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# Studies on Antigenic Competition

## III. EFFECT OF ANTIGENIC COMPETITION ON ANTIBODY AFFINITY*

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**Summary.** The effect of antigenic competition on antibody affinity was studied using a haptenic system in guinea-pigs. A moderate depression in the amount of antibody formed, as a result of antigenic competition, had relatively little effect on affinity. Increasing the dose of the competing antigen resulted in a greater degree of competition. Under these conditions a large amount of low affinity antibody was produced by the animals while essentially no high affinity antibody was detectable. Thus, marked competition appeared to result in a failure to select for high affinity antibody synthesis.

## INTRODUCTION

Antigenic competition refers to the depression in antibody synthesis which may occur when two antigens are injected simultaneously or within a relatively brief time span. This phenomenon was first described by Michaelis (1902, 1904) and the literature dealing with it has been reviewed by Adler (1959, 1964), by Taylor and Iverson (1971) and by Taussig (1973). The mechanism of antigenic competition is still unknown, although recent studies have tended to suggest that it is due to the local production of a nonspecific inhibitor of antibody synthesis, perhaps by T lymphocytes (Radovich and Talmage, 1967; Möller and Sjöberg, 1970; Waterston, 1970; Gershon and Kondo, 1971; Monier and Salussola, 1971; Sjöberg and Britton, 1972; Katz, Paul and Benacerraf, 1973). Alternatively, Kerbel and Eidinger (1971) have suggested that competition is the result of a deficiency of T- and B-cell interactions in the immune response to a second antigen as a result of the marked cell proliferation occurring in response to the first antigen injected. The massive cell proliferation is viewed as altering the population distribution of specific cells in the lymphoid organs rendering cell–cell interactions inefficient. Finally, it has been proposed (Taussig and Lachmann, 1972; Feldmann and Nossal, 1972; Taylor and Iverson, 1971) that the locus for antigenic competition is at the level of the macrophage and that antigens, or antigen–antibody complexes, 'compete' for available 'macrophage space' or arrangement on the macrophage surface.

Brody and Siskind (1969) and Eidinger, Pross, Kerbel, Baines, Ackerman and Khan (1971) reported that the average affinity of the antibody produced when two antigens are given simultaneously is the same as that produced by an animal given a single antigen. In

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contrast, Harel, Ben-Efraim and Liacopoulos (1970) reported a significant depression in average affinity as a result of antigenic competition. It was suggested by Harel *et al.* (1970) that this difference in results might be due to the fact that the degree of antigenic competition was greater under the conditions of their studies.

It was the purpose of the experiments reported here to clarify the effect of antigenic competition on the affinity of the antibody synthesized. It was found that with marked antigenic competition the usual increase in affinity seen with time after immunization did not occur. Marked antigenic competition is thus primarily characterized by a failure to select for high affinity antibody synthesis. Low affinity antibodies are formed in relatively normal amounts. Moderate degrees of competition have little or no effect on antibody affinity.

## MATERIALS AND METHODS

*Animals*

Guinea-pigs weighing 300–400 grams were used.

*Antigen preparations*

Dinitrophenylated egg albumin (DNP-EA) was prepared essentially as described by Eisen, Belman and Carsten (1953) by reacting egg albumin with 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals, Rochester, New York) at room temperature under alkaline conditions. Arsanilate bovine gamma-globulin (R-Azo-BGG) was prepared by coupling *p*-arsanilic acid to BGG by a diazo linkage as previously described by Brody, Walker and Siskind (1967). In both cases the product was purified by acid precipitation and extensive dialysis. The concentration of the hapten-conjugated protein was determined by drying a known volume to constant weight at 95°. The degree of hapten substitution was estimated spectroscopically as described previously (Brody *et al.*, 1967; Siskind, 1964).

*Hapten preparation*

Dinitrophenylated ε-amino-caproic acid (DNP-EACA) was prepared by the reaction of DNFB with ε-amino-*n*-caproic acid (EACA; Sigma Chemical Company, St. Louis, Missouri) under alkaline conditions. Tritiated DNP-EACA was prepared by the reaction of $^{3}$H-labelled DNFB (Amersham Searle, Arlington Heights, Illinois) with EACA. The detailed procedures for both preparations have been presented previously (Werblin and Siskind, 1972a).

*Immunization procedures*

Guinea-pigs were immunized with a single injection of the indicated amount of antigen or antigens emulsified in Freund's complete adjuvant (CFA) containing 0·5 mg/ml of killed *Mycobacteria butyricum* in the final emulsion. A total of 0·5 ml of emulsion was injected, divided equally among the four footpads and subcutaneously into the back of the neck. Animals were bled at 2 weeks or 6 weeks after immunization.

