GENE INTERACTION AND THE A-O BLOOD-GROUP SYSTEM IN PIGS¹

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SINCE the report by SPRAGUE (1958a) that red blood cells of pigs may be divided into three groups (A, O, and A-O negative) on the basis of hemolytic reactions with bovine normal sera, other workers (ANDRESEN 1962, 1963; SAISON and INGRAM 1962) have suggested that the genetic basis for A-O phenotypes in pigs may be analogous to that proposed for the R-O (*RrIi*) system in sheep by RENDEL, NEIMANN-SØRENSEN and IRWIN (1954). This report is concerned with the A-O system in pigs and presents additional evidence that interaction of alleles at the A locus with other genes is responsible for the A, O, and “—” (“dash”) phenotypes.

MATERIALS AND METHODS

Blood samples: Blood samples were collected from 1524 pigs in 235 litters, including 476 Durocs, 592 Yorkshires and 456 Duroc-Yorkshire crosses or first and second generation descendants of such crosses. There were 29 purebred Durocs and 39 purebred Yorkshires, assembled as foundation stock with no regard to their blood groups, in the original generation from which all these pigs descended. Matings in the original and two subsequent generations, either within breeds or crosses of Duroc $\times$ Yorkshire and within the F_1 or F_2 offspring of these crosses, were made at random with respect to blood groups. Blood samples from sows and boars of the original generation were tested in the same test as samples from their offspring. The boars chosen as sires of subsequent generations were disposed of before their offspring were born, so that it was not possible to retest them when their offspring were tested. Blood samples from some of these boars were collected and retested after the matings were completed, shortly before the boars were sold. Samples from the sows chosen as dams for the succeeding generation were retested with few exceptions when samples from their offspring were tested. Except for the original generation, all pigs were bled as “juveniles” (when they were between four and ten weeks of age in almost every case) and 190 animals were bled again and retested as “adults” (at approximately one year of age). The samples were tested between March 1961 and October 1963 in hemolytic tests similar to those described by RASMUSEN (1958) for blood typing of sheep.

Serum and saliva samples: Preliminary studies were undertaken to test the ability of serum and saliva samples from pigs to inhibit the reactions of the different reagents with appropriate red cells. Whole, unheated serum samples were tested in doubling dilutions to a final dilution of 1/64. Saliva samples were collected by drawing saliva from the mouth of the pig into a syringe, approximately $\frac{1}{2}$ hour after the pig had received a subcutaneous injection of 50 mg pilocarpine hydrochloride (in 4 ml saline) per 100 kg body weight to stimulate salivation. Salivas were boiled 10 min soon after collection, centrifuged, and the supernatant was tested in quadrupling dilutions to a final dilution of 1/16,384. The inhibition tests were performed as described by SPRAGUE (1958a).

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Sources of reagents: Serum samples from sheep, pigs, and Hereford cattle were tested for the presence of antibodies which might be of use to differentiate A-O groups in pigs. The normal serum (coded S8) of a group-O sheep was selected for use in tests as an A reagent at a dilution of $\frac{1}{4}$. Fresh guinea-pig serum diluted $\frac{1}{12}$ or $\frac{1}{15}$ was used as complement with S8. Undiluted fresh rabbit serum absorbed at 0°C to remove natural heterohemolysins for pig red cells was used as complement with all other reagents used in this study.

A normal serum (diluted $\frac{1}{64}$ and coded C4) from a Hereford cow was also used as an A reagent for all tests. This serum was found to react more strongly with group-A cells than the sheep serum. Beginning in October 1962 a normal serum (diluted $\frac{1}{4}$ and coded P62) from a "—" pig was used as a third A reagent. P62 paralleled C4 quite closely, but was slightly more reactive, especially with cells which reacted only weakly with C4 and not at all with S8.

