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The Secondary Response of Chickens Given a Primary Inoculation of Bovine Serum Albumin at Different Ages (1)

By:

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It is a well known fact that often when an animal is given a second injection of an antigen to which it has previously been exposed, antibodies will appear earlier in the circulation and the titer of the antiserum will at any given time following the second injection be higher than after the primary one. Reviews referring to this phenomenon can be found in BURNET AND FENNER'S (1) monograph and in an article by FREUND (2).

MACLEOD et al. (3) have recently reported on the response to poliovirus under different vaccination schedules at different ages. Their results indicated that infants (3 to 12 months of age) do not respond as well as children or adults to a primary inoculation but the differences are small. There were no significant differences after the booster dose. PERKINS et al. (4) found that in general infants vaccinated with a poliovirus vaccine intramuscularly at 10 weeks of age respond better to a booster dose than those starting at ages of 1 and 6 weeks. They conclude that immunization against poliomyelitis in the first few weeks of life is unsatisfactory.

There are many questions that have not been answered in regard to the nature of this "secondary" response. This paper attempts to clarify one of the points of interest. The chicken is used as an experimental animal because of its excellent response to certain kinds of antigen, particularly to bovine serum albumin.

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One explanation for the secondary response has as its basis the assumed or supposed existence of a functional immune mechanism at the time of the primary stimulus. It would infer that young animals whose immune mechanisms are only partially developed would probably not be sensitized in the same way as mature animals at the time of the primary stimulus. The primary response of the growing chicken has been studied in our laboratory. It has been shown that there is a steady increase in the ability of chickens to produce antibodies from hatching until about 22 weeks of age (5, 6). Using bovine serum albumin as the antigen and a quantitative assay method for antibody nitrogen we have found that twelve-week-old chickens are good producers but the titers are not as high as those produced by 22-week-old birds. Six-week-old animals produce antisera of relatively low titer. Two- and 3-week-old birds produce only very weak antisera and a large percentage yield no measurable amounts of precipitins. At 22 weeks of age, the chicken reaches serological maturity, and, though it may produce a larger quantity of antibodies with increasing age, the increase has been of doubtful statistical significance. The present experiments describe the secondary response of chickens receiving a primary stimulus at the ages of 20 days, 6 weeks and 12 weeks and a secondary homologous injection at 6, 12 and 22 weeks of age.

Methods and Materials

Arbor Acre White Rock (AAWR) chickens, a heavy breed, were used in these experiments. These were secured at one day of age from a local hatchery. The birds were almost entirely (97%) males. At 20 days of age, 37* of these chickens were injected intravenously with 40 milligrams of bovine serum albumin (BSA) per kilogram body weight (KBW). Thirteen of the chicks so injected and 35 others that had not been injected previously were inoculated at 6 weeks of age with 40 milligrams of BSA per KBW. At 22 weeks of age forty-eight birds were injected; among these were 11 that were injected at 20 days only, 11 that were injected at 6 weeks only, 13 that were injected at 12 weeks only, and 13 that were not previously injected.

All of the birds injected at 6 weeks or later were bled daily from day 4 to day 9 following the inoculation and then on either day 11 or day 12. The 22-week-old birds were also bled on day 15. Each bird was starved for 18 hours before a blood sample was taken from the wing vein. Bleedings of days 4, 5 and 6 were tested for both circulating antigen and antibody; later

* The number of animals referred to in all groups is the actual number used in the experiment. There some deaths due ot natural causes.

bleedings were tested for antibody only. Antibody content was determined by the quantitative precipitin method of HEIDELBERGER et al. (7). One ml. of reaction mixture with a final salt concentration of 8% (8) was made in the region of equivalence; each tube contained 0.25 ml. antiserum with BSA-antigen at one gamma intervals. High titered antisera were diluted and a correction factor was used in accordance with the findings of GENGOZIAN AND WOLFE (9). Mean antibody nitrogen values were compared for significant differences using the "student" — FISHER t-test (10) applied to the $\log_{10}$ of the original antibody N values expressed as μ g. Ab N/ml.

