*Immunology*, 1963, **6**, 592.

# Ontogenesis of Immunity to Erythrocyte Antigens in the Chick

J. B. SOLOMON AND D. F. TUCKER

*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W.3*

(*Received 17th December* 1962)

**Summary.** Erythrocytes labelled with chromium$^{51}$ were injected into embryos and young chicks and a similar challenge given soon after the majority of the initial dose had been eliminated. While tolerance was the typical response in embryos and chicks up to 3 days of age, immunity could be induced in most chicks from this time onwards. The intensity of the immune response increased rapidly until the 12th day of age. No maternal antibody could be detected. Pretreatment of the embryo with testosterone impaired the immune response of 14-day-old chicks.

## INTRODUCTION

The elimination of erythrocytes in an immune manner is a sensitive method for the detection of low levels of antibody which would not be detectable by the usual serological methods (Chaplin, 1957; Mitchison, 1962a). Ottesen (1955) first demonstrated immune rejection of allogeneic (homologous) $^{32}$P-labelled erythrocytes by the adult chicken, and more recently Mitchison (1959) has shown both immunological tolerance and rejection by the use of $^{51}$Cr-labelled erythrocytes in the chicken. Previously, immunological tolerance to allogeneic chicken erythrocytes had been measured by depression of haemagglutinin formation after parabiosis (Hašek, 1953) and after injection of chick embryos (Billingham, Brent and Medawar, 1955; Hašek, 1956).

Tolerance to turkey erythrocytes can be induced in 17-day-old chick embryos and 1-day-old chicks; the immune response appears at 2 days of age, and in 2-week-old chicks is as vigorous as in the adult (Beckitt, 1958). However, a large proportion of the turkey erythrocytes were removed instantly from the circulation in Beckitt's experiments, presumably by the natural haemagglutinin known to circulate in the chick embryo (Ryle, 1957; Mitchison, 1962a).

Recently Mitchison (1962a) has shown that whereas newly hatched chicks could readily be made tolerant of $^{51}$Cr-labelled allogeneic erythrocytes, this was difficult, with the same antigenic dosage, at 4 days of age. Two- to 6-day-old chicks showed some type of accelerated elimination of erythrocytes, but the typical immune response did not appear until 8 days of age.

In the present work the transition of responsiveness from tolerance to immunity, using allogeneic erythrocytes as an antigen, has been followed during the development of the young chick. Second injections have been made to confirm the degree of immunological response. While our results confirm Mitchison's (1962a) finding that tolerance-responsiveness to a moderate dose of antigen ends at 2 days of age, they also show the clear-cut onset

of the normal immune response at this period in development. Evidence is presented to show that the chick embryo may sometimes be capable of weak immune response just before hatching, and that the recently hatched chick can become sensitized by foreign erythrocytes.

## EXPERIMENTAL METHODS

One Rhode Island Red hen was used as the sole source of erythrocytes. Although the same antigenic material was used throughout, the strength of the antigenic stimulus presumably depended upon the antigenic difference between the donor and the recipient; this, together with slight variation in the number of the recipient chick's lymphocytes could account for the variation of immune response within a particular age-group. Fresh blood was obtained by cardiac puncture, 4 vols. of blood being collected into a syringe containing 1 vol. of sterile acid citrate dextrose solution (ACD). The ACD solution consisted of disodium citrate monohydrate (2 g.) and anhydrous dextrose (3 g.) in 120 ml. of water (pH 5·5). The blood cells were packed by centrifugation at 1200 rev./min. on a M.S.E. minor centrifuge for 4 minutes. Plasma, the buffy coat layer and the upper portion of the red cell layer were drawn off by pipette. The packed erythrocytes were incubated for 1–2 hours at 20° with sodium chromate ($Na_2{}^{51}CrO_4$) solution in the proportion of 70–100 μc./ml. packed cells. The concentration of chromate did not exceed 10 μg./ml. packed cells. After incubation the unbound chromate was removed by washing the cells twice in sterile Ringer phosphate solution (10 vols.). After each centrifugation any 'buffy coat' layer was removed. Finally, the erythrocytes were resuspended in Ringer phosphate solution to approximately the original concentration of erythrocytes in the blood. Such erythrocyte suspensions did not contain sufficient lymphocytes to elicit splenomegaly when injected into 15-day-old embryos.