Satisfactory sources of anti-O are much more difficult to find than sources of anti-A. Since STORMONT described bovine antisheep O in 1951, the presence of anti-O in the serum of certain Hereford cattle (as revealed by tests with red cells from sheep of group O) has been reported by SPRAGUE (1958b) and RASMUSEN (1962). RENDEL (1957) found antisheep O in the serum of two group-i sheep, and SUZUKI and STORMONT (1961) reported the presence of anti-O in the serum of four "—" goats. For the present study, an O reagent was prepared from the normal serum of a Hereford cow. This serum (coded C1) also had some anti-A activity, and absorption with red cells of group-R sheep or group-A pigs was necessary to remove the anti-A. The serum was diluted $\frac{1}{4}$ for absorption, and the absorbed serum was diluted $\frac{1}{2}$ for testing. The normal sera (coded P15 and P44) from each of two group-A pigs were also used as O reagents from July 1961 to July 1962, and a normal serum (coded G1) from a "—" French Alpine goat has been used as an O reagent since July 1961. Serum P15, used at a dilution of $\frac{1}{16}$, was not dependable as an O reagent since its reactions with red cells of group-O pigs were weak and variable. P44 (diluted $\frac{1}{4}$) and G1 (diluted $\frac{1}{16}$) each lysed red cells from the same 287 samples of a total of 603 tested with both reagents. If 1+ hemolysis is considered a positive reaction, the two reagents failed to react alike in only seven cases: three bloods showed 1+ or 2+ hemolysis with P44 and were negative with G1, two showed 1+ hemolysis with G1 and were negative with P44, and two showed 2+ hemolysis with G1 and trace with P44. In inhibition tests P15 and P44 strongly inhibited the reaction of anti-A with pig A cells, as expected of sera obtained from pigs of group A.

The reactive patterns of two other sera appeared to be related to A-O phenotypes. One of these, P3, was obtained from a group-A crossbred pig. This pig had received a series of five injections of 10 ml of whole blood of another group-A crossbred pig, but failed to produce antibodies reactive with red cells of the donor. When this serum was absorbed with the red cells of group-O pigs to remove anti-O, certain of the absorbed samples contained a residual fraction of antibodies which reacted more frequently with the red cells of "—" pigs than with those of group A or group O. The reactive pattern of serum P18 (diluted $\frac{1}{4}$), a normal serum from a group-A Hampshire pig, was almost identical with that of P3 absorbed by group-O red cells (final dilution $\frac{1}{16}$). Neither of these sera completely lysed any samples, but both reacted in almost every case to very nearly the same degree with the same red cells. Serum P18 was chosen as a standard serum, and the symbol P18 is used to designate a positive reaction with this reagent. Both P18 and P3 (absorbed by red cells of a group-O pig) were used in all tests, and reactions of the latter reagent were extremely useful in interpreting the reactions with P18.

Interpretation of reactions of A and O reagents: There was some variability in the degree of reaction of the A and O reagents with different bloods, and it was necessary in some cases to decide on the basis of partial hemolysis whether a blood should be designated as A or "—", or as O or "—" (red cells were never definitely positive for both A and O). Red cells showing 1+ or greater hemolysis in reactions with C4 or 2+ or greater with P62 at 5 hours were classified as group A. Red cells showing 1+ or greater hemolysis with C1 and/or G1 were classified as group O. Red cells showing lesser degrees of hemolysis or no hemolysis with A or O reagents were classified as "—". There was little variation in degree of reaction among tests on different days, so that the standards chosen were generally applicable. In every test, at least two A and two O reagents were included, and the A-O classification was made after comparing results of

tests with the different A and O reagents. In some cases, the classification remained doubtful after such comparisons, and samples were retested.

RESULTS

Family studies and inhibition tests: The A-O blood groups of pigs from various kinds of matings are given in Table 1. The 44 offspring from 11 “—” × “—” matings were all “—”, as would be expected if the “—” phenotype results when pigs are homozygous for a recessive gene which can be designated *s*. The occurrence of some “—” offspring from all possible kinds of matings confirms the recessive nature of this phenotype. GOODWIN and COOMBS (1956) have reported that A substance is present in the saliva of group-A pigs but not in A-negative pigs, and the presence of A substance in the serum of group-A pigs was demonstrated by ANDRESEN (1963). In the present study, saliva from each of 11 group-A pigs was found to contain A substance as measured by inhibition test (titer 1/256 to 1/16384), whereas salivas of nine group-O and four “—” pigs inhibited the A anti-A reaction weakly, if at all (titer 0 to 1/16). The results with serum were similar; sera from the group-A pigs inhibited the A anti-A reaction (titer 1/16 to 1/64) whereas serum from group O and “—” pigs did not inhibit the reaction. The symbol *S* (for serum substance) seems appropriate for the gene which together with the *A*⁴ gene results in the presence of A substance in the serum and saliva.

