

CYTOLOGICAL AND CYTOPHOTOMETRIC ANALYSIS OF
BINUCLEATED RED BLOOD CELL MUTANTS (*bn*) IN
TURKEYS (*MELEAGRIS GALLOPAVO*)¹

STEPHEN E. BLOOM, EDWARD G. BUSS AND G. K. STROTHER

Department of Poultry Science, Cornell University, Ithaca, New York 14850
and
*Departments of Poultry Science and Biophysics, The Pennsylvania State University,
University Park, Pennsylvania 16802*

Received September 9, 1969

BINUCLEATED cells occur in a variety of tissues in plants and animals normally and under varying physiological conditions (e.g., anemia, stress, ageing, regeneration, malignancy and viral infection) (BLOOM 1968). Among animals, naturally occurring binucleated cells have been observed in organisms of the following classes: Protozoa, Nematoda, Crustacea, Arachnida, Insecta, Agnatha, Pisces, Amphibia, Aves and Mammalia. Binucleated red blood cells (BRBC's) have been noted only in Pisces (African lungfish and trout), Amphibia (Necturus, frog and Newt), Aves (turkey and chicken) and Mammalia (man).

Genetic control of binucleated cell formation has been reported for *E. coli* (ADLER and HARDIGREE 1965; ADLER, FISHER and STAPLETON 1966) and the turkey (BUSS 1965). One case reported in man may be genetically controlled (WOLFF and VON HOFE 1951). A gene that uncouples the relation between the dividing macronucleus and the cleavage furrow in *Paramecium aurelia* was described by NOBILI (1961). Binucleated and anucleated cells were occasionally formed.

BUSS (1965) identified binucleated red blood cells in the peripheral blood of turkeys from six sources. These included birds of the black-winged bronze and bronze strains of turkeys. Later, Shearer bronze turkeys were found to have BRBC's (BLOOM 1968). It was shown by BUSS that this condition was inherited as an autosomal recessive gene designated *bn*. In adults, about eight percent of circulating red blood cells (RBC's) were reported to have two nuclei.

Amitosis or direct nuclear division has often been considered as the mechanism for the origin of binucleated cells (MARSHALL 1908; MACKLIN 1916; KATER 1940, 1951; HUGHES 1952; CLELAND 1961; LAPHAM 1962). However, several other studies have suggested that mitosis without cytokinesis produces binucleated cells (WOLFF and VON HOFE 1951; ALLEN 1962; GODINA, BARASA and TIZZANI 1966). Preliminary work on the effects of the *bn* gene mutation on cell division (BLOOM, BUSS and STROTHER 1969) suggests that this gene causes some abnormality in mitosis that leads to the production of binucleated and other abnormal

¹ Authorized for publication on August 1, 1969 as paper no. 3640 in the journal series of the Pennsylvania Agricultural Experiment Station.

RBC's. Thus, a unique opportunity is presented for learning more about specific gene control of events in mitosis. The present investigation was undertaken to characterize the mutant phenotype at the cytological and cytochemical levels.

MATERIALS AND METHODS

Experimental material: Turkey embryos, newly hatched poults, growing, and mature birds of three genotypes (*Bn Bn*, *Bn bn*, *bn bn*) were used in this study.

In embryos, blood was obtained from the heart or peripheral blood vessels. At 0 to 11 D.O. (days old after hatching) the toenails were cut just short enough to yield one or two drops of blood. In older birds, blood was obtained from a wing vein.

Blood smears were stained with Wright's stain (LUCAS and JAMROZ 1961) and examined using a Leitz Ortholux microscope with bright field or phase contrast optics. Nuclear and cell areas were determined by measuring camera lucida tracings of Wright-stained blood cells with a *K&E* polar planimeter.

Cytochemistry: The DNA (deoxyribonucleic acid) in RBC nuclei was revealed using the Feulgen nuclear reaction with a 20 minute (HCl, 1N) hydrolysis at 60°C (BLOOM 1968; DEITCH 1966; DEROBERTIS, NOWINSKI and SAEZ 1960; LEUCHTENBERGER 1958; SWIFT 1950; VENDRELY and VENDRELY 1956). Azure B staining for DNA and RNA (ribonucleic acid) was also used (DEITCH 1966; FLAX and HIMES 1952). The following were the controls for the Feulgen reaction and Azure B staining:

1. Unhydrolyzed RBC (Feulgen).
2. TCA (trichloroacetic acid) extraction of DNA and RNA (Feulgen and Azure B) (ALFERT and GESCHWIND 1953; DEITCH 1966).
3. Cell components stained (i.e., nucleus, nucleolus and cytoplasm) with Feulgen and Azure B stains.

