*Immunology*, 1968, **14**, 21.

# Studies on the Control of Antibody Synthesis

## EFFECT OF ANTIBODY AFFINITY UPON ITS ABILITY TO SUPPRESS ANTIBODY FORMATION

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(*Received 6th March* 1967)

**Summary.** Suppression of anti-DNP antibody formation by passively administered anti-DNP antibody was studied quantitatively. The ability of an antiserum to suppress antibody formation was found to be related to the affinity of the antibody for the homologous antigenic determinant (ε-DNP-L-lysine), high affinity antibody being capable of causing suppression at far lower concentrations than low affinity antibody. In addition, very low concentrations of high affinity antibody were found to bring about enhancement of antibody formation. The results are discussed with respect to the significance of circulating antibody in the control of antibody synthesis.

Partial suppression of antibody formation by a single injection of passive antibody slightly lowered the affinity of the antibody synthesized. The affinity of the antibody synthesized was independent of the affinity of the passive antibody used to bring about partial suppression.

## INTRODUCTION

It is clear from the work of a number of investigators that passively administered antibody can specifically depress the immunological response to concomitantly administered antigen (Glenny and Südmersen, 1921; Park, 1922; Osborn, Dancis and Julia, 1952; Uhr and Baumann, 1961; Rowley and Fitch, 1964; Sahiar and Schwartz, 1964; Smith and Eitzman, 1964; Crowle and Hu, 1965; Möller and Wigzell, 1965). As originally suggested by Uhr and Baumann (1961), it appears probable that circulating antibody serves as one of the mechanisms involved in the control of antibody synthesis. While the mechanism of the suppressive effect of passive antibody upon antibody synthesis has not been definitively determined, it is generally assumed that suppression is the result of the binding of antigen by circulating antibody. If this is correct, then the ability of an antibody to suppress should be related to both its concentration and affinity. It has been shown that antiserum produced late in immunization is more effective in bringing about suppression than is antibody formed early in the immune response (Finkelstein and Uhr, 1964; Wigzell, 1966). This might be related to the increased affinity of antibody formed late in the course of immunization (Eisen and Siskind, 1964).

The present study was undertaken to evaluate quantitatively the relationship between antibody affinity and its ability to suppress antibody formation.

In addition, the affinity of the antibody synthesized by a partially suppressed animal was determined and any effect of the affinity of the passive antibody on the affinity of the antibody synthesized was looked for.

## MATERIALS AND METHODS

*Dinitrophenylated protein*

Dinitrophenylated bovine γ-globulin (DNP-BGG) was prepared by the method of Eisen, Belman and Carsten (1953) by reacting the protein with 2,4-dinitrobenzene sulphonic acid under alkaline conditions. In order to obtain a highly substituted product, the reaction was carried out overnight at 37°. The dinitrophenylated protein was purified by repeated acid precipitation and extensive dialysis. DNP-BGG concentration was determined by drying known volumes of protein solution to constant weight at 95–100°. The 'dry weights' were corrected for the weight of buffer present. The extent of substitution with DNP groups was estimated spectrally. It was assumed that all DNP groups were coupled to ε-amino groups of lysine and that their absorbance in the protein was equal to the molar absorbancy of free ε-DNP-L-lysine (17,530 at 360 mμ) (Ramachandran and Sastry, 1962).

One preparation of DNP-BGG was used as immunizing antigen throughout this work. This preparation had approximately sixty DNP groups per mole of protein. With this highly substituted antigen, no antibody was formed which was precipitable by native BGG and the amount of precipitate obtained with DNP-bovine fibrinogen (DNP-BF) was the same as that obtained with the immunizing antigen.

Highly substituted DNP-BF was prepared by the reaction of bovine fibrinogen with 2,4-dinitrofluorobenzene at room temperature under alkaline conditions. The conjugated protein was purified by extensive dialysis. Spectrally these preparations were found to have approximately 150 DNP groups per mole of protein.

*Immunization of rabbits*

New Zealand rabbits (2–2·5 kg) were used throughout. In all experiments rabbits were immunized by a single injection of 5 mg DNP-BGG emulsified in complete Freund's adjuvant. The antigen was given in a total volume of 2·5 ml of emulsion divided among five sites: the four footpads and the subcutaneous tissues in the back of the neck. Animals in which the suppressive effect of antiserum was to be tested were injected intravenously with an appropriate volume of rabbit anti-DNP antiserum 1 day before antigen injection.

