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Ontogeny of Haemolytic Plaque-forming Cells in Newborn Rabbit's Spleen in Response to Different Erythrocyte Antigens

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Summary. The ability of rabbits to respond to relatively high doses of sheep erythrocytes appears within 5 days after birth. Young rabbits exhibited an apparent sequential maturation of responsiveness to sheep, mouse, rat and guinea-pig erythrocyte antigens. The delay in response to these antigens can be correlated with different levels of specific haemagglutinin, presumably of maternal origin, in the circulation of young rabbits.

INTRODUCTION

The ontogenesis of immunological function in mammals and birds has been integrated by using physiological, rather than chronological age, in a theory of age equivalence (Solomon, 1970, 1971). In this way various immunological capabilities can be compared across the species and the time when a given species becomes immunocompetent predicted with a considerable degree of accuracy. The rabbit's thymus begins to become lymphoidal around 20 days of gestation (Ackerman and Hostetler, 1970); immunoglobulin-producing cells have been detected 10 days later at birth (Wainer, Robbins, Bellanti, Eitzman and Smith, 1963) and plaque-forming cells (PFC) have been detected after a further 5 days (Tlaskalová, Šterzl, Hájek, Pospíšil, Říha, Marvanová, Kamarýtová, Mandel, Kruml and Kovářů, 1970). Age-equivalence theory predicts the onset of ability to produce haemolytic antibody by the time rabbits are born. However, before embarking on studies involving the immunization of the rabbit foetus we investigated the PFC response in newborn rabbits to different erythrocyte antigens.

Although it is now generally accepted that many species are immunocompetent before birth (Solomon, 1971) it is still controversial whether the ability to react with antigen mosaics arises virtually simultaneously in the developing foetus (Solomon, 1973) or whether there is a real sequential maturation to different antigens at different stages of foetal development (Silverstein, Uhr, Kraner and Lukes, 1963). Our rabbit model overcomes two of the major criticisms of Silverstein's experiments, as we use similar types of antigen (xenogeneic erythrocytes) and a single method for measurement of responsiveness (plaque-forming cells).

The apparent sequential maturation of responsiveness of young rabbits to different erythrocyte antigens seems to be due to the presence of haemagglutinating antibodies of maternal origin causing differential delay in the onset of PFC responses to different erythrocyte antigens.

MATERIALS AND METHODS

Litters were obtained from matings of outbred New Zealand White rabbits. Sheep erythrocytes (SRBC) in Alsever's solution were obtained weekly from Wellcome Research Laboratories, Beckenham. All other erythrocytes were obtained fresh daily by cardiac puncture and stored in Alsever's solution. Immediately before they were required, the erythrocytes were washed three times with ice-cold physiological saline and the number of packed cells determined by haemocytometer count. Initially, we investigated the onset of PFC response using a body-weight adjusted dose of 3×10^{10} cells/kg body weight. At this dose a 2-day-old rabbit received 2×10^{9} erythrocytes while a 5-day-old rabbit received 3×10^{9} erythrocytes. In experiments to detect the earliest onset of PFC response to SRBC we also used a 10-fold higher dose (3×10^{11} cells/kg body weight).

Four days after an intraperitoneal injection of washed xenogeneic erythrocytes the spleens were removed from the rabbits, and cell suspensions prepared as previously described (Solomon, Leiper and Reid, 1972). Usually individual spleens were assayed separately. All cells were maintained at 4° during preparation. The method of detecting PFC was that of Cunningham and Szenberg (1968) incorporating modifications described by Solomon, Leiper and Reid (1972).

Rabbit haemagglutinating and haemolytic antibody titres were measured after complement inactivation of the sera. Haemagglutination titres were detected as previously described (Solomon, Riddell and Whyte, 1972). To measure haemolytic titres duplicate series of aliquots (0·1 ml) of doubling dilutions of the sera were transferred to wells on WHO agglutination plates, and 0·1 ml of a 1 per cent (v/v) suspension of erythrocytes containing 1 per cent (v/v) absorbed guinea-pig complement were added. The plates were read after 1 and 2 hours incubation at 37°, endpoints were recorded according to the erythrocyte sedimentation pattern or 50 per cent haemolysis.

