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Time of Appearance of Antigenic Factors on Cattle Erythrocytes.* (27716)

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The time of appearance of antigenic factors‡ on erythrocytes (red cells) during ontogeny has long been of interest. Now, it is generally accepted that in humans most, if not all, of the blood factors are sufficiently developed in early embryonic life to be recognizable(1,2). However, the reactions are usually weaker in the embryo and in the newborn than in adults(3).

Similar results have been reported for rats (4) rabbits(5), chickens(6), pigeons and doves(7,8) and cattle(9,10). In (unpublished) studies on cattle, we were able to detect most of the blood factors on the red cells of newborn calves, although some of them were considerably weaker than those on adult red cells. For example, the H′ factor was found, both by Stormont *et al.*(10) and ourselves, as weakly reactive in embryos and newborn. It reached adult reactivity at about 6 months of age. The so-called J factor is an exception, because it has not been found on the red cells of embryos or newborn, but develops gradually within the first week *postpartum*. Since the J factor is primarily a constituent of the serum from which it may be acquired secondarily by the cells, this exceptional behavior is understandable(11,12).

Observations on the development of the H-2 red cell factors in mice indicate that they appear at about 3-8 days after birth and attain the concentrations found in adults by the 10-12th day(13,14,15). A recent report by Pizarro *et al.*(16) on the development and distribution of the histocompatibility (H-2) antigens (factors) of mice presented evidence that they were not present in tissues and on the erythrocytes of embryos or newborn, but appeared about the third day after birth and reached adult levels at about 6 days. In adults, the H-2 factors were found in most tissues except brain, but not in the same con-

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‡ The term antigenic factor or blood factor is used to denote an attribute of the erythrocyte surface responsible for the specific interaction of the erythrocyte with the antibodies in a blood typing serum. The terms antigen, antigenic determinant, haptenic group, etc., have been avoided deliberately because much confusion already exists in the literature as to their exact meanings.

centration. However, the same tissues from different mice of the same age and inbred strain had about equal concentration of factors. Significantly, these authors found a close correlation between the different H-2 factors that comprise an H-2 antigenic complex (phenogroup) with respect to age of appearance, rate of increase of individual factors within a given H-2 phenogroup and distribution in adult tissues. They interpreted these data as evidence that the H-2 locus is "one gene" in the sense that it behaved as a "physiological unit" or a "unit of function". In other words, these authors suggested that the complex antigenic system or phenogroup (consisting of multiple antigenic factors that are usually but not always inherited as one block) should be thought of as controlled by a gene defined in terms of function only.

Because these studies are pertinent to an understanding of the relationships between genes and antigens—an area of concern to our laboratory for a number of years(17)—we wish to present some of our data, gathered several years ago, concerning the time of appearance of blood factors on the red cells of cattle embryos. The so-called B system (locus) of cattle has characteristics paralleling those of the H-2 system of mice, although there is no evidence that the antigenic factors of the B system are involved in histocompatibility. In the B system there are more than 20 typing fluids (reagents) that detect over 250 different combinations (phenogroups) of these factors. Unlike the H-2 locus of mice (16), recombination among the factors of a phenogroup has not been observed in cattle so that it is reasonable to consider these 250 phenogroups as the products of multiple alleles(18).

Our purpose is to inquire if the various factors within a given phenogroup are detectable at the same age of embryologic development, and if the same factors occurring in different phenogroups are detectable likewise at the same age.

Materials and methods. Blood samples and gravid uteri were obtained from cows at slaughter. Most of the cows were Holstein-Friesians. The uteri were dissected and the embryos removed. Embryos with a crown-rump length in excess of 70 mm (approximately 60 days old) were bled immediately by severing the umbilical cord. Smaller embryos were brought back to the laboratory for bleeding. Uncoagulated blood samples were collected from dams and their embryos in test tubes containing sodium citrate solution. They were stored in the refrigerator until the time of test, usually within 24 hours of collection.

The blood samples were washed 3 times in isotonic saline and made up to a 2.5% suspension for typing. The red cell samples were typed by the standard hemolytic test described in detail elsewhere(19). Readings were made after 30 minutes, 2 hours and 4 hours. Two operators alternated in reading and recording the results. Special care was taken to read accurately the degree of reactivity so that gross quantitative differences might be obtained. Blood samples from a dam and her embryo were always included in the same test.

Results. Complete blood typing tests were performed on blood samples from 93 embryos and their dams. Although data were gathered on all 9 of the antigenic systems of cattle blood, only the results concerning the B system will be presented here. The embryos ranged in size from 23 mm to 884 mm crown-rump length which is equivalent in age from about 40 days to 260 days.