Cytophotometry: On each slide, 50 mononucleated (MRBC's) and 50 binucleated red blood cells were measured for relative DNA contents with a microspectrophotometer (BLOOM and STROTHER 1966; STROTHER and WOLKEN 1959; STROTHER 1966). The two-wavelength method was employed to correct for nonrandom distribution of dye molecules in a nucleus (GARCIA and IORIO 1966; GRASSO, WOODWARD and SWIFT 1963; MENDELSON 1958, 1966; PATAU 1952).

At each wavelength ($\lambda_1 = 490 \text{ m}\mu$ and $\lambda_2 = 510 \text{ m}\mu$) (BLOOM 1968), transmission (T) through a whole nucleus (I_n) was recorded using a fixed aperture setting. The microscope slide was then moved to an immediately adjacent "blank area" of the slide, and transmission through the slide, mounting medium, and cover glass (I_0) was determined. The ratio of these readings (I_n/I_0) times 100 gave the percentage transmission for the nucleus at that wavelength (T_1 at 490 m μ and T_2 at 510 m μ). From T_1 and T_2 the amount of DNA in a nucleus (γ) was determined from a set of tables (MENDELSON 1958). The area component in the calculations was a constant. Comparable results were obtained using a plug method for DNA determinations.

Chromosomes: A previously reported technique was employed for preparing chromosomes in mitotic bone marrow (femur and tibiotarsus) cells from seven homozygous turkeys at 11 D.O. (BLOOM 1969).

Significance of differences in the mean values was determined using the null hypothesis with Student's t-test employed to calculate probability levels (ALDER and ROESSLER 1964) (Tables 4 and 5).

RESULTS

Cytology of embryonic blood: Embryonic blood was sampled from *Bn bn* and *bn bn* genotypes. Only mononucleated RBC's were noted in heterozygous embryos. Of 25 homozygous recessive embryos at 3 to 27 D.I. (days of incubation), 23 had only MRBC's. The two embryos that had BRBC's may have been approach-

ing death since 24 of 30 dead embryos representing all three genotypes had been found to have BRBC's.

Cytology of blood and bone marrow (post-hatch): The abnormal cells observed in the blood of *bn bn* poultts included RBC's with greatly enlarged nuclei (LMRBC's) and RBC's with partially or completely constricted nuclei (binucleated or BRBC's) (Figure 4A, B, E). The abnormal circulating RBC's exhibited stages of development from mid-polychromatic (as defined by LUCAS and JAMROZ 1961) through mature RBC's. Other blood cell types (e.g., eosinophils, basophils, heterophils, lymphocytes, monocytes, thrombocytes) appeared normal morphologically and numerically.

By sampling both the blood and bone marrow of newly hatched *bn bn* poultts it was possible to determine that BRBC's and LMRBC's were first formed in the marrow and then released about one day later into circulation.

To study the development of BRBC's in the circulation of newly hatched turkeys, peripheral blood was sampled in poultts (*Bn Bn*, *Bn bn*, *bn bn*) from day of hatch (day 0) to 11 D.O. In poultts of the *Bn Bn* genotype only MRBC's were observed at 0-11 D.O. In heterozygous (*Bn bn*) poultts, small numbers of BRBC's (< 0.1%) were observed. However, at 36 weeks of age only two of 42 female and none of the 23 male heterozygous poultts had BRBC's. In homozygous recessive (*bn bn*) poultts, five of ten on the second day, nine of ten on the third day and all ten on the fourth day after hatching had abnormal RBC's (Table 1). By the eleventh day after hatching four percent of RBC's counted in blood smears taken from ten poultts were binucleated.

In addition to BRBC's, the homozygous recessive poultts had greater percentages of anucleated RBC's (ARBC's) than *Bn Bn* or *Bn bn* genotypes. The percentage of ARBC's in homozygous recessives was 1.5% and was greater in females than in males (ratio ♀/♂ = 1.4).

