THE EFFECT ON THE OFFSPRING OF MATERNAL IMMUNIZATION IN MICE

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Introduction

Since the original discovery that haemolytic disease of the newborn in man could be caused by immunization of the mother against the red cell antigens of the foetus (Levine, Newark & Stetson, 1939; Landsteiner & Weiner, 1940), various analogous cases have been reported. Naturally occurring haemolytic diseases of the newborn have been shown to be due to immunization against isoantigens for the human blood groups *D*, *c*, *C*, C^w, *E* and Kell (Race & Sanger, 1950), and in the horse (Dimcock, Edwards & Bruner, 1947; Bruner, Hull & Doll, 1948; Coombs, Crowhurst, Day, Heard, Hinde, Hoogstraten & Parry, 1948). It has also been suggested that certain naturally occurring diseases of the newborn are due to maternal immunization, in the dog (Young, Ervin, Christian & Davis, 1949), the pig (Bruner, Brown, Hull & Kinkaid, 1949), and the rabbit (Nachtsheim & Klein, 1948). Naturally occurring haemolytic disease of the newborn in an inter-specific cross, between the horse and donkey, has been shown by Caroli & Bessis (1947) to be due to maternal immunization. The death of the second and third offspring of a female *Mandrillus leucophaeus*, crossed successively to *Macaca nemestrina*, *M. maurus* and *M. irus*, is suggestive of haemolytic disease (Knottnerus-Meyer, 1904). In addition, haemolytic disease has been caused by experimental immunization of the mother in the dog (Eyquem, 1948; Young *et al.* 1949), and ambiguous results have been obtained with the rabbit (Heard, Hinde & Mynors, 1949).

These are all cases which are clinically recognizable as haemolytic disease. Since the discovery of *Rh* immunization, there has been considerable discussion of the fact that haemolytic disease does not occur in the same way in an infant belonging to groups *A* or *B* when the mother has circulating anti-*A* or anti-*B* antibody. In these cases clinically recognizable disease may occur, but only very rarely (Race & Sanger, 1950). Nevertheless, there is strong evidence for the loss of some *ABO*-incompatible offspring (Levine, 1943; Waterhouse & Hogben, 1947). Levine put forward the hypothesis that loss of *ABO*-incompatible offspring occurs early in pregnancy, possibly at implantation. At later stages, the foetus and newborn infant would be protected from haemolysis by the possession of soluble *A* or *B* antigens. Protection by soluble blood-group antigens has also been put forward as an explanation of the low incidence of haemolytic disease in pregnancies incompatible for the *Rh* group (Witebski & Mohn, 1945). The death of antigenically incompatible offspring without haemolytic disease has never been demonstrated in animals. The experiment described here is a search for the death of antigenically incompatible offspring, whether recognizable haemolytic disease occurs or not.

Mice were the animals chosen, since they are the only animal which breeds sufficiently rapidly, and which can be kept sufficiently economically, for this experiment. The experiment could be carried out in several ways, but only two kinds of breeding procedure need be seriously considered. In the first, mice of different antigenic types would

be crossed. In some of the matings, the female would be immunized against the antigens of the male. A smaller mean litter size among the group immunized would constitute evidence for death of incompatible offspring. The second possible procedure would provide evidence strictly analogous to the evidence for the loss of *ABO*-incompatible offspring in man, where *O* mothers and *A* fathers produce a smaller proportion of *A* offspring than *A* mothers and *O* fathers. Mice homozygous for different genes controlling red-cell antigens would be crossed, and the F_1 would be backcrossed to one of the parental types. The backcross generation would show segregation of antigens, and some of the offspring of females of the parental type would be antigenically incompatible. If a group of these females were immunized, a smaller proportion of antigenically incompatible types among their offspring would constitute evidence for the death of incompatible offspring.

The second procedure was finally chosen, for two reasons. First, the variation in litter size of crosses between lines of mice would have been difficult to control; variation due to season and maternal age would make the data inhomogeneous, and since genetical stocks were started in this laboratory for the first time after a long interval, the variation in litter size reported in the literature would probably have been exceeded. Furthermore, the processes of injection or inoculation during immunization might themselves affect litter size. Secondly, considerable replacement of incompatible offspring lost early in pregnancy was expected. Average values for replacement by normal or heterozygous litter-mates of lost homozygotes, have been calculated for the following genes A^y, 11·6 % (Cuénot, 1908; Castle & Little, 1910; Ibsen & Stiegleder, 1917), *Ki*, 5·6 % (Caspari & David, 1940), *T*, 4·5 % (Dobrovolskaia & Kobozieff, 1930; Kobozieff, 1935). The objections of Ibsen & Stiegleder to the replacement hypothesis are unfounded, and their own data have been recalculated to show 8·8 % replacement.

