

Antibody Formation in Germfree Chickens¹

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Introduction

Germfree animals are characterized by an underdevelopment of the lymphoid tissues (2). In the germfree chicken, fewer plasma cells and secondary nodules are found, and the serum gamma globulin concentration is less than that found in the conventional bird (6). The available lymphoid tissue has, by virtue of the germfree state, never been exposed to the specific and non-specific stimuli of a viable microbial flora and its metabolites. In this study we investigated how such a tissue will react to the challenge of a single injection of bovine serum albumin (BSA). This entailed a comparison of anti-BSA precipitin production and serum gamma globulin levels in germfree and conventional chickens. Later, in one of the experiments, we also determined the turnover of gamma globulin in both groups after *in vivo* labeling with glycine-C.¹⁴

Materials and Methods

Adult 90-110 days old male and female white Leghorn germfree and conventional chickens derived from the same egg clutches were sensitized with 40 mgm BSA/Kgm body weight. For one month the animals were bled on subsequent designated days from the cupital vein to obtain 1 ml of anti-BSA chicken plasma.

Antibody determination was done using the Preer double diffusion agar gel technique (5). The data obtained with the Preer technique was converted to a quantitative mgm% antibody basis via the following standardization procedure. In one experiment, germfree and conventional anti-BSA chicken plasmas were collected on designated days in a sufficient amount to enable both Preer tests and quantitative precipitin tests to be made. The quantitative precipitin tests were done in the manner described by Banovitz et al. (1). For the Preer test, all antiplasmas were tested with three dilutions of BSA (6.0×10^{-2} mgm BSA/ml, 3×10^{-2} mgm/ml and 1.5×10^{-2} mgm/ml) in triplicate to insure obtainment of proper antigen/antibody rates. The antigen dilution that produced satisfactory Preer factors for all antiplasma samples was chosen for correlation with the quantitative precipitin results. Then, the Preer factor was plotted on semi-log paper against the quantitative amount of antibody for each antiplasma sample to establish the relationship. The values obtained with the germfree and with the conventional chickens showed the same straight line relationship. Hence, Preer factors (obtained with that antigen dilution) for all anti-BSA chicken plasmas obtained during the course of antibody production were transposed to mgm% antibody.

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Electrophoresis to determine gamma globulin fluctuations during the antibody production period was done using cellulose acetate paper and a Sodium Barbitol buffer of pH 8.6 and ionic strength of 0.06. Recordings were made using the Photovolt Densitometer and values were converted to mgm% protein. Protein values were determined by the Kagen falling drop method.

Half-life determination of conventional and germfree chicken gamma globulin was done *in vivo* using glycine 1-C-14. Eighty microcuries were injected into the capital vein and animals were bled on 1, 2, 3, 5, 7, 9 and 12 days after injection. All plasma samples were fractionated at 33 $\frac{1}{3}$ % $(\text{NH}_4)_2\text{SO}_4$ saturation to obtain the gamma globulin. This fractionation process was repeated four times and the final product was tested for purity by agar gel electrophoresis. The gamma globulin was dialyzed against 1% NaCl at 4°C until absolutely free of $(\text{NH}_4)_2\text{SO}_4$. Measured aliquots were precipitated on planchets under infra-red light. Radioactivity was determined and protein nitrogen of the sample was determined by Kjeldall digestion and Nesslerization, and results recorded as cpm/mgm protein. All values were recorded on semi-log paper to determine the gamma globulin half-life.

Results

The data on anti-BSA precipitin production are depicted in Figure 1 and Table 1. They show that in the conventional group, precipitating