*Farr technique*

Measurements of the concentration and affinity of antibody were carried out, in some cases, by the Farr technique (Farr, 1958; Stupp, Yoshida and Paul, 1969) using $^{3}$H-

labelled DNP-EACA as ligand. Bound and free ligand were separated by precipitation at 50 per cent cold saturated $(NH_4)_2SO_4$. The methods for carrying out the Farr assays, and the procedures for computation of affinities have been described in detail in previous publications (Werblin and Siskind, 1972a; Werblin, Kim, Quagliata and Siskind, 1973). The concentration of antibody was determined by saturation of antibody-combining sites at high free hapten concentration. Five points ranging from $2 \times 10^{-7}$ to $5 \times 10^{-6}$ M free hapten were used. The data were plotted as $1/R$ versus $1/C$ (where $R$ and $C$ are the equilibrium concentrations of bound and free ligand respectively) and the plot extrapolated by linear regression analysis to infinite free hapten concentration. Antibody-binding site concentration is determined as the reciprocal of the $y$ intercept. Antibody concentration is calculated from binding site concentration assuming a mol. wt of 75,000 per antibody binding site. This procedure essentially defines antibody site concentration in terms of the ability of the antibody to bind hapten at a designated free hapten concentration. The determination of antibody concentration was carried out on samples diluted so as to contain between 30 and 300 $\mu$g antibody/ml. After the antibody concentration was determined by hapten saturation the samples were diluted to 1·0 $\mu$g/ml for the measurement of the remainder of the binding curve. Measurements were made at ten free hapten concentrations ranging from $8 \times 10^{-12}$ to $7 \times 10^{-10}$ M. The data are plotted according to the method of Sips (1948). 'Average' association constants ($K_{I\ 10\ per\ cent}$) are calculated from the binding data falling in the range between 0·1 and 10 per cent of antibody sites occupied by hapten. A straight line is fitted to these data by linear regression analysis and the average affinity is expressed as the reciprocal of the free hapten concentration at which this line indicates 50 per cent of the antibody sites occupied by hapten. Restriction of the data used for calculation of K to a clearly defined portion of the binding curve tends to eliminate uncertainties caused by the non-linearity of the binding curves, and to make the data obtained on different samples more comparable. The justification for these procedures has been presented previously (Werblin and Siskind, 1972a). This technique for expressing average affinity emphasizes the highest affinity antibody subpopulations present in the sample. The data are presented as free energy changes ($\Delta F^{\circ}_{I\ 10\ per\ cent}$) which are calculated from the association constants using the relationship:

$$\Delta F^{\circ} = -RT\ ln\ K$$

where R is the gas constant and $T$ is the absolute temperature.

*Equilibrium dialysis*

Antibody concentration and affinity have also been measured by equilibrium dialysis using $^3H$-labelled DNP-EACA as ligand by methods previously described in detail (Werblin and Siskind, 1972a). Measurements were carried out on globulin fractions of antisera prepared by precipitation twice at 50 per cent saturated $(NH_4)_2SO_4$. Determination of antibody concentrations was carried out on samples diluted so as to contain between 50 and 500 $\mu$g of antibody per millitre based upon preliminary measurements. The antibody-binding site concentration was generally calculated from the amount of hapten bound at high free hapten concentration ($5 \times 10^{-6}$ M). The extrapolation procedure described above was not used in these cases since the presence of considerable amounts of low affinity antibody in most of these samples makes the extrapolation procedure somewhat unreliable (Werblin and Siskind, 1972a). Based on this determination, the samples were diluted to an antibody concentration of 3·0 $\mu$g/ml and the remainder of the binding curve was obtained using sixteen points ranging from $6 \times 10^{-13}$ to $7 \times 10^{-8}$ M. Eight addi-

tional points ranging from $3 \times 10^{-7}$ to $5 \times 10^{-6}$ M were obtained using antibody diluted to approximately 100 μg/ml. The data were plotted according to the procedure of Sips (1948) and the average affinity was expressed as described above. Distributions of affinities were computed by a curve-fitting procedure using a series of Fortran computer programs written by Werblin (1972). This procedure has been described and justified previously (Werblin and Siskind, 1972a).

Since we define antibody by its ability to bind hapten at a pre-established free hapten concentration, what will be called specific antibody is, in effect, determined by the free hapten concentration chosen for measurement. It should be noted, in this regard, that any binding of hapten by normal globulin is always subtracted from the amount of hapten bound by immune globulin before the antibody binding site concentration is calculated. In general, antibody concentration measurements are carried out at $5 \times 10^{-6}$ M free ligand.