THE EFFECTS OF MATERNAL IMMUNIZATION

Experimental

Mice from Strong's *A* line (albinos) and *CBA* line (agoutis) were used in this experiment. The *A* line was started from a stock received from Prof. P. B. Medawar at Birmingham University, and the *CBA* line from a stock received from Dr T. C. Carter at the University of Edinburgh. Both lines were maintained by sib mating.

Most of the experimental matings were between F_1 hybrids of these two lines, with male and female parents from either line, and the *CBA* line. These backcross matings were in three groups:

(1) F_1 ♀ × *CBA* ♂. Thirty-eight of these matings were set up, which produced 618 mice in 100 litters. The offspring of this group constituted controls to the offspring of groups (2) and (3).

(2) *CBA* ♀ × F_1 ♂. Eighteen of these matings were set up, which produced 235 young in thirty-nine litters. The females in this group were immunized with repeated grafts of a tumour from the *A* line. This tumour occurred spontaneously in a female from the *A* line, and has been identified as a mammary carcinoma. It was maintained during this experiment by transplantation approximately every 5 weeks to susceptible mice. It takes in all mice from the *A* line; thirty-one tested were all susceptible. It does not take in mice from the *CBA* line; thirty-seven mice tested were all non-susceptible. The usual trocar technique of transplantation was employed.

Each of the females in this group received repeated grafts of the carcinoma in alternate flanks, at intervals of approximately 6 weeks. The course of grafts was commenced at least a month before the mice were set up in matings. The total number of grafts received depended on how long the mouse survived in the experiment. Some of the mice received six grafts before giving birth to their last litters.

(3) *CBA* ♀ × F_1 ♂. Twenty-seven of these matings were set up, which produced 160 young in thirty-four litters. The females in this group were immunized with repeated injections of *A* line red cells. Blood was obtained by cardiac puncture of mice of the *A* line. A measured volume of blood, usually 0·4 ml., was withdrawn into a 2 ml. syringe containing some 3·2 % citrate. This blood was washed out into a large volume of saline and centrifuged. The cells deposited were then resuspended in a volume of saline sufficient to make up a 1 % suspension. This suspension was always used fresh for immunization. The females received intraperitoneal injections of 0·5 ml. of the 1 % suspension, repeated at intervals of approximately 14 days. The course of injections was commenced at least a month before the mice were set up in matings. The total number of injections received depended on how long the mouse survived in the experiment. Some of the mice received twenty injections before giving birth to their last litters.

Two smaller groups of experimental matings were set up between F_1 hybrids of the *A* and *CBA* lines, and the *A* line.

(4) F_1 ♀ × *A* ♂. Fifteen of these matings were set up, producing 167 mice in twenty-six litters. The offspring of this group constituted controls to the offspring of group (5).

(5) *A* ♀ × F_1 ♂. Fourteen of these matings were set up, producing 93 mice in nineteen litters. The females of this group were immunized with repeated grafts of a *CBA* line tumour. This tumour is lymphosarcoma 6C 3HED, described by Gardner, Dougherty & Williams (1944). It was maintained during this experiment by transplantation approximately every 16 days to *CBA* line mice. It takes in all mice from the *CBA* line; 188 mice tested were all susceptible. It does not take in *A* line mice; twenty-five tested were all non-susceptible. The females in this group received grafts of tumour at intervals of approximately 6 weeks. Most of the mice died after one or two grafts; mice from the *A* line seemed to be particularly susceptible during an epidemic of ectromelia in the colony.

The *CBA* females in groups (2) and (3) above, immunized with *A*-line tumour and *A*-line red cells respectively, were expected to produce antibody capable of agglutinating *A*-line red cells. The mice which had received grafts of tumour were expected to produce haemagglutinins in accordance with the theory of Gorer (Gorer, 1937, 1938). Various attempts were made to detect this antibody. Preliminary tests on blood from *CBA* females which had received five grafts of the carcinoma showed that saline agglutinins were present, but in small and variable amounts. No saline agglutinins could be detected in the blood of *CBA* females which had received three intraperitoneal injections of *A*-line blood.

Since conventional agglutination tests gave doubtful results, Gorer's technique of agglutination in the presence of human serum was tried (Gorer, 1950). Reasonably high titres of antibody could be detected in this way. The blood to be tested was collected by cardiac puncture, and allowed to clot in tubes. Serum was taken after the clots had retracted, and the tests carried out immediately. About 0·3 ml. serum can usually be obtained in this way without killing the mouse.

Although Gorer was able to show that with different donors of human serum, the same sample of antibody gave different titres, no attempt was made in these experiments to find a particularly efficient human serum. All the tests in this series were in fact carried out with serum from a single donor, though in some tests on the development of iso-antigens, described below, the serum from a second donor was employed. 4 ml. of the human serum was absorbed against a mouse red cell pool according to the method of Gorer, and Gorer's procedure was followed in the rest of the test. The serum was first inactivated for 30 min. at 56° C. It was then incubated for an hour with 2 ml. packed mouse red cells, which had been washed with saline. The absorption was repeated with 2·5 ml. fresh packed cells. A 1 % suspension of *A*-line red cells was then made up in this serum.

