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Induction of Antibody Formation to Goat Erythrocytes in the Developing Chick Embryo and Effects of Maternal Antibody

J. B. SOLOMON*

Chester Beatty Research Institute, Institute of Cancer Research, Fulham Road, London

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Summary. The formation of antibody to goat erythrocytes has been studied in embryos and young chicks. Opsonizing antibody was measured by accelerated clearance of antigen and the sensitivity of this technique compared with haemagglutination tests. A wide range of immunizing doses of goat erythrocytes was used; all induced immunity. Small amounts of antibody were detected as early as the day of hatching but vigorous antibody production did not occur until 3 days later. An immune response could be induced in 12-day-old embryos and the most vigorous response was obtained by injection of 14-day-old embryos. Antibody production was relatively poor between the fifteenth day of embryonic life and 15 days after hatching. This was due to the presence of maternal antibody.

INTRODUCTION

Many previous attempts to induce antibody production in the chick embryo appear to have been unsuccessful, probably because of the insensitivity of the serological tests employed. The earliest recorded production of haemagglutinating antibody in the developing chick was at 15 days of age in response to multiple injections of guinea-pig erythrocytes given during the first week after hatching (Bailey, 1923). The chick embryo has been shown to be capable of homograft rejection (Isacson, 1959; Solomon and Tucker, 1963a) and sensitization to foreign cells so that a second-set response was obtained soon after hatching (Solomon, 1963). The use of antigen clearance techniques to detect small amounts of antibody in chicks (Mitchison, 1962) enabled antibody to chicken erythrocytes to be detected as early as 5 days after hatching (Solomon and Tucker, 1963b). It has been found that antibody to goat erythrocytes can be induced in the chick embryo provided there is little maternal antibody in the circulation. As the level of maternal antibody increases the immune response is increasingly suppressed. Even a trace of maternal antibody produces a marked suppression of immunity in older embryos. This may provide a mechanism for the induction of partial tolerance to heterologous antigens.

METHODS

White Leghorn embryos and chicks of various ages were divided into control and experimental groups (4–5 per group). The experimental groups were injected intravenously with 1×10^4 to 1×10^{10} goat erythrocytes (in a few experiments, guinea-pig erythrocytes were used for both the immunizing and test doses). Both control and experimental groups were later given a test dose of goat erythrocytes sufficient to establish a 1 : 1 ratio of goat

* Present address: Department of Microbiology, University of Adelaide, Adelaide, South Australia.

to chicken cells. Methods of obtaining erythrocyte suspensions and measuring the rate of clearance of goat cells from the circulation have been described previously (Solomon, 1966).

In one series of experiments the test injection was given 7 days after the immunizing dose, the time of maximum antibody response to goat erythrocytes in older chicks (Delhanty and Solomon, 1966). In another series, test injections were made 5 days after hatching, regardless of the age of immunization.

The opsonizing antibody produced in response to an immunizing dose of goat erythrocytes was estimated by subtracting the mean phagocytic index (K_1) of the control group from the mean index (K_2) of the immune group (Biozzi, Stiffel, Halpern, Le Minor and Mouton, 1961). It has already been established that immune antibody passively transferred to embryos increases the phagocytic index in direct proportion to the amount of antibody added, and also that the test dose of erythrocytes depresses the phagocytic index in direct proportion to the dose (D) so that $K \times D$ = constant (Solomon, 1966). In the series of experiments where antibody was measured at 5 days after hatching, the standard test dose was 5×10^9 erythrocytes; any variation in this dose was corrected by taking D as unity (when $D = 5 \times 10^9$) in the formula expressing opsonizing antibody units as:

$$\frac{100(K_2 - K_1)}{D}.$$

In the other series of experiments, where antibody was estimated at different ages, a sliding standard test dose related to the change in blood volume with age was used; this was based on the test dose of $D = 5 \times 10^9$ cells at 5 days after hatching. The method of measuring chick blood volumes using ^{51}Cr-labelled autologous erythrocytes has been described previously (Solomon and Tucker, 1963b).

In three experiments sera was obtained immediately prior to the test injection of erythrocytes. All such sera were heated at 56° for 30 minutes to destroy complement before haemagglutination tests which were carried out at 37°, and in some cases, at 4° in normal saline with 0·05 per cent suspension of goat erythrocytes.

RESULTS

In the first series of experiments opsonizing antibody was measured at 7 days after the immunizing injection of erythrocytes (Table 1 and Fig. 1). When 14-day-old embryos were injected with 1×10^4 cells no antibody was produced at hatching; however, immunization of 15-day-old embryos produced minute amounts of antibody at hatching (6 days later). Injection of 1×10^4 erythrocytes into 17- or 19-day-old embryos elicited virtually no response; hatching and 2-day-old chicks producing only minute amounts of antibody. When very large immunizing doses were given at late stages of embryonic development (17 and 19 days) much larger amounts of antibody were produced.