Data on 90 embryos could be used in these studies, since it was possible to determine their B phenogroups. If the mother's genotype as contrasted with her phenotype could be ascertained, it was usually possible to assign a phenogroup to her embryo. Fortunately, the majority of embryos had phenogroups that were clearly inherited from their dams. If the mother's genotype could not be ascertained, the embryo's phenotype was postulated on the basis of the array of reactions of its red cells with the panel of B reagents(20). Blood types of the sires were not available.

Among the 90 embryos tested, 34 different phenogroups could be clearly delineated. For brevity, detailed data on only 34 embryos, representing 12 different phenogroups, are

shown in Table I. These selected data illustrate the major points of this paper. The remaining data on 56 embryos are summarized in Tables II and III.

At a given age all of the factors that comprise a phenogroup may not be detectable (Table I). Presumably the failure to detect them means that they are not sufficiently developed on the surface of the red cells. Thus, in the phenogroup $BGKO_3Y_1A'E'_3G'K'2$, factors B,G,O_3,Y_2 and E'3 were developed on the cells of the youngest embryo tested (No. 41 at 146 mm, about 90 days). In contrast, factors K, G', K' and 2 were not present on the cells of this embryo, but were present weakly on the cells of the next oldest embryo No. 76 which was 157 mm in length (about 93 days).

In the 3 remaining embryos showing this phenogroup (No. 57, 62 & 82) whose lengths were 224, 278 and 296 mm respectively, these factors were clearly present and showed a tendency to react more strongly with increasing age.

The factor Y_1 was not developed in any of the embryos presumably containing it. In fact, Y_1 seemed to be very slow in developing irrespective of the phenogroup in which it appeared. The data of Stormont *et al.*(10) indicated this same peculiarity of the Y_1 factor. This is of special interest because Y_1 and Y_2 are sub-groups; that is, ordinarily adult cells possessing Y_1 also possess Y_2, whereas cells may possess Y_2 alone. This linear sub-grouping relationship is also typical of the factors I_1I_2, O_1O_3, T_1T_2, and $E'_1E'_2E'_3$(21).

The factor A' appeared slow in developing; it was not detectable until 278 mm (embryo No. 62) and then only weakly. The failure to detect A' in the older embryo, No. 82 (296 mm), may have been due to inaccuracies of the crown-rump measurements as an index of age(22). It should be emphasized that the crown-rump measurements are only approximate indices of age. Two embryos of equal crown-rump lengths may be of different ages and *vice versa* because of the differences in sex, breed and individual physiologic development. Therefore, a factor in one embryo may be recorded as strongly reactive at an early age, whereas in another it may be recorded as weakly reactive or even absent at an older age. For example, the K factor was strongly reactive in embryo No. 62 at 278 mm, whereas it was only weakly reactive in the "older" embryo No. 82 at 296 mm. More serious discrepancies occurred when a factor was absent at one age but present at an earlier age, as for example the factor K in embryos No. 95 and 40. Obviously, errors in performing and reading the tests could account for some of these apparent inconsistencies.

It is clear from these data, that at a given age *all* of the factors that comprise a phenogroup may not be developed. Further, there is rather wide variation in the age at which the factors of a given phenogroup develop. Similar variation is evident from an analysis of the other phenogroups also shown in Table I. In fact, for each of the 12 phenogroups listed, except O_1A', there is at least one age at which one or more of the factors representative of that phenogroup were not present.

Additional data compatible with these findings are summarized in Table II. Ten phenogroups are represented among the 14 embryos. Considering each of these 10 phenogroups at a given age, it was found that the various factors comprising that phenogroup were not developed simultaneously. To cite but one example, the embryo in Table II of 105 mm in length (about 80 days old) which inherited the phenogroup PI' from its dam, showed only the I' reaction.

Table III contains a summary of data on 42 embryos representing 12 different phenogroups. All of the factors within each of these phenogroups were developed simultaneously. For example, there were 15 embryos with the phenogroup $GY_2E'_1$ ranging in size from 54-554 mm (60-195 days). All of the factors were present at the earliest age tested (although not all equally reactive). These data, in contrast to those shown in Tables I and II, suggest that the factors of certain phenogroups closely parallel one another in time of appearance on the red cells.

Since many of the different phenogroups contain one or more of the same factors, we can consider the question of whether or not the same factor occurring in different phenogroups is detectable at the same age. Re-

TABLE I. Reactions* of Embryo Red Cells with Reagents Detecting Blood Factors and Various Phenogroups of the B Locus of Cattle.