In Table 1 data from matings of O × O show that the *S* gene is also necessary for the O phenotype; 42 offspring of O × O (from 14 matings where both parents were presumably heterozygous *Ss*) had “—” red cells. Failure to obtain any group-A offspring from O × O matings confirms that the gene for A (*A*⁴) is dominant to its allele for O (*a*^o) (ANDRESEN 1962, 1963). These results indicate that the gene *S* is necessary for the O reactivity of red cells, and the results of inhibition tests for serum O substance might be expected to reveal the presence of O in the serum of pigs with group-O red cells and its absence in the serum of other pigs. However, serum and saliva inhibition tests did not differentiate A, O, and “—” pigs with respect to O inhibition; saliva and sera from all three groups inhibited the O anti-O reaction to approximately the same extent. Differences

TABLE 1
Blood groups in the A-O system in pigs

Mating	Number of matings	Number of offspring of group			Total number of offspring
		A	O	“—”	
“—” × “—”	11	0	0	44	44
O × “—”	30	22	95	73	190
O × O	50	0	298	42	340
A × “—”	49	121	38	118	277
A × O	61	218	159	45	422
A × A	34	158	28	50	236
Total	235	519	618	372	1524

between sera and salivas of group-O and other pigs which may exist could not be detected by the use of tests for inhibition of available O reagents.

If the gene for A (A^A) is dominant to its allele for O (a^o), and a dominant gene (S) at another locus is necessary for the expression of A and O, genotypes possible for group-A pigs would be $A^A A^A SS$, $A^A A^A Ss$, $A^A a^o SS$, or $A^A a^o Ss$; group-O pigs would $a^o a^o SS$ or $a^o a^o Ss$, and "—" pigs would be $A^A A^A ss$, $A^A a^o ss$, or $a^o a^o ss$. In offspring of three different A $\times$ A matings all three types, A, O, and "—" were represented, as might be expected from crosses of dihybrids. Three matings of O $\times$ "—" produced A, O, and "—" pigs, as expected from matings of $a^o a^o Ss \times A^A a^o ss$. From a mating of "—" Duroc boar 40B and group-O Yorkshire sow 260S, all seven offspring were group-A, as expected from mating $A^A A^A ss \times a^o a^o SS$. The proposed genotypes of 40B and 260S were confirmed by other matings.

Gene frequencies: There were 16 group-A, four group-O, and nine "—" Durocs in the original generation, and nine group-A, 27 group-O, and three "—" Yorkshires. Gene frequency estimates are given in Table 2, based on the original generation which is assumed to consist of a random sample of each breed.

Comparison of "juvenile" and "adult" types: GOODWIN and COOMBS (1956) reported that newborn pigs have A-negative red cells, and observed that the red cells may not become strongly reactive with anti-A until the pigs are 30 days old. SAISON and INGRAM (1962) stated that A and O substances are not present on red cells at birth, although they can be detected by the sixth or seventh day. It is possible that errors in classification may result when red cells from young pigs are tested, so that some pigs which will eventually be of group-A or O are classified as "—" when immature. Comparisons of juvenile and adult types were made for 190 pigs for which the adult classification was 69 group A, 86 group O, and 35 "—". In only one case was a pig with red cells which were strongly A-positive as an adult erroneously classified as "—" in the earlier test, but seven bloods which were classified as "—" in the earlier test were later classified as group A on the basis of 2+ or 3+ hemolysis when tested with P62. P62 was not available for five of the eight earlier tests, when these bloods were negative for A reagents C4 and S8.

It is possible that the nonreactivity of cells for A in the earlier tests is associated with increased reactivity with the P18 serum. Six of the eight juvenile bloods classified as "—" which were later classified as group A reacted with P18 (out of a total of 14 group-A, P18-positive bloods); two of these also reacted with P18 as adults (out of a total of four group-A, P18-positive bloods).

