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THE TIME OF APPEARANCE OF SPECIES-SPECIFIC ANTIGENS OF COLUMBA GUINEA IN THE EMBRYOS OF BACKCROSS HYBRIDS[1]

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ANTIGENS of the red blood cells specific to the triangular spotted pigeon, *Columba guinea*, in contrast to the common domestic pigeon, *C. livia*, were identified singly in backcross hybrids by Irwin, Cole, and Gordon (1936). These antigens were designated A, B, C (formerly called CD), E, and F. Each of these species-specific antigens has behaved as if produced by the action of one or more genes on a single chromosome. Each species-specific antigen is expressed whether the gene producing it is present in the heterozygous or homozygous combination. Birds possessing these antigens have been maintained following backcrosses to *livia* or mating *inter se* and were available for the current study.

The time of appearance of antigens during ontogeny is of interest. Kemp (1930) and Bornstein (1942) found that early human fetuses may possess known agglutinogens on the red blood cells.

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Keeler and Castle (1934) found that rabbit embryos at an early stage may possess certain antigens of the cells of the adult. In contrast, Briles, McGibbon, and Irwin (1948) reported that some antigens of the red blood cells of chickens were present early in embryonic development, while others were not found until after hatching. The following experiments represent comparable tests upon the blood cells of embryos and squabs from backcross hybrids originally resulting from the mating of *guinea* and *livia*.

MATERIAL AND METHODS

Most of the backcross hybrid parents were heterozygous for the gene or genes producing a particular antigen of *guinea* (A, B, C, E, or F), and they were mated *inter se* or to *livia*. Some hybrids were later demonstrated by genetic tests to be homozygous for the gene producing a particular antigen of *guinea*.

The general serological technique has been described elsewhere (Irwin and Cole, 1936). Specific antisera to the red blood cells of *guinea* were obtained from rabbits. The majority of the present tests for antigens B, C, and E were conducted with one potent antiserum to *guinea* cells, No. 473F7. The diluted antiserum (1/60) was absorbed by washed *livia* cells to remove antibodies to antigens held in common between *livia* and *guinea*, thus making a "reagent" specific for the antigens of *guinea*. This "reagent" reacted with A or F cells to a dilution of 1/240, with B or E cells to a dilution of 1/480, and with C cells to a dilution of 1/1,920.

Other kinds of reagents were used to a limited extent. The antiserum to *guinea* could be absorbed by a combination of cells from several different backcross birds, each possessing a different antigenic character specific to *guinea*. For example, the antiserum to *guinea* absorbed by the pooled red blood cells from *livia* and from birds possessing A, C, E, and F, respectively, was presumed to be specific for "B" cells. Such antisera reacted specifically with all B cells, as expected. It is possible that not all the species-specific antigens of *guinea* in contrast to *livia* were found. Thus anti-*guinea* absorbed with cells of backcross birds possessing each of the known antigens of *guinea* (A, B, C, E, and F) might still contain antibodies to *guinea*. However, only birds possessing known antigens of *guinea* were used in this study.

Antisera to backcross birds possessing an antigen specific to *guinea*, when absorbed with *livia* cells, would yield reagents which would react only with that *guinea* antigen carried by the backcross birds. Such birds were the offspring of four or more backcrosses to *livia* and presumably were identical to *livia* in antigenic composition except for one of the antigens of *guinea*. The A and F antigens of *guinea*, when present in unit form in heterozygous backcross hybrids, have never induced detectable antibodies when injected into rabbits or pigeons. These antigens (A and F) were detected only by using antiserum to *guinea* or by "cross-reaction" with appropriately absorbed antisera (absorbed with cells of *livia*) against one of several species of pigeons (e.g., *C. rufina* or *C. picazuro*). In the majority of the tests the antigens A and F were detected by such reagents from an antiserum to *picazuro*. The reagent from anti-*picazuro* was used because the antibodies withstood thawing and freezing better than the antibodies to A and F in the anti-*guinea* serum. The titer of this reagent from anti-*picazuro* with A or F cells was the same as that from anti-*guinea*, 1/240. Reagents prepared from antisera to backcross birds possessing B, C, or E were not so strong

as that prepared from anti-*guinea* and so were used only in a few tests to show that reagents prepared from either source paralleled one another in their reactions.