TABLE 1

Percentages of circulating BRBC's in poultts of bn bn genotype at 0 to 11 days of age

Wing band No.	Days after hatching										Number of cells
	0	1	2	3	4	5	6	7	9	11	
J 275 A	0	0	0	0	0.6	0.6	2.0	2.9	3.1	7.0	1934
J 275 B	0	0	0.3	0.3	1.0	1.4	2.4	2.5	2.8	3.0	2545
J 275 C	0	0	0.3	0.6	0.3	1.3	2.3	1.4	1.4	2.6	2654
J 275 G	0	0	0	1.3	1.4	3.2	4.2	3.2	3.9	4.3	2268
J 275 I	0	0	0.3	0.6	2.2	2.0	2.1	3.5	3.3	4.9	2594
J 275 J	0	0	0	0.3	0.6	0.9	0.7	1.0	1.3	2.5	2167
J 275 O	0	0	0.3	0.9	1.1	1.8	2.7	1.8	2.5	3.8	2608
J 275 P	0	0	0	0.8	0.9	1.8	1.8	2.8	4.5	5.9	1611
J 275 R	0	0	0	1.0	1.5	1.4	1.0	1.8	2.1	2.6	1530
J 275 S	0	0	0.3	0.5	1.0	1.2	0.9	1.6	3.1	3.0	1871
Total											21,782
Mean	0	0	0.3	0.7	1.1	1.6	2.0	2.3	2.8	4.0	
SD	0	0	0	0.3	0.5	0.7	1.0	0.8	1.0	1.6	

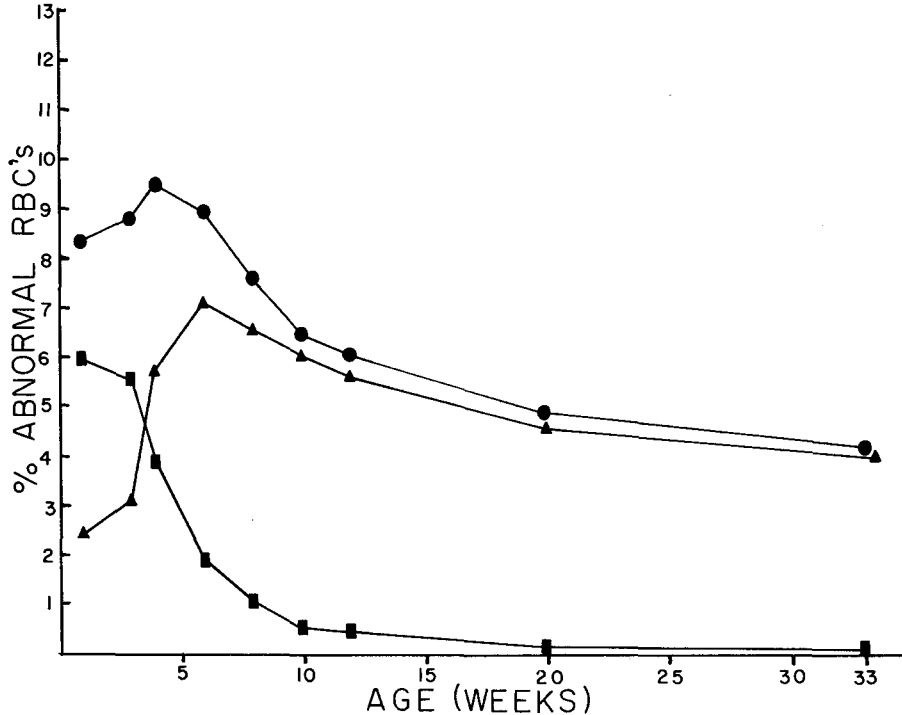


FIGURE 1.—The effect of age on the production of binucleated red blood cells (BRBC's), red blood cells with enlarged nuclei (LMRBC's) and total abnormal red blood cells (TARBC's). A sample of 60 male and 40 female *bn bn* turkeys at 1 to 33 weeks was used. A total of 157,500 cells were counted.

- Percent LMRBC's
- ▲ Percent BRBC's
- Percent TARBC's

Peripheral blood from 100 (60 males and 40 females) homozygous recessive turkeys was sampled at weekly intervals to determine the effects of age and sex on the production of BRBC's, LMRBC's and the total number of abnormal RBC's (TARBC's = BRBC's + LMRBC's) (Figures 1 and 2). Mean values were computed and presented in graph form. Variability about the means was considered by determining standard deviations and ranges. Since primary consideration was given to minimum and maximum percentages of abnormal RBC's in individual turkeys, ranges were tabulated (Tables 2 and 3).