Results

Figure 1 is a graphic representation of the results. Included in the graphs are the controls that received only one injection. The abscissa shows the day of bleeding and ordinate, the corresponding mean antibody nitrogen values per milliliter of antiserum.

The chickens injected at 20 days of age and reinjected at 6 weeks responded in the same way as those injected for the first time at 6 weeks; figure 1 shows these results. It appears that the mean peak titer was reached one day earlier in the group of birds previously inoculated at 20 days but the difference in the titers for days 6, 7 and 8 and the differences in the maximum titers are not statistically significant. One of the birds injected at 20 days had a high antibody content at day 6 which decreased by the 8th day; its behavior resembled a typical secondary response.

The data for the animals given a secondary injection at 12 weeks of age are shown in fig. 1b. Antibody production appears to be the same in the three groups; that is, there are no statistically significant differences among them. The group injected at 20 days produced the lowest average titer; those injected at 6 weeks and reinjected at 12 weeks of age showed the same response as those injected at 12 weeks only.

Four groups of chickens were injected with 40 mg. of BSA per KBW at 22 weeks of age. These included one group of birds previously injected at 20 days, another previously injected at 6 weeks, a third previously injected at 12 weeks, and a fourth that had not previously been injected. The results are shown graphically in fig. 1 c and the statistical analyses are recorded in table I. Because of the great variation (table I) in response, the $\log_{10}$ of the antibody nitrogen values were used for statistical analysis. Those birds previously injected at 20 days and the controls reacted quite similarly. The controls showed higher average antibody nitrogen values on days 7 through

11 post injection but the differences are not statistically significant. Only one bird in each of these two groups had circulating antibody on the fifth day but all contained circulating antibody on the sixth day. The peak of

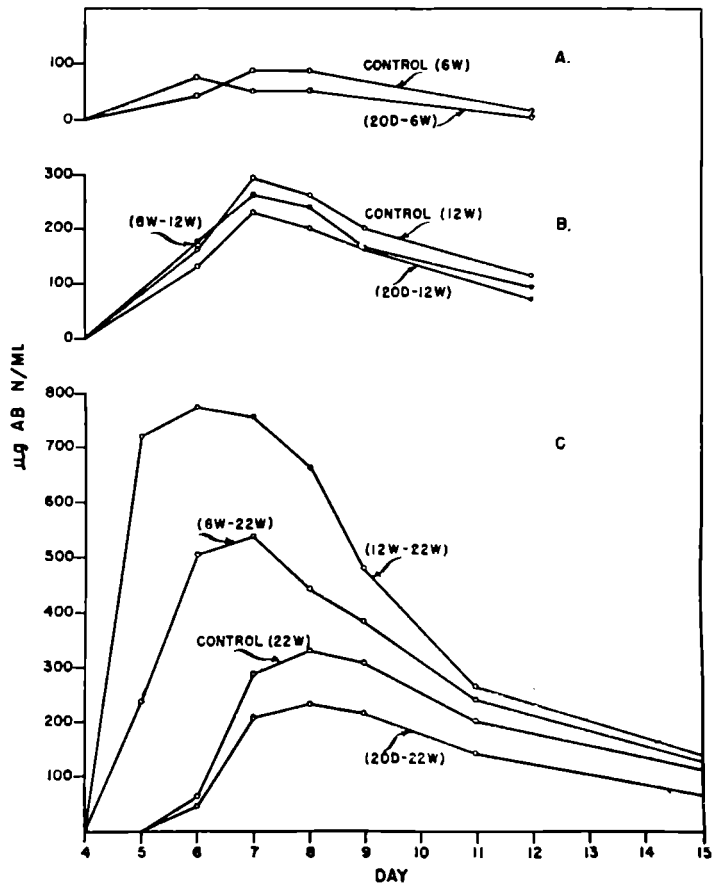

Fig 1. The antibody production curve of chickens injected at various ages with 40 mg. bovine serum albumin. Number in parenthesis is the age at which the birds were given the intravenous injection (s).

the mean titers was found in both groups on the eighth day following the injection.