Embryo†				B reagents																		
No.	Sex	mm	Age‡	B	G	K	O_1	O_3	Q	T_1	T_3	Y_1	Y_2	A′	D′	E_1′	E_2′	E_3′	G′	I′	J′	K′	2	Phenogroup
41	♂	146	90	W	+	—		W				—	W	—				W	—			—	—	
76	♀	157	93	W	+	W		+				—	W	—				W	W			W	W	
57	♂	224	115	W	+	W		W				—	W	—				W	W			W	W	$BGKO_3Y_1A'E_3'G'K'2$
62	♀	278	130	+	+	+		+				—	+	W				+	+			+	+	
82	♀	296	134	+	+	W		+				—	+	—				W	+			+	W	
95	♂	58	62	W	W	W		—				—	—	—				—	+	—			W	
40	♀	189	105	W	—	—		W				—	W	—				—	W	—			—	$BGKO_3Y_1A'E_3'G'I'2$
23	♀	395	155	+	+	W		+				—	W	W				W	W	+			W	
36	♂	262	125				+	+		—	—							W	W	+		W	—	$O_1T_1E_3'G'I'K'2$
65	♂	156	92				+	+		—	—							—	—			+		$O_1T_1E_3'G'K'$
33	?	44	54					—											W		—	W	W	
11	♂	112	80					W											+		W	W	W	
46	♀	124	84					W											+		+	+	+	$O_3G'J'K'2$
18	♂	208	111					W											+		+	W	W	
45	♂	295	133					W											+		+	+	+	
50	♂	86	70									—	W		W					+				
70	♂	149	91									—	+		W					+				
83	♂	207	110									—	+		+					+				$Y_1D'I'$
78	♀	211	112									—	+		+					+				
84	♀	213	113									—	+		+					+				
29	♂	222	114									—	+		W					+				
85	♂	278	130									—	+		+					+				
28	♀	884	259									W	+		+					+				
98	♂	154	92									W	W		+									Y_1D'
1	♂	276	128									—	W		W									
11	♂	112	80												—			W	W				W	
90	♀	132	85												W			—	—				—	
57	♂	224	115												W			W	W				W	$D'E_3'G'2$
3	♀	300	135												+			+	W				W	
51	♂	369	148												W			W	W				—	
4	♂	734	231												+			+	+				W	
92	♂	45	55						W						+	W	W	—						$QD'E_1'$

TABLE I (continued)

Embryo† No.	Sex	mm	Age‡	B reagents B	G	K	O_1	O_3	Q	T_1	T_2	Y_1	Y_2	A′	D′	E_1'	E_2'	E_3'	G′	I′	J′	K′	2	Phenogroup
44	♂	246	118						W						+	+	+	+	+	—				$QE_1'I'$
60	♀	377	150				W	W						W										O_1A'
25	♂	419	160				+	+						W										
24	♀	461	170									—	+		—	—	—	W	—		—	—		$Y_1A'D'E_1'G'2$

* + = Complete hemolysis after 4 hr; W = partial hemolysis after 4 hr; — = no reaction. The blank spaces are not pertinent to the phenogroup being tested.

† There are actually only 34 different embryos listed, but 2 of them (No. 11 & 57) are included twice since each is heterozygous for a different phenogroup.

‡ Estimated days after breeding, from Dukes(22).

TABLE II. Phenogroups of the B System of Cattle Blood Groups in Which all of the Factors Are Not Developed Simultaneously.

Phenogroup	Number of embryos	Range in length (mm)
$BGKO_3Y_2A'G'2$	1	500
$BGKO_3A'G'K'2$	1	74
$BO_3Y_1A'E_3'G'$	2	326-465
I_1 (I_1I_2)	1	232
$BGKE_2'G'2$	3	251-281
O_3Y_2A'	1	265
$I_1A'I'$	1	105
$E_3'G'2$	2	222-316
PI'	1	105
PI_1	1	373

turning to Table I, it can be seen that the factor Y_1 occurs in 5 different phenogroups. In the phenogroup Y_1D' it is detectable weakly on the cells of embryo No. 98 at 154 mm (about 92 days), but is not detectable in the other 4 phenogroups even though many of the embryos showing these phenogroups were much older. (The only exception is the Y_1 reaction in the phenogroup $Y_1D'I'$ exhibited by embryo No. 28.) We cannot explain why Y_1 was not detectable on the cells of embryo No. 1 which was clearly older (128 days) than embryo No. 98 and which also contained the Y_1D' phenogroup. This was not likely an error in technic, since we have confirmed the results in retests. Similar discrepancies for Y_1 were found in the data of Stormont *et al.*(10). The peculiar behavior of Y_1 as compared to its sub-factor Y_2 is apparent also in Table I. Note that Y_1 ap-

TABLE III. Phenogroups of B System of Cattle Blood Groups in Which all of the Factors Are Developed Simultaneously.