RESULTS

ONTOGENY OF THE PLAQUE-FORMING CELL RESPONSE TO ERYTHROCYTE ANTIGENS

The ontogeny of the PFC response in spleens of rabbits antigenically stimulated with sheep erythrocyte antigen is shown in Fig. 1. There was no true onset of the PFC response until rabbits injected with 3×10^{10} erythrocytes/kg body weight were 9 days of age. However, when the dose of erythrocytes injected was increased 10-fold, a weak response was detected 4 days after immunization of rabbits less than 4 hours old and a vigorous response when rabbits less than 16 hours old were injected It is interesting to note this larger dose did not evoke a greater response in 9-day-old rabbits.

Suspecting that specific maternal antibody might be suppressing the response in rabbits to SRBC which we could overcome by injecting huge doses of SRBC, we tested the response of young rabbits to different erythrocyte antigens in an attempt to find an antigen for which the neonates possessed no antibody of maternal origin (Solomon, Riddell and Whyte, 1972). The erythrocyte antigens used were mouse (MRBC), rat (RRBC) and guinea-pig (GPRBC) at a dose of 3×10^{10} erythrocytes/kg body weight. The PFC response to these erythrocytes in developing rabbits was measured from birth to 22 days (Fig. 2) and 45 days (Table 1). SRBC elicited the earliest and most vigorous response, while the onset to MRBC did not appear until 11 days after birth and to RRBC 3 days later,

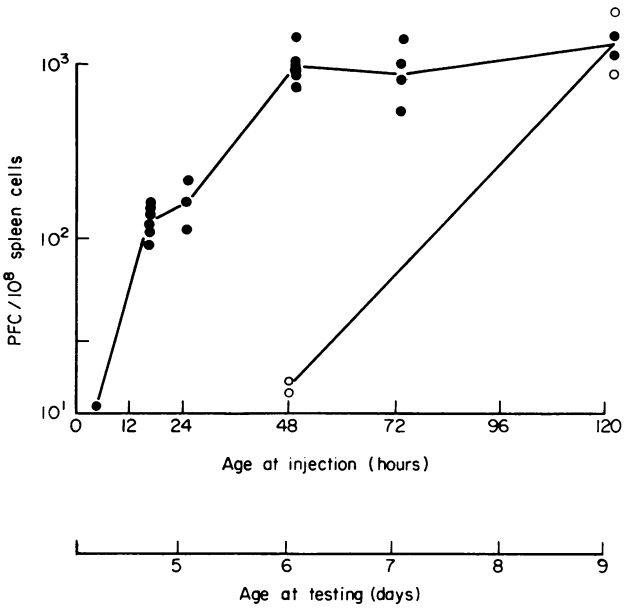

FIG. 1. Ontogeny of haemolytic plaque-forming cells in the spleen of rabbits antigenically stimulated with sheep erythrocytes, immunizing dose (○) 3×10^{10} SRBC/kg body weight, (●) 3×10^{11} SRBC/kg body weight. Each point represents individual spleens except for newborn rabbits injected with 3×10^{11} SRBC/kg and those injected with 3×10^{10} SRBC/kg where litters were pooled.

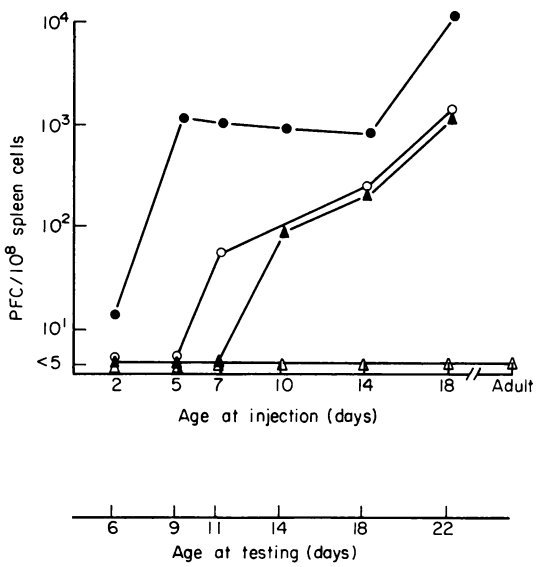

FIG. 2. Ontogeny of haemolytic plaque-forming cells in the spleen of rabbits in response to different erythrocyte antigens: (●) SRBC; (○) MRBC; (▲) RRBC; (△) GPRBC. Each point is the geometric mean PFC obtained from individual spleens.