LMRBC's predominated at first, but after three weeks declined rapidly and approached zero by 33 weeks (Figure 4E and Figure 1). The percentage of BRBC's increased rapidly in both sexes at 1 to 4 weeks, peaked at six weeks (7.1%) and declined thereafter. At 20 to 33 weeks the BRBC's began to level off at about 4%. At 4 to 6 weeks females developed a clearly greater percentage of BRBC's than males. At the peak (six weeks), females had 3% more BRBC's than males (Figure 2). After 20 weeks the sexes differed by only 1.2%. The data on sex differences were confirmed on three separate occasions with different populations of turkeys

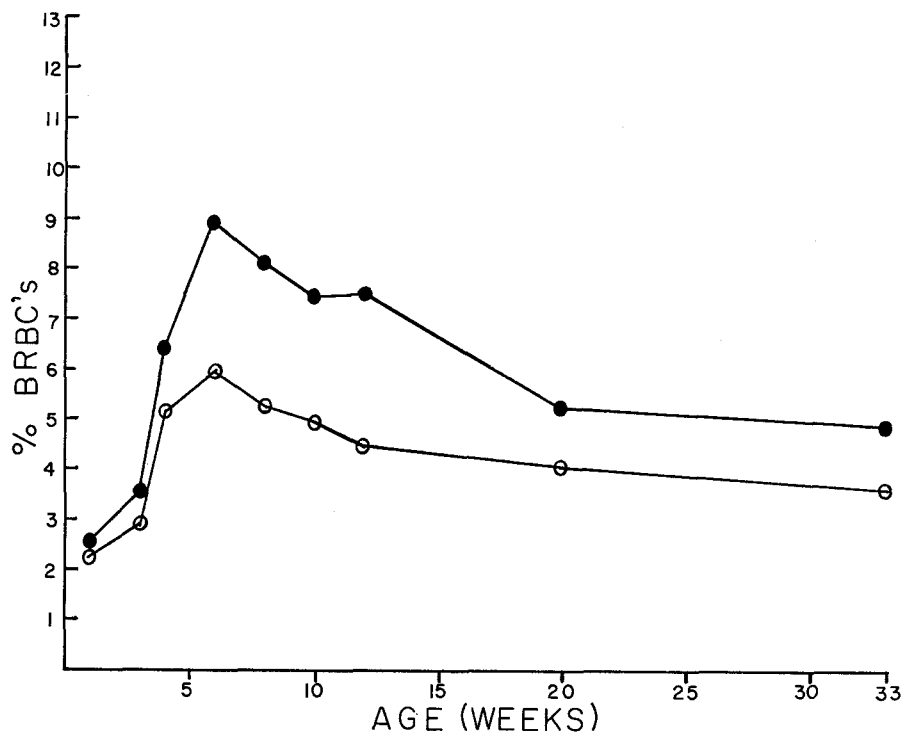


FIGURE 2.—The effect of sex on the production of binucleated red blood cells (BRBC's) in 60 male and 40 female *bn bn* turkeys at 1 to 33 weeks of age (same turkeys as in Figure 1).

- Percent BRBC's in females.
- Percent BRBC's in males.

TABLE 2

Minimum and maximum percentages (expressed as ranges) of BRBC's, LMRBC's and TARBC's in 100 male and female bn bn turkeys at 1 to 33 weeks of age

Age (weeks)	Range in percentages for		
	BRBC's	LMRBC's	TARBC's (BRBC's + LMRBC's)
1	0.6- 6.1	1.0-19.6	1.5-22.8
3	0.5- 7.0	1.8-13.2	2.7-18.4
4	1.2-16.0	0 - 8.8	3.4-19.2
6	1.9-21.4	0 - 5.2	2.2-26.5
8	0.5-17.1	0 - 4.5	0.5-20.5
10	1.0-15.0	0 - 2.9	1.0-17.9
12	0.6-19.8	0 - 2.9	0.6-19.8
20	0.6-16.6	0 - 1.2	0.6-17.0
33	0.5-14.3	0 - 1.3	0.5-14.8
Overall ranges	0.5-21.4	0 -19.6	0.5-26.5

TABLE 3

Minimum and maximum percentages (expressed as ranges) of BRBC's in 100 male and female *bn bn* turkeys at 1 to 33 weeks of age

Age (weeks)	Range in percentages of BRBC's for		Difference in maximum values ($\bar{\sigma} - \bar{\sigma}$)
	Males	Females	
1	0.6- 5.9	0.6- 6.1	+ 0.2
3	0.5- 5.5	0.7- 7.0	+ 1.5
4	1.2-10.5	1.2-16.0	+ 5.5
6	1.9-12.7	2.2-21.4	+ 8.7
8	0.5-11.7	2.1-17.1	+ 5.4
10	1.0-13.9	1.9-15.0	+ 1.1
12	0.6-11.1	2.4-19.8	+ 8.7
20	0.6-15.7	0.6-16.6	+ 0.9
33	0.5-11.9	0.5-14.3	+ 2.4
Overall ranges	0.5-15.7	0.5-21.4	0.2-8.7

and different laboratories handling the material and data. The curve for TARBC's showed a high level at one week (8.3%), peaked at four weeks (9.5%) and declined thereafter. From eight weeks on the BRBC curve and TARBC curve were almost identical.