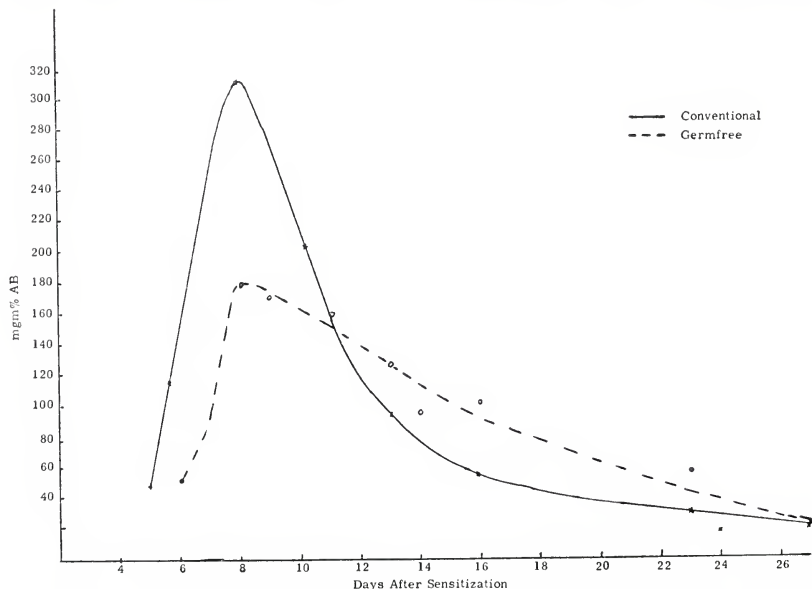


Fig. 1. Comparison of anti-BSA precipitin production in germfree and conventional chickens.

antibodies are detected on the fifth day after BSA administration, presumably the time when detectable amounts of BSA have been eliminated

TABLE 1
Ratio of Number of Positive Precipitin Plasmas to Total
Number of Samples*

Days After Sensitization	Germfree	Conventional
4	0/6	0/12
5	0/6	8/15
6	5/11	15/15
7	11/11	15/15
9	12/12	14/14
13	7/7	14/14
16	7/7	13/14
20	7/7	12/14
27	2/6	4/8

* Numerator=number of positive precipitin plasma.

Denominator=number of animals bled.

from the circulation. Peak titers are reached 3 days later, after which antibody production declines rapidly. This response agrees entirely with the findings of Wolfe et al. (9) and Patterson et al. (4). In the germ-free birds the antibody can be detected by the sixth day, but peak titers are reached at approximately the same time after sensitization as in the conventional chickens. Possibly in agreement with the lower amount of lymphoid tissue originally present, peak titers reach about one-half the values found in the conventional animals. However, decline of antibody concentration is much slower in the germfree birds.

The total increase in gamma globulin over presensitization levels, brought about by the BSA challenge, presents a different picture than in the case of precipitating antibody. Within the limits of error the response in both groups seems comparable (Table 2). Comparing antibody and gamma globulin production especially at peak titer, obviously less of the gamma globulin increase can be accounted for as a precipitating antibody in the germfree group.

After peak titers have been reached, both gamma globulin content and antibody titers decrease, but the decline in antibody titer was found to be much slower in the germ free than in the conventional groups. A determination of the half-life of gamma globulin during a subsequent experiment depicted no difference in metabolic degradation of this protein. Within the limits of error, the values were the same in both groups with 4.2 days for germfree and 4.3 days for conventional birds.

The slopes of the precipitin curves (Fig. 1) taken between the eighth and twentieth days indicate the difference in recession of antibody titers. When charted on semi-log paper they reveal that the half value factor of 7.5 days for germfree animals is twice that of the conventional animals (Fig. 2) while the factor for the latter group agrees within limits of error with the half-life of the gamma globulin for both groups.

TABLE 2

Gamma Globulin and Precipitating Antibody (Ab) After Sensitization With 40 mgm BSA per Kgm Body Weight. Number of Observations in Parenthesis. S.D.M. Values Given.

Day	Germfree				Conventional			
	y glob.	Δ y glob.*	Ab	Ab/ Δ y glob.	y glob.	Δ y glob.*	Ab	Ab/ Δ y glob.
	mg%	mg%	mg%	%	mg%	mg%	mg%	%
0	469 (5) ± 36	0	0	...	862 (5) ± 47	0	0	...
4	643 (5) ± 50	174	0 (6)	0	947 (5) ± 111	85	0 (12)	0
8	865 (5) ± 90	396	179 (7) ± 27	45.5	1314 (5) ± 117	479	311 (14) ± 45	64.5
16	697 (5) ± 56	228	101 (7) ± 24	44.3	1070 (5) ± 119	208	53 (13) ± 9.4	25.5

* Increase in gamma globulin level over 0 day value.