Pools of anti-DNP antiserum of widely differing affinities were prepared by bleeding groups of fifteen to twenty-five rabbits at various times after immunization. For these pools, animals were bled by cardiac puncture daily for 3 days, and the serum pooled. Antibody was assayed by quantitative precipitation reaction using DNP-BF as precipitating antigen (see below). Anti-DNP antibody was purified from a small aliquot of each pool and its affinity determined (see below). Three pools of antiserum were used in these experiments. They were prepared and characterized as indicated in Table 1. Although association constants were determined using purified antibody which represents only approximately 40 per cent of the total antibody present in the serum, it has been shown by Eisen and Siskind (1964) that such purified antibody preparations have essentially the same average association constant for ε-DNP-L-lysine as does the total antibody present.

*Precipitin curves*

Antibody concentrations were determined by quantitative precipitin reactions according to the methods of Farah, Kern and Eisen (1960). DNP-BF was used as precipitating

TABLE 1
CHARACTERISTICS OF ANTISERUM POOLS

| Pool No. | Time of bleeding (days) | Antibody concentration (mg/ml) | Affinity* (l/mole) |
|---|---|---|---|
| VIII | 27–29 | 2·04 | $6{\cdot}1 \times 10^7$ |
| IX | 9–11 | 1·01 | $1{\cdot}9 \times 10^6$ |
| X | 56–58 | 2·11 | $\sim 10^{11}$ |

* Association constant ($K_0$) in l/mole for the binding of ε-DNP-L-lysine measured at 21° in 0·15 M NaCl, 0·01 M phosphate buffer, pH 7·5.

antigen. Reaction mixtures were incubated at 37° for 1 hour and then held overnight at 4°. Precipitates were collected by centrifugation, washed and dissolved in 0·02 M sodium lauryl sulphate. Antibody present in the precipitates was assayed spectrally from their absorbancy at 278 and 360 mμ. The 360-mμ absorbancy was used to correct for antigen present in the precipitate. $E_{1\,cm}^{1\%}$ at 278 mμ for rabbit anti-DNP antibody was taken as 14·0.

*Purification of antibody*

Anti-DNP antibody was purified by the method of Farah *et al.* (1960). Briefly, this involves extraction of antibody with 0·1 M 2,4-dinitrophenol, in the presence of 35 mg/ml streptomycin sulphate, from a specific precipitate formed at equivalence with DNP-BF. The extracted antibody is then purified by dialysis and passage through Dowex I anion exchange resin.

*Measurement of antibody–hapten association constants*

Antibody–hapten association constants were determined by the method of fluorescence quenching developed by Velick, Parker and Eisen (1960). The technique of titration and method of calculation were identical to that described in detail by Eisen and Siskind (1964) using the same hapten–antibody system as employed in the present work. All association constants presently reported are for the binding of ε-DNP-L-lysine by antibody at 21° in 0·15 M NaCl, 0·01 M phosphate buffer, pH 7·5.

## RESULTS

### EFFECT OF ANTIBODY AFFINITY ON ITS ABILITY TO SUPPRESS ANTIBODY FORMATION

The ability of various doses of anti-DNP antisera, of different affinities, to suppress active antibody synthesis was determined. In each case passive antiserum was administered 1 day before antigen injection. The results are displayed in Table 2. It is clear that the higher the affinity of the passive antibody the greater is its ability to suppress active antibody formation. With each antiserum tested, regardless of affinity, the degree of suppression is dose-dependent: a larger dose of passive antibody resulting in a more profound and longer lasting suppression.