Analysis of ranges at 1 to 33 weeks of age showed that individual *bn bn* turkeys had 0.5 to 21.4% BRBC's, 0 to 19.6% LMRBC's and 0.5 to 26.5% TARBC's (Table 2). Divergence by individual females occurred at four weeks, when a difference in maximum values of + 5.5% BRBC's occurred (Table 3). From then on, individual females exceeded the highest males.

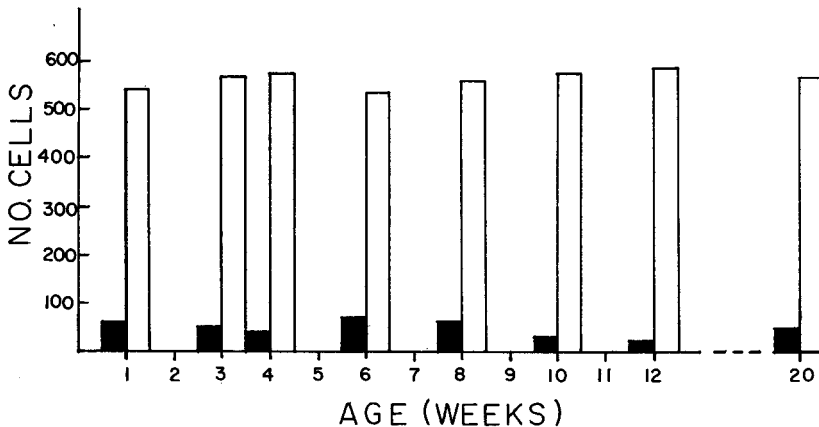


FIGURE 3.—The effect of age on the equality of nuclear division in binucleated red blood cells (BRBC's) from 12 male and 12 female *bn bn* turkeys at 1 to 20 weeks of age. A total of 4,880 cells were studied.

- BRBC's with equal sister nuclei.
- BRBC's with unequal sister nuclei.

TABLE 4

Nuclear sizes in mononucleated (MRBC's) and binucleated (BRBC's) red blood cells
(relative area expressed as cm² on camera lucida drawings)

MRBC's	BRBC's	Nuclear areas for	
		LBRBC's†	SBRBC's‡
1.19 ± 0.25*	1.64 ± 0.39*	0.99 ± 0.26	0.65 ± 0.22

* P (for difference between 50 MRBC's and 50 BRBC's) < .001 (highly significant).

† LBRBC's = larger nucleus of BRBC's.

‡ SBRBC's = smaller nucleus of BRBC's.

To determine the equality of nuclear division in circulating BRBC's at a gross level, cells were classified as having either equally divided nuclei or unequally divided nuclei (Figure 4A and 4B). In 8% of BRBC's, sister nuclei were of about equal size, while 92% of BRBC's had unequal sister nuclei. Age (also sex) did not significantly alter the proportion of cells with equal or unequal sister nuclei (Figure 3). Nuclear and cell sizes (areas) were determined by planimetric measurements of camera lucida tracings. The mean total nuclear area (two nuclei combined) for 50 BRBC's was significantly greater than the nuclear area for 50 MRBC's (Table 4). The larger nucleus of a BRBC (LBRBC) was just smaller than the nucleus of an MRBC, while the smaller nucleus of a BRBC (SBRBC) was two-thirds the size of the LBRBC. In contrast to the unequal nuclear sizes, the total cell areas for 50 MRBC's and 50 BRBC's were not significantly different (Table 5). However, the standard deviation for BRBC's was greater than for MRBC's. The BRBC's therefore had a greater proportion of nuclear material per cell than MRBC's and showed a greater diversity in cell sizes.

BRBC's (mid-polychromatic to mature) were found in bone marrow (i.e., all wing and leg bones, scapula, coracoid, ribs, ischium and sternum) as well as in the circulating blood. The percentage of BRBC's in marrow (femur and tibiotarsus) was 7.3 versus 4.0 in peripheral blood at 11 D.O. In addition to BRBC's, RBC's with other abnormalities were observed in small frequencies in bone marrow. Cells with 3 to 5 nuclear lobes were observed. Figure 4, C and D, shows an abnormally large immature RBC with a large irregular nucleus and a multinucleated RBC, respectively. BRBC's were also observed with cytoplasmic constrictions suggestive of cytokinesis.