TABLE 1

EFFECT OF ANTIGEN DOSE ON ANTIGENIC COMPETITION BY THE FARR TECHNIQUE*

| Dose DNP-EA (mg) | Dose R-Azo-BGG (mg) | Anti-DNP antibody response: 14 days Concentration (mg/ml) | 14 days Affinity $\Delta F^{\circ}_{1\ 10\ per\ cent}$ (kcal/mole) | 42 days Concentration (mg/ml) | 42 days Affinity $\Delta F^{\circ}_{1\ 10\ per\ cent}$ (kcal/mole) |
|---|---|---|---|---|---|
| 0·05 | 0 | 1·10 ± 0·11(8) | 10·95 ± 0·32(8) | 1·16 ± 0·20(9) | 11·76 ± 0·29(5) |
| 0·05 | 5·0 | 0·24 ± 0·12(5)‡ | 8·57 ± 1·06(6) | <0·03 – –(6)§ | Not measurable** |
| 0·5 | 0 | N.D.† | N.D. | 2·14 ± 0·32(10) | 11·73 ± 0·08(10) |
| 0·5 | 5·0 | N.D. | N.D. | 0·38 ± 0·10(6) | 11·62 ± 0·14(6) |
| 5·0 | 0 | 0·68 ± 0·13(7) | 10·05 ± 0·41(7) | 0·77 ± 0·16(7) | 10·92 ± 0·44(4) |
| 5·0 | 5·0 | 0·62 ± 0·15(8) | 9·69 ± 0·36(7) | 0·37 ± 0·14(6) | 10·36 ± 0·29(6) |

* Guinea-pigs were injected with one or both antigens, in the dose indicated, emulsified in CFA and were bled either 14 or 42 days later. Antibody concentration and affinities for DNP-EACA were determined by the Farr technique at 20°. Data are presented as mean ± standard error of the mean (number of animals studied).

† N.D. = not done.

‡ One of the five samples was below the accurate measurement range (<0·03) and was not included in calculating the mean.

§ The lower limit for accurate assay of antibody concentration by saturation using the Farr technique as performed here is 0·03 mg/ml. Six samples studied here had less than this concentration of antibody.

** Affinity too low to be measured by the Farr technique in all six samples studied. Based upon equilibrium dialysis studies (see Table 3) these samples are known to contain significant amounts of low affinity antibodies. Assays on these samples by the Farr technique showed only a minimal amount of binding at the highest concentrations of reagents that could be studied. These findings are consistent with the interpretation that the affinity of the antibody present is very low although a precise value cannot be given.

In a few cases antibody 'concentration' was determined at several different free hapten concentrations in order to illustrate how this affects the apparent antibody site concentration. The details are indicated in the text and in the footnote to the appropriate table.

*Haemagglutination technique*

Anti-DNP antibody was also assayed by a modification of the haemagglutination technique described by Levine and Levytska (1967) using DNP-conjugated sheep red blood cells. Cells were prepared by reaction with DNFB at pH 8·0. Results are reported as the reciprocal of the maximum dilution (log base 2) giving detectable agglutination.

*Scintillation counting*

Samples were counted in 5 ml Aquasol (New England Nuclear, Boston, Massachusetts), using an ambient temperature Beckman Scintillation counter, for sufficient time to accumulate enough counts to give a percentage standard error of counting not exceeding ±3 per cent. Samples were screened for quenching by use of an external standard and no significant quenching was observed in the studies reported here.

## RESULTS

### EFFECT OF ANTIGEN DOSE ON ANTIGENIC COMPETITION

Guinea-pigs were immunized with varying amounts of DNP-EA alone, or mixed with 5·0 mg of R-Azo-BGG in CFA. The concentration of anti-DNP antibody was assayed by the Farr technique and the results are presented in Table 1. Animals immunized with 5·0 mg R-Azo-BGG and 5·0 mg DNP-EA simultaneously showed no antigenic competition at

TABLE 2

EFFECT OF DOSE OF COMPETING ANTIGEN ON ANTIGENIC COMPETITION MEASURED BY HAEMAGGLUTINATION*

| Dose DNP-EA (mg) | Dose R-Azo-BGG (mg) | Anti-DNP HA titre† 14 Days | Anti-DNP HA titre† 42 days |
|---|---|---|---|
| 0·05 | 0 | 15·4±0·2 (5) | 16·6±0·3 (5) |
| 0·05 | 0·05 | 13·0±0·4 (4) | 15·0±0·4 (4) |
| 0·05 | 0·5 | 11·6±0·2 (5) | 13·3±0·2 (5) |
| 0·05 | 5·0 | 5·6±0·2 (5) | 8·0±0·3 (5) |

* Guinea-pigs were injected with one or both antigens, in the doses indicated, emulsified in CFA and were bled either 14 or 42 days later.