For the agglutination tests to detect the presence or absence of the antigen on the red blood cells, an antiserum or reagent at a 1/60 dilution was mixed with a 2–3 per cent washed suspension of red blood cells in the proportion of 2 drops to 1 drop of cells, from a pipette delivering 30–36 drops per cubic centimeter. The cells involved were previously washed and suspended in 0.75 per cent saline solution. The mixture was incubated at approximately 31° C. for 1½–2 hours, and the agglutinations were read immediately after incubation. The tests were read again after standing overnight at 2°–5° C. Agglutination of the test cells with the reagent indicated the presence of the antigen. About half of those cells which failed to agglutinate with the specific reagents, and therefore were presumed to lack the particular antigen of *guinea*, were tested with unabsorbed antisera against *guinea* or *livia* cells to ascertain whether or not such cells could be agglutinated. The unabsorbed antisera always strongly agglutinated the cells of embryos or squabs of any age. This was expected, since unabsorbed antisera contain many antibodies to those antigens which are held in common by *guinea* and *livia*.

Blood samples were collected from squabs by cutting the wing vein and allowing a few drops to fall into a solution of anticoagulant (2 per cent sodium citrate, 0.42 per cent NaCl, and 97.58 per cent distilled water). A pipette was necessary to remove the blood from the wing vein of the youngest squabs. Some squabs were bled and tested as often as five times during their growth.

Blood from embryos was obtained from the heart or blood vessels, after removing a portion of the shell of the egg and of the inner membranes by puncturing the heart or blood vessel with a fine needle. The blood was removed with a capillary pipette and immediately placed in citrate. Care was exercised so that little or no yolk was removed with the blood. The majority of fertile eggs at 68–72 hours of incubation would not yield enough blood for testing. Those listed under the 68–72-hour column in Table 1 gave barely enough blood to make one drop of a 2 per cent suspension of erythrocytes.

The accuracy of knowing the age of the embryos depends on when and how often the eggs were collected. Eggs which were to be tested after 4 days of incubation were collected at least once a day. Those eggs which were to be tested at 4 days of incubation or earlier were collected at least twice daily, at noon and at 6:00 P.M. Cole and Kirkpatrick (1915) found that the first egg is laid about 5:00 P.M. (range 1:00–7:00 P.M.) and the second egg about 1:00 P.M. (range 8:00 A.M. to 4:00 P.M.), approximately 44 hours later. Pigeons seldom incubate the first egg until the second egg is laid. Early embryos in the present study probably deviated from the stated age up to 5 hours of additional incubation, usually less than 5 hours. The erythrocytes of young squabs and of embryos from eggs incubated at 40° C. were tested at different stages of development.

Seventy embryos were examined under a binocular microscope for the number of somites and for the heart rate. Should the antigens appear "suddenly" on the erythrocytes of the early embryo, it might be possible to correlate the somite number or heart rate with appearance of the antigen. After having been bled, these embryos were fixed in Kleinenberg's picrosulfuric fixative and stained with Delafield's haematoxylin,

and the somite counts confirmed. Counts of the heart rate were made on each embryo during the second minute after removal from the incubator.

RESULTS

From previous results it may be concluded that the genetic ratios to be expected from backcrosses to *livia* and *inter se* matings of backcross hybrids involving a species-specific antigen of *guinea* are the same as expected in a monohybrid backcross or mating *inter se*. In those cases where one parent is homozygous for an antigen of *guinea*, all offspring are expected to possess the antigen of *guinea*. Since this work was finished, it has been found possible, by serological means, to differentiate heterozygotes from homozygotes for the antigens of *guinea* (Miller and Bryan, 1951). However, many of the parents for the present experiment died before their genotype could be determined. Since the homozygosity or heterozygosity of the hybrid parents for the genes producing the species-specific antigen of *guinea* was not known, an exact genetic ratio of the offspring is not expected in the following data. Therefore, the data were pooled from various families carrying any one specific *guinea* antigen. Thus, between 50 and 100 per cent of the offspring in each such family would be expected to possess an antigen of *guinea*.

TABLE 1*

NUMBER OF EMBRYOS AND SQUABS OF DIFFERENT AGES FROM BACKCROSS MATINGS AND *inter se* MATINGS OF BACKCROSS BIRDS POSSESSING OR LACKING SPECIES-SPECIFIC ANTIGENS OF *guinea*

Columba guinea Antigen	A. Age of Embryo at Bleeding								Total	B. Age of Squab at Bleeding (Days after Hatching)				Total
	Hours			Sub-total	Days									
	68–72	73–84	85–96		2–4	5–8	9–12	13–16		1–10	11–20	21–30	31–48	
A	3/3	4/4	1/1	8/8	10/12	6/14	4/7	2/3	23/37	13/19	4/5	7/10	10/11	34/45
A†	...	...	...	...	...	...	...	...	...	(5)‡	(6)‡	(1)‡	...	...
B	2/4	3/4	5/6	10/14	13/21	11/19	6/12	2/3	32/55	13/28	6/8	1/1	1/5	21/43
C	3/3	6/6	6/7	15/16	24/29	28/31	10/14	4/4	66/78	28/34	13/16	4/4	6/6	51/60
E	1/1	1/5	0/1	2/7	12/22	20/28	5/8	1/3	38/61	19/26	8/12	2/2	3/4	32/44
F	2/2	5/5	6/7	13/14	17/18	13/16	6/7	2/2	38/43	12/15	12/16	13/14	6/6	43/51
F†	...	...	...	...	...	...	...	...	...	(8)‡	(4)‡	(1)‡	...	...