Chromosome counts: Counts of prometaphase and metaphase colchicized chromosomes were made in bone marrow cells of seven *bn bn* turkeys at 11 D.O.

TABLE 5

Cell sizes of mononucleated (MRBC's) and binucleated (BRBC's) red blood cells
(relative area expressed as cm² on camera lucida drawings)

MRBC's	Cell sizes for	
	BRBC's	
8.12 ± 0.82*	8.22 ± 2.04*	

* P (for difference between 50 MRBC's and 50 BRBC's) > 0.1 (not significant).

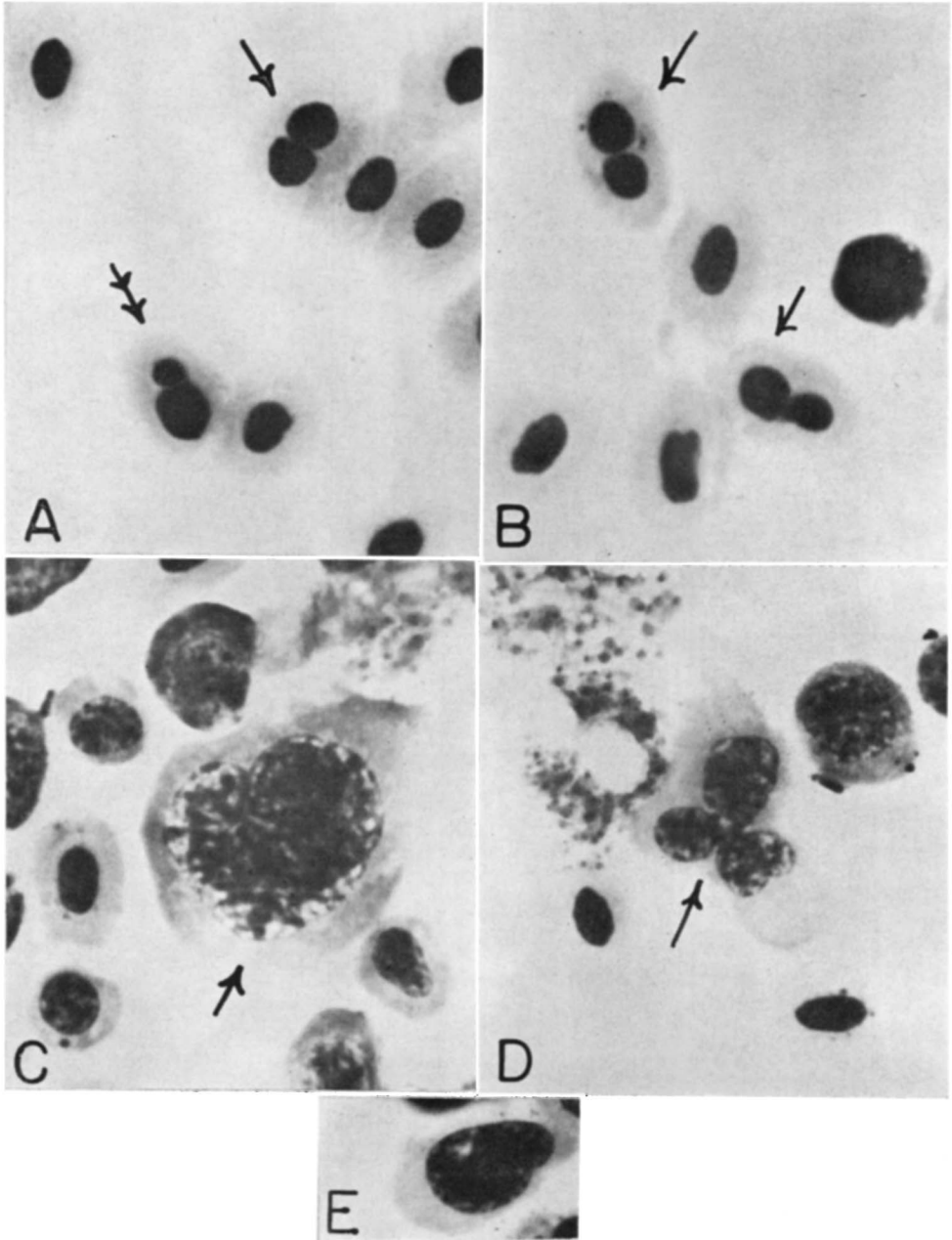


FIGURE 4.—Wright-stained smears from homozygous recessive turkeys showing (A) binucleated red blood cells from the peripheral circulation with equally (single arrow) and unequally (double arrow) divided nuclei, (B) binucleated red blood cells (arrows) with nuclei divided to degrees of inequality intermediate between the binucleated cells shown in (A), (C) abnormally large immature red blood cell with a massive, irregular nucleus (arrow) from bone marrow, (D) multinucleated red blood cell with an irregular cytoplasm from bone marrow (arrow), and (E) immature red blood cell with an enlarged nucleus. Figures magnified 1800 \times .