TABLE 2
Frequencies of alleles in two breeds of pigs

Allele	Frequency in	
	Durocs	Yorkshires
A^A	0.45	0.13
a^o	0.55	0.87
S	0.44	0.74
s	0.56	0.26

Only two of the 87 group-O pigs were classified as “—” as juveniles on the basis of reactions with G1. C1 failed to react to 1+ hemolysis with these same two bloods and seven others, as well as six adult bloods classified as group O by reactions with G1. One of the two group-O bloods, that from a young Duroc pig, which failed to react with G1 gave 1+ hemolysis with P18, whereas the adult blood reacted with G1 and not P18. The other, from a young Yorkshire, gave a trace reaction with P18.

The P18 factor: The reactions of the A, O, and “—” bloods with P18 are given in Table 3. A reaction of 1+ or stronger was used as the criterion for the presence of the P18 factor. The A, O, or “—” classification is based on the adult test. Bloods from the same 190 pigs were classified as “juveniles” and as “adults.” Only one blood, that of a “—” Duroc female, was classified as P18-negative in the first test and P18-positive in the adult test, whereas 26 bloods which were originally P18-positive were P18-negative in the later test.

The P18 factor is more frequent in Durocs than in Yorkshires, and is more frequent in “—” than in group-A or O bloods. These observations agree with results of tests with P18 of 196 blood samples from pigs which were four months old or older and of various breeds or breed mixtures, but not related to the Durocs and Yorkshires for which family data are reported. Of these 196, four of 82 group-A, two of 177 group-O, and 19 of 37 “—” bloods had the P18 factor. Although the P18 serum appears to be detecting a hereditary blood factor related to A-O phenotypes in its expression, the mode of inheritance of this factor is not clear. The graded reactions with P18 made classification as positive or negative for the P18 factor somewhat arbitrary, and the change in phenotype, usually in the direction of less reactivity with P18 with increasing age, contributed to the difficulty in classification. It is clear from family studies that two adults lacking the P18 factor may have offspring which have the factor as juveniles, but there have been no cases (among the 18 adult pigs possessing the P18 factor for which both parents are known) of a P18-positive adult which is the offspring of P18-negative parents. Seven of the eight P18-positive adult Durocs and crossbreds

TABLE 3
Reactions of pig bloods with serum P18

Age and breed	Number of bloods with P18 factor/total of group			Total
	A	O	“—”	
Juvenile Yorkshires	0/17	2/52	0/1	2/70
Juvenile crossbreds	5/28	5/20	6/8	16/56
Juvenile Durocs	9/24	2/14	14/26	25/64
Juvenile total	14/69	9/86	20/35	43/190
Adult Yorkshires	0/17	1/52	0/1	1/70
Adult crossbreds	0/28	2/20	1/8	3/56
Adult Durocs	4/24	0/14	10/26	14/64
Adult total	4/69	3/86	11/35	18/190

in generation one were offspring of 222B, the only “—”, P18-positive boar among the seven Duroc boars of generation zero. Six offspring of 222B lacked the P18 factor as adults.

Inhibition tests to detect a P18-inhibiting substance in serum or saliva have been unsatisfactory; reactions are weak at best and it is difficult to interpret the results of the tests. Sera and saliva from A, O, and “—” pigs all appear to inhibit the P18 reaction weakly, and the inhibiting power of serum or saliva from individual pigs does not appear to be related to the reactivity of the red cells with P18.

DISCUSSION

In order to determine the inheritance of A-O blood groups in pigs, it was necessary to classify red blood cells from individual pigs as A, O, or “—”. There was no problem of erroneous classification of A as O or vice versa since no bloods were positive for both A and O. Gene A^4 for group A appears to be completely dominant to its allele a^o for group O, just as in sheep the gene for R is dominant to its allele for O (STORMONT 1951). When A-O negative cells and cells only weakly reactive with the A or O reagents used were classified as “—”, all data were in agreement with the hypothesis that a gene designated S which allows expression of A or O on red cells is dominant to its allele s for “—”, just as gene I in sheep is dominant to i (RENDEL *et al.*, 1954). The juvenile and adult phenotypes of some of the pigs differed, so that some of the bloods from young pigs were misclassified, if the adult type is accepted as the correct one. When juvenile bloods are misclassified, the error will most likely be in the direction of classification of A or O as “—”, and this will result in an excess of “—” bloods. No family data in disagreement with the hypothesis will be observed, since “—” offspring may result from any of the matings. However, if an error is not corrected by retyping an animal as an adult, two pigs classified as “—” may, when mated, have A or O offspring, if one or both is actually of group A or O.