TABLE 1

ONSET OF PFC RESPONSE TO VARIOUS ERYTHROCYTE ANTIGENS IN YOUNG RABBITS

Age tested (days)	Erythrocyte antigen* Sheep	Mouse	Rat	Guinea-pig
	(mean PFC/10^8 spleen cells)			
6	15	0	0	0†
9	1,287	0	0	0
11	1,100	61	0	0
14	982	209	95	0
18	894	273	226	0
22	12,800	1,500	1,300	0
32	17,700	1,800	1,500	0
45	35,500	2,600	2,000	0

* Dose of erythrocytes = 3×10^{10}/kg body weight.
† Less than five PFC/10^8 spleen cells.

although by 18 days of age the RRBC evoked a response of virtually the same magnitude as the MRBC.

Low levels of 'natural' PFC to SRBC only, appeared in non-immunized rabbits at 18 days of age (10 PFC/10^8 spleen cells). At 22 days of age the levels of 'natural' PFC were SRBC 440, MRBC 57 and RRBC 22 PFC/10^8 spleen cells and in adults 90, 420 and 60 PFC/10^8 spleen cells respectively.

LEVEL OF HAEMAGGLUTINATING ANTIBODY AT THE TIME OF ONSET OF THE PFC RESPONSE

Haemagglutination titres of serum from non-injected rabbits taken at the ages of the onset of the PFC response (>100 PFC/10^8 spleen cells) to the various xenogeneic erythrocytes are given in Fig. 3. Haemagglutination titres to SRBC, MRBC and RRBC dropped progressively during the first 18 days after birth. Only the anti-GPRBC titre remained

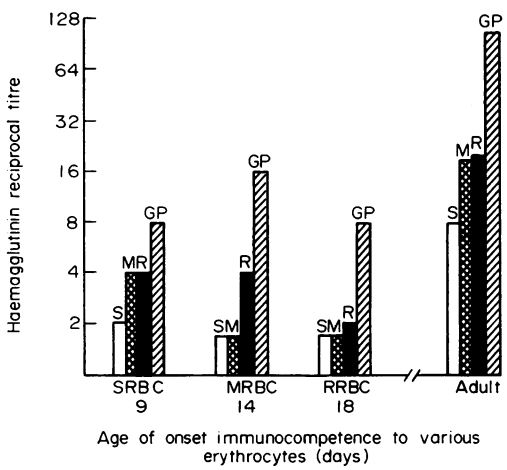

FIG. 3. Haemagglutination titres (geometric means) of non-immunized rabbits to (□) sheep erythrocytes, (▩) mouse erythrocytes, (■) rat erythrocytes and (▨) guinea-pig erythrocytes measured at the age of onset of PFC (>100/10^8 spleen cells) response to the various erythrocytes.

about 1/8 during this period of development. It appears that the onset of the PFC response cannot be detected until the specific haemagglutination titre has dropped to 1/2 or below; this occurs at 9 days for SRBC, 14 days for MRBC and 18 days for RRBC.

EARLY HAEMAGGLUTINATING AND HAEMOLYTIC ANTIBODY RESPONSES TO GUINEA-PIG ERYTHROCYTE ANTIGENS

The most surprising finding was the complete unresponsiveness of rabbits to GPRBC in young and even adult rabbits. When guinea-pig erythrocytes are injected into rats they elicit mainly haemagglutinating antibody (Gery and Davies, 1963). We therefore examined the ontogeny of humoral antibody response to GPRBC in rabbits to test whether the lack

TABLE 2

ONSET OF DETECTABLE HAEMOLYTIC PLAQUE-FORMING SPLEEN CELLS

Species	IgM PFC observed (days post-coitus)	Age-equivalent k(t-t′)	Reference
Rabbit	34	0·69	
Rat	27	0·50	Solomon, Riddell and Whyte (1972)
Mouse			
CFW, CDI	25	0·40	Hargis and Malkiel (1970)
NZB	27	0·45	Playfair (1968)
Balb/c	29	0·52	Playfair (1968)
Hamster	22	0·78	Solomon, Leiper and Reid (1972)

of PFC to GPRBC in rabbits was due to haemagglutinating rather than haemolytic antibody being produced. Sera were taken 4 days after injection of antigen just before the spleen was removed for PFC assay. Not until the rabbits were 45 days of age could an active antibody response to GPRBC be detected 4 days after injection (haemagglutination titre 1/16 and haemolysin titre 1/4); by this age, maternal antibody had dropped to a haemagglutinin titre of 1/4. The humoral antibody response is much delayed in comparison with the other erythrocyte antigens and although the main antibody component of the response is haemagglutinating antibody, haemolysins are also produced. When $1{\cdot}4 \times 10^{11}$ GPRBC were injected into adult rabbits their natural anti-GPRBC titres were reduced within one day from 1/64 to 1/2. There appears to be so much natural antibody in the circulation that even this huge dose of GPRBC is complexed by specific antibody and removed by macrophages so no antigenic stimulus occurs.