Karyotype analysis of 371 metaphases revealed only diploid cells. Several isolated cases of monosomy involving chromosomes in pairs nos. 7 to 9 were noted. This is currently under investigation.

Cytophotometric analysis: The Feulgen reaction applied to peripheral blood and marrow smears revealed that only the nuclei of the cells stained purple-red while the unhydrolyzed blood cells, TCA extracted blood cells, nucleoli and cytoplasm of RBC's and WBC's in bone marrow were unstained.

As a control, telophase nuclei in bone marrow were compared to MRBC's with respect to DNA contents. MRBC nuclei and telophase nuclei had similar DNA contents, and, based on chromosome counts (BLOOM 1968), were presumably diploid (2C).

In *Bn Bn* and *Bn bn* genotypes, single symmetrical populations were observed for DNA values of MRBC's. In the *bn bn* genotype at 0 D.O. (hatching) single populations were also observed for MRBC's. At 11 D.O. substantial numbers of BRBC's could be measured in the *bn bn* turkeys. BRBC's had more DNA than MRBC's (Figure 5). The MRBC populations were usually symmetrical and varied to the same extent as MRBC's in the *Bn Bn* genotype. DNA ratios were computed by taking the ratio of mean BRBC/mean MRBC DNA. For nine birds at 11 D.O. to 16 weeks of age a ratio of 2.2 ± 0.1 was obtained (Table 6). The BRBC's were therefore about 4C or tetraploid. Consideration of the sources of error in the cytophotometric procedure does not permit us to conclude that a ratio of 2.2 is significantly different than a ratio of 2.0.

Azure B differentially stains DNA (green-blue), RNA (blue-purple) and acid polysaccharides (purple-red). This stain applied to blood revealed only nuclear staining in MRBC's and BRBC's. The cytoplasm of MRBC's and of BRBC's was unstained indicating little or no RNA as expected of mature RBC's.

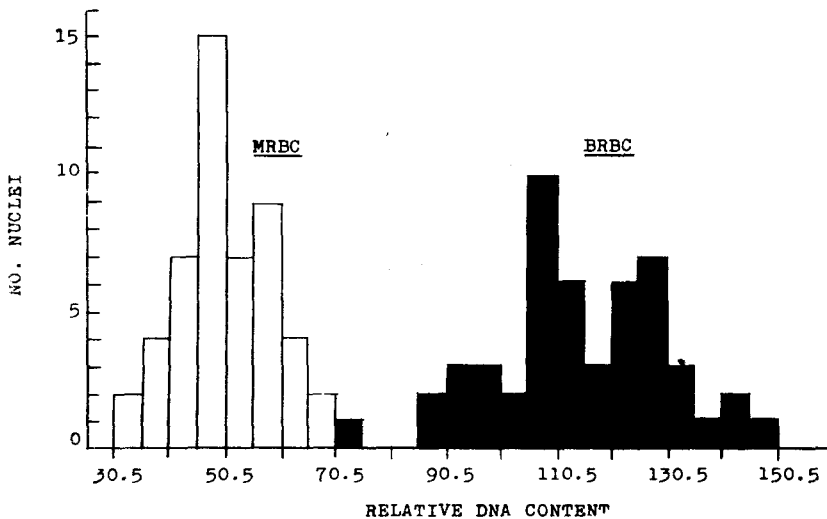


FIGURE 5.—Relative DNA contents of MRBC and BRBC nuclei in a homozygous recessive male turkey at 9 weeks of age.

TABLE 6

DNA ratios from RBC's in peripheral blood

Number of birds	Ratio Mean BRBC DNA/Mean MRBC DNA	Number of cells
9	2.2	900
Range	2.0-2.3	
Standard deviation	± 0.1	

DISCUSSION

The bn gene and development: In this study it was observed that the gene (*bn*) was not expressed in the embryo, at least not at the level of the BRBC phenotype. Abnormal cells were first formed in bone marrow at 1 D.O. and by 2-3 D.O. were released into circulation. The expression of this gene (involving only RBC's) was not an all-or-nothing phenomenon but rather a gradual development with age. In the first six weeks of life, the percentage of circulating BRBC's increased rapidly and then began a slow decline. In adult turkeys, the number of BRBC's did not exceed 21 percent. In females, higher percentages of BRBC's were found than in the males after 4 to 5 weeks of age. In contrast, LMRBC's predominated at first but faded out with age. Thus, peak production of abnormal cells occurred at about four weeks of age with a maximum of 27 percent abnormal cells.