Serial dilutions of antibody were made up in tubes containing 0·25 ml. saline. 0·1 ml. of the dilutions was then transferred to precipitin tubes by a marked Pasteur pipette, and 0·1 ml. of the suspension of *A*-line red cells added. 0·1 ml. of the same series of dilutions was transferred to a second series of precipitin tubes, and 0·1 ml. of a 1 % saline suspension of *A*-line red cells added to each tube. All the tubes were read after the cells had settled at the bottom of the tubes at 37° C. In all tests for agglutinins, the tubes were first read directly by eye. In positive tubes, the cells are spread out in a characteristic cap over the bottom of the tube, instead of collecting in a button. The tubes were then read microscopically, by gently pipetting the sediment of cells, and transferring some of the suspension to a microscope slide. Attention was paid to the behaviour of the cells during the pipetting, and to the behaviour of the sediment of cells when the tube was tipped. This procedure follows closely the method of Gorer, and the author later had an opportunity of checking with Dr Gorer that the mouse haemagglutination tubes were being read in the same way. Positive results for these haemagglutinations are shown by one or two pluses. (The second shows that the tubes showed agglutination on direct examination, without disturbing the deposit. The first indicates the result of microscopic examination.)

This test was carried out on four *CBA* females from group (3), each of which had received eighteen intraperitoneal injections of *A*-line red cells. The mice were bled 12 days after the last injection. Control serum was collected from two unimmunized *CBA* females. The results of the test are shown in Table 1. It can be seen that the *CBA* females had developed antibody which could be detected in the presence of human serum at dilutions of 1/64 to 1/512. The saline agglutinations show only traces of antibody.

The offspring of the backcross matings, in groups (1)–(5) above, were scored for the isoantigens separating *A* and *CBA* lines. A preliminary attempt was made to score red cell antigens in the backcross by haemagglutination, which was later abandoned. Strong antibody was not formed in *CBA* mice, in response to single injections of *A*-line cells, or single grafts of the *A*-line tumour. Test antisera produced in mice are in any case unsatisfactory, because of the small yield of serum. Various attempts to produce antisera in rabbits, following Gorer (1937), also proved unsatisfactory. The following are some courses of immunization which were carried out.

(1) A rabbit received three weekly injections of 1 ml. of a 10 % suspension of *A* cells, washed three times in saline. It was bled from the median ear artery 4 days after receiving the last injection. Antibody titre against *A* and *CBA* cells in the serum was measured, using 1 % suspensions of washed red cells as antigen. The titre against both

A and *CBA* cells was 2560. After absorption with an equal volume of 1 % suspension of *A* red cells for an hour at 37° C., the titre against *CBA* cells was 2048, and the titre against *A* cells had only dropped to 1024. Following a similar absorption against *CBA* cells, the titre against both *CBA* and *A* cells was 1024.

(2) A rabbit received a similar course of injections with *CBA* cells, and was bled after the same interval. Antibody titre against both *CBA* and *A* cells was 4000. Following a similar absorption with *A* cells, antibody titre against *CBA* cells was 200, against *A* cells 100. Following absorption with *CBA* cells, the titre against *CBA* cells was 100, against *A* cells 400.

(3) Two further rabbits received four weekly injections of 1 ml. of 10 % suspension of washed *CBA* cells, and were bled 6 days after the last injection. Antibody titres against both *CBA* and *A* cells were 800 in one rabbit, and 400 in the other.

Table 1. *Antibody in immunized CBA females*

	Antibody dilution									
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
	(1) Agglutination in presence of human serum									
Serum 1	+ +	+ +	+ +	+ +	+ −	+ −	− −	− −	− −	− −
Serum 2	+ +	+ +	+ +	+ +	+ +	+ ±	+ −	+ −	± −	− −
Serum 3	+ +	+ ±	+ −	+ −	+ −	+ −	− −	− −	− −	− −
Serum 4	+ +	+ +	+ +	+ −	+ −	+ −	− −	− −	− −	− −
Control serum 1	− −									
Control serum 2	− −									
	(2) Saline agglutination									
Serum 1	± −	± −	− −	− −						
Serum 2	− −	− −	− −	− −						
Serum 3	+ ±	− −	− −	− −						
Serum 4	± ±	− −	− −	− −						
Control serum 1	− −									
Control serum 2	− −									

The attempt to produce satisfactory line-specific antisera in rabbits was abandoned at this point. The possibility of preparing antisera by absorption had not been fully eliminated, and it is possible that sera which could have been used for scoring the backcross generations might have been obtained.