In another series of experiments, the antibody was measured at 5 days after hatching (Table 2 and Fig. 2). Induction of antibody to small doses of cells occurred at 13 days and reached a maximum at 14 days of incubation. The large immunizing doses induced antibody as early as 12 days and the response was greatest at 13–14 days of incubation. Intermediate doses of 1×10^6 or 1×10^8 erythrocytes produced intermediate amounts of antibody but failed to elicit a response at 19 days of incubation.

TABLE 1

OPSONIZING ANTIBODY AT 7 DAYS AFTER INJECTION OF EMBRYOS AND CHICKS WITH GOAT ERYTHROCYTES

Immunizing		Opsonizing antibody units $100(K_2-K_1)/D$	Immunizing		Opsonizing antibody units $100(K_2-K_1)/D$
Embryo's age (days of incubation)	Dose		Chick's age (days after hatching)	Dose	
14	1×10^4	0	0	1×10^4	3·3
				1×10^6	10·1
15	1×10^4	6·2			
		2·9	2	1×10^4	6·2
		5·3		1×10^8	20·0
17	1×10^4	0	5	5×10^9	26·8
	1×10^{10}	16·5			
			8	1×10^8	71·5
19	1×10^4	0			
	1×10^6	0			
	1×10^8	2·2			
	5×10^9	26·6			

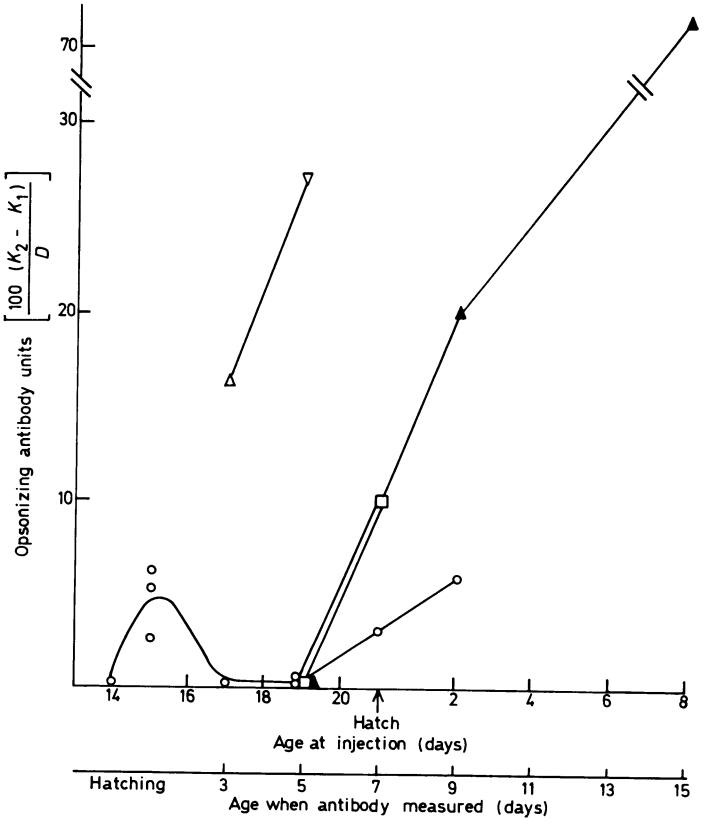

FIG. 1. Opsonizing antibody produced 7 days after injection of goat erythrocytes. Doses of erythrocytes: ○, 1×10^4; □, 1×10^6; ▲, 1×10^8; ▽, 5×10^9; △, 1×10^{10}.

TABLE 2

OPSONIZING ANTIBODY AT 5 DAYS AFTER HATCHING PRODUCED BY INJECTING CHICK EMBRYOS AT VARIOUS STAGES OF DEVELOPMENT WITH GOAT ERYTHROCYTES

Immunizing		Opsonizing antibody units $100(K_2-K_1)/D$
Age (days of incubation)	Dose	
11	1×10^4	0
12	1×10^4	0
	1×10^{10}	20·9
13	1×10^4	25·2
	1×10^6	35·6
	1×10^{10}	69·5
14	1×10^4	133·0
		102·0
	1×10^{10}	60·2
15	1×10^4	70·0
17	1×10^4	0·8
	1×10^{10}	46·0
19	1×10^4	0
	1×10^6	0
	1×10^8	2·2
	5×10^9	26·6