† Anti-DNP haemagglutination titres are the reciprocal of the highest dilution of the antiserum giving detectable haemagglutination expressed as a logarithm to the base 2. Data are presented as mean titre± standard error of the mean (number of animals).

2 weeks after immunization but exhibited approximately a 50 per cent depression of their anti-DNP response at 6 weeks after immunization as compared to control animals which were injected with only DNP-EA. The animals immunized with 5·0 mg R-Azo-BGG and 0·5 mg DNP-EA showed an 82 per cent depression in anti-DNP antibody concentration at 6 weeks after immunization. The most marked antigenic competition was observed among the animals immunized with 5·0 mg R-Azo-BGG and 0·05 mg DNP-EA. In this case there was a 78 per cent depression in the anti-DNP response at 2 weeks, while at 6 weeks anti-DNP antibody was unmeasurable in the competed animals (greater than 97 per cent depressed) by the Farr technique. Thus, when the dose of the competing antigen is held constant, greater competition is seen with smaller doses of the antigen to which the antibody response is being evaluated.

Similarly, if the dose of DNP-EA is held constant at 0·05 mg and the dose of the competing antigen (R-Azo-BGG) is varied, then greater competition is observed with larger doses of the competing antigen. This is illustrated by data presented in Table 2.

TABLE 3

EFFECT OF ANTIGEN DOSE ON ANTIGENIC COMPETITION MEASURED BY EQUILIBRIUM DIALYSIS*

| Dose DNP-EA (mg) | Dose R-Azo-BGG (mg) | Anti-DNP antibody response | | | |
|---|---|---|---|---|---|
| | | 14 days | | 42 days | |
| | | Concentration (mg/ml) | Affinity $\Delta F^{\circ}_{1\ 10\ \text{per cent}}$ (kcal/mole) | Concentration (mg/ml) | Affinity $\Delta F^{\circ}_{1\ 10\ \text{per cent}}$ (kcal/mole) |
| 0·05 | 0 | 4·34 | 8·96 | 1·93 | 9·71 |
| 0·05 | 0 | 3·24 | 9·00 | 1·03 | 12·18 |
| 0·05 | 5·0 | 4·12 | <5·00 | 3·28 | 8·12 |
| 0·05 | 5·0 | 2·90 | 7·00 | 4·12 | 8·02 |

* Serum samples were from representative animals from the groups studied by the Farr technique in Table 1. Antibody concentration and affinity were measured by equilibrium dialysis at 2° using DNP-EACA as ligand. Antibody concentration was calculated from the number of hapten molecules specifically bound at $5 \times 10^{-6}$ M assuming a mol. wt of 75,000 per antibody-binding site.

## EFFECT OF ANTIGENIC COMPETITION ON ANTIBODY AFFINITY

The affinity of anti-DNP antibody for DNP-EACA was determined by the Farr technique and the results are presented in Table 1. As has been reported by previous workers (reviewed in Siskind and Benacerraf, 1969; Werblin and Siskind, 1972b) the rate of increase in average affinity is more rapid after lower doses of antigen. Thus, at both 2 and 6 weeks after immunization the affinity of the anti-DNP antibody produced by control animals immunized with 0·05 mg DNP-EA is higher than that produced by controls receiving 5 mg of antigen. In the groups immunized with 5 or 0·5 mg DNP-EA antigenic competition had little or no effect on antibody affinity although there was significant depression in the concentration of anti-DNP antibody. The groups immunized with 0·05 mg of DNP-EA showed the most profound depression in the amount of anti-DNP antibody

TABLE 4

EFFECT OF DOSE OF COMPETING ANTIGEN ON ANTIGENIC COMPETITION MEASURED BY EQUILIBRIUM DIALYSIS*

| Dose DNP-EA (mg) | Dose R-Azo-BGG (mg) | Anti-DNP antibody response | | | |
|---|---|---|---|---|---|
| | | 14 days | | 42 days | |
| | | Concentration (mg/ml) | Affinity $\Delta F^{\circ}_{1\ 10\ \text{per cent}}$ (kcal/mole) | Concentration (mg/ml) | Affinity $\Delta F^{\circ}_{1\ 10\ \text{per cent}}$ (kcal/mole) |
| 0·05 | 0 | 7·33 ± 1·34(5) | 6·77 ± 0·23(5) | 14·91 ± 1·32(5) | 9·39 ± 0·06(5) |
| 0·05 | 0·05 | 6·43 ± 0·73(4)† | 7·00 ± 0·44 | 10·26 ± 1·89(4) | 8·65 ± 0·25(4) |
| 0·05 | 0·5 | 4·42 ± 0·92(5) | 6·58 ± 0·16(5) | 7·18 ± 2·33(5) | 8·75 ± 0·36(5) |
| 0·05 | 5·0 | 4·85 ± 0·44(5) | <5·00 (5) | 9·45 ± 2·93(5) | 5·54 ± 0·08(5) |

