STUDIES ON THE CONTROL OF ANTIBODY SYNTHESIS

II. Effect of Antigen Dose and of Suppression by Passive Antibody on the Affinity of Antibody Synthesized*

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It has been frequently observed that the immune response is characterized by a progressive increase in the avidity of the antibody for the antigenic determinant (for references see reference 1). The effect of antigen dose and time after immunization on antibody affinity was systematically studied in a haptenic system by Eisen and Siskind (1), where it was found that affinity increased progressively with time after immunization and that this increase was greater with lower doses of antigen. While the mechanism which controls these variations in affinity has not been definitely established it has been suggested that the observed changes in affinity could be explained on the basis of preferential selection, by antigen, of cells capable of synthesizing antibody of high affinity. As antigen concentration decreases progressively after immunization, only cells bearing antibody molecules of a high affinity would be able to capture antigen and be stimulated to divide and to synthesize antibody. Such a theory assumes the existence of lymphoid cells precommitted to synthesis of a particular antibody molecule. This antibody would presumably be represented on the cell surface. The interaction of this cell-associated antibody with antigen, or "processed antigen," is presumed to lead to proliferation and antibody formation. Evidence for a selection of populations of antibody-forming cells on the basis of the energetics of the antigen-antibody interaction has been presented for a variety of different immunological systems (2-6).[1]

It is well known that passive antibody can specifically suppress an active antibody response to concomitantly administered antigen (7-12). We have

[1] Theis, G. A., and G. W. Siskind. Selection of cell populations in induction of tolerance: affinity of antibody formed in partially tolerant rabbits. *J. Immunol.* In press.

previously shown that the ability of an antibody to suppress is related to the affinity of the antibody for the haptenic determinant (13). While the mechanism of suppression is not definitively established, it is generally assumed that suppression is mediated through the binding of antigen, by circulating antibody, which prevents it from stimulating antibody-forming cells. If this theory is correct, one would expect that passive antibody would preferentially suppress low affinity cells resulting in an increase in the affinity of the antibody synthesized. This prediction was tested in the experiments to be reported here. In addition, the effect of antigen dose on the kinetics of antibody synthesis and on the affinity of the antibody formed was studied in detail in order to gain further insight into the mechanisms involved in the control of antibody synthesis.

Materials and Methods

Antigens and Haptens.—Dinitrophenylated bovine gamma globulin (DNP-BGG) and dinitrophenylated bovine fibrinogen (DNP-BF) were prepared by the reaction of either 2,4-dinitrobenzene sulfonic acid or 2,4-dinitrofluorobenzene with protein, at room temperature under alkaline conditions as described previously (14–16). DNP-proteins were purified by acid precipitation and extensive dialysis. The concentration of protein solutions was determined by drying a known volume to constant weight, at 95°C. Dry weights were corrected for the weight of buffer present. The degree of DNP substitution was estimated spectrally from the absorbancy at 360 mμ assuming all DNP to be present on the epsilon-amino groups of lysine and using the molar extinction coefficient for free N,ϵ-2,4-dinitrophenyl-L-lysine (ϵ-DNP-L-lysine) at 360 mμ (17,530) (17). A single preparation of DNP-BGG containing 60 DNP groups/mole and a single preparation of DNP-BF containing 150 groups/mole were used throughout.

ϵ-DNP-L-lysine was purchased from Cyclo Chemical Corp., Los Angeles, Calif. 2,4-dinitrophenol (DNP-OH) was obtained from Fisher Scientific Corporation, N. Y., and recrystallized from water before use. Sodium lauryl sulfate was obtained from Fisher Scientific Corporation, and recrystallized from 95% ethanol before use. 2,4-dinitrofluorobenzene was obtained from Eastman Organic Chemicals, Rochester, N. Y., and 2,4-dinitrobenzene sulfonic acid from Fisher Scientific Corporation.

Immunization Procedures.—2–2 ½ kg female rabbits were used throughout. Rabbits were immunized by a single injection of varying amounts of DNP-BGG emulsified in a total volume of 2.5 ml of complete Freund's adjuvant divided among five sites (four foot-pads and subcutaneously into the back of the neck). If passive antibody was to be administered it was given intravenously, using a single pool of rabbit anti–DNP-BGG antiserum. Details are indicated in footnotes to appropriate tables.

Precipitin Reactions.—Measurements of antibody concentration were made, in all cases, by quantitative precipitin reaction using DNP-BF as precipitating antigen. Complete precipitin curves, involving at least five points, were carried out on every serum. Only antihapten antibody was measured since the carrier used for precipitation was different than that used for immunization. Precipitin reactions were carried out in phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M potassium phosphate buffer, pH, 7.4) by the method of Eisen et al. (15, 18). Washed specific precipitates were dissolved in 0.02 M sodium lauryl sulfate and their absorbancy determined at 360 and 278 mμ. The 278 mμ absorbancy was corrected for absorbancy due to antigen by use of the absorbancy at 360 mμ and the 278 mμ/360 mμ absorbancy ratio for the antigen. $E_{1cm}^{1\%}$ for rabbit anti-DNP antibody at 278 mμ was taken as 14.0. Data is expressed as milligram of antibody protein per milliliter of serum.