The recipients of labelled erythrocytes were White Leghorn embryos and chicks. Transfusions were made with a 30-gauge needle on a tuberculin syringe into a vein of the chorio-allantoic membrance of embryos, into the leg or wing vein of chicks up to 4 days of age, and into wing veins of older chicks.

Embryos received 0·2 ml. at first injection (larger doses caused a marked reduction in hatchability) and 0·6 ml. erythrocyte suspension at second injection. Newly hatched chicks were given 0·4–0·8 ml., 2-day-old chicks 0·7–1·0 ml., 3–10-day-old chicks 1·0 ml. and 12-day-old chicks 2 ml. erythrocyte suspension at first and second injections. The blood volume of the 2-day-old chick was calculated at about 2·6 ml., and of the 12-day-old chick at 9·8 ml. The erythrocyte doses in chicks up to 2 days old were graded so that the dose of antigen per unit blood volume remained the same as in the embryo. Chicks were between 10 and 16 days old at the second (challenge) injection, which was given 1 day after about 95 per cent of the original dose of erythrocytes had been eliminated.

Blood specimens (0·03 ml.) were obtained from the wing vein at 1–2 hours after injection of labelled erythrocytes with a 0·25 ml. syringe fitted with a 30-gauge needle. Specimens were taken daily, if immune elimination was anticipated, and otherwise at 2–3 day intervals. Blood was spread over planchettes with washings from the syringe (3 per cent w/v trisodium citrate solution), and dried to a film under an infra-red lamp. The specimens were counted twice by a gas-flow detector.

The percentage survival of the injected erythrocytes was determined in the same manner as that used by Mitchison (1959) and Hašek (1962). The radioactivity of the specimen

taken 1–2 hours after injection was taken as 100 per cent and radioactivities of subsequent specimens were calculated as a percentage of this after correction for radioactive decay. The percentages were plotted on semi-logarithmic paper and straight lines sight-fitted. The time of elimination of 75 per cent of the erythrocytes has been used to compare the various degrees of immune reaction. The figures given in parentheses in the text below indicate the mean time taken to eliminate 75 per cent of the erythrocytes injected; this proportion of the antigenic dose has been taken to include as much elimination time as possible without danger of including in the mean those eliminations which show a 'tail'.

## RESULTS

### CLASSIFICATION OF REACTIONS

We have made a somewhat arbitrary classification of the immune reaction into types, based on the rate of elimination of antigen. Typical examples of immune reactions and

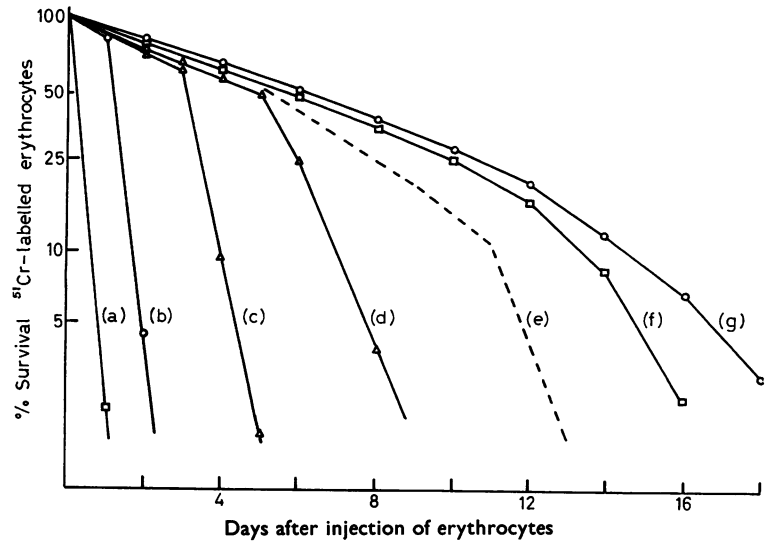


FIG. 1. Typical examples of elimination of $^{51}$Cr-labelled erythrocytes by tolerant and immune chicks. (a) Free antibody; (b) secondary; (c) normal primary; (d) weak primary; (e) partially tolerant; (f) tolerant; (g) autologous.