DISCUSSION

The earliest PFC response to sheep erythrocytes in our rabbits was detected by injecting newborn rabbits with a single huge dose of sheep erythrocytes, less than 16 hours after birth and assaying the spleen 3·5 days later. This is a slightly earlier onset of response than found by Tlaskalová *et al.* (1970) who used daily injections of sheep erythrocytes. This onset of the PFC response compares favourably by age-equivalence with those of rodents (Table 2); the 'physiological age', or k(t-t′) value of 0·69 being less than the hamster's (0·78) but greater than the mouse and rat (0·4–0·5).

It is well known that newborn animals require much larger doses of erythrocyte antigen

(relative to body weight) to elicit a response than adults (Tlaskalová *et al.*, 1970). The increased number of erythrocytes may be necessary to effect the minimal collision frequency necessary for induction as there may only be a few lymphocytes of the reactive clones present at this age. In our rabbits, this effect had disappeared by 5 days after birth when the lymphocyte population will certainly have enlarged, also any interfering anti-sheep maternal antibody begins to be eliminated by catabolism. So the earlier onset of the PFC response to SRBC produced by giving a huge dose of erythrocytes is probably achieved by ensuring sufficient antigen is present not only to complex with any specific maternal antibody but also to leave an immunogenic level of erythrocytes to stimulate active haemolytic antibody production.

Natural anti-SRBC or Forssman haemolysins are transmitted from mother to foetus exclusively via the yolk sac in rabbits and appear in the newborn at similar titres. Although its half-life in the young rabbit is only 8–9 days, maternal antibody can still be detected up to 5 weeks after birth (Aitken, 1964). If maternal antibody is interfering with an ostensibly immunogenic number of erythrocytes young rabbits will be unable actively to produce specific antibody to certain erythrocytes until the titre of maternal antibody has declined to a low level.

Haemagglutination titres to sheep, mouse and rat erythrocytes in young rabbit sera declined from quite low levels to $<1/2$ and we have attempted to equate this with the onset of PFC response. The two extreme responses—the early anti-sheep PFC response and the failure of guinea-pig erythrocytes to induce a response—can be explained by the low titres of anti-sheep haemagglutinins soon after birth and the relatively high anti-guinea-pig titres at all ages. However, the differences in haemagglutination titres are quite small and any such differences needed to be confirmed by measurement of the relative blood clearance rates of these two erythrocytes. Such experiments revealed that guinea-pig erythrocytes are cleared twelve times faster than sheep erythrocytes from the blood of 10-day-old rabbits (Solomon, 1974). The rapid clearance of guinea-pig erythrocytes from the circulation by opsonization and phagocytosis (99 per cent in 1 minute) may mean they have little chance of encounter with lymphocytes, and there is no induction of an antibody response.

Interference by antibody of maternal origin in the induction of PFC responses to mouse erythrocytes has been experimentally designed in rats suckling hyperimmune mothers (Solomon, Riddell and Whyte, 1972). Not until the titre of haemagglutinating antibody in the serum of the offspring had fallen to 1/2 (at 60 days of age) could normal adult responses be obtained. This experiment is a model which can be used to explain why our rabbits were unresponsive to GPRBC.

An active haemagglutination response to GPRBC has been recorded in 10-day-old rabbits which gave little or no response to bovine erythrocytes (Říha, 1961). Likewise, plaque-forming cells could not be detected in adult rabbits immunized with human erythrocytes (Benezra, Gery and Davies, 1970). This lack of response to bovine and human erythrocytes may be due to the method of detection used because Gery and Davies (1963) have shown that human erythrocytes elicit almost exclusively haemagglutinating antibody in the rat while bovine erythrocytes produced chiefly haemolysins.

We believe this apparent sequential maturation of the young rabbit to respond to xenogeneic erythrocytes is due to newborn rabbits possessing differential concentrations of antibody derived from the mother. If the onset of responsiveness to such antigen mosaics is indeed a one step event (Solomon, 1973) the generation of antibody diversity according

to the germ-line theory will only be manifest by experimentation with truly agammaglobulinaemic foetuses injected with antigens stimulating a single cell clone.

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