* Tabular headings indicate the age of the embryos or squabs when they were first tested. The fractions represent the ratio of individuals possessing the antigen to the total tested (i.e., the numerator represents those with the antigen; the denominator represents those with the antigen plus those lacking the antigen).

† Individuals lacking A or F were tested repeatedly to confirm their classification.

‡ The numbers in parentheses represent the indicated number of birds negative with the specific reagent at the time indicated but giving a positive reaction later. These birds are in a separate category from others indicated in the table.

The results of the agglutination tests on embryos and squabs are summarized in Table 1 and are listed as fractions under a column heading specifying the age of the individuals when they were first tested. The numerator indicates the number of individuals which gave positive agglutination with the reagent, showing that they possessed the antigen. The denominator represents the total number of individuals tested. The difference between the numerator and denominator indicates those individuals whose red blood cells were not agglutinated by the respective reagents and,

therefore, presumably did not possess the antigen of *guinea*. Embryos and squabs were examined at various ages to make reasonably certain that the specific antigens could be found at all stages. Species-specific antigens (A, B, C, E, and F) of *guinea* were detected in the embryo as early as blood cells were present in quantities sufficient for testing, which was at approximately 72 hours of incubation and at all times thereafter.

Part A of the table indicates the hours of incubation of very young embryos grouped into three periods, 68–72 hours, 73–84 hours, and 85–96 hours. The grouping decreases the occasional error of incubation time due to parental incubation. The data in the column showing the subtotals indicate that all of 8 embryos, 68–96 hours of age, possessed antigen A; 10 out of 14 possessed B; 15 out of 16 had C; 2 out of 7 had E; and 13 out of 14 had F.

The rest of part A of Table 1 deals with the number of days of incubation divided into four periods, 2–4 days, 5–8 days, 9–12 days, and 13–16 days. The first period, 2–4 days, includes all of the embryos 68–96 hours of incubation plus a few more embryos of this age but with less exact knowledge of the hours involved. It may be noted in part A that 23 out of 37 embryos had A; 32 out of 55 had B; 66 out of 78 had C; 38 out of 61 had E; and 38 out of 43 had F.

After the sixth day of incubation, there was usually enough blood available from the embryo to use the red blood cells of the embryo in a titer test with cells from a mature bird known to possess the antigen. A titer test was performed several times for each unit antigen of *guinea*, and in no case was there a difference between the titer of embryo cells and positive control cells from mature birds. The titers were 4 to 6 doubling dilutions of the reagent for the antigens B, C, and E, and 1 to 3 doubling dilutions for antigens A and F (the starting dilution for each was 1/60). The only difference noted between the cells from embryos and adults was that cells of the embryos appeared to be slower in reaching the same agglutination strength and titer than did cells from mature birds.

Part B of the table gives the results of testing the blood of squabs at from 1 to 10, 11 to 20, 21 to 30, and 31 to 48 days after hatching. The number and classification of squabs are given for 10-day periods, except for the last period, which includes a few additional days. Additional confirmatory tests for those individuals tested more than once are not included. It may be seen in the last column of the table that 34 out of 45 squabs had A; 21 out of 43 had B; 51 out of 60 had C; 32 out of 44 had E; and 43 out of 51 had F. Within parentheses is given the number of squabs whose red blood cells later were shown to possess A and F antigens but which did not react with the specific antiserum during these periods. These birds are listed in the table only in parentheses. In general, it was observed that immediately after hatching and during the first 2 weeks to 2 months thereafter red blood cells of squabs containing A and F antigens tended to be weaker in agglutination and titer than did embryonic cells or control cells from adults. A few A or F squabs (those in parentheses in the table) appeared to acquire the antigen gradually. Recent data indicate that squabs homozygous for A or F may "acquire" the antigen more quickly than those heterozygous for A or F.