tolerance from our results are shown in Fig. 1. The elimination rate of tolerated erythrocytes was always somewhat faster than that of autologous cells (see also Mitchison, 1962a). Transfusion of labelled blood (3 ml.) between two adult hens of the homozygous Iowa strain (Reaseheath) showed typical autologous elimination (13 days). In this work complete tolerance was considered to have been induced when the elimination time of 75 per cent of the allogeneic erythrocytes was not less than 9·0 days (80 per cent of the mean 75 per cent elimination time of autologous erythrocytes). Partial tolerance (Fig. 1) is shown by a slightly steeper curve than that of complete tolerance with no sharp decrease after a lag phase. Primary immune responses usually have a lag phase of 3–4 days but in older chicks the primary response was sometimes so vigorous that no lag phase could be

detected. Secondary immune responses usually have a lag phase of only 1–2 days. Elimination of antigen during the lag phase proceeded at the same rate as in tolerant birds. The most rapid elimination (1 day) of erythrocytes has been ascribed to circulating antibody (Mitchison, 1962a) because erythrocyte chimaerism in tolerant birds can be rapidly abolished with immune sera (Hašek, 1962; Mitchison, 1962b), but this rapid reaction may equally well be due to a vigorous reaction (of the same strength as a secondary response) in young birds. Total elimination within 1 day usually only occurs after a second injection of antigen; this has been found in chicks (Table 2), in adult Brown Leghorn chickens (Mitchison, 1962a) and turkeys (Silber, Hedberg, Akeroyd and Feldman, 1961). However, Hort, Hašek and Knizetova (1961) found that at first injection nearly half of their 2-month-old White Leghorns completely eliminated foreign erythrocytes in less than 1 day and the rest showed vigorous immune rejection with very short lag phases. In one of our experiments four White Leghorns (4 months old) showed complete elimination in 2 days (75 per cent in 0·7 days) and the rest exhibited vigorous immune responses (2–3 days). These differences in intensity of the primary immune response are probably due to breed differences.

## INDUCTION OF TOLERANCE

Tolerance or partial tolerance was observed in most 16-day-old embryos (Fig. 2), in all the newly hatched chicks, in most of the 2-day-old chicks, but in only half of the 3-day-old chicks (Table 1). Two of the 16-day-old chick embryos were exceptional in showing a weak primary response with a 7-day lag phase (Table 1 and Fig. 2). Assuming a 4-day lag phase, these embryos would have been about 19 days old when they began to react immunologically. It is probable that these embryos were abnormally mature and that the antigenic difference between donor and host may have been greater than usual in these two embryos. Challenge at 6 days after hatching showed that these two birds had become

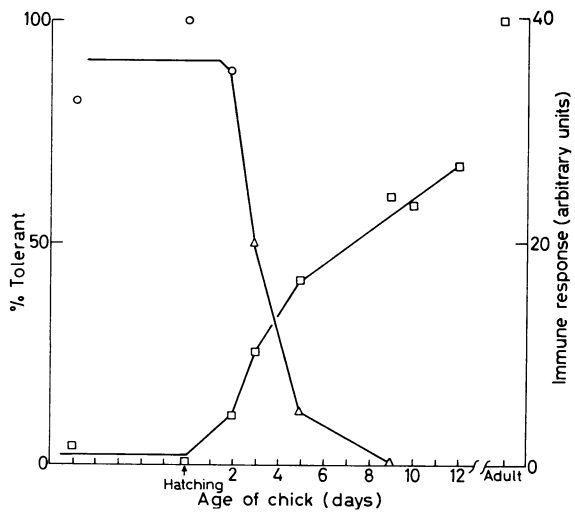


FIG. 2. Loss of tolerance responsiveness and onset of the immune response to erythrocyte antigens during development of the young chick. Values are taken from data obtained after first injection. ○, complete and partial tolerance; △, partial tolerance only; □, immune response (calculated by scoring 1 for each weak primary, 2 for normal primary, 3 for secondary response and 4 for each case of circulating antibody; expressed as a score per ten chicks).