On the other hand some of the chickens previously injected at 6 weeks and all of those previously injected at 12 weeks and reinjected at 22 weeks gave a definite secondary response. High titered antisera were found on the 5th day post injection and the peak mean titer was reached on the

6th day among birds previously injected at 12 weeks and reinjected at 22 weeks. These showed highest mean titer on the sixth day but this was only slightly different from that of the fifth day. Birds injected at 6 weeks and 22 weeks showed a peak mean titer at 7 days. This peak was only slightly greater than the average on the sixth day but much higher than the mean titer of the fifth day. When the titers of antisera of chickens injected at 12 and reinjected at 22 weeks are compared with antisera produced in the other 22-week-old groups statistically significant differences occur in all but two instances (exceptions are the 7 and 9 day bleedings of the 6-and 22-week-old group).

The graphic representation of the data in fig. 1 and the statistical analyses given in tab. I show only a partial picture of the secondary response. The characteristics of the secondary response are an earlier disappearance of antigen from the circulation, an earlier appearance of antibody, and usually a higher titered antiserum. Tab. II records the data for the time of disappearance of antigen and the appearance of antibody in the circulation. The data for the different groups of chickens given a second inoculation at 6 or 12 weeks of age show practically no difference in these characteristic responses; on the other hand, the 22-week-old birds showed much variation. On the fourth day following the injection all control chickens and all chickens previously injected on the 20th day post-hatch had circulating antigen. Of the eleven chickens that were given a primary injection at 6 weeks and a secondary injection at 22 weeks, 8 had circulating antigen and 3 did not; 1 of the latter had antibody. Of the 12 chickens previously injected at 12 weeks, only 2 had circulating antigen and 5 had circulating antibody present on the 4th day. The fifth day bleeding shows the results more dramatically. All of the 13 chickens previously injected at 12 weeks contained antibody in their circulation on this day, as did 5 out of 11 of those previously injected at 6 weeks. Five of those previously injected at 6 weeks of age still had circulating antigen. All but one of the 13 controls and 9 of the 11 birds previously injected at 20 days had circulating antigen.

The above clearly indicates (using antigen disappearance as a criterion that some of the chickens previously injected at 6 weeks and reinjected at 22 weeks gave a primary type of response while others gave the secondary type of response. All the chickens injected at 12 weeks and reinjected at 22 weeks gave a typical secondary response.

It should be stated that there was a great deal of variation among the antibody responses of the various animals. The highest value secured was 3000 micrograms antibody nitrogen per milliliter serum; this was produced by an animal that was primarily injected at 12 weeks and reinjected at 22

Table I

The secondary response of chickens given a primary inoculation at different ages.

Age at time of injections	Day of bleeding	Ab μg N/ml Mean ± S.E.	Range	Statistical significance of differences between means of $\log_{10}$ sample values (P values)		
		6 weeks groups				
20 days and	6	75 ± 34	15—376	No statistical significant difference between		
6 weeks	7	55 ± 15	15—164	20 day-6-week group and 6 weeks		
(13)*	8	52 ± 12	15—152			
6 weeks	6	42 ± 13	0—135			
only	7	90 ± 19	15—236			
(13)	8	91 ± 14	15—172			
		12 weeks groups				
20 days and	6	131 ± 45	0—492	No statistical significant differences among		
12 weeks	7	239 ± 63	0—549	3 groups injected at 12 weeks.		
(13)	8	205 ± 55	0—569			
	9	168 ± 46	15—449			
6 weeks and	6	176 ± 40	15—382			
12 weeks	7	264 ± 34	100—392			
(11)	8	241 ± 25	100—357			
	9	169 ± 20	56 —320			
12 weeks	6	168 ± 39	0—540			
only	7	296 ± 45	40—666			
(15)	8	262 ± 28	108—492			
	9	206 ± 26	68—387			
				6 and 22 weeks	12 and 22 weeks	22 weeks only
		22 weeks groups				
20 days and	6	47 ± 17	0—140	.045	.0001	.818
22 weeks	7	202 ± 52	0—444	.041	.0004	.560
(11)	8	229 ± 48	15—511	.126	.0004	.841
	9	208 ± 39	15—521	.099	.0060	.880
6 weeks and	6	502 ± 245	0—2644		.014	.058
22 weeks	7	534 ± 200	96—2367		.054	.144
(11)	8	438 ± 153	108—1867		.024	.197
	9	375 ± 112	108—1396		.2186	.141
12 weeks and	6	771 ± 193	274—2698**			.0001
22 weeks	7	757 ± 150	301—2375			.0036
(13)	8	664 ± 148	299—2336			.0010
	9	479 ± 107	226—1512			.0104
22 weeks only	6	60 ± 23	0—252			
(13)	7	283 ± 120	0—1637			
	8	329 ± 123	15—1730			
	9	301 ± 119	15—1652			