* Same serum samples as were studied by haemagglutination in Table 2. Antibody concentration and affinity were measured by equilibrium dialysis at 2° using DNP-EACA as ligand. Antibody concentration was calculated from the number of hapten molecules specifically bound at $5 \times 10^{-6}$ M free hapten assuming a mol. wt of 75,000 per antibody binding site. Data are presented as mean ± standard error of the mean (number of animals).

† The four animals studied had antibody concentrations of 6·82, 5·03, 7·46 and 0·51 respectively. In calculating the average presented in the Table the value 0·51 was not included.

TABLE 5

COMPARISON OF ANTIBODY CONCENTRATION DETERMINED BY 'SATURATION' AT DIFFERENT FREE HAPTEN CONCENTRATIONS IN EQUILIBRIUM DIALYSIS*

| Dose DNP-EA (mg) | Dose R-Azo-BGG (mg) | Anti-DNP antibody concentration | | | | | |
|---|---|---|---|---|---|---|---|
| | | 14 days Free hapten concentration (M) | | | 42 days Free hapten concentration (M) | | |
| | | $5 \times 10^{-6}$ | $4 \times 10^{-7}$ | $7 \times 10^{-8}$ | $5 \times 10^{-6}$ | $4 \times 10^{-7}$ | $7 \times 10^{-8}$ |
| 0·05 | 0 | 7·33 ± 1·34(5) | 0·83 ± 0·10(5) | 0·09 ± 0·04(5) | 14·91 ± 1·32(5) | 1·57 ± 0·16(5) | 0·94 ± 0·12(5) |
| 0·05 | 0·05 | 6·43 ± 0·73(4) | N.D.† | 0·18 ± 0·03(4) | 10·26 ± 1·89(4) | N.D. | 0·55 ± 0·15(4) |
| 0·05 | 0·5 | 4·42 ± 0·92(5) | N.D. | 0·04 ± 0·01(5) | 7·18 ± 2·33(5) | N.D. | 0·30 ± 0·07(5) |
| 0·05 | 5·0 | 4·85 ± 0·44(5) | 0·90 ± 0·10(5) | Not measurable‡ | 9·45 ± 2·93(5) | 1·79 ± 0·59(5) | 0·014 ± 0·002(5) |

* Serum samples are the same as were studied in Table 2. Measurements were carried out by equilibrium dialysis at 2° using DNP-EACA as ligand. Antibody concentration was calculated from the number of hapten molecules specifically bound at the free hapten concentration indicated in the headings assuming a mol. wt of 75,000 per antibody-binding site. For determination of binding at $5 \times 10^{-6}$ M free hapten, 1 ml of antibody at a concentration of between 50 and 500 μg/ml was used. For measurement at $7 \times 10^{-8}$ M free hapten an antibody concentration of approximately 3 μg/ml was used. For measurement at $4 \times 10^{-7}$ M free hapten, an antibody concentration of between 5 and 50 μg/ml was used. Data are presented as mean ± standard error of the mean (number of animals).

† N.D. = not done.

‡ Under conditions of assay no binding detectable.

as a result of competition. In this case a marked depression in affinity was also observed. Thus, it appears that only when competition causes a very marked depression in antibody synthesis is there a significant effect on the affinity of the antibody formed.

### EFFECT OF ANTIGENIC COMPETITION ON LOW AFFINITY ANTIBODY SYNTHESIS

Haemagglutination and the Farr technique, as carried out in our laboratory, appear mainly to detect relatively high affinity antibodies. This technical problem will be discussed extensively in a separate publication (Kim, Werblin and Siskind, 1973a). We, therefore, studied serum from normal and competed guinea-pigs using equilibrium dialysis. This method permits a more critical evaluation of the total antibody content of a serum sample since concentration of antibody is defined purely in terms of the number of antibody sites binding hapten at high free hapten concentration. In addition, the binding affinity of the sample can be studied over a wide range of ligand concentration which permits a more complete evaluation of the actual distribution of affinities present.