TABLE 1

INCIDENCE OF TOLERANCE AND IMMUNITY DURING EARLY DEVELOPMENT OF THE CHICK

| *Age of recipient at first injection* | *Injection of erythrocytes* | *Tolerance* | | *Primary response* (4–5 *days*) | *Secondary response* (2 *days*) | *Circulating antibody* (<1 *day*) |
|---|---|---|---|---|---|---|
| | | *Complete* (>9 *days*) | *Partial* (7–8 *days*) | | | |
| 16-day-old embryo | First | 9/11 | — | 2/11* | — | — |
| | Second | 5/11 | 4/11 | — | 2/11 | — |
| Newly hatched chick | First | 6/9 | 3/9 | — | — | — |
| | Second | 5/8 | 3/8 | — | — | — |
| 2-day-old chick | First | 7/9 | — | 2/9 | — | — |
| | Second | 7/8 | — | 1/8 | — | — |
| 3-day-old chick | First | — | 5/10 | 5/10 | — | — |
| | Second | — | 3/10 | 2/10 | 1/10 | 4/10 |
| 5-day-old chick | First | — | 1/8 | 7/8 | — | — |
| | Second | — | — | 1/8 | — | 7/8 |
| 9-day-old chick | First | — | — | 3/5 | 2/5 | — |
| 10-day-old chick | First | — | — | 4/6 | 2/6 | — |
| | Second | 1/5 | — | — | 1/5 | 3/5 |
| 12-day-old chick | First | — | — | 1/3 | 2/3 | — |

Figures in parentheses give 75 per cent elimination time.
* 7-day lag phase.

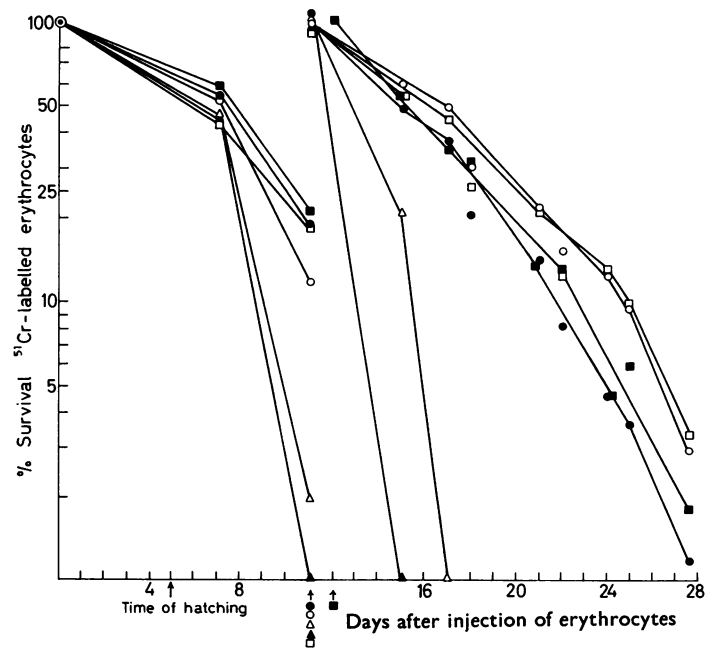


FIG. 3. Tolerance and immunity to erythrocyte antigens in 16-day-old embryos.

sensitized (2·4 days) and some of the others had lost complete tolerance (8·7 days). Tolerance was maintained by second injection in newly hatched and 2-day-old chicks (Fig. 3); two of the latter group showed primary-response (4·3 days) without sensitization (Table 1).

