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The Maintenance of Transferrin Polymorphism in Pigeons

(Saccharomyces cerevisiae/eggwhite)

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ABSTRACT Transferrin, a nonheme iron-binding protein, is polymorphic in most vertebrate species that have been examined. In pigeons, it is controlled by an autosomal gene, with two known codominant alleles, Tf^A and Tf^B. The two alleles are found in nearly equal frequencies and the three genotypes are at Hardy–Weinberg equilibrium in all populations studied. This report shows that ovotransferrins from heterozygous females inhibit microbial growth, by use of yeast as an assay organism, better than ovotransferrins from either of the homozygous types, or those from a mixture of homozygous types. Heterozygous females hatch a larger percentage of their eggs than homozygous females. This difference is probably accounted for by the transferrin effect. The failure of the mixture of the homozygous types to act like the heterozygous type calls into question the currently accepted structure of transferrin as a monomeric protein. The greater fecundity of heterozygous females can account for the maintenance of transferrin polymorphism in pigeons.

How genetic polymorphism is maintained is one of the major unanswered questions in genetics today. The belief that it depends upon a selective advantage of heterozygotes is widely held but not well supported by data. This paper presents evidence for maintenance of transferrin polymorphism in pigeons by the differential inhibition of microbial growth by different transferrin phenotypes.

Transferrin is a nonheme iron-binding protein found in the plasma of vertebrates and the eggs of birds. It is found to be polymorphic in most species examined. In each case appropriately studied, transferrin is found to be controlled by codominant alleles of an autosomal gene (1, 2). There have been many attempts to find mechanisms for the maintenance of this polymorphism. Ashton and his associates (3, 4) have reported evidence in some cattle for excess of heterozygotes, and postulated superior heterozygote fitness. This is not confirmed in other groups of cattle (5, 6), although lowered milk production of one homozygous class has been reported (6). In other domestic animals (pigs, mice), no effect of transferrin type on fertility has been found (7, 8). In *Microtus*, lowered fitness of both one homozygous and one heterozygous type has been suggested by field studies, but no selective mechanism has been proposed (9, 10). Recently, Morton and Gilmour have reported an association between maternal transferrin type and hatchability in chickens (11).

Mueller *et al.* (12) reported that there are two alleles, which act as Mendelian codominants, present in pigeon populations. These observations have been confirmed in population studies by others (13, 14) and in this laboratory by family studies. Starch gel electrophoresis used in our laboratory displays no difference in mobility between transferrins of different tissue sources in the same bird, so the term *transferrin* will be used to describe the molecule regardless of the tissue source. In all populations of pigeons that have been studied, frequencies of the two alleles have been close to 0.5 for each allele (13, 14). The fact that only two alleles exist, and that they exist in similar frequencies with the genotypes at Hardy–Weinberg equilibrium in such diverse places as Ireland, Wisconsin, Missouri, and California, suggests that powerful selective pressures may be operating to maintain this polymorphism.

The data reported here suggest that in pigeons, the polymorphism is maintained by fertility differences among females, due to greater resistance of the offspring of heterozygous females to embryonic and early posthatching infection. I have reported (15) that young squabs express, for a considerable period, not their own transferrin type but the maternal type. This interval corresponds closely to the period of immuno-incompetence, thus giving the squabs of heterozygous females an extended period of enhanced protection from microbial infection. Consideration of this kind of selection leads to a model that predicts maintenance of polymorphism in the absence of a discernible excess of heterozygotes in the population (Frelinger and Crow, manuscript in preparation).

MATERIALS AND METHODS

Pigeons. Two breeds of pigeon (*Columba livia*) were used in these studies, White Kings and Tumblers. The birds were maintained in pairs in mating cages or in flypens. All nests were checked for eggs three times a week. Eggwhites were collected from infertile eggs and stored frozen at −20°C until use. Eggwhites and sera were typed by starch gel electrophoresis as described (15).

Transferrin Purification. Pigeon transferrin was purified by dialyzing eggwhite against 5 mM sodium acetate (pH 5), centrifuging, and discarding the precipitate. The supernatant was chromatographed on carboxymethyl Sephadex C-25 in the same buffer. The breakthrough was pooled, 1 ml of 1 mM $FeCl_3$ in 0.5 M sodium carbonate was added, and the solution was dialyzed against 0.01 M Tris–0.06 M NaCl, and chromatographed on DEAE Sephadex A-25. This gave a product about 80% pure as judged by A_{280}/A_{470} ratios and by immuno-electrophoresis.