The variation within the A and O groups appears to be due in part to genetic differences within these groups due to multiple alleles at the A-O locus which subdivide A^4 and a^o . No attempt was made in this study to make fine distinctions within A and O, but it appears that inhibition tests with serum and saliva, as well as titrations of red cells with several A or O reagents and absorption tests would be helpful in distinguishing A-O subtypes. It appears quite possible that weak A reactions in some sire families are associated with positive reactions with P18. It may be that P18 is detecting the product of a gene which is using the same substrate as the A^4 gene product, so that competition for substrate results in a weaker reaction for A when the P18 factor is present.

If it is assumed that A or O reactivity of red cells is due to the acquisition of soluble blood-group substances by the cells from the serum (SAISON and INGRAM 1962) it is clear that genes S and A^4 are necessary for the production of A substance and its acquisition by red cells so that it may be detected by appropriate reagents. S is also necessary for sufficient O substance to be acquired by red cells to be detected by a simple hemolytic test, and ss pigs will have A- and O-negative (“—”) red cells.

STONE and IRWIN (1954) proposed that in cattle a threshold level of J substance in the serum is necessary for its acquisition by red cells. In pigs, it appears that animals with A substance in their serum have A-positive red cells as adults. The relationship between O substance in the serum of pigs and its presence on red cells is not clear; A, O, and "—" pigs all appear to have nearly the same or the same amount of O substance in their serum as measured by inhibition tests. Pigs and sheep apparently differ in this respect; RENDEL *et al.* (1954) and RENDEL (1957) demonstrated the presence of O substance in the serum of group-O but not of group-R and group-i sheep, and RENDEL (1957) and TUCKER (1962) demonstrated O substance in the saliva of group-R and group-O but not of group-i sheep.

Family data confirm the hypothesis that the inheritance of A-O groups in pigs can be explained using a genetic model comparable to that of RENDEL *et al.* (1954) for sheep. ANDRESEN (1962, 1963) and SAISON and INGRAM (1962) have reported that group-A offspring may result from mating of two A-negative parents. In the present study, eight different A-negative matings produced at least one group-A offspring. Such group-A pigs did not result from "—" $\times$ "—" or O $\times$ O matings, but only from O $\times$ "—" (as shown in Table 1). There is no evidence from this study that more than one genotype (designated *ss*) is responsible for the "—" phenotype in Durocs, Yorkshires, and their crosses; all "—" $\times$ "—" matings gave only "—" offspring.

The apparent similarity between the genetic basis for the A-O system in pigs and the R-O system in sheep is not unexpected, since sera which are used to classify pig cells as A, O, and "—" can also be used to classify sheep cells for R, O, and i, as demonstrated by SPRAGUE (1958a). Bovine serum C4, ovine S8, and porcine P62 used in this study as A reagents may also be used as reagents for sheep R (P62 must be carefully absorbed with R-negative sheep cells to remove heterohemolysins against sheep red cells). G1 and C1 are antisheep O as well as antipig O.

The reaction of C1 with group-O sheep or pig cells is inhibited by saliva from human secretors, and C1 agglutinates red cells of humans of group A, AB, B or O. It appears that this serum is also antihuman H. The bovine antipig A serum (C4) can also be used as a source of antihuman A. Complex interactions have been described among genes for the Bombay, Secretor, Lewis, and ABO systems in man (see RACE and SANGER 1962 for discussion and references), and further study of the genetics and serology of the A-O and related systems in pigs may reveal similar complex interactions in this species. The P18 reactions appear to be related to such interactions, and the increased frequency of reaction of P18 with A-negative bloods and with juvenile bloods suggests parallels with the Lewis system of man.

SUMMARY

Data from 235 families of Duroc, Yorkshire, and Duroc-Yorkshire crossbred pigs are in accord with the hypothesis that phenotypes in the A-O blood-group system are controlled by genes at two loci. A^A-S- results in group-A red cells, $a^o a^o S-$ results in group O, and *ss* pigs have "—" (A-negative, O-negative) red cells.

Serum P18 detects a blood factor which is more common in young pigs than in adults and is more common in "—" bloods than in group A or group O.

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