### ONSET OF THE IMMUNE RESPONSE

The onset of response occurs at 2–3 days post hatching (Fig. 3). There is a sharp difference in response among chicks at these ages. Only 30 per cent of 2-day-old chicks showed immune response without sensitization and the rest were tolerant; but among 3-day-old chicks, half showed an immune response which led on to sensitization and production of circulating antibody, and the remainder became only partially tolerant (Table 1, Fig. 2). Four-day-old chicks gave primary responses (four normal, two weak) and 5-day-old chicks gave more vigorous elimination than 3-day-old chicks (Table 1). In young Brown Leghorn chicks no such sharply defined immune reaction occurred and the erythrocytes were removed in a linear manner with no lag phase (Mitchison, 1962a). Nine- and 10-day-old chicks showed very rapid response at first injection (Table 1). The 12-day-old chicks showed even more rapid elimination. One 10-day-old chick after giving a primary response (4·5 days) then became fully tolerant after the second injection (10·5 days). This extraordinary finding is an example of the phenomenon described by Mitchison (1962b) in 13-day-old chicks.

### INDUCTION OF IMMUNITY IN EMBRYOS AND NEWLY HATCHED CHICKS

Embryos (15- and 19-day-old), newly hatched chicks and 4-day-old chicks were injected with a diluted blood (1/100 to 1/250th of the tolerance-inducing dose of erythrocytes) and then challenged at 7 days of age with 0·5 ml. of labelled blood. In all groups, except the 15-day-old embryo, the initial dose induced sensitization (Table 2). Whereas

TABLE 2

INDUCTION OF IMMUNITY TO ERYTHROCYTE ANTIGENS IN EMBRYOS AND NEWLY HATCHED CHICKS

| *Age of recipient* | *Immunizing dose of diluted blood* | *Mean 75 per cent elimination time (days) after challenge at 7 days of age* | | | |
|---|---|---|---|---|---|
| | | *Partially tolerant* | *Primary response* | *Secondary response* | *Circulating antibody* |
| Not immunized | | | | | |
| 17-day-old embryo | 0·05 ml. Ringer | — (0) | 3·9 (3) | 2·6 (2) | — (0) |
| Immunized | | | | | |
| 15-day-old embryo | 0·05 ml. erythroctyes | 7·0 (2) | 4·8 (3)* | 1·9 (1) | — (0) |
| 19-day-old embryo | 0·02 ml. erythrocytes | — (0) | 3·8 (2) | 2·1 (5) | 1·0 (1) |
| Newly hatched chick | 0·05 ml. erythrocytes | — (0) | — (0) | 2·8 (4) | 1·0 (4) |
| 4-day-old chick | 0·05 ml. erythrocytes | — (0) | — (0) | 3·2 (4) | 1·5 (4) |

Figures in parentheses indicate the number in each group.
* Includes two weak primary responses.

80 per cent of normal 7-day-old chicks gave primary responses, newly hatched and 4-day-old chicks which had been exposed to diluted blood showed no primary responses; 50 per cent gave secondary and 50 per cent circulating antibody responses. Injection of 19-day-old embryos with diluted blood did not produce such vigorous responses after challenge as those of the chicks.

PARTIAL INHIBITION OF THE IMMUNE RESPONSE BY PRETREATMENT WITH TESTOSTERONE

Antibody production in chicken can be greatly reduced by treating the eggs with testosterone (Mueller, Wolfe and Meyer, 1960; Glick, 1961). The method of Glick consists of dipping the pointed end of eggs in an alcoholic solution of testosterone propionate (2 g. per 100 ml.) for 5 seconds to a depth of 1½ in. on the 3rd day of incubation. Glick (1961) states that this causes very considerable retardation of growth of the Bursa of Fabricius, and a failure of response to polyvalent K *Salmonella pullorum* antigen at 5 weeks of age. We repeated the procedure of Glick but in our experiment there must have been only slight absorption of hormone as the birds did not show the symptoms characteristic of 'chemical bursectomy', and only a mild inhibition of immune response to erythrocyte antigen when such birds were injected at 2 weeks of age. The inhibition was shown by partial tolerance in three out of five cases, whereas the controls all gave vigorous immune responses.