Yeast Growth. Wild-type diploid yeast, *Saccharomyces cerevisiae*, was grown on 1% casein hydrolysate, 1%

yeast nitrogen base (Difco), 2% dextrose, 2 mg of tryptophan per ml, 0.1 mg of biotin per ml, and 100 μg of streptomycin per ml. For the eggwhite experiments, this medium was made 10% eggwhite from a single typed eggwhite, filtered through a 0.3 μm Millipore filter, and 10 ml of this solution was inoculated with about 10^5 colony-forming units of yeast per ml. The yeast was grown in suspension at 30°C, in a shaking water bath. When purified transferrin was used, the protein was first dialyzed against 1000-fold excess of 0.01 M citric acid (pH 4.7) to remove the iron, followed by dialysis against 0.05 M morpholinoprane sulfonic acid buffer (pH 7.0). The medium was also made up in 0.05 M morpholinopropane sulfonic acid buffer and sterilized by filtration as before. The inoculum used for this experiment gave a final concentration of about 7×10^3 colony-forming units/ml. The final transferrin concentration was 2 mg/ml, approximately the same as a 10% eggwhite solution. Yeast growth was monitored by serial dilution and duplicate plating on 2% agar plates (1% yeast extract, 2% dextrose, and 2% peptone) at intervals during the growth period. Tests of statistical significance were "two-tailed" t tests.

RESULTS

Following the current convention for naming electrophoretic variants, the alleles described by Mueller as Tf^{L1}, Tf^{L2} (12) have been renamed as Tf^{A} and Tf^{B}, Tf^{A} controlling the faster-migrating transferrin. For the pigeons in this laboratory the allele frequencies are A = 0.52 and B = 0.48 (n = 97). These genotypes do not differ significantly from Hardy–Weinberg equilibrium in their distribution in the flock, or in either breed. The allele frequencies reported by Ferguson (14) in Northern Ireland were A = 0.592 and B = 0.408. Brown and Sharp (13) in Missouri reported A = 0.381 and B = 0.619. It appears that this polymorphism is both relatively stable and widespread.

Hatchability

The hatching records of the female birds in our colony were correlated with transferrin type of the females. The values for Tumblers are shown in Table 1. Kings have poorer performance in our flock, but show similar differences among female genotypes. A significantly greater portion of the eggs of AB females hatch than do the eggs of the two homozygous types.

Inhibition of yeast growth

Fig. 1 shows typical curves for the growth of yeast in medium containing individual eggwhites, all at the same eggwhite concentration. Variability of the AB inhibition was common among individual AB eggwhites, but in no case did AB inhibit growth less than the homozygous types. Table 2 shows

TABLE 1. *Hatchability of eggs*

Genotype of female	Number of eggs laid	Number of eggs hatched	% Hatched
AA	128	59	46
BB	144	75	52
AB	267	180	67

Chi square = 7.99; $P < 0.02$.