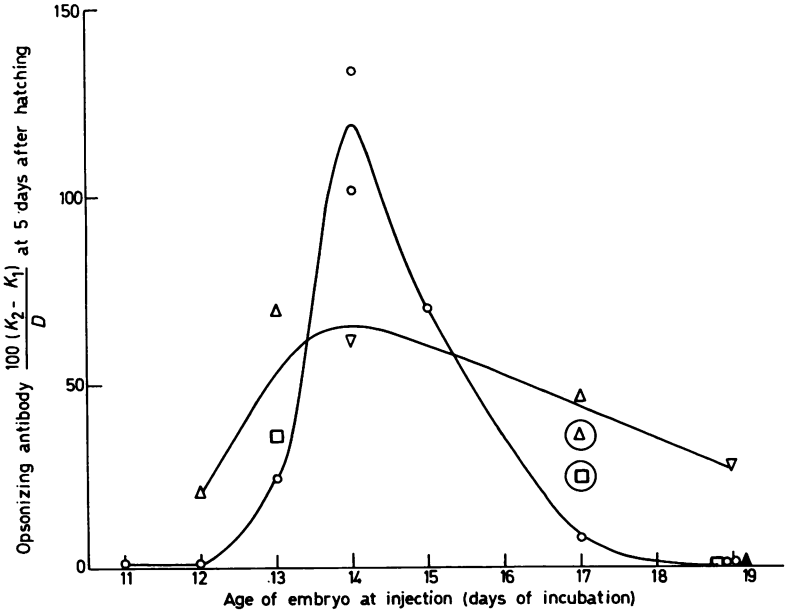

FIG. 2. Opsonizing antibody at 5 days after hatching produced by injecting chick embryos at various stages of development with goat erythrocytes. Doses of goat erythrocytes: ○, 1×10^4; □, 1×10^6; ▲, 1×10^8; ▽, 5×10^9; △, 1×10^{10}; guinea-pig erythrocytes, symbols inside circle.

It is believed that the inhibition during late embryonic life is caused by small amounts of maternal antibody and this was confirmed by the following experiment. Adult chicken serum (0·1 ml) containing IgG anti-goat antibody (titre 1 : 64) was diluted 1 : 4 and injected into 14-day-old embryos 2 hours before an immunizing dose of 3×10^8 goat erythrocytes. The antibody response measured at 5 days after hatching was completely suppressed.

In such developmental studies it is clearly desirable to follow the course of antibody production in order to assess any delay in the response. A large immunizing dose (1×10^{10}) of guinea-pig erythrocytes given to 17-day-old embryos produced only 9·3 units of opsonizing antibody 7 days later (3 days after hatching) but after a further 2 days 34·2 units had been produced.

In the above experiments no antibody had been measured in the embryo. Thirteen-day-old embryos were injected with 1×10^4 goat or guinea-pig erythrocytes and test injections of the appropriate erythrocytes were given at 4, 5 and 8 days after immunization. However, the clearance rates of the immunized groups were always considerably less than that of the controls. Not until 11 days after immunization (3-day-old chick) could opsonizing antibody be measured (11 units), during the next 2 days this rose to 25 units; in contrast, injection of the 15-day-old embryo produced minute amounts of antibody at hatching (Table 1).

TABLE 3

COMPARISON OF THE OPSONIZING AND AGGLUTINATING ABILITY OF SERUM FROM IMMUNE CHICKS

Age of chicks (days)	Reciprocal haemagglutination titres		Test dose of erythrocytes ($\times10^9$)	Mean K	$100(K_2-K_1)/D$
	37°	4°			
5 (normal)	<2	<2	5	0·189	60·2
5 (immune)*	2, <2	2, <2	5	0·791	
15 (normal)	<2	2	22	0·369	96·3
15 (immune)‡	3·5†	9·2†	22	0·588	
22 (normal)	2, <2	2	35	0·405	(1820)
22 (immune)§	27·9†	64·0†	35	(>3·000)	

* Immunized at 14 days of incubation with 1×10^{10} goat erythrocytes.
‡ Immunized at 8 days with 1×10^8 goat erythrocytes.
§ Immunized at 15 days with 2×10^8 goat erythrocytes.
† Geometric mean.

In order to compare the opsonizing and agglutinating abilities of anti-goat antibody, small amounts of serum were obtained from chicks before testing for antigen clearance. In order to compare the relative capacities of different age groups to clear erythrocytes from the circulation, opsonizing antibody was expressed as the amount required to clear a given test dose (5×10^9). The increasing ability of serum to opsonize and agglutinate during the early development of the chick is shown in Table 3. Serum from 5-day-old chicks, showed little if any agglutination although highly immune with respect to opsonization. Clearance of goat erythrocytes by immune 22-day-old chicks was so rapid that it could no longer be measured quantitatively (90 per cent of the test dose was removed in 20 seconds).