## DISCUSSION

The clearance of foreign erythrocytes from the circulation appears to be brought about by an immunological reaction. The rate of elimination of $^{51}$Cr-labelled erythrocytes has been shown to be directly related to the amount of haemagglutinin production (Drury and Tucker, 1958; Smith, Odell and Caldwell, 1959) Primary and secondary types of immune clearance of foreign erythrocytes in adult birds have been described by Mitchison (1962a) and are further illustrated in the present work on young chicks. In common with immune responses to other antigens, erythrocyte clearance from the circulation is inhibited when the chick embryo is 'chemically bursectomized' with testosterone. It might be considered that the cases of 'instant' elimination of foreign erythrocytes in the present work were due to maternal antibody present in the circulation of the embryo. However, as no iso-haemagglutinins have been detected until long after absorption of the yolk sac, so it is unlikely that maternal haemagglutinins play a part. This is confirmed by our present results, where no 'instant' elimination of the erythrocytes could be detected at first injection. Furthermore, very young chicks can be sensitized with small doses of antigen which implies that small numbers of erythrocytes are not instantly removed as inactive antigen–antibody complex.

The present results indicate that tolerance is maintained so long as erythrocytes remain in the circulation (see also Mitchison, 1959), but a state of complete tolerance can become one of partial tolerance (e.g. in the 16-day-old chick embryo group). While 2-day-old chicks can become almost completely tolerant, partial tolerance was produced in only half of the 3-day-old chick group and in hardly any of the 5-day-old chicks (Fig. 2). The 'tolerance-responsive' phase in development in this work is therefore very similar to that previously found by Mitchison (1962a) and the rapid acquisition of immunity with no 'null period' is similar to that for turkey erythrocytes (Beckitt, 1958). The tolerance-responsive phase ends at 1–2 days after hatching, which is the same as has been found for transplantation antigens (Billingham, Brent and Medawar, 1956; Simonsen, 1957; Solomon and Tucker, 1963; Solomon, 1963b). These timings depend to a certain extent on antigenic dosage, for tolerance can be induced in older birds by large doses; Mitchison (1962a) has induced tolerance in 8-day-old chicks with doses equivalent to 10–13 ml. of blood and Hašek and Puza (1962) have similarly induced tolerance in adult ducks. Our results roughly conform to the rule (Smith and Bridges, 1956) that a given dose of antigen

will induce either tolerance or immunity depending upon whether the antigen is given at an early or late stage of development.

A characteristic type of reaction to antigen given at the end of the tolerance-responsive phase is the development of partial tolerance. This is probably due to a proportion of the recipients' lymphocytes being immunologically competent at the time of injection of antigen. These competent cells become immune and produce antibody while other lymphocytes not so well differentiated become tolerant (Hašek, 1962). Partial tolerance can be maintained by second injection as shown in our experiment with newly hatched chicks and can last indefinitely (Mitchison, 1962a). However, if sufficient time is allowed to lapse between the disappearance of antigen and the second injection, tolerance is lost in those cells which were previously tolerant (Mitchison, 1959). In 3- and 5-day-old chicks partial tolerance breaks down even when further doses of antigen are given, and on challenge some chicks show vigorous immune responses. Further support for Hašek's interpretation of partial tolerance is provided by our experiment with partially 'bursectomized' chicks, in which the number of lymphocytes capable of producing antibody had presumably been reduced by testosterone to such an extent that partial tolerance would be obtained in 14-day-old chicks.

Antibodies to bacterial antigen have not been detected in chicks by serological agglutination techniques until 11–30 days after hatching (Buxton, 1954). The detection of small amounts of antibody by these methods may be prevented by the removal of antibody in the form of an antigen–antibody complex. For this reason, perhaps, many workers believe that responsiveness to heterologous antigens develops later than the homograft reaction. However, we have shown that young chicks develop the capacity to become immunized rapidly and by 12 days of age can act nearly as fast, although not quite as vigorously, as the adult. While the onset of immunological competence in the chick blood and spleen towards transplantation antigens occurs at 8–11 days after hatching (Simonsen, 1957; Solomon, 1960), it has recently been shown that lymphocytes in the skin of 2–3-day-old chicks can already react to transplantation antigens (Solomon, 1963a), a time of onset of immunological competence which is identical with that found for erythrocyte antigens. Transplantation immunity and the ability to produce haemagglutinins therefore differ only quantitatively in their development. Whereas the power of homograft rejection appears to be as strong in young animals as in the adult (Schinckel and Ferguson, 1953; Solomon, 1963a), the ability to produce antibodies increases gradually during post-natal development (Baumgartner, 1934; Buxton, 1954; Freund, 1930; Wolfe and Dilkes, 1948).