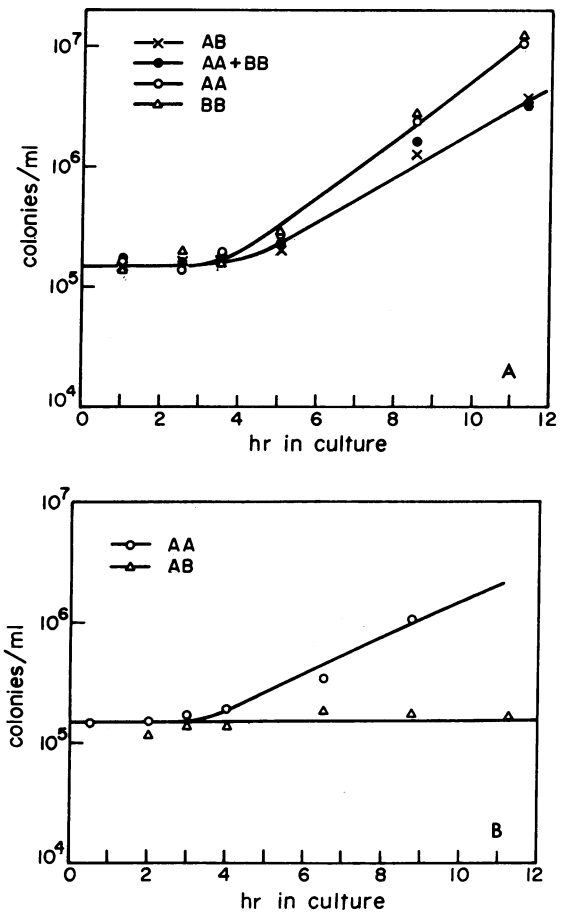

FIG. 1. Growth of yeast in medium containing eggwhite. (*A*) shows one of the experiments in which AB transferrin did not inhibit yeast growth greatly. (*B*) shows an experiment in which the AB transferrin totally inhibited the yeast growth, while the yeast grown in A tranferrin grew at its characteristic rate.

a composite of growth rates of these growth curve experiments. These rates are calculated by computation of the number of yeast doublings during the culture period divided by the elapsed time in hours minus the 2-hr lag phase. Addition of $FeCl_3$ at a concentration of 0.01 mg/ml abolished the differences among transferrin genotypes. The two homozygous types and, most interestingly, mixtures of the two types, give similar yeast growth rates. This finding suggests an interaction in the heterozygote, absent from the simple mixture of the homozygous eggwhites. However, mixtures of the two homozygous types are indistinguishable from the heterozygous type on starch gels.

Early experiments with purified transferrin in phosphate buffer failed to show the difference found with crude eggwhite. Apparently, this was due to iron contamination of the reagent-grade phosphate used. When the iron-free synthetic buffer morpholinopropane sulfonic acid was used, the difference again became apparent (Fig. 2). In these experiments it was necessary to pool 5 or 6 eggwhites of each type for the purification procedure. The yeast growth rates calculated in this experiment (Table 2) are almost identical to those calculated from the experiments with pooled raw eggwhite. This indicates that the effect observed in raw eggwhite is in fact due to transferrin.

DISCUSSION

These experiments show for the first time *in vitro* a differential inhibition of microbial growth by transferrin from different phenotypes. The bacteriostatic and fungistatic properties of transferrin are well documented, predating, in fact, the modern description of transferrin (16). Transferrin has been shown to inhibit a wide variety of iron-dependent microorganisms including *Saccharomcyes cerevisiae, Staphlococcus aureus, Candida albicans, Shigella dysenteria, Pasturella septica, Pseudomonas, Clostridium welchii, Lysteria moncytogens,* and *Salmonella typhimurium* (17). In chickens, embryonic death is frequently associated with microbial contamination of the egg (18). *Salmonella* can penetrate through the egg shell to contaminate the egg (19). Levi (20) has reported that while *Salmonella* infection may be asymptomatic in adults, it is a frequent cause of death in young squabs. He also cites *Candida albicans, Pasturella,* and *Mycobacterium tuberculosis* as common in pigeons. Thus, it appears that the opportunities for the infection of the egg and young squab are substantial, and the bacteriostatic and fungistatic properties of transferrin constitute, presumably, an important early line of defense. Because the transferrin both in the egg and the young bird is of maternal origin, this critical selective period is based on the maternal genotype rather than the embryo's own constitution. This leads to the survival of a greater proportion of the progeny of heterozygous mothers, but produces distributions of surviving genotypes indistinguishable from the Hardy–Weinberg equilibrium (Frelinger and Crow, manuscript in preparation).

TABLE 2. *Yeast growth in the presence of eggwhite and of purified transferrin of three types**

Transferrin type	Material	Growth rate	Number of egg-whites†
A (Tf^A/Tf^A)	Eggwhite	0.495 ± 0.0637	6
	Purified transferrin	0.34	5 (pool)
B (Tf^B/Tf^B)	Eggwhite	0.393 ± 0.0374	13
	Purified transferrin	0.30	5 (pool)
Mixture of A and B (Tf^A/Tf^A + Tf^B/Tf^B)	Eggwhite	0.425 ± 0.0509	4
AB (Tf^A/Tf^B)	Eggwhite	0.162 ± 0.067	10
	Purified transferrin	0.14	6 (pool)

* Gosset's t test for significance revealed no significant difference ($0.5 > P > 0.3$) between A and B. These data were therefore pooled and tested against the mixtures of A and B. This comparison also yielded no significant difference ($0.7 > P > 0.5$). Therefore, these data (A, B, mixture of A and B) were pooled and compared to AB. The difference is highly significant ($P < 0.0003$).