Phenogroup	Number of embryos	Range in length (mm)
BO_1Y_2D'	3	74-266
$O_1Y_2E_3'$	2	45-392
GY_2E_1'	15	54-554
O_3A'	2	179-281
$I'E_1'$	2	173-266
PY_2	1	208
BO_1	3	54-278
BI'	1	156
GD'	1	47
I′*	7	23-346
A′*	2	24-554

* These phenogroups are composed of only one factor.

peared to develop slowly in each of the 5 different phenogroups listed, but its sub-factor Y_2 developed at very early ages in all but one of the phenogroups ($BGKO_3Y_1A'E'_3G'I'2$ in embryo No. 95). As previously mentioned, this differential rate of development seems characteristic of sub-factors. Further examples may be seen with factor E'_3 which is a sub-group of E'_1 and E'_2. In the phenogroup $QD'E'_1$, the factor E'_3 was not detectable (embryo No. 92 at 45 mm), whereas, it was detectable in the phenogroup QE'_1I' (embryo No. 44 at 246 mm). Also, it was detectable weakly in the phenogroup $Y_1A'D'E'_1G'2$ (embryo No. 24 at 461 mm), but neither E'_1 nor E'_2 was detectable. It would appear, therefore, that the factors comprising a sub-group need not develop sequentially.

The D′ factor serves to illustrate further the differences in the behavior of the same factor in different phenogroups. Thus, D′ was not expressed in the phenogroup $D'E'_3G'2$ of embryo No. 11 (112 mm about 80 days), but was expressed in 2 other phenogroups of younger embryos ($Y_1D'I'$ and $QD'E'_1$). Similarly, each of the factors E'_3, G′, I′, K′, and 2 showed at least one example of their development in one phenogroup *at an earlier age* than in another phenogroup. Taken together, the evidence is highly suggestive, if not compelling, that certain factors differ in time of appearance of red cells depending upon the phenogroup in which they occur.

Discussion. The classical view of the gene as an indivisible unit of inheritance is no longer valid now that it has been shown that there is "structural differentiation" within the so-called allelic gene. Evidence for this comes from studies on pseudoalleles(23) and on subgenic units such as the muton and recon(24).

One major concern to immunogenetics is how the "fine structure" of the gene is related to the complexity of the antigen. For our purpose, the question is whether a phenogroup, consisting of a complex array of antigenic factors, is controlled by an allelic gene or by clusters of subgenic units with a one-to-one relationship between "sub-genes" and antigenic factors. Unfortunately, in so far as the genes controlling the red cell antigens of humans and cattle are concerned, there are no data that clearly differentiate between these hypotheses, although certain authors strongly favor one over the other(1,18,25,26).

In mice, recombination has been observed between antigenic factors of the H-2 phenogroups(16), consequently, the locus has been considered as pseudoallelic. However, Pizarro, *et al.*(16) propose that the "H-2 chromosome region behaves as a 'physiological unit', *i.e.*, as one gene", because they found that there was a close quantitative correlation between different H-2 antigens (factors) with respect to age of appearance, development, and distribution in various adult tissues. Since these authors were careful to point out that the word gene, locus or allele was used in the restricted sense of a "unit of function", they did not discuss their data from the point of view of differentiating between the two hypotheses previously mentioned. But they suggested that if the genes controlling the phenogroups are viewed as functional units, the two hypotheses are reconciled.

Our findings that the antigenic factors within a given phenogroup of the B system of cattle blood are not correlated in development differs from the findings, if not the interpretations, of Pizzaro and co-workers(16). If we assume, as these workers did, that a correlation in age of development is evidence for physiologic unity, then we must conclude that the B locus of cattle does not behave as a physiologic unit. This is surprising, since definite evidence of recombination has not been observed among the factors comprising the B phenogroups. Indeed, all of the evidence, with a few possible exceptions(27,28, 29), suggests that the B phenogroups are controlled by allelic genes(18). The dilemma here stems from the unanswered question of whether the factors of a phenogroup represent distinct molecules and therefore are produced probably by distinct sub-genic units, or whether they are simply attributes of a large macromolecule with multiple reactive sites (determinant groups) produced probably by allelic genes. In the absence of data on the chemical nature of the different antigenic factors, this question remains unsolved. However, when there is no evidence of recombination, our interpretation is that a correlation in