It has recently been suggested by Solomon and Tucker (1963) that chick embryos possess sufficient lymphocytes after 15 days of incubation to give a weak homograft response against foreign lymphocytes proliferating in the spleen. Chick embryos can be sensitized to transplantation antigens by injections of whole blood at as early as 15 days of incubation, at a time shortly after lymphocytes appear in the spleen (Solomon, 1963b). The present experiments do not confirm the capacity of embryonic lymphocytes to become sensitized; erythrocyte antigens can sensitize 4-day-old chicks, and possibly newly hatched chicks, but not embryos. Sensitization of embryos to foreign erythrocytes may be difficult to achieve because the dosage for sensitization during this period of development is very critical (Solomon, 1963b), and because the challenge dose cannot be given until the chicks are all old enough to give an immune response. The newborn mouse, for example, can be sensitized with small doses of antigen or rendered tolerant with large doses of antigen (Howard and Michie, 1962; Thorbecke, Siskind and Goldberger, 1961).

Kalmutz (1962) has found that 27–30-day-old opossum in the pouch can react against bacteriophage as rapidly as older animals; the ability to do so follows closely lymphoid infiltration of the spleen, in much the same manner as has been suggested for the chick embryo's reactivity towards transplantation antigen (Solomon and Tucker, 1963; Solomon, 1963b). However, even with the sensitive method used in the present work only two embryos (16-day-old) reacted to erythrocyte antigens; we have therefore confirmed that the chick embryo even in its late stages of development is practically incapable of producing antibodies (Needham, 1942; Tyler, 1959).

## ACKNOWLEDGMENTS

We wish to thank Professor A. Haddow, F.R.S., for his encouragement and support. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

## REFERENCES

Baumgartner, L. (1934). 'The relationship of age to immunological reactions.' *Yale J. Biol. Med.*, **6**, 403.

Beckitt, A. M. (1958). 'Some Studies on the Development of the Immune Response in Chicken.' B.Sc. thesis, Edinburgh.

Billingham, R. E., Brent, L. and Medawar, P. B. (1955). 'Tolerance of red cell antigens and transplantation immunity in chickens.' *Experientia*, **11**, 444.

Billingham, R. E., Brent, L. and Medawar, P. B. (1956). 'Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance.' *Phil. Trans.* B, **239**, 357.

Buxton, A. (1954). 'Antibody production in avian embryos and young chicks.' *J. gen. Microbiol.*, **10**, 398.

Chaplin, H. (1957). 'Shortened survival of incompatible red cells in patients with "aggammaglobulinemia"; a sensitive test for the presence of trace of isoantibody.' *J. lab. clin. Med.*, **50**, 800.

Drury, A. N. and Tucker, E. M. (1958). 'The relationship between natural and immune haemolysins and incompatability of $^{51}$Cr-labelled red cells in the sheep.' *Immunology*, **1**, 204.

Freund, J. (1930). 'Influence of age upon antibody formation.' *J. Immunol.*, **18**, 315.

Glick, B. (1961). 'Influence of dipping eggs in male hormone solutions on lymphatic tissue and antibody response of chickens.' *Endocrinology*, **69**, 984.

Hašek, M. (1953). 'Vegetativni hybridiscae zivocichu spojenim krevnich obehu v embryonalnim vyvoji.' *Ceskoslov. Biol.*, **2**, 265.

Hašek, M. (1956). 'Tolerance phenomenon in birds.' *Proc. roy. Soc.* B, **146**, 67.

Hašek, M. (1962). 'Quantitative aspects of immunological tolerance.' *Folia Biol. (Praha)*, **8**, 73.

