

THE PRODUCTION OF IMMUNOLOGICAL UNRESPONSIVENESS BY THE INTRAVENOUS INJECTION OF BOVINE SERUM ALBUMIN INTO THE CHICK EMBRYO*

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Received for publication February 27, 1958

MANY investigators have reported the successful reduction of the immunological response to soluble protein antigens (Hanan and Oyama, 1954; Dixon and Maurer, 1955; Cinader and Dubert, 1955; Smith and Bridges, 1956). These workers were able to bring about this effect by injecting large quantities of these antigens into foetal and new-born rabbits. Recently Wolfe *et al.* (1957) reported that by injecting newly hatched chicks (8–50 hr. of age) intraperitoneally with bovine serum albumin (BSA) antibody production to the homologous antigen was lessened. The duration and degree of this reduced responsiveness seemed to depend upon the amount of the original injection.

Simonsen (1956) was able to produce immunological unresponsiveness by injecting human erythrocytes directly into the chick embryo. He observed that this effect depended upon the day the initial injection was made and the interval between this injection and the challenging injection. Chick embryos injected prior to the 15th day of incubation failed to show "partial immunological tolerance". Animals injected between the 17–19th days gave the best results. Billingham, Brent and Medawar (1956) were able to prolong the life of tissue transplants by injecting Rhode Island Red (RIR) blood cells into 10–11-day White Leghorn (WL) chick embryos. The injection of human serum albumin into the allantoic cavity of chick embryos will produce partial tolerance (Stevens, Pietryk and Ciminera, 1958).

Hašek (1956) was unable to bring about a suppression of agglutinins when he intravenously injected chick embryos at 13 days with turkey, guinea-fowl or duck blood. The injection of goose blood into 15-day chick embryos led to a suppression of agglutinins but injections of duck embryos with chicken blood led to no suppression.

Cohn (1957) using several different antigens, reported that intravenous injection of 14-day chick embryos failed to inhibit the ability of these animals to respond to the homologous antigen when challenged at 10–12 weeks of age.

This investigation is concerned with immunological responsiveness of chickens as affected by injecting bovine serum albumin intravenously into the developing chick embryo.

MATERIALS AND METHODS

Arbor Acre White Rock chickens, approximately 50 per cent males, were used in these experiments. All embryos except 6 were injected on the 15th day of incubation; six were injected at 18 days. The experimental birds in Experiment I received 37.5 mg. (in 0.2

* This project was supported by the National Science Foundation.

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ml.) bovine serum albumin (BSA) supplied by Pentex, Inc., and those in Experiment II, 46.8 mg. (in 0.25 ml.) BSA. All injections were made into the chorio-allantoic vein of the embryos. These embryos were hatched and maintained in our laboratory until they were challenged and subsequently sacrificed.

Groups of previously embryonically injected and uninjected animals were inoculated intravenously with 40 mg. of BSA per kg. body weight (KBW) at 6 and 12 weeks of age. On the 4th and 6th days following the injection a small amount of blood was removed from the wing vein in both groups and the sera were tested for the presence of antigen or antibody. The presence of antigen was determined by the flocculation technique employing a strong homologous antiserum. On the 8th day all animals were bled by cardiac puncture and sacrificed. The serum was collected in the usual manner and allowed to stand for 3-4 weeks in the refrigerator at 4° for stabilization (Gengozian and Wolfe, 1957).

Where antibody was present the amount of antibody nitrogen was determined by the method developed by Heidelberger, *et al.* (1933). The reaction mixture of 1.0 ml. contained 0.25 ml. of antiserum, 0.25 ml. of the antigen solution in 8 per cent saline and 0.5 ml. of 11.5 per cent NaCl. This brought the salt concentration of the reacting mixture up to 8 per cent which was shown by Goodman, *et al.* (1951) to be optimal for the chicken system. The antigen concentrations employed were at 1 μ g. N intervals and were in the region of equivalence. These mixtures, run in duplicate, were incubated for 3 hours at 37° and then kept in the cold (4°) for approximately 18 hr. They were then centrifuged at 4° for 30 min. at 3000 r.p.m. The supernatant fluids were saved and tested for the presence of antigen and antibody. The precipitates were washed with 5.0 ml. of cold 8 per cent NaCl and recentrifuged. The nitrogen determinations of the precipitate were made by the method of Johnson (1940). The antibody nitrogen per ml. of serum was determined by using the region of slight antigen excess. When necessary, because of high antibody content, the antiserum was diluted. The determinations were then calculated using the formula devised by Gengozian and Wolfe (1956) for diluted chicken antisera. It was not possible to measure antibody contents accurately below 25 μ g. antibody N/ml. All sera tested that gave a positive flocculation test but contained less than 25 μ g. N/ml. were arbitrarily recorded as 15 μ g. N/ml. and this figure was used in the calculations.