* Number in parenthesis is number of animals.

** On day 5 this animal had antibody content of 3000 μg. Ab N/MI.

Table II

Antigen disappearance from, and antibody appearance in circulation of chickens receiving a secondary inoculation of 40 mg. bovine serum albumin intravenously in one injection.

Groups	Day of bleeding following injection 3	4	5	6	7	
			Injected at 6 weeks of age			
days and	13 ag+	12 ag+,	1 ag—	8 ag+,	5 ag—	13 ag—		13 ab+	
weeks (13)		1 ab—		5 ab—		10 ab+,	3 ab—		
weeks only				8 ag+,	5 ag—	13 ag—			
(13)*	13 ag+	13 ag+		5 ab—		11 ab+,	2 ab—	13 ab+	
			Injected at 12 weeks of age						
days and	13 ag+	13 ag+		11 ag+,	2 ag—	13 ag—		13 ag—	
weeks (13)				2 ab+		8 ab+,	5 ab—	12 ab+,	1 ab—**
weeks and	11 ag+	10 ag+,	1 ag—	10 ag+,	1 ag—	11 ag—			
weeks (11)		1 ab+		1 ab+		11 ab+		11 ab+	
weeks only	15 ag+	15 ag+		13 ag+,	2 ag—	15 ag—		15 ab+	
(15)				1 ab+,	1 ab—	14 ab+,	1 ab—		
			Injected at 22 weeks of age						
0 days and	11 ag+	11 ag+		9 ag+,	2 ag—	11 ag—			
2 weeks (11)				1 ab+,	1 ab—	11 ab+		11 ab+	
6 weeks and	11 ag+	8 ag+,	3 ag—	5 ag+,	6 ag—	11 ag—		11 ab+	
2 weeks (11)		1 ab+,	2 ab—	5 ab+,	1 ab—	11 ab+			
2 weeks and	13 ag+	2 ag+,	11 ag—	13 ag—					
2 weeks (13)		5 ab+,	6 ab—	13 ab+		13 ab+		13 ab+	
2 weeks only	13 ag+	13 ag+		12 ag+,	1 ag—	13 ag—			
(13)				1 ab+		11 ab+,	2 ab—	12 ab+,	1 ab—***

* Number in parenthesis is number of animals.

** Responded weakly on 9th day.

*** Responded weakly on 8th day.

weeks. But even in the group to which this individual belonged there were animals that did not give titers higher than 400 micrograms antibody N/ml. of serum.

Discussion

The experiments described in this paper evaluating the basis for the secondary response (also called the homologous anamnestic response), strongly suggest that in the chicken the secondary response to bovine serum albumin

is dependent upon the age of the animal at the time of the primary response. They also permit the inference that the mechanism for the production of antibodies must be well developed at the time of the primary exposure to the antigen if a secondary response is to appear. The criteria used here for recognizing a typical secondary response are 1) an earlier disappearance of antigen, 2) an earlier appearance of antibody, 3) an earlier peak titer. Often the titer resulting from the secondary inoculation is much greater than that following a primary injection.