Purification of Antibody.—Anti-DNP antibody was purified by the procedure described by Farah et al. (18). Briefly, antibody was eluted with 0.1 M DNP-OH, in the presence of 35 mg/ml streptomycin sulfate from specific precipitates formed at equivalence with DNP-BF. Hapten was removed by extensive dialysis and chromatography on Dowex I-X8 ion exchange column.

Measurement of Antibody-Hapten Affinity.—Measurements of the association constant (affinity) for the reaction of purified anti-DNP antibody with its homologous antigenic determinant ε-DNP-L-lysine, were carried out by the method of fluorescence quenching described

TABLE I

Immune Response of Normal Rabbits to Varying Doses of DNP-BGG

Antigen dose	Anti-DNP concentration,‡ mg/ml (No. of animals)					
	Time after immunization, *days*					
	4	7	13	20	27	41
mg 0.05	—	—	0.02 (12)	0.08 (12)	0.44 (9)	0.54 (9)
0.5	—	0.07 (3)	0.26 (8)	0.61 (11)	2.31 (6)	4.23 (7)
5.0	0.01 (10)	0.07 (10)	1.06 (25)	1.16 (18)	1.80 (24)	1.98 (7)
50.0	0.04 (6)	0.18 (6)	1.78 (6)	1.14 (5)	1.09 (5)	1.36 (4)

* Rabbits were immunized by a single injection of antigen emulsified in complete Freund's adjuvant.

‡ Antibody concentration was measured by quantitative precipitin reaction using DNP-BF as antigen. The antibody concentrations listed are the mean values from a series of individual animals tested. The numbers in parenthesis indicate, in each case, the number of animals tested.

by Velick et al. (19). The details of titration and methods of calculation, were precisely as described by Eisen and Siskind (1). Measurements were, in all cases, carried out in PBS, at 21°C in a thermostated Aminco-Bowman Spectrophotofluorometer using an exciting wavelength of approximately 280 mμ and recording fluorescent emission at approximately 350 mμ. For convenience in presentation of data affinities were generally expressed as the standard free energy change ($\Delta F°$) for the reaction between antibody and hapten calculated from the equilibrium constant by use of the usual thermodynamic relationship: $\Delta F° = - R T \ln K_0$, where R is the gas constant, T the absolute temperature, and ln K_0 the natural logarithm of the average intrinsic association constant for the reaction. Thus, the more negative $\Delta F°$ the greater the affinity.

RESULTS

Effect of Varying Antigen Dose on the Kinetics of Antibody Formation.—Table I indicates the immune response of rabbits to doses of DNP-BGG varying

from 50 μg to 50 mg in complete Freund's adjuvant. It is immediately apparent that with higher antigen doses levels of circulating anti-DNP antibody, detected by precipitin reactions, appeared earlier after immunization than with lower antigen doses. With 50 mg of antigen, peak antibody concentrations were observed at 13 days after immunization followed by a fall in titer and a plateauing at relatively low antibody concentration. In sharp contrast was the

TABLE II

Affinity of Anti-DNP Antibody Formed by Normal Rabbits in Response to Varying Doses of DNP-BGG*

Antigen dose	Affinity of anti-DNP antibody,‡ $-\Delta F^\circ$ in kcal/mole (No. of animals)			
	Time after immunization, *days*			
	13	20	27	41
mg				
0.05	—	9.88 (3)	10.0 (9)	11.1 (8)
0.5	8.72 (6)	10.3 (7)	11.2 (7)	12.7 (7)
5.0	8.96 (17)	9.70 (8)	10.2 (5)	11.0 (5)
50.0	8.46 (6)	8.06 (5)	8.52 (5)	9.54 (4)

* Rabbits were immunized by a single injection of antigen emulsified in complete Freund's adjuvant.