In Tables 3 and 4, data are presented on the effect of antigenic competition on the amount and affinity of anti-DNP antibody as assayed by equilibrium dialysis. The samples studied are the same as were studied by the Farr technique or by haemagglutination (Tables 1 and 2). It is immediately clear that the equilibrium dialysis data are superficially quite different from those obtained by haemagglutination or by the Farr technique. When measured by direct binding of hapten using equilibrium dialysis, antibody concentration of the competed animals appears to be only slightly lower than that of the control animals, while the antibody affinity of the samples from the competed animals is markedly lower than that of the control animals. These differences can be explained by considering the limitations of the different techniques used for the measurement of antibody concentration. The haemagglutination test and the Farr technique detect mainly high affinity antibody. These assays fail to detect the significant amounts of low affinity antibody actually present in both the normal and the competed animals. Antigenic competition thus appears to result in a lack of selection for high affinity antibody synthesis. Low affinity antibodies appear to be synthesized in near normal amounts in the competed animals.

In essence, the above data suggest that a considerably greater amount of low affinity antibody is formed by guinea-pigs immunized with antigen in CFA than is generally appreciated. This is based upon a definition of antibody as a globulin capable of binding hapten at a free hapten concentration of $5 \times 10^{-6}$ M. Any 'non-specific' binding by normal globulin is subtracted in calculating antibody concentration. One could, of course, define antibody by its ability to bind the antigenic determinant at any arbitrarily chosen hapten concentration. If a lower hapten concentration is chosen, then only relatively high affinity antibody will be detected. In Table 5 data are presented comparing the concentration of antibody detected by measurements at different free hapten concentrations. When measurements are carried out at $4 \times 10^{-7}$ M or $7 \times 10^{-8}$ free hapten considerably less 'antibody' is detected than when 'saturation' at $5 \times 10^{-6}$ M hapten is employed. When binding at $7 \times 10^{-8}$ M free hapten concentration is used to measure antibody concentration the data obtained by equilibrium dialysis become very similar to that obtained with either the Farr technique or haemagglutination. A marked decrease in the concentration of antibody as a result of antigenic competition is seen. The results are consistent with the view that in antigenic competition there is a failure to synthesize high affinity antibody.

EFFECT OF ANTIGENIC COMPETITION ON THE DISTRIBUTION OF ANTIBODY AFFINITIES

It is well known that antibody present in the serum of an individual animal is generally heterogeneous with respect to its affinity for the antigenic determinant (Karush, 1962; Siskind and Benacerraf, 1969; Werblin and Siskind, 1972b). The distribution of antibody in a serum sample with respect to its affinity for the antigenic determinant can be computed by an approximation procedure which we described previously (Werblin and Siskind, 1972a). In Table 6 data are presented illustrating the effect of antigenic competi-

TABLE 6

EFFECT OF ANTIGENIC COMPETITION ON THE DISTRIBUTION OF ANTIBODY AFFINITY*

| Average affinity of subpopulations (log K) | Distribution of affinities (percentage)† | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | | | | Competed | | | | |
| 11·00 | 0·17 | 0·38 | 0·01 | 0·13 | 0·13 | 0·01 | 0·01 | 0·01 | 0·20 | 0·08 |
| 9·57 | 4·47 | 3·40 | 9·63 | 3·48 | 3·46 | 0·01 | 0·01 | 0·03 | 0·07 | 0·08 |
| 8·33 | 1·49 | 3·40 | 0·04 | 1·16 | 1·15 | 0·16 | 0·04 | 0·10 | 0·20 | 0·72 |
| 7·00 | 0·05 | 0·38 | 0·36 | 0·39 | 1·15 | 3·12 | 3·12 | 8·32 | 1·80 | 2·15 |
| 5·67 | 13·40 | 91·93 | 86·63 | 94·06 | 93·34 | 84·26 | 84·32 | 74·89 | 48·56 | 58·18 |
| 4·33 | 40·21 | 0·38 | 3·21 | 0·39 | 0·38 | 9·36 | 9·37 | 8·32 | 48·56 | 19·39 |
| 3·00 | 40·21 | 0·13 | 0·12 | 0·39 | 0·38 | 3·12 | 3·12 | 8·32 | 0·60 | 19·39 |

* Distributions of antibody affinities computed from the equilibrium dialysis data summarized in Table 4. Control guinea-pigs were immunized with 0·05 mg DNP-EA. Competed animals received 0·05 mg DNP-EA together with 5·0 mg R-Azo-BGG. Data are presented for the 42-day bleeding.

† The data are presented as the percentage of the total antibody which is present in the particular affinity subpopulation. Each column of numbers indicates the distribution of affinities in an individual animal.

tion on the distribution of antibody affinities in a series of individual animals. In both control and competed animals a large proportion of the antibody formed is of low affinity. Clearly antigenic competition is characterized by a failure to form the high affinity anti-DNP antibody sub-populations which are present in the control animals.