From Table 2 it is clear that with an antiserum of intermediate affinity ($K_0 = 6{\cdot}1 \times 10^7$) 20·4 mg of passively administered antibody markedly suppressed antibody synthesis to DNP. Following this dose, almost complete suppression of antibody formation is seen 13 days after immunization and some degree of suppression is still present 4 weeks after

TABLE 2

EFFECT OF PASSIVELY ADMINISTERED ANTI-DNP ANTIBODY ON ANTIBODY FORMATION*

| Passive antibody | | Anti-DNP antibody concentration (mg/ml) | | | |
|---|---|---|---|---|---|
| | | Days after immunization | | | |
| $K_0$ | mg | 13 | 20 | 27 | 41 |
| — | 0 | 1·06 (25)† | 1·16 (18) | 1·69 (9) | 1·98 (7) |
| $1·9 \times 10^6$ | 20·4 | 0·76 (8) | 1·34 (5) | — | — |
| | 30·6 | 0·72 (4) | — | — | — |
| | 67·3 | 0·33 (6) | 1·06 (5) | — | — |
| $6·1 \times 10^7$ | 0·6 | 1·07 (3) | 1·16 (3) | 1·46 (3) | 1·92 (2) |
| | 2·0 | 0·78 (11) | 1·06 (4) | 1·04 (4) | 1·27 (4) |
| | 6·1 | 0·73 (11) | 1·42 (10) | 1·11 (4) | 1·34 (4) |
| | 13·5 | 0·48 (11) | 1·59 (6) | — | — |
| | 20·4 | 0·10 (14) | 0·52 (12) | 0·66 (7) | 1·93 (8) |
| | 40·8 | 0·15 (3) | 0·49 (3) | 0·80 (3) | 1·13 (3) |
| $\sim 10^{11}$ | 0·6 | 1·07 (5) | — | — | — |
| | 2·0 | 1·22 (14) | 1·68 (11) | 1·50 (5) | 1·84 (3) |
| | 6·1 | 0·47 (10) | 1·63 (6) | 1·65 (2) | — |
| | 13·5 | 0·10 (14) | 0·40 (5) | — | — |

* Passive antibody given 1 day before immunization with 5 mg DNP-BGG in complete Freund's adjuvant.

† Mean (number of animals).

administration of antigen. By 6 weeks most animals have begun to attain serum levels of antibody similar to the control group. With decreased doses of antiserum lesser degrees of suppression are observed. With 2·0 mg or with 6·1 mg of passive antibody only minimal suppression is observed at 13 days, and no suppression is present 3 weeks after immunization. With still less passive antibody no suppression is observed. Thus, the degree and duration of suppression is related to the dose of passive antibody employed.

It can be seen from Table 2 that an antibody of relatively low affinity ($K_0 = 1·9 \times 10^6$) is far less effective in suppression than is the antibody of intermediate affinity just discussed. With even 67·3 mg of low affinity anti-DNP antibody only moderate suppression is observed at 13 days and none at 20 days. At 13 days the degree of suppression observed with 67·3 mg of low affinity antibody is approximately equal to that observed after 13·5 mg of the intermediate affinity antibody.

Finally, an antiserum pool of very high affinity ($K_0 \sim 10^{11}$) was found to be more effective in suppression of antibody synthesis than are the lower affinity antisera described above. About 13·5 mg of high affinity antibody causes suppression approximately equivalent to 20·4 mg of intermediate affinity antibody and greater than that produced by 67·3 mg of the low affinity antibody. From a comparison of the results obtained with the three different pools of antisera, it is clear that at 13 days, 6·1 mg of high affinity antibody is approximately equivalent in its suppressive effect to 13·5 mg of intermediate affinity antibody and to 67·3 mg of low affinity antibody. With doses of high affinity antibody below 6 mg no suppression is observed. On the contrary, with 2·0 mg of high affinity antibody an apparent increase in antibody concentration is observed at 2 and 3 weeks after immunization. At 3 weeks the increase in antibody concentration can be shown to be significant at the 5 per cent level of confidence when evaluated by Student's *t*-test. With 6·1 mg of high affinity antibody the initial suppression observed at 2 weeks

is followed by increased antibody synthesis 3 weeks after immunization. This seeming increase in antibody synthesis is also observed 20 days after 13·5 mg of intermediate affinity antibody. Thus, 13·5 mg of intermediate affinity antibody is comparable to 6·1 mg of high affinity antibody in that both cause a 55 per cent suppression of antibody production 13 days after immunization followed by an approximately 50 per cent increase in antibody present 20 days after immunization. No significant increase in antibody synthesis is observed following injection of low affinity antibody at any dose level.