‡ Free energy change for the reaction of anti-DNP antibody with ϵ-DNP-L-lysine at 21°C in PBS· ΔF°, in kcal/mole, was calculated from the association constant for hapten as determined by fluorescence quenching by use of the relationship: $\Delta F^\circ = -R\ T\ \ln K_0$. The free energies listed are the mean values from a series of individual animals tested. The numbers in parenthesis indicates, in each case, the number of animals tested.

response to 0.5 mg of antigen. In this case the response began slowly so that at 13 days the mean antibody concentration was only 15 % of that observed at 13 days after 50 mg of antigen. However, after 13 days the amount of antibody present in the circulation of rabbits immunized with 0.5 mg of antigen increased rapidly so that by 41 days after immunization the mean concentration of antibody was more than three-fold greater than that resulting from immunization with 50 mg of antigen. 5 mg of antigen led to a response intermediate between 50 and 0.5 mg while 0.05 mg resulted in a slowly developing and weak immune response. The kinetics of the appearance of antibody in the serum of immunized rabbits is thus markedly effected by the dose of antigen used. Higher

doses of antigen result in an initial accentuated burst of antibody snythesis followed by an apparent inhibition of antibody formation as compared with the response to lower doses of antigen.

Effect of Antigen Dose on the Affinity of the Antibody Synthesized.—In order to further evaluate the effects of antigen dose on the immune response the affinities of the antibody synthesized at various times, in response to different doses of antigen, were determined. The data obtained is presented in Table II. As has

TABLE III

Effect of Passive Anti-DNP Antibody on the Affinity of the Anti-DNP Antibody Formed by Rabbits in Response to Immunization with DNP-BGG*

No passive antibody			Received passive antibody‡		
Antibody concentration	K_0§	$-\Delta F°$‖	Antibody concentration	K_0§	$-\Delta F°$‖
mg/ml			*mg/ml*		
2.16	9.0×10^7	10.7	0.26	3.1×10^8	11.5
0.89	7.7×10^6	9.30	0.68	1.4×10^8	11.0
1.53	9.5×10^6	9.42	0.71	5.7×10^7	10.5
0.54	1.2×10^7	9.56	0.28	8.0×10^6	9.31
1.40	2.7×10^6	8.66	0.15	2.3×10^8	11.3
0.98	3.5×10^7	10.2	0.26	3.3×10^8	11.5
1.20	1.4×10^7	9.65			
1.36	3.0×10^7	10.1			
Average 1.26	—	9.70	0.39	—	10.9

* Rabbits were immunized with 5 mg DNP-BGG emulsified in complete Freund's adjuvant and bled 20 days after immunization.

‡ Received 20 mg rabbit anti-DNP antibody intravenously 1 day prior to immunization and 10 mg anti-DNP antibody intravenously at weekly intervals.

§ K_0 in liters/mole as determined by fluorescence quenching for the reaction of anti-DNP antibody with ϵ-DNP-L-lysine at 21°C in PBS.

‖ Free energy change for the reaction of antibody with ϵ-DNP-L-lysine at 21°C in PBS expressed in units of kcal/mole.

been observed by previous workers (1) there is a progressive increase in affinity with time after immunization. The antibody found on day 13, the earliest time after immunization at which sufficient antibody was present to be purified, was of the same affinity regardless of the dose of antigen used for immunization. Following immunization with 0.5 mg of antigen a rapid increase in affinity occurred from day 13 to day 41. With 5 mg of antigen the affinity of the antibody increased with time after immunization but the rate of increase was less than after immunization with 0.5 mg. With 50 mg of antigen no significant change in affinity was observed between days 13 and 27 after immunization. Thus, while with antigen doses between 0.5 and 50 mg the affinity of the antibody formed is the same at day 13, at day 41 there is a marked difference in as-

sociation constants: the lower doses resulting in antibody of significantly greater affinity (between 2 and 3 kcal/mole more negative $\Delta F°$). This difference in free energy corresponds to approximately two orders of magnitude in terms of association constants. In contrast to the pattern of increased affinity with decreased antigen dose seen between 50 and 0.5 mg of antigen the antibody response to 0.05 mg DNP-BGG was of somewhat lower affinity than the response to 0.5 mg of antigen (in terms of equilibrium constants approximately 10-fold lower).

The affinity increased with time after immunization and the increase was delayed when high doses of antigen were used. With a very low antigen dose affinity was somewhat reduced.

Effect of Suppression with Passive Antibody on Antibody Affinity.—Rabbits were injected with 20 mg rabbit anti-DNP antibody 1 day prior to immunization with 5 mg DNP-BGG in complete Freund's adjuvant. The animals received additional injections of 10 mg rabbit anti-DNP antibody 6 and 13 days after immunization. Animals were bled 20 days after immunization, antibody purified, and affinities determined. As can be seen in Table III passive antibody resulted in approximately 70% depression of anti-DNP antibody formation. The amount of passive antibody given was such that unimmunized control animals receiving only passive antiserum had serum antibody concentrations below 0.03 mg/ml. Thus, the passive antibody did not contribute significantly to the total antibody present on day 20 and thus could not influence the affinities observed. It is clear that suppression by passive antibody resulted in an increase of about 1 kcal/mole in the affinity of the antibody synthesized (approximately a 10-fold increase in association constant).