The method of scoring finally used was grafting the offspring of groups (1), (2) and (3) above with the *A*-line carcinoma, and grafting the offspring of groups (4) and (5) with the *CBA* line lymphosarcoma. The tumours were expected to take only in mice of the backcross generation possessing the antigens which were present in the tumours and which separated the *A* and *CBA* lines.

The mice of the backcross were weaned at about 21 days. Litters were kept in separate cages until the tumour susceptibility or non-susceptibility was definitely established. The method of grafting was as described for the immunizing grafts. All the pieces of tumour were prepared, before operating on a batch of mice, and carefully mixed in the Petri dish to ensure randomization of the grafts. At first single grafts were given into the right flank. Later, bilateral grafts of the carcinoma were given as a check on the reliability of this method of scoring. The mice were grafted between 4 and 8 weeks of age. More accurate timing of the transplantations was not used, since it would have meant splitting the batches of mice receiving grafts into smaller groups.

The carcinoma grows rather slowly. After a fortnight, only a small proportion of grafted mice have palpable tumour masses, and some of these are in non-susceptible mice which

later regress. After 4 weeks almost all susceptible mice have a palpable tumour, and after 6 weeks all the mice in which the tumour is going to grow have palpable masses. The mice to be scored by this tumour were kept for 6 weeks, and those with palpable masses at that time scored as positives. This timing is open to criticism; the scoring could have been made more certain by keeping the mice for a longer time, but this was not done because of shortage of cage space. Any error introduced in this way of course affects the experimental and control groups equally.

The lymphosarcoma grows more rapidly. All mice have palpable tumours after 10 days, often more than 1 cm. in diameter; these regress in a proportion of the mice from the backcross. The regression is slow and variable, and scoring with the tumour therefore rather uncertain. Only a small sample of mice was finally tested, and no great weight need be attached to the exact results.

RESULTS

(1) *Results from the cross $F_1 \times CBA$*

A total of 1013 mice were born from this cross, offspring of unimmunized females, and offspring of females immunized with grafts of tumour or injections of red cells. 629 of these mice were tested for susceptibility to the *A*-line carcinoma. Before going on to give the results of this experiment, two tests for the adequacy of the technique of scoring by tumour graft can be made.

Table 2. *Behaviour of bilateral grafts in backcross mice showing at least one take*

Mice with grafts taken on right-hand side only	10
Mice with grafts taken on both sides	34
Mice with grafts taken on left-hand side only	4

If some of the grafts of tumour do not grow in mice which are susceptible, we should expect some mice which receive bilateral grafts of tumour to grow lumps on one side only. This is the first test for the adequacy of the technique, and the results of bilateral grafts in a group of backcross mice are shown in Table 2. The probability of a mouse being miss-scored can be calculated, on the hypothesis that a tumour can fail to grow either (*a*) because the mouse is non-susceptible, or (*b*) because of some fault in the grafting. Among susceptible mice, we must assume that all grafts are equally likely to be successful. This assumption is not valid if, for instance, an inhomogeneous mass of tumour is cut up to provide grafts, and a single host receives two grafts from neighbouring parts of the tumour mass. This is the reason for the precautions taken during the course of the experiment to randomize grafts. Then let p be the probability of a graft growing in a susceptible animal; and let n_r, n_b and n_l be the number of mice in a sample which received bilateral grafts, which have respectively grown tumours on the right-hand side only, on both sides, and on the left-hand side only. Imagine that the right-hand side grafts have been carried out first, and that n_b+n_r mice have been found to be susceptible. If these mice are then tested with left-hand side grafts, only a proportion $n_b/(n_b+n_r)$ will take, and this fraction is an estimate of p. The fraction $n_b/(n_b+n_l)$ provides a similar estimate of p, and the best estimate of p is the weighted mean of these two fractions. Thus

$$p = 2n_b/(2n_b+n_r+n_l).$$

If the values shown in Table 2 are substituted in this equation, $p = 0{\cdot}83$. Thus 17 % of the grafts in susceptible mice fail to grow. In the remaining analysis of the results of this experiment, account will only be taken of the results of the right-hand side grafts, so that results in batches of mice which received single or bilateral grafts will be comparable.

All the mice of the backcross were not grafted at the same time. A series of thirteen batches were grafted separately, spread over 18 months, with each batch containing approximately equal numbers of experimental and control mice. The homogeneity between these batches is therefore a second test of the adequacy of tumour grafting as a method of scoring. Variation might be expected between batches. For example, the carcinoma was maintained through sixteen transplantations during this time, and it might have lost by 'mutation' some of the antigens controlling its ability to grow in the backcross generation. If this had occurred, the proportion of susceptible mice would have risen. Mutations of this sort occur rather rarely (see review by Bittner, 1935). The results for the control mice in different batches are shown in Table 3. A test for heterogeneity on this table gives $\chi^2_{(15)} = 17{\cdot}3$, $0{\cdot}5 > p > 0{\cdot}3$. This test therefore detects no significant heterogeneity between batches of mice grafted separately.