### EFFECT OF PASSIVE ANTIBODY ON THE AFFINITY OF THE ANTIBODY SYNTHESIZED

The affinity of the anti-DNP antibodies produced by rabbits which had received various doses of anti-DNP antibodies of varying affinity was determined. A single dose of passive antibody was given intravenously 1 day prior to immunization with 5 mg DNP-BGG in complete Freund's adjuvant. Animals were bled periodically, their anti-DNP antibodies purified, and the affinity of the antibody for ε-DNP-L-lysine determined by fluorescence quenching. Affinities, expressed as free energy change ($\Delta F°$) associated with the binding of ε-DNP-L-lysine by antibody, are indicated in Table 3. It is clear

TABLE 3
EFFECT OF ANTI-DNP ANTIBODY ON THE AFFINITY OF THE ANTI-DNP ANTIBODY SYNTHESIZED

| Passive antibody | | Affinity ($-\Delta F°$) anti-DNP antibody (kcal/mol) | | | | | |
|---|---|---|---|---|---|---|---|
| $K_0$ | mg | Days after immunization | | | | | |
| | | 13 | | 20 | | 27 | |
| — | 0 | 8·95 | (17)† | 9·70 | (8) | 10·54 | (5) |
| $1·9 \times 10^6$ | 20·4 | 8·45 | (5) | — | | — | |
| | 30·6 | 9·60 | (4) | — | | — | |
| | 67·3 | 8·17 | (3) | 8·29 | (3) | — | |
| $6·1 \times 10^7$ | 0·6 | 8·71 | (3) | 9·83 | (2) | — | |
| | 6·1 | 9·00 | (4) | 9·71 | (3) | 9·86 | (2) |
| | 13·5 | 8·88 | (5) | — | | — | |
| | 20·4 | 7·21 | (2) | 8·43 | (8) | 9·99 | (5) |
| $\sim 10^{11}$ | 2·0 | 8·44 | (5) | 9·39 | (4) | — | |
| | 13·5 | 7·83 | (5) | 9·38 | (6) | — | |

* Passive antibody given intravenously 1 day before immunization with 5 mg DNP-BGG in complete Freund's adjuvant.

† $-\Delta F°$ in kcal/mol measured in PBS at 21° for the reaction of antibody with ε-DNP-L-lysine. $\Delta F°$ calculated from the equilibrium constant determined by fluorescence quenching by use of the relationship: $\Delta F° = -RT \ln K_0$; where $R$ is the gas constant, $T$ the absolute temperature and $\ln K_0$ the natural logarithm of the equilibrium constant. The data are presented as: mean (number of animals measured).

from the table that amounts of passive antibody sufficient to produce marked suppression result in a moderate decrease in the affinity of the antibody produced. At the time of bleeding the amount of passive antibody remaining in the animals was too small to affect significantly the affinity of the antibody present. The decrease in affinity seen under conditions of marked suppression is the same whether the suppression is brought about by use of high, intermediate or low affinity passive antibody. The affinity of antibody made by both normal and suppressed animals increases progressively with time after immunization.

## DISCUSSION

It is clear from the data presented that the effectiveness of passive antibody in suppressing active antibody formation is related to the affinity (equilibrium constant) of the antibody for the homologous antigenic determinant. That is, a smaller amount of high than low affinity antibody is required to achieve an equivalent degree of suppression. It should be emphasized that this is a purely quantitative difference since sufficient low affinity antiserum is capable of bringing about profound suppression of antibody formation. These findings suggest a mechanism of suppression involving the binding of antigen by the passive antibody. If the mechanism of suppression were independent of antigen binding, then the ability to suppress would not be expected to be related to the affinity of the passive antibody. Provided antigen binding is involved in suppression, the effect of affinity observed here is quite explicable since it follows from the 'law of mass action' that the amount of antigen bound at any given concentration of antibody is a function of the affinity of the antibody.

All data reported here were obtained using a single injection of passive antiserum 1 day prior to immunization. Immunization was always carried out using Freund's adjuvant and thus a depot of antigen was present from which antigen might be slowly released over a protracted period of time. As the concentration of passive antibody in the animal decreased with time, the suppressive effect disappeared, and the animals developed serum antibody concentrations equal to controls in the majority of cases.