All sera that were negative by the flocculation test (in 8 per cent saline) were retested using the interfacial test at a salt concentration of 2 per cent.

RESULTS

In Experiment I, 40 6-weeks-old Arbor Acre White Rocks were injected intravenously with 40 mg. KBW of BSA; the data for these are recorded in the table. Twenty of these birds received 37.5 mg. (in 0.2 ml.) BSA as embryos on the 15th day of incubation. Approximately 3.0 ml. of blood were drawn from several of the control and experimental animals on the 4th and 6th days. These sera were tested for the presence of antigen and antibody. The results of these tests indicated that the time of antigen disappearance and antibody appearance in the circulation was about the same in both groups. Antigen disappeared from the circulation sometime between the 4th and 6th day with antibody being observed in those animals producing antibody by the 6th day. The results of the 6-weeks-old group of animals indicate that it is possible to produce partial immunological unresponsiveness by this method. The average mean titre of the experimental animals was 108 ± 19 μ g. antibody nitrogen per ml. (N/ml.) as compared with the control values which averaged 197 ± 33 μ g. N/ml. of serum. When these two groups of animals were compared statistically using the *Z*-score test for significance the *P*-value was 0.0102 (Mode, 1951).

Similarly at 12 weeks of age another group of 40 animals was injected with 40 mg. BSA per KBW. This group consisted of an equal number of control and experimental animals. Eight days after the injection the experimental animals had antibody nitrogen values similar to those of the control birds. The mean

titre of the animals was $336 \pm 94 \mu\text{g. N/ml.}$ in contrast to $389 \pm 96 \mu\text{g. N/ml.}$ for the control animals. The two groups compared statistically by the *Z*-score test showed no significant differences ($P = 0.3483$).

In Experiment II all experimental embryos received 46.8 mg. (in 0.25 ml.) of BSA. At 6 weeks of age 13 birds injected at 15 days and 6 injected on the 18th day of embryonic development along with 19 control animals were injected with 40 mg./KBW of BSA. As in the previous experiment the time of antigen disappearance and antibody appearance was determined. The antigen disappearance in the experimental groups was the same whether the animals produced antibody or not. It was possible by the use of 46.8 mg. of BSA injected into the chick embryo to produce almost complete unresponsiveness in 6-weeks-old animals. Of the total of 19 experimental animals, 14 failed to produce antibody (10 were injected as 15-day and 4 as 18-day embryos). All of the negative animals were tested by both the interfacial and flocculation tests. In this group all of the control animals produced antibody. The mean titres for the experimental animals were $11 \pm 6 \mu\text{g. N/ml.}$ for the 15-day group and $25 \pm 17 \mu\text{g. N/ml.}$ for the 18-day animals. This was in contrast to $152 \pm 18 \mu\text{g. N/ml.}$ for the control group. When the two experimental groups were compared statistically with the controls, using the *Z*-score test for significance, they both had a *P*-value of < 0.0001 (see the table).

An additional group which received 46.8 mg. BSA on the 15th day of embryonation was challenged at 12 weeks of age. Here, as in the previous experiment, the control and experimental groups responded in a like manner. The mean titre was $304 \pm 68 \mu\text{g. N/ml.}$ and that for the control animals $317 \pm 74 \mu\text{g. N/ml.}$

A number of chickens were bled as late as 14 days after the challenging injection. There was no indication that a delayed response occurred.