the age of development is consistent with the latter view (allelic genes), whereas, no correlation in the age of development is more consistent with the former view (sub-genic units). In support of this interpretation are data that show a series of physiologically interdependent genic units controlling sequential steps in biosynthesis(30). Paradoxically, our results in cattle are more compatible with the hypothesis that the B locus is composed of sub-genic units, despite no definite evidence for recombination, whereas the results in mice are more compatible with the hypothesis that the H-2 locus is composed of allelic genes, despite evidence for recombination. It should be pointed out that if we had obtained only the data on the phenogroups listed in Table III, in which all of the factors within each of the 12 phenogroups were developed simultaneously, our data would have paralleled those of Pizarro *et al.*(16). Since their study included data on only 4 (D,E,H,K) of 15 antigenic factors of an H-2 phenogroup, an investigation of the entire array of factors comprising that phenogroup might possibly have revealed that *all* factors were not developed simultaneously. Furthermore, the studies of mice and of cattle may not be strictly comparable since the H-2 factors of mice are not detectable on the red cells of embryos.

The significance of our finding that the same antigenic factor occurring in different phenogroups is *not* developed at the same age is not clear. Unfortunately, no data on this point are available in mice. Under the hypothesis of allelic genes, some kind of *interallelic* interaction might account for this phenomenon, as for example the D^u effect in the Rh system of humans(1). On the other hand, in an intensive study (unpublished) we found no interallelic interactions between the B locus phenogroups. Under the hypothesis of sub-genic units, it is conceivable that *intra-allelic* interaction between the sub-genic units may be operating. The phenomenon of position effect is clearly pertinent here. However, in mice no *cis, trans* differences have been observed(16) at the H-2 locus. Finally, we shall resist the temptation to speculate on the relationship between phenogroups and operons(31).

Summary. Blood typing of cattle embryos of various ages indicated that the different antigenic factors that comprise a phenogroup (complex of antigenic factors) of the B locus do not develop simultaneously in ontogeny. Further, the same factor develops at different ages depending upon the phenogroup in which it occurs. It is concluded from these results that, in so far as age of development is concerned, the B locus of cattle does not behave as a physiologic unit.

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Immunological Unresponsiveness Produced in Adult Guinea Pigs by Parenteral Introduction of Minute Quantities of Hapten or Protein Antigen.* (27717)

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As has long been known (1-8), immunological unresponsiveness to haptenic chemicals may be established in adult guinea pigs by repeatedly feeding the chemicals. Animals fed a particular hapten no longer respond to injection of the hapten (*i.e.,* to the derivative antigens formed by conjugation *in vivo*) either with production of specific circulating antibody or delayed-type contact hypersensitivity. Early attempts to make animals unresponsive by dermal or intravenous hapten administration produced sporadic rather than reproducible results(1,7,9). Parenteral inoculation of adult animals with proteins and polysaccharides effects immunological tolerance but only when the antigens are injected in massive amounts(10,11).

The present authors have recently succeeded in inducing unresponsiveness to picryl chloride by introducing minute doses of the hapten into the mesenteric veins of adult guinea pigs(12). The following report shows the effectiveness of this method for making adult animals immunologically tolerant not only to a simple chemical hapten (picryl chloride) but also to a protein antigen (bovine gamma globulin).

Experimental. In a typical experiment demonstrating induction of unresponsiveness to a hapten, sexually mature guinea pigs were inoculated *via* mesenteric veins with 50γ of picryl chloride in 2 ml of 1% ethanol-0.85% NaCl solution on 2 occasions, one week apart; control animals were similarly injected with 2 ml of saline. To make the inoculations, an animal was positioned on its side upon a restraining board, the hair about its midsection clipped and, under ether anesthesia, a 2 cm ventrolateral incision made just posteriorly to the last rib. A loop of small intestine was exposed sufficiently to allow entry into one of the smaller tributaries of the mesenteric vein with a 30-gauge, 3/8-inch needle. Upon completing the injection, made slowly to prevent rupture of the tiny vessel, the vein was ligated above and below the puncture site; after ascertaining that no bleeding occurred, the intestine was returned to the peritoneal cavity, and the incision sutured. The wound was covered with a thin layer of bacitracin ointment; but left unbandaged. The second inoculation, 7 days later, was made from the opposite side.

After resting 25 days, the animals were examined for capacity to respond with delayed

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