## DISCUSSION

The data reported in the present paper indicate: (1) increasing the dose of the competing antigen results in greater competition; (2) if the dose of the competing antigen is held constant then competition is greater with a lower dose of the test antigen; (3) with a minimal degree of competition, relatively little effect on the affinity of the antibody produced is observed; (4) when the degree of competition is increased, the main effect is a depression in synthesis of high affinity antibodies. With a marked degree of competition one sees essentially a complete absence of detectable high affinity antibody; (5) competed animals, while failing to form high affinity antibodies, do synthesize near normal amounts of low affinity antibodies.

The observations, summarized above, dealing with the effect of antigen dose on antigenic competition are consistent with previous studies on this phenomenon (Adler, 1959, 1964; Taussig, 1971; Eidinger *et al.*, 1971). It is clear that when two antigens are injected

simultaneously the degree of competition is determined by the nature and the relative doses of the two antigens.

Conflicting findings have previously been reported with respect to the effect of antigenic competition on the affinity of the antibody formed. Brody and Siskind (1969) and Eidinger *et al.* (1971) reported that antigenic competition had no detectable effect on the affinity of the antibody formed. In contrast, Harel *et al.* (1970) found a significant depression in antibody affinity as a result of antigenic competition. Harel *et al.* (1970) interpreted this difference in results as a consequence of the fact that the degree of competition in the system which they were studying was far greater than that observed under the experimental conditions employed by Brody and Siskind (1969). The data reported in the present paper support this interpretation. Clearly, as the relative antigen doses are varied so as to alter the degree of competition one can see varying effects of competition on affinity. With a modest degree of competition, relatively little effect on affinity is noted. As the degree of competition becomes more profound, a marked depression in affinity is observed.

The mechanism for the failure in selection for high affinity antibody synthesis is not completely clear. It is generally assumed (Siskind and Benacerraf, 1969; Werblin and Siskind, 1972b) that the progressive increase in antibody affinity with time after immunization results from the selective proliferation of high affinity antibody-forming cells. When antigen concentration is low only cells capable of synthesizing high affinity antibody will interact sufficiently with antigen to be stimulated to proliferate and secrete antibody. Under the selective pressure of decreasing antigen concentrations, a type of microevolutionary process occurs with selection for high affinity antibody-forming cells taking place. According to such a selectional theory, one would predict that non-specific depression of antibody synthesis would have relatively little effect on antibody affinity since no special sub-population of antibody-forming cells would be turned off. This appears to be true for immunodepression with anti-metabolites (Harel *et al.*, 1970; Mond, Kim and Siskind, 1973). In contrast, it would be predicted that specific immunodepression by B-cell tolerance induction should markedly depress antibody affinity. This prediction has been found to be true (Theis and Siskind, 1968; Davie, Paul, Katz and Benacerraf, 1972). In fact, a 50 per cent depression of antibody synthesis as a result of neonatal tolerance induction, resulted in a very marked depression in antibody affinity (Theis and Siskind, 1968). Thus, specific tolerance induction (presumably B-cell tolerance) does tend to cause a greater depression in affinity, for an equivalent degree of depression in the amount of antibody synthesized, than is observed with antigenic competition.

It should, however, be noted that in order to see a selective evolution towards the synthesis of high affinity antibody two factors are required: the selective pressure of decreasing antigen concentration; and a vigorous proliferative response. If, as a result of non-specific inhibition, little proliferation of B lymphocytes occurs, then efficient selection is not possible. Thus, one might predict that any condition which very markedly depresses antibody synthesis would *pari passu* depress the selection for high affinity antibody synthesis. High affinity antibody-forming cells are presumably rare relative to low affinity producers at the onset of the immune response. It is only as a result of the proliferative expansion of the high affinity subpopulation of cells that these cells come to represent a significant fraction of the total antibody-forming cell population, and the average affinity of the antibody increases. Depression of proliferation would thus be expected to depress the efficiency of selection for high affinity antibody synthesis.