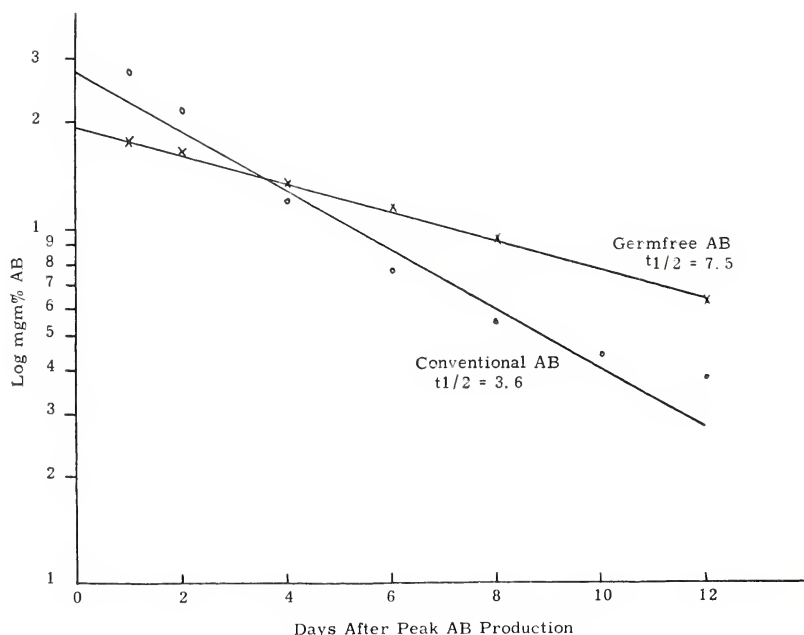


Fig. 2. Comparison of half value factors for degradation of BSA precipitin antibodies in germfree and conventional chickens.

Discussion

Previous literature reports that in the germfree chickens the reticuloendothelial system (RE) is deficient to the extent that less

plasma cells and less secondary nodules are found in the spleen, liver, thymus, ileocecal-colic junction and ceca (6). These underdeveloped potentialities may be interpreted to mean that germfree animals have less ability to react to an antigenic stimulation and therefore may be more susceptible to a microbial attack. Early research with chickens monoassociated with *Streptococcus fecalis* and *Clostridium perfringens*, demonstrates that the RE system can react to such stimulation and provide protection, but the sequence of cellular and humoral changes leading to this protection was not recorded (7, 10). Figure 1 and Table 1 both demonstrate the duration and magnitude of circulating precipitin titers in germfree and conventional chickens, sensitized to a relatively simple antigen, and show that the response of the humoral defense mechanism of the germfree birds differs from that in conventional animals in three aspects.

First, the conventional animals are able to produce a detectable level of precipitins quicker than the germfree chickens. This 24 hour difference in detection could be attributed to the original underdevelopment of the RE system of the germfree and the actively conditioned state of the conventional system. The conventional RE system has been subjected to antigenic attack since the early days of life and consequently many of its more exposed elements have obtained a well developed cellular level in comparison to those in the system of the germfree birds. It is recorded that antibody production is dependent upon the mitotic division of immature pyroninophilic cells and their subsequent development into antibody producing entities (8). Hence, in conventional animals, this mitotic division of antibody forming cells with the resulting geometric progression may furnish a detectable antibody level earlier than does the germfree's lower quantity of untrained cells.

The second difference in the antibody response is concerned with the quantity of precipitins produced at the peak of circulating antibody response. Although the germfree group demonstrates a 24 hour delay in detectable response, it reaches its peak titer on the eighth day as does the conventional animal, but at that time depicts approximately one-half the amount of antibodies produced in the conventional system. Again this difference suggests a greater proliferation of cells with a specific anti-BSA antibody potential in the conventional animals.

The third distinction between the two responses is concerned with the disappearance of detectable antibodies from the circulation. Here the conventional group follows the well defined literature values of declining sharply in concentration right after the day of peak titer, but in the germfree animals the antibody concentration shows a more gradual decline. The conventional half value factor falls within the determined gamma globulin half-life time of four days, while the half value factor for the germfree animals shows a much higher value of 7.5 days. Antibody degradation thus becomes quite evident in both groups after the eighth day but the slope of the lines in Figure 2 suggests that as antibody production virtually ceases in the conventional group, it continues to a certain extent in the germfree chickens.

One possible explanation for this difference is that the RE system of the germfree BSA sensitized animal reacts to only one antigen, and

once this antibody producing mechanism is stimulated it will produce until it becomes metabolically inert, whereas the RE system of the conventional bird is constantly being subjected to environmental antigenic stimulations. Therefore, some of the potential BSA-antibody producing cell lines may encounter an additional, more demanding antigen during or following mitotic division, which suppresses the BSA-antibody instruction, and stimulates the cell line to produce a second type of antibody.

This reasoning is in accordance with the concept that a cell can only produce one antibody at one given time (3). Hence, the conventional system becomes deprived of an elevated, longer lasting antibody titer because of competitive interactions of different antigenic stimuli for the existing or potential antibody producing cells.

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