The dosage (40 mg. BSA/KBW in a single intravenous injection) of antigen used was constant in all the experiments. This amount of antigen has yielded antisera of high titer when injected into adult chickens in our earlier experiments (6, 11). The same dosage has also resulted in typical secondary responses when the adult animals were reinoculated at a later date (unpublished data). Our present results show that chickens, 6 weeks of age or younger, produce low titered antisera thus verifying our previous reports (5, 6). The chickens injected at 20 days of age failed to yield the secondary response when reinoculated at 6, 12 or 22 weeks of age (one possible exception). Only a small number of the chickens inoculated at 6 weeks gave the secondary response at 12 or 22 weeks of age. The three 6-week-old birds that gave a good secondary response at 22 weeks were the ones that had given higher antibody titer than the other 8 of the group at 6 weeks of age. On the other hand, all of the chickens injected at 12 weeks of age gave the characteristic secondary response when reinoculated at 22 weeks of age.

The heightened response that results from a secondary injection has been attributed to the sensitization of the immune mechanism by a previous homologous inoculation. But since a secondary response results, it must be assumed that at the time of the primary injection an animal must have had an immune mechanism. Our results further suggest that the degree of development of this mechanism is important. Incomplete or a partial development is not sufficient to insure the secondary effect since a large percentage of the chickens that were 6 weeks of age or younger did not respond with a typical anamnestic response at an older age.

HOFSTAD (12) vaccinated chicks with 1 or 2 doses of inactivated Newcastle virus at different ages, and subsequently challenged them with the live virus. The vaccinations were made with an alumina-gel containing formalin-inactivated virus. The singly vaccinated chicks were inoculated at either 2 days or 4, 8, 12, 16 or 20 weeks of age. The doubly vaccinated animals were all given the initial inoculation at 2 days and then revaccinated at the weeks listed above. A challenging dose of live virus was given 16 weeks after the vaccinated dose was completed. The resistance of the chicks given

two vaccinations increased steadily from the 4th up to the 12th week and remained high with the 16-and 20-week-old groups. On the other hand a single vaccination given at 4 and 8 weeks resulted in a rather high survival of chicks when challenged 16 weeks later but those given a single vaccination at either 2 days or 12 weeks or 16 or 20 weeks had a low survival. HOFSTAD stated, "...the degree of immunity obtained following two doses of inactivated Newcastle disease vaccine depends to a great extent upon the interval between doses". He further states, "In addition the data indicate that an initial dose given at 2 days of age prepares the birds for an anamnestic immune response to a subsequent dose of vaccine which is comparable to the response observed previously when the initial dose was given at 3 weeks of age."

HOFSTAD's findings that the longer the interval between doses resulted in greater anamnestic response is contradictory to our data. The results that we report do not show this with the age groups used. The interval between the 12-week-old groups and the 22 week was ten weeks and this group gave a better anamnestic response than the animals injected at 6 weeks and reinjected at 22 weeks. We also have evidence (unpublished data) that when adult chickens are given a primary inoculation of bovine serum albumin and a secondary injection at varying intervals there is a typical secondary type response when the intervals range from 1 month to about a year.

It is difficult to try compare and assess the results of our work with those of HOFSTAD. The antigens used, the injection procedures, and the end point of the reaction were all different. It is quite possible that the use of a particulate antigen such as a virus with an adjuvant can lead to an anamnestic response when the antigen is given at a very early age.

SMITH AND BRIDGES (13) found that 50% of the rabbits injected with a massive dosage of bovine serum albumin at 15 days of age failed to produce antibodies when reinjected at 4 months, but 50% of those that did respond had titers 2-5 times the control group. They state, "These data suggest that in rabbits a period of life from 3-15 days is marked by a transition from a state in which exposure to heterogenous protein will condition specific failure of response to one in which a specific response is induced." This indicates the development of the mechanism for the immune response occurs earlier in the rabbit. On the other hand, the injection of massive dose at the early age may have been the significant factor in the two types of responses that they secured.

Summary

The age at which chickens (also apparently the strength of the first

response) are given a primary inoculation of 40 mg. bovine serum albumin per KBW determines whether or not a secondary response can be produced. Chickens injected at 20 days of age do not give a secondary response if reinoculated at 6, 12 or 22 weeks of age. A small percentage of chickens injected at 6 weeks of age give a secondary response at 12 and 22 weeks of age but chickens given a primary inoculation at 12 weeks of age give a good secondary response when reinjected at 22 weeks of age.

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