Table 3. *Homogeneity between batches of mice grafted*

Susceptible	8	12	5	4	5	3	6	0	2	2	2	1	13	3	3	2
Total	34	39	11	12	29	32	42	9	15	25	14	15	76	30	18	12

Table 4. *Frequency distribution of litter size*

	Litter size										
	1	2	3	4	5	6	7	8	9	10	11
Controls	1	3	6	8	20	20	15	11	10	5	1
Mothers immunized with tumour	0	2	3	2	6	10	9	4	2	0	1
Mothers immunized with red cells	0	1	8	9	5	7	2	1	1	0	0

We can now proceed to give the results of the experiment which provides evidence whether any young are lost due to maternal immunization. The first effect we may search for is a smaller mean litter size among immunized mothers than among the controls. The frequency distribution of litter size among the three experimental groups is set out in Table 4. The mean litter size of the controls is $6{\cdot}2 \pm 2{\cdot}1$, of the mice immunized with grafts of tumour $4{\cdot}0 \pm 1{\cdot}9$, and of the mice immunized with red cells $4{\cdot}7 \pm 1{\cdot}6$. The difference between the two experimental groups is not significant, but both the differences between the experimental groups and the controls are significant. For the group immunized with tumour, $t_{(135)} = 5{\cdot}5$, $p < 0{\cdot}01$. For the group immunized with red cells, $t_{(132)} = 3{\cdot}6$, $p < 0{\cdot}01$. These differences can, however, be accounted for in other ways than by the loss of young of immunized mothers. All the control mothers are F_1 hybrids, and all the immunized mothers are members of the *CBA* line. The difference is therefore no doubt due to heterosis.

The proportions of susceptible offspring of each immunized female are shown separately in Table 5. Since some mice develop higher titres of antibody than others, heterogeneity between offspring of different mothers would be evidence for our hypothesis. A test for heterogeneity on this table gives for the offspring of mothers immunized with tumour, $\chi^2_{10} = 8{\cdot}6$, $0{\cdot}7 > p > 0{\cdot}5$. For offspring of mothers immunized with red cells, $\chi^2_{17} = 14{\cdot}0$, $0{\cdot}7 > p > 0{\cdot}5$. Since the cell numbers in Table 5 are small, the χ^2 test overestimates the

heterogeneity, and these results give no indication of heterogeneity between the offspring of immunized females.

Table 6 (*a*) shows the different proportions of susceptible offspring during the course of immunization of their mothers with grafts of tumour, and Table 6 (*b*) shows the proportions during the course of immunization of the mothers with red cells. If any young are lost as a result of maternal immunization, we should expect the proportion lost to rise as the mothers develop antibody. Holt's test for regression is an appropriate test for a progressive change during the course of immunization (Holt, 1947–9). The proportion of susceptible offspring appears to increase as the mother receives tumour grafts, $\chi^2_{[1]} = 0{\cdot}22$, $0{\cdot}7 > p > 0{\cdot}5$. The proportion of susceptible offspring appears to increase as the mother receives injections of red cells, $\chi^2_{[1]} = 0{\cdot}08$, $0{\cdot}8 > p > 0{\cdot}7$. However, neither of these results is greater than might be expected by chance.

Table 5. *Homogeneity between offspring of different mothers*

(*a*) Mothers immunized with tumour

Susceptible	1	2	1	5	2	1	0	0	5	1	2
Total	10	10	8	11	10	5	5	4	11	7	8

(*b*) Mothers immunized with red cells

Susceptible	2	1	0	3	0	0	1	1	2	0	1	1	1	0	1	3	1	1
Total	18	5	3	15	4	3	5	17	6	1	3	4	4	4	7	8	7	13

Table 6. *Proportion of susceptible offspring during course of maternal immunization*

(*a*) Mothers immunized with tumour

Number of grafts received by mother	1	2	3	4	5
Susceptible	2	9	2	2	5
Total	11	36	14	13	15

(*b*) Mothers immunized with tumour

Number of injections received by mother	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Susceptible	2	3	3	2	0	.	.	1	2	3	2	.	0	.	.	1
Total	5	19	8	22	4	.	.	6	18	13	24	.	2	.	.	6

Table 7. *Total proportion of susceptible offspring from immunized mothers*

	Mothers immunized with tumour	Mothers immunized with red cells	Controls
Susceptible	20	19	73
Total	89	127	413
% susceptible	22·5	15·0	17·7

Table 7 shows the results which are most critical for a test of the hypothesis that maternal immunization causes loss of young. If the hypothesis is correct, we should expect fewer susceptible mice among the offspring of immunized mothers. There is, in fact, a higher proportion of susceptible offspring from females immunized with grafts of tumour, and a lower proportion from females immunized with red cells. Neither of these differences is, however, significant. For the difference between the offspring of females immunized with tumour and controls, $\chi^2_{[1]} = 1{\cdot}05$, $0{\cdot}5 > p > 0{\cdot}3$. For the difference between offspring of females immunized with red cells and controls, $\chi^2_{[1]} = 0{\cdot}51$, $0{\cdot}5 > p > 0{\cdot}3$.