† For purified transferrin, number of eggwhites is the number of different eggwhites in the pool from which the transferrin was purified. In the case of eggwhites, it represents the number of individual egg whites that were separately tested. The results are given as the mean growth rate ± the standard error.

Several workers have reported that transferrin is a monomeric protein (1, 2). The failure of the mixture of the two homozygous types to display the functional characteristic of the heterozygous type suggests the presence of an interaction product in the heterozygote. This is difficult to explain in terms of a monomeric protein, if the alleles represent a structural locus controlling the primary structure of the protein. The difference in the action of the heterozygous protein suggests the presence of dimeric protein, so that "hybrid" molecules can be produced in the heterozygote. In cattle, a second gene affecting the carbohydrate portion of the transferrin molecule has recently been reported by Spooner (21). If the difference between the allelic transferrins of pigeons is in their carbohydrate rather than in their primary protein structures, it then becomes possible to resolve the difference between heterozygous transferrin and the mixture of the two homozygous types. Experiments are currently in progress to determine if the inherited difference is in the amino acid or in the carbohydrate portions of the molecules.

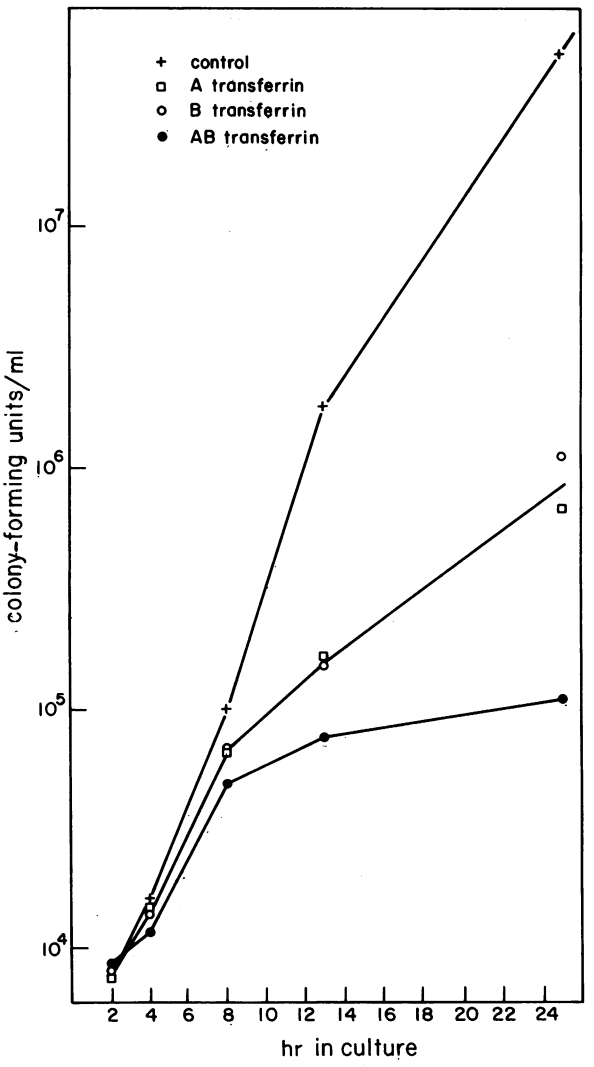

FIG. 2. Growth of yeast in purified transferrin. This shows clearly that while both A and B transferrins inhibit the growth as compared with the control, the AB type inhibits more than either homozygous type.

The results report for the first time a direct *in vitro* test of a proposed selective mechanism for the maintenance of a polymorphism. The data are consistent with the hypothesis that the polymorphism is maintained by selection against the offspring of homozygous females because of their greater susceptibility to infection both as embryos and as young birds. This interpretation is suggested by both the *in vitro* data on inhibition of yeast growth and the *in vivo* results represented by the hatchability data.

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