Realization of the approximate expected proportion (that is, between 50 and 100 per cent) of embryos and squabs possessing the antigen to those that do not supports the direct demonstration, as described above, that an antigen of

guinea first appears at the same time and stage in every individual. It is pertinent to note that two birds whose parents possessed antigen C and one whose parents possessed antigen A were probably homozygous for genes producing the antigen of *guinea*. In backcrosses to *livia* these three birds produced 23, 15, and 10 embryos and offspring, respectively, all possessing the antigen. Thirty-six embryos from the backcross matings of these birds were tested at from 68 hours to 13 days of incubation; all possessed the antigen as just stated, regardless of the age.

SOMITES AND HEART RATES IN EMBRYOS

The somites and heart rates were counted for over 70 embryos of 19–100 hours of incubation at 40° C. The period of greatest somite development seemed to occur at from 25 to 72 hours of age. Sixteen embryos 71–73 hours of age (by which time the antigens of *guinea* are present) gave an average of 29 somites (28.8 ± 2.00). Less change in somite number was observed through the 95th hour. Three 96-hour embryos had 35, 35, and 36 somites, respectively.

Initiation of regular heartbeat occurs between 42 and 48 hours of incubation (about the 12th somite stage). Twenty-seven embryos at 48–73 hours of age had an average heart rate of 42.7 ± 5.34 beats per 15 seconds. Seventeen embryos 74–100 hours of age had an average heart rate of 47.2 ± 5.7 per 15 seconds. No difference in somite number or heart rate was observed among the various backcross hybrids of *guinea* or embryos of *livia*.

DISCUSSION

Keeler and Castle (1934) found that rabbit embryos of the 4-mm. stage possessed antigens called H_1 and H_2 on the nucleated blood cells. Kemp (1930) found that a 37-day-old human fetus may have an agglutinogen on the red blood cells. Bornstein (1942) found agglutinogens A, B, M, N, and "Rh positive" in 7- to 21-cm. human fetuses. It is a comparatively recent finding that some cellular antigens are not detectable in the embryo or the newborn. Briles, McGibbon, and Irwin (1948) showed that certain antigens in chickens may not appear in the red blood cells of early embryos but may be expressed during a later stage of development. In two series of multiple alleles determining cellular antigens, all six antigens of the B series were found after the third day of incubation. In the D series the D^1 factor was found at the third day of incubation, but three others appeared only 6 days after hatching. These authors noted that the cell suspensions of antigens D^2, D^3, and D^4 (especially D^3) only gradually attained the degree of agglutination characteristic of a homogeneous suspension of adult cells. They suggested that this slow increase in the degree of reaction of the chick cell suspension may be due to the increasing proportion of reactive cells overcoming the dilution effect of the nonreactive cells initially present.

Although A and F antigens of *guinea* appear strongly in the embryo in contrast to D^2, D^3, and D^4 of chicks, which did not appear until after hatching, there was a resemblance in the period following hatching, in that a gradual rise in the degree of agglutination occurred until it reached a level characteristic of adult cells.

It may be that erythrocytes from different hemopoietic tissues differ in their antigenic composition at a particular stage or stages of a young animal. Sabin (1920) and Sugiyama (1926) describe stages of development of erythrocytes in the chicken embryo. Best and Taylor (1950) present a general outline of the

formation of red blood cells of an embryo. According to Sugiyama, cytological maturation of erythrocytes may not be noticed until the 17th day in the chicken embryo. It has already been proposed by Andresen (1947) that erythrocytes from more mature individuals in man may lack a specificity possessed by younger individuals. Andresen found that the Lewis factor (Le^a) of human erythrocytes usually was detected in infants which were both heterozygous and homozygous for the gene producing the Lewis factor but that in adults apparently only homozygotes reacted with the antisera detecting the Lewis factor.

The gradual rise in agglutinability of red blood cells of the A and F squabs after hatching has a slight resemblance to the behavior of the "J" substance in cattle (Stormont, 1949) and the R antigen in the lamb (Ycas, 1949). The red blood cells of cattle and sheep probably acquire these antigens from the serum, since it has been demonstrated that the serum in each species contains the particular antigenic substance by virtue of its ability to inhibit the reaction of the respective antisera with known positive cells. However, plasma from mature A and F birds will not inhibit agglutination of A or F and, seemingly, therefore does not contain the A and F antigens. It is possible that an environmental or physiological change is responsible for the "suppression" or "partial disappearance" of A and F at hatching. Regardless of the causative factor or factors, the phenomenon is consistent in direction and approximate time.

SUMMARY

The species-specific antigens A, B, C, E, and F of *C. guinea* appear in backcross hybrids with *C. livia* by 72 hours of incubation, or approximately the 29-somite stage, which is as early as enough blood can be obtained for testing. Antigens A and F tend to be weak or "absent" after hatching of the squab and during the first 2 months thereafter.

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