The grafts which fail to grow in susceptible animals may do so for a variety of reasons, reviewed in Bittner (1935). In order to test the agreement of the segregation in the backcross with Mendelian ratios, the data presented in Table 7 must be corrected by our

factor of 17 % for grafts which fail to grow in susceptible animals. The segregation after recalculation is shown in Table 8, together with the Mendelian expectations for 1, 2 and 3 genes controlling susceptibility. The data for the offspring of normal, tumour immunized, and red cell immunized females all fit a two-gene ratio reasonably well. In no case is the difference between observation and expectation greater than twice the standard error. These two genes were not tested for identity with any of the genes controlling the isoantigens discovered by Gorer. According to Gorer (1938), Strong's *A* line possesses antigen II, which is weak or absent in the *CBA* line, and this antigen may correspond to one or both of the genes controlling susceptibility to our carcinoma.

Table 8. *Comparison of susceptibility of offspring with Mendelian expectations*

	Mothers immunized with tumour	Mothers immunized with red cells	Controls
Number susceptible	20	19	73
Corrected number	24·1	22·9	88·0
Expectation:			
1 factor	.	.	$206·5 \pm 10·2$
2 factors	$22·3 \pm 4·1$	$31·8 \pm 4·9$	$103·5 \pm 8·8$
3 factors	.	.	$51·6 \pm 6·7$
Difference/standard error	0·45	1·82	11·62 1·76 5·43

Table 9. *Proportion of susceptible offspring from cross $F_1 \times A$*

	Mothers immunized with tumour	Controls
Susceptible	3	5
Total	9	30

(2) *Results from the cross $F_1 \times A$*

A total of 260 mice were born from this cross, of which thirty-six were tested for susceptibility to the *CBA*-line lymphosarcoma. The numbers are too small to permit the full analysis used for the offspring of the previous cross.

The mean litter size of the unimmunized mothers is $6·4 \pm 2·2$, and of the immunized mothers $4·9 \pm 2·5$. This difference is significant: $t_{(43)} = 2·17$, $0·5 > p > 0·2$. As with the other cross, this difference can no doubt be assigned to the difference between hybrid and inbred mothers.

Table 9 shows the difference in the proportion of susceptible offspring between the immunized and control females. On these small numbers there is clearly no significant difference between the two groups.

The development of isoantigens in young mice

According to Gorer (1938) the red cells of newborn mice of Strong's *A* line do not react with the strongest obtainable isoagglutinating sera. One possible reason for the absence of damage from the maternal antibody is that foetal and newborn red cells are protected by lack of antigens. In order to test this hypothesis, the technique of iso-agglutination in the presence of human serum was employed. Three series of experiments

were therefore carried out on the red cells of mice at various ages, in order to confirm Gorer's results and determine at what age the isoantigens of young mice appear. The results of these tests are shown in Table 10.

In the first series, four *CBA* females which had been used as mothers of the backcross generation were bled to provide anti-*A* serum. Each of these mice had received seventeen intraperitoneal injections of 0·5 ml. of 1 % suspension of *A* red cells at intervals of approximately 10 days. They were bled 10 days after receiving the last injection, and the serum pooled. This serum is labelled serum *A* in Table 10.

Table 10. *The development of isoantigens in young mice*

Age of red cell donor	Antibody dilution							
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
	(1) Anti-*A* serum prepared in *CBA* mice. Serum *A*							
2 days	– –	– –	– –	– –	– –	– –	– –	– –
5 days	– –	– –	– –	– –	– –	– –	– –	– –
8 days	– –	– –	– –	.	.	.	.	.
8 days	± –	– –	– –	.	.	.	.	.
12 days	+ –	+ –	+ –	– –	– –	.	.	.
12 days	+ –	+ –	+ –	– –	– –	.	.	.
Adult	+ +	+ +	+ +	+ ±	+ –	± –	– –	– –
Adult	+ +	+ +	+ +	+ +	+ +	+ –	– –	– –
Adult	+ +	+ +	+ +	+ +	+ –	– –	– –	– –
	(2) Anti-*A* serum prepared in *CBA* mice. Serum *B*							
12 days	– –	– –	– –	– –	– –	– –	– –	– –
16 days	+ +	+ +	+ +	± +	± ±	– –	– –	– –
25 days	+ +	+ +	+ +	± ±	– –	– –	– –	– –
Adult	+ +	+ +	+ +	+ +	+ ±	± ±	– –	– –
	(3) Anti-*CBA* serum prepared in *DBA* mice							
4 days	– –	– –	– –	.	.	.	.	.
7 days	– –	– –	– –	.	.	.	.	.
12 days	± –	± –	– –	– –	.	.	.	.
12 days	± –	– –	– –	– –	.	.	.	.
17 days	+ –	± –	± –	– –	– –	.	.	.
Adult	+ –	+ –	– –	– –	– –	– –	.	.
Adult	+ –	+ –	± –	– –	– –	– –	.	.