Nature of gene expression at the cytological level: There were two phenotypes observed at the cytological level. One was in the peripheral circulation where BRBC's were found with nuclei divided to varying degrees. The two nuclei of a majority of BRBC's appeared unequal in size. This suggested some gross disturbance in the mechanism of chromosome separation in mitosis. Also, cytokinesis must be impaired if a single cell results having two nuclei. The affected cells were either late polychromatic RBC's or mature RBC's. Secondly, greater abnormalities of RBC's were found in the bone marrow than in the circulating blood, including large bi-, tri-, tetra- and possibly pentanucleated RBC's. Cells with two or more nuclei were sometimes seen to have bud-like formations, suggesting that some cytoplasm with or without nuclear material was being released. The BRBC's and other abnormal RBC's in the marrow were classified as about mid to late polychromes. Thus, the *bn* gene acts relatively late in the development of the RBC. In marrow about 7% of RBC's were binucleated as compared to nearly 4% in the blood at 11 D.O. This difference may be related to the rate of release of BRBC's from the marrow, the stability of marrow BRBC's or the longevity of the BRBC's.

In bone marrow, as in the circulating blood, BRBC nuclei were usually unequal in size, and when BRBC's showed signs of cytokinesis, the division products (nuclei and cells) were usually unequal. Thus, the cells affected by the mutant gene did not exhibit a uniform pattern of nuclear and cell division characteristic of normal mitotic cells. While BRBC's and other abnormal forms were often in interphase, abnormal mitotic cells were also observed (BLOOM, BUSS and STROTHER 1969). These included abnormal anaphase and telophase cells. Chromosome bridges were particularly frequent. These preliminary results suggest some ab-

normality in the mitotic apparatus of mutant cells. These observations are currently under study.

In terms of the activity of the gene in the heterozygote (*Bn bn*), it was observed that few BRBC's were produced. However, additional studies are required for the further characterization of the heterozygote.

An interesting observation was that the percentage of BRBC's was greater in females than males. One speculation is that the female hormones act to enhance the effect of the gene. Therefore, increasing the level of a hormone such as estrogen might increase the percentage of BRBC's.

Anucleated RBC's, which occurred in higher frequencies in *bn bn* turkeys, were most likely produced as by-products of abnormal cell division involving nuclear elimination. From an evolutionary point of view this is interesting since the mature mammalian erythrocyte does not possess a nucleus.

Nature of gene expression at the cytochemical level: The circulating BRBC's contained about twice the DNA as MRBC's. Age and sex did not appear to be correlated with DNA ratio. The BRBC's were, on the average, 4C or tetraploid. This quantity of DNA is expected if the BRBC's are the result of mitosis in which the DNA has been duplicated in S-phase of interphase, but the chromosomal material (tetraploid) remains in one cell as two nuclei rather than being distributed to two daughter cells. The results of the DNA determinations and chromosome counts (at metaphase) suggest that chromosome duplication in S-phase is normal and the chromosomes proceed to metaphase. It is then in subsequent events (anaphase, telophase) that the mitotic errors occur. Details of this are currently being examined.

SUMMARY

Binucleated turkey red blood cells (BRBC's), inherited as an autosomal recessive gene (*bn*), were studied cytologically, cytochemically and cytophotometrically. The gene was first expressed 1 to 2 days after hatching with the appearance of BRBC's and other abnormal RBC's in bone marrow. About 1 day later, abnormal RBC's were found in the peripheral blood. An increasing percentage of BRBC's appeared in the peripheral circulation with advancing age with a peak of 7% at 6 weeks of age. In adults about 5% of RBC's were binucleated. In some individual turkeys as many as 21% of the RBC's had two nuclei. After 4 to 5 weeks of age females had more BRBC's than males. Sister nuclei in BRBC's were most often unequal in size.—Circulating BRBC's contained about twice as much DNA (mean = 2.2) as circulating mononucleated RBC's.—Other abnormal nuclear and cell forms noted in bone marrow included immature tri- and tetra-nucleated RBC's and forms suggestive of nuclear and cytoplasmic budding. This study and other preliminary cytological work on dividing bone marrow cells suggest that the mutant gene affects some postmetaphase event(s) in mitosis.

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