DISCUSSION

The sensitivity of the antigen clearance method lies in the measurement of the total immune opsonins in the circulation, whereas other methods depend upon the measurement of only aliquots of serum. This factor alone can increase the sensitivity by about 100-fold. However, this is partly offset by the enormous amount of antigen that needs to be given in a test dose in order that sufficient antigen may be detected in an aliquot of blood. Relatively high levels of immune opsonizing antibody may be measured before antibody is detectable by haemagglutination. This is not surprising as agglutination may require antibody for several antigenic determinants or a minimal level of antibody for a single determinant: whereas opsonization may involve the combination of only a few antibody molecules with one determinant (Rowley, Thöni and Isliker, 1965).

Early failures to induce antibody formation in the chick embryo (e.g. Burnet, Stone and Edney, 1950) could have been partly due to inhibition by maternal antibody. However, these failures played their part in fashioning the concept of immunological tolerance (Burnet and Fenner, 1949) although this concept, itself, obscured the embryo's ability to initially react immunologically to foreign antigens.

The choice of the goat erythrocyte as the immunogen in this work has been fortunate, as these erythrocytes are rapidly removed from the circulation of the embryo (Solomon, 1966) and are no longer immunogenic after ingestion by liver and spleen cells (Solomon, 1965). These erythrocytes are thus eminently suitable for determining at which stage of development induction of antibody formation actually occurs.

The present work shows that opsonizing antibody to goat erythrocytes may be measured as early as hatching and that antibody synthesis may be most readily induced at 15 days of incubation before the level of maternal antibody increases (Solomon, 1966). Induction of antibody can take place as early as 12 days of incubation which is as soon as, or even earlier than, lymphoid cells have been identified in the spleen (Sandreuter, 1951). This supports previous findings that transplantation immunity (measured by reduction of splenomegaly) is manifest in the chick embryo after 13 days of incubation (Solomon and Tucker, 1963a). Also, the embryo may be sensitized to foreign cells during embryonic life as evidenced by second-set homograft reactions (Solomon, 1963) and secondary responses to chicken erythrocytes (Solomon and Tucker, 1963b; Solomon, 1965) soon after hatching. Other evidence that the chick embryo is capable of furnishing an immune response is provided by van Alten and Schechtman (1963) who found that introduction of rabbit serum or human γ-globulin into 12-day-old embryos sensitized the embryos at some time before 3 days after hatching when anaphylactic shock was obtained following a second injection.

In the absence of maternal antibody, for example when chicken erythrocytes were used as antigen, semi-quantitative estimates of antibody levels indicated that there was a rapid maturation of response within 12 days after hatching (Solomon and Tucker, 1963b). However, the presence of maternal antibody in the embryo has been shown to inhibit antibody formation. It is significant that this inhibition could only be partially overcome by the use of very large immunizing doses of goat erythrocytes; in such experiments high levels of antibody were produced as early as 3 days after hatching. Within 3 weeks the chick is nearly mature as far as its IgM response to goat erythrocytes is concerned.

It is well known that antibody, passively administered before an immunizing injection of antigen, will suppress active production of antibody (Nossal, 1957; Uhr and Baumann, 1961; Neiders, Rowley and Fitch, 1962). Passive immunization of human babies results

in the inhibition of the active response to diphtheria toxoid (Barr, Glenny and Randall, 1950) and flagellar antigen of *Salmonella* (Smith and Eitzman, 1964); examples of similar inhibition in animals are given by Hoerlein (1957) and Dray (1962).

Similar inhibition has been found in chick embryos for protein antigens (Stevens, Pietryk and Ciminera, 1958; Hirata and Schechtman, 1960), *Shigella* vaccine (Friedman and Gaby, 1960) and heterologous blood cells (Billingham, Brent and Medawar, 1955; Hasek, 1956). Simonsen (1956) obtained the greatest suppression of antibody formation (at 6 weeks of age) when 19-day-old chick embryos were injected with human blood cells and only slight suppression at 15 days of incubation or at 3 days after hatching. It has been shown in this work that the immediate response to heterologous cells is the production of antibody; this is later followed by a state of partial tolerance. Maternal antibody may thus play a major role in not only suppressing the early immune response but also in 'switching off' cells which, under normal conditions, would later become immunologically mature thus resulting in a state of partial tolerance.

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