Red cells were obtained from adults by cardiac puncture. Blood was obtained from mice under 14 days old by cardiac puncture, but instead of using a larger syringe, a 1 ml. tuberculin syringe was used, with the plunger replaced by a piece of rubber tubing so that it could be sucked. About 0·05 ml. blood could be collected from a young mouse, and this was made up to a 1 % suspension by eye.

The same technique of agglutination was used, as described for the titration of antibody in the mothers of the backcross generation, except that smaller quantities were used. Antiserum dilutions were made up in 0·25 ml. saline, and the agglutination carried out in precipitin tubes containing 0·05 ml. antiserum dilution plus 0·05 ml. of 1 % suspension of red cells in human serum. The human serum used in this experiment was from the same pool as in the previous experiment.

The results of this experiment are set out in Table 10 (1). The agglutination is described by the same conventions as in Table 1. Antigens appear to develop 8–12 days after birth. In order to check this, three further pools of antiserum were prepared. *CBA* cells were injected into groups of *A* and *DBA* mice, and *A* cells were injected into a group of *CBA* mice. Each mouse received an intraperitoneal injection of 0·5 ml. of 5 % suspension of

red cells, followed after intervals of 20 and 8 days by two further injections. The mice were bled 6 days after the last injection, and the serum pooled. The most powerful antibody was formed in the *CBA* mice immunized with *A* red cells; the titre of antibody, using the human serum technique, was 64. The *DBA* mice immunized with *CBA* cells developed a weak antibody, with a titre of 4. The *A* mice immunized with *CBA* cells developed hardly any antibody, which confirms Gorer's results with rabbit sera (Gorer, 1936).

In the experiments with these sera, the same technique of agglutination was used as before. Human serum from a different donor was, however, used. Non-specific mouse red cell agglutinins were absorbed out by the same procedure.

The results with the *CBA* serum, labelled serum *B*, and the *DBA* serum, are set out in Table 10 (2) and (3). The results from all the experiments are similar, and the *CBA* antigens appear to develop at the same time as the *A*-line antigens.

Discussion

It is of some interest to compare these results on the time of development of red cell isoantigens in mice with the rates of development in other animals. Unpublished observations by Burhoe on the development of the *rat* groups *A* and *B* (Burhoe, 1947) have been reported by Keeler (in '*The Rat*', 1949). At birth the agglutinating power of rat red cells is low for *A* and *B*, and this power develops rapidly during the first 2 weeks. During the second month it reaches adult level.

The *rabbit* agglutinogens H_1 and H_2 (Levine & Landsteiner, 1929; Fischer & Klinkhardt, 1929) have been shown by Keeler & Castle to be as fully developed in the newborn as in the adult rabbit (Keeler & Castle, 1933). Furthermore, these agglutinogens were demonstrated in the large nucleated red blood corpuscles of 15-day-old embryos averaging 14 mm. in length.

Most of the *human* red cell isoantigens can be detected at birth, but their time of development is variable. The *MNS* and *Rh* antigens are present at birth, and the *D* antigen has been detected as early as 11 weeks after conception (Stratton, 1943). *ABO* antigens are present at birth, but their agglutinating power is not as strongly developed as in adults (Tovey, 1945; Kemp, 1930). Henningsen (1949) has shown that about 20 % of *P*-positive persons lack the *P* antigen at birth, and that the antigen when present is weaker than in adults. The Lewis antigen is weaker at birth than among adults (Andresen, 1947), and the development of Lewis heterozygotes is of particular interest. Le^a heterozygous adults are Le^a-positive, but Le^a heterozygous babies in their first month are Le^a-negative. The adult condition develops progressively during the first year and is only reached after about 14 months. The antigen Le^b bears an almost completely antithetical relationship to Le^a, and Andresen has suggested that Le^a and Le^b are alleles. Le^b heterozygotes are Le^b-positive at birth, but not when adult.

There has been no work reported specifically on the development of isoantigens in *cattle*. But there has been some work on blood grouping among calves (Owen, Davis & Morgan, 1946), and no reference is made to difficulty with young animals.

Unpublished work of W. G. Black on the development of antigens in *doves* has been mentioned by Irwin (1946). Antigens specific to differences between *Columba guinea* and *C. livia* can be detected almost as soon as red cells can be obtained from the embryos.