A striking finding in the present studies is the presence of a large amount of low affinity

antibody in both the control and the competed animals. It should be noted that this low affinity antibody is not present in preimmunization serum or in serum of animals immunized only with CFA (Kim, Werblin and Siskind, 1973b). Furthermore, appropriate controls for non-specific binding are included with all equilibrium dialysis measurements and any non-specific binding to normal globulin is subtracted in the course of calculation of the number of antibody sites present. All assays for antibody are, to some extent, dependent upon antibody affinity. Different assays vary significantly with regard to their ability to detect low affinity antibodies (Kim *et al.*, 1973a). The Farr technique and haemagglutination detect mainly high affinity antibodies. As a consequence, when studied by these techniques, severely competed animals appear to have little or no anti-DNP antibody in their serum. However, when examined by equilibrium dialysis a high concentration of low affinity antibody-binding sites can be readily detected in these sera. Thus, the *apparent* effect of various experimental procedures on the immune response may be greatly influenced by the technique used to measure antibody concentration.

The mechanism for antigenic competition is not clear although evidence from a number of sources has been consistent with the theory that it is mediated by the production, perhaps by T lymphocytes, of a non-specific, locally active inhibitor of antibody synthesis (Radovich and Talmage, 1967; Brody and Siskind, 1969; Möller and Sjöberg, 1970; Waterston, 1970; Gershon and Kondo, 1971; Monier and Salussola, 1971; Sjöberg and Britton, 1972; Katz *et al.*, 1973). The primary effect of marked antigenic competition appears to be a depression in selection of high affinity antibody-forming cells. In part, the difficulty in understanding the mechanism for the depression in affinity as a result of antigenic competition is a consequence of our lack of understanding of the detailed mechanism of antigenic competition. It is reasonable to assume, as discussed above, that any profound depression of proliferation would make selection for high affinity antibody production inefficient. However, the observation that a large amount of low affinity antibody is produced by competed animals is clearly not fully consistent with this rather simplistic interpretation.

An alternative explanation following from the 'macrophage-space' theory (Taussig and Lachmann, 1972; Taylor and Iverson, 1971) might be that in the absence of antigen, in a form that could stimulate antibody synthesis, tolerance induction to the antigen may predominate. As has been previously shown (Theis and Siskind, 1968) tolerance induction tends to affect mainly high affinity antibody-forming cells with a consequent decrease in the average affinity.

A clonal theory of antibody formation such as described above, would predict that high affinity antibody-forming cells are initially very rare but have a selective advantage in terms of antigen capture especially at low antigen concentration. However, the details of how the selective shift towards a population of high affinity antibody-forming cells is accomplished is not clear. Obviously the intact animal does not function at thermodynamic equilibrium. A highly complex system exists which cannot be closely approximated by any simple model based on thermodynamic considerations. Antigen is not uniformally distributed throughout the animal but tends to be localized at special sites (e.g. on the surface of dendritic macrophages as demonstrated by Nossal, Abbot, Mitchell and Lummus, 1968). There is, in addition, a continual generation of new lymphoid cells from the bone marrow and recirculation of existing cells. Finally, the interaction between two or perhaps three different cell types (T lymphocytes, B lymphocytes and macrophages) appears to be involved in the normal immune response. The selection from the circulat-

ing pool of lymphoid cells, by localized antigen, of high affinity antibody-forming cells is a dynamic process which obviously involves many factors in addition to the interaction of antigen with 'cell-associated' antibody. It is possible that it is at this stage in the immune response that antigenic competition occurs. A vigorous immune response to one antigen, or the localization of excessive amounts of one antigen, might alter the subtle factors involved in the localization of a second antigen and the subsequent selection of high affinity antibody-forming cells to proliferate in regional lymphoid tissue. Such a mechanism would account for many of the *in vivo* properties of antigenic competition including: (1) the relationship of antigen dose to competition (e.g. Eidinger *et al.*, 1971); (2) the time relationship between injection of the two antigens and competition (e.g. Radovich and Talmage, 1967; Möller and Sjöberg, 1970; Waterston, 1970); (3) the greater ease of eliciting competition if both antigens are injected into the same footpad (Brody and Siskind, 1969); (4) the observed increase in competition as a result of prior immunization with the competing antigen (Brody and Siskind, 1972); (5) the failure to select efficiently for high affinity antibody production in competed animals (this paper and Harel *et al.*, 1970); (6) the results of cell transfer studies into pre-immunized animals (Möller and Sjöberg, 1970); (7) the fact that DNP-poly-L-lysine can cause competition in non-responder guinea-pigs (Ben-Efraim and Liacopoulos, 1969).

We do not feel that the data available at the present time are adequate definitively to differentiate between the type of formulation we have briefly described here and the possibility of a non-specific mediator produced by 'suppressor' T lymphocytes. Our main reason for suggesting an alternative to the inhibitor hypothesis is the difficulty in understanding how the formation of large amounts of low affinity antibodies by competed animals could be accounted for by this mechanism. It is, furthermore, possible that competition is actually a complex phenomenon with different mechanisms operative under different circumstances.

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