TABLE.—*Bovine Serum Albumin Injected into Chick Embryos and the Statistical Analyses of the Antibody Response of these Animals*

Exp. No.	Preliminary treatment	Six weeks of age				Twelve weeks of age			
		No. of birds	Range ($\mu\text{g. N/ml.}$)	Mean \pm S.E.	Statistical evaluation*	No. of birds	Range ($\mu\text{g. N/ml.}$)	Mean \pm S.E.	Statistical evaluation
1	37.5 (in 0.2 ml.) on 15th day incubation	20	0†-260	108 ± 19	$Z=2.32$ $P=0.0102$	20	0§-1068	336 ± 94	$Z=0.39$ $P=0.3483$
	None (controls)	20	0‡-612	197 ± 33		20	64-792	389 ± 96	
2	48.6 mg. (in 0.25 ml.) on 15th day	13	0 -64	11 ± 6	$Z=7.38$ $P=0.0001$	13	15-704	304 ± 68	$Z=0.13$ $P=0.4483$
	48.6 mg. (in 0.25 ml.) on 18th day incubation¶	6	0**-96	25 ± 17	$Z=5.14$ $P=0.0001$				
	None (controls)	19	32-304	152 ± 18		16	0-1089	317 ± 74	

* Using *Z*-score test for significance and the single tailed test for probability.

† Two animals failed to produce antibody.

‡ Two animals failed to produce antibody.

§ One animal failed to produce antibody.

|| Ten animals failed to produce antibody.

¶ Only 6 chickens survived to be challenged.

** Four animals failed to produce antibody.

DISCUSSION

The results reported in this investigation compare favourably with our previous report of the effects of injected, newly hatched chicks (Wolfe *et al.*, 1957). We were unable to bring about complete unresponsiveness with the procedures we employed. In the two experiments reported here, the unresponsiveness produced was transitory. Experimental group I demonstrated partial unresponsiveness while in those in Experiment II this inhibition was almost complete at 6 weeks of age. However, by the 12th week this inability to respond was lost in both experimental groups and they responded as well as the control groups. It appears that the injection of 15-days-old chick embryos with the dosage used in these studies does not produce any greater degree of unresponsiveness or prolong the duration of this effect than a similar injection given soon after hatching (Wolfe *et al.*, 1957). In fact the duration is shorter since when injecting with large doses at post-hatching the effect lasted 12–22 weeks. These results with embryos compare with the results obtained by Simonsen (1956) and Stevens *et al.* (1958). They, too, were unable to bring about complete unresponsiveness and Simonsen showed that by the second month this inability to respond had disappeared.

However, these results differ from those reported by Cohn (1957). Certain reasons may account for Cohn's inability to alter the immune mechanism. The amount of antigen he injected into the chick embryo was small. In addition, he waited until the 10–12th week to challenge his animals and in view of the studies reported here and those of Simonsen (1956), this seems to be too long an interval to wait to test for immunological unresponsiveness. The day of injection may also be of significance, because Simonsen (1956) was unable to produce any effect earlier than the 15th day and Cohn chose the 14th day for his injections. Further work is needed on determining the time of the initial injection which will produce the maximum effect in chickens.

The rate of antigen disappearance in the partially or completely unresponsive animals was similar to the control animals. Antigen was present in all animals on the 4th day bleeding but was absent by the 6th day. No reason can be given for the rapid rate of disappearance in the experimental animals. Preliminary tests do not indicate the presence of incomplete antibody. This is not in agreement with other workers who worked with rabbits (Hanan and Oyama, 1954; Cinader and Dubert, 1955; Smith and Bridges, 1956). They all reported a slower disappearance of antigen from the circulation in their non-responding animals. It should be noted that these other workers used mammals.

SUMMARY

A reduced immunological responsiveness at 6 weeks of age was produced in chickens when 15-days-old embryos were intravenously injected with 37.5 mg. BSA and almost a complete inhibition when the dosage was 46.8 mg. BSA. This effect disappeared by the 12th week.

The time of antigen disappearance from the circulation of the experimental groups was similar to the control group, whether or not the experimental animals produced antibody.

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