Hašek, M. and Puza, A. (1962). 'On the induction of immunological tolerance in adult recipients.' *Folia Biol. (Praha)*, **8**, 55.

Hort, J., Hašek, M. and Knizetov, F. (1961). 'Further immunological analysis of chicken embryonic parabionts.' *Folia Biol. (Praha)*, **7**, 301.

Howard, J. G. and Michie, D. (1962). 'Induction of transplantation immunity in the newborn mouse.' *Transplant. Bull.*, **29**, 1.

Kalmutz, S. E. (1962). 'Antibody production in the opossum embryo.' *Nature (Lond.)*, **193**, 851.

Mitchison, N. A. (1959). 'Blood transfusion in the fowl: an example of immunological tolerance requiring the persistence of antigen.' *Biological Problems of Grafting* (Ed. by F. Albert and G. Lejeune-Ledant), p. 239. Blackwell Scientific Publications, Oxford.

Mitchison, N. A. (1962a). 'Tolerance of erythrocytes in poultry: induction and specificity.' *Immunology*, **5**, 341.

Mitchison, N. A. (1962b). 'Tolerance of erythrocytes in poultry: loss and abolition.' *Immunology*, **5**, 359.

Mueller, A. P., Wolfe, H. R. and Meyer, R. K. (1960). 'Precipitin production in chickens. XXI. Antibody production in bursectomized chickens and in chickens injected with 19-Nortestosterone on the fifth day of incubation.' *J. Immunol.*, **85**, 172.

Needham, J. (1942). *Biochemistry of Morphogenesis.* Cambridge University Press.

Ottesen, J. (1955). *The Life Cycle of Hen Erythrocytes.* Munksgaard, Copenhagen.

Ryle, M. H. (1957). 'Studies on possible serological blocks to species hybridization in poultry.' *J. exp. Biol.*, **34**, 365.

Schinckel, P. G. and Ferguson, K. A. (1953). 'Skin transplantation in the foetal lamb.' *Aust. J. biol. Sci.*, **6**, 533.

Silber, R., Hedberg, S. E., Akeroyd, J. H. and Feldman, D. (1961). 'The transfusion behaviour of avian erythrocytes: the lack of functional transplantation antigens in a nucleated cell.' *Blood*, **18**, 207.

Simonsen, M. (1957). 'The impact on the developing embryo and newborn animal of adult homologous cells.' *Acta path. microbiol. scand.*, **40**, 480.

Smith, R. T. and Bridges, R. A. (1956). 'Response of rabbits to defined antigens following neonatal injection.' *Transplant. Bull.*, **3**, 145.

SMITH, L. H., ODELL, T. T. and CALDWELL, B. (1959). 'Life span of rat erythrocytes as determined by $Cr^{51}$ and differential agglutination methods.' *Proc. Soc. exp. Biol. (N.Y.)*, **100**, 29.

SOLOMON, J. B. (1960). 'The onset and maturation of the graft versus host reaction in chickens.' *J. Embryol. exp. Morph.*, **9**, 355.

SOLOMON, J. B. (1963a). 'Ontogenesis of immunological competence of lymphocytes in chicken skin.' (In press).

SOLOMON, J. B. (1936b). 'Actively acquired transplantation immunity in the chick embryo.' (In press).

SOLOMON, J. B. and TUCKER, D. F. (1963). 'Immunological attack by adult cells in the developing chick embryo: effect on splenomegaly of spleen differentiation and of homograft rejection by the embryo.' *J. Embryol. exp. Morph.*, **11**, 179.

THORBECKE, G. J., SISKIND, G. W. and GOLDBERGER, W. (1961). 'The induction in mice of sensitization and immunological unresponsiveness by neonatal injection of bovine γ-globulin.' *J. Immunol.*, **87**, 147.

TYLER, A. (1959). 'Ontogeny of immunological properties.' *Analysis of Development* (Ed. by B. H. Willier, P. A. Weiss and V. Hamburger), p. 556. Saunders, London.

WOLFE, H. R. and DILKES, E. (1948). 'Precipitin production in chickens. III. The variation in antibody response as correlated with the age of the animal.' *J. Immunol.*, **58**, 245.