in response to O-ps six days after primary vaccination and primary agglutinating antibody response measured at day 6 and 13. Antibody and CMI responses may be controlled by different effector mechanisms since selection of commercial pigs or inbred mice for antibody response does not affect the degree of their CMI response (17,19). In the miniature pigs the relationship between the antibody and cellular response varies for the stimulating antigen (8).

A striking aspect of the present study is the observed similarities of the dd, dg and gg pigs, which are homozygous for class II genes. Because the miniature pigs were generally refractory to challenge in this experimental model it is still not known whether or not the class II^d genes confer any advantage in disease resistance. This study does however confirm the results of several previous experiments which found that pigs with class II^d genes compared to pigs of the other haplotypes, produced similar antibody and cell-mediated immune responses (7), similar monocyte bactericidal activities (9) and similar immunoglobulin avidity indexes during secondary antibody response (18).

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