It appears probable that in the naturally occurring immunization process the presence of circulating antibody would serve as a control mechanism to limit the extent of the immune response upon repeated exposure to the same antigen. As the affinity of antibody increases with time, after immunization (Eisen and Siskind, 1964) such a mechanism would become increasingly effective in preventing recruitment of additional antibody synthesizing cells. It seems probable, however, that cells synthesizing high affinity antibody are very efficient in capturing antigen and would, therefore, be highly resistant to suppression. It has not as yet been determined definitively what role suppression plays in the natural history of the immune response. It is theoretically likely that low affinity antibody producing cells are more sensitive to suppression than are 'high affinity cells'. As a result, circulating antibody might be important in controlling the progressive increase in antibody affinity observed during the course of the immune response.

It should be emphasized that small amounts of high affinity passive antibody were capable of profound suppression: thus 13·5 mg of high affinity antibody almost completely suppressed the immune response of a 2-kg rabbit for 2 weeks. Two weeks after intravenous injection of 20 mg of antibody to similar normal, non-immunized rabbits the serum antibody concentration was found to be less than 0·03 mg/ml.

The apparent increase in antibody formation observed following low doses of high affinity antibody deserves further mention. This phenomenon was not seen with low affinity antibody. Several previous workers have reported an increased immune response following immunization with appropriate antigen-antibody complexes (Campbell, 1953; Terres and Wolins, 1959; Morrison and Terres, 1966). The mechanism of this effect is most likely to be related to distribution of the antigen–antibody complexes formed with high affinity antibody under conditions of antigen excess. It may be that such complexes are very efficient in stimulating antibody formation. It is probable that this effect can only be seen with high affinity antibody because only with such antibody

can sufficiently stable complexes be formed at the extremely low antibody concentrations apparently required to observe this effect. Proof of the mechanism of stimulation is, however, still lacking. Thus, in addition to its role in controlling antibody synthesis, through a suppression mechanism, circulating antibody might also serve to increase antibody formation when antigen is presented for a second time long after the primary response, i.e. when low concentrations of high affinity antibody are present. It thus appears possible that besides the cellular mechanisms undoubtedly involved in immunological 'memory', an extracellular mechanism, in the form of high affinity circulating antibody (present in very low concentrations), may contribute to the secondary response.

It was found that marked suppression produced by a single injection of passive antibody resulted in the synthesis of antibody of lower average affinity than is usually present at that time after immunization. This moderate decrease in affinity was seen regardless of the affinity of the antibody used to bring about suppression. If the mechanism of suppression were some type of 'feedback' effect in which antibody molecules directly inhibited antibody-forming cells from making more antibody molecules of the same type, then one would expect that high affinity antibody would preferentially suppress high affinity antibody formation, and low affinity antibody would preferentially suppress low affinity antibody formation. The results obtained were not consistent with such a theory. Both high and low affinity antibody produced a moderate decrease in antibody affinity. Passive antibody appears to introduce a delay in immunization. In suppressed animals affinity increases with time, as in normals, but appears to lag behind normals especially early in immunization. This is precisely what would be expected with the experimental design used here if one assumes that the mechanism of suppression is the binding of antigen by the passive antibody. The current experiments were carried out using a single injection of antibody and an antigen emulsified in Freund's adjuvant. Under these conditions there is a continuous source of antigen which is slowly released from depots in the foot pads. In addition, the concentration of passive antibody is greatest immediately after injection and falls rapidly thereafter. As long as sufficient circulating antibody is present all antigen is effectively bound and no cells are stimulated. As the passive antibody concentration falls antigen reaches immunologically competent cells and stimulates them to carry out an essentially normal, but delayed, immune response. The low affinity observed for the antibody present in suppressed animals is due to the fact that these animals are, in essence, at a somewhat earlier stage in their immune response than are normal animals at the same time after antigen injection.

## ACKNOWLEDGMENTS

This study was supported by U.S. Public Health Service Grant No. 5-R01-AM08805, and was carried out while J.G.W. was a holder of a Sir Henry Wellcome Travelling Scholarship. One of us (G.W.S.) is Career Scientist of the Health Research Council of the City of New York under Career Scientist Award I-464.

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