We may summarize this information on the development of red cell isoantigens thus:

Rat } Isoantigens absent at birth
Mouse }

Man

Rabbit }
(Cattle) } Isoantigens present at birth
Dove }

These times of development are clearly related to the general states of development of the various species at birth. This relation deserves closer examination. Enough is known of the general state of the red cells at birth of the rat, mouse, rabbit and man to permit a comparison with their isoantigens.

In the early development of all mammals there is a gradual transition from megaloblastic to normoblastic erythropoesis. At the same time, the main sites of erythropoesis change from the liver and spleen to the bone marrow. There is also a corresponding change in the circulating blood. In the foetus there is a relatively small concentration of large red cells, with a relatively high haemoglobin content per cell, and a high proportion of reticulocytes. This transition occurs sometime before birth in man, but is not

Table 11. *The erythrocytes of rats, mice, rabbits and man at birth*

(1) Erythrocytes/mm.^{3} at birth as percentage of erythrocytes/mm.^{3} in adult

		Source
Rat	25	(1)
Mouse	37	(2)
Rabbit	78	(3)
Man	100	(4)

(2) Mean haemoglobin content of erythrocytes at birth as percentage of mean in adult

Rat	269	(1)
Mouse	176	(2)
Rabbit	157	(3)
Man	117	(5)

(3) Percentage of reticulocytes among erythrocytes at birth

Rat	89	(1)
Mouse	53	(2)
Rabbit	35	(3)
Man	6	(6)

(1) Bruner, van der Erve & Carlson (1938).
(2) Grüneberg (1942).
(3) Kunde, Green, Changnon & Clark (1931–2).
(4) Lucas, Hoobler, Cox & Jones (1921).
(5) Lucas & Dearing (1921).
(6) Seyfarth & Jürgens (1927–8).

completed until after birth in rats, mice and rabbits. The state of development of the red cells at birth is shown for these animals in Table 11. This table shows that at birth the red cells of the rat, mouse, rabbit and man form a series in their state of development, with the cells of the rat least well developed. A comparison with the values for human foetuses given in Knoll (1928) shows that at birth the rat and mouse correspond roughly to 3-month human foetuses, and the rabbit to a 5-month human foetus. Few data are

available on the state of development of cattle red cells at birth. According to Storch (1901) the red cells of calves are similar to those in adult cows.

The appearance of red cell isoantigens after birth in the mouse is therefore in line with the development of other features of the mouse red cells at birth. In general, the time of appearance of red cell isoantigens in different species fits fairly well with the general development of the cells. The early appearance of rabbit isoantigens is no doubt partly due to the strength of rabbit antisera.

The results given in the earlier part of this paper show that foetal and newborn mice are able to survive, in spite of the presence in the maternal blood of antibody against antigens which the young mice will possess as adults. This maternal antibody is detectable at high titres only in the presence of serum, and is therefore strikingly similar to incomplete antibody in man. There is evidence (Pickles, 1949) that incomplete antibody is particularly effective in causing haemolytic disease in man. It has been shown that the young mice may be protected by the late appearance of red cell isoantigens. This explanation would be difficult to prove definitely. One difficulty is the very small quantities of blood which can be obtained from newborn mice.

The failure to demonstrate selection *in utero* against antigenically incompatible offspring confirms the interpretation which Cloudman (1941) set on his own work. He found that the proportions of mice susceptible to certain tumours were raised following the passage of the mice through the uterus of susceptible females, using the technique of oval transplantation. Now any experiment of this sort, where there is uncontrolled mortality (for discussion of the mortality in oval transplantation, see Fekete & Little, 1942), is open to two interpretations: 'selection' or 'adaptation'. The position is precisely analogous to the current controversy over bacterial adaptation. Cloudman tends to interpret his results in terms of adaptation. If susceptibility to his tumours is governed by host tumour antigenic differences, his results could have been interpreted to mean that selection was occurring *in utero* against antigenically incompatible foetuses. Since sufficiently strong selection does not occur in our experiment, where the mothers were strongly immunized, it is unlikely to have occurred with the oval transplantation work, where the mothers were not deliberately immunized.

Summary

1. F_1 hybrids between Strong's *A* and *CBA* lines are backcrossed to the *CBA* line, and susceptibility to an *A*-line carcinoma is shown to be controlled by two factors in the backcross generation.

2. Groups of *CBA* mothers of the backcross generation are immunized with *A*-line red cells, or *A*-line tumour, and are shown to develop agglutinins to *A*-line red cells.

3. The offspring of the immunized *CBA* females which possess *A*-line antigens are not killed by the maternal antibody.

4. Similar indications are obtained with the offspring of *A*-line mice immunized with a *CBA* lymphosarcoma.

5. Isoantigens are shown not to develop in young mice until 8–12 days after birth.

6. This is shown in the discussion to be in line with the general state of development of the mouse red cells at birth. It is suggested that this protects the young mice from being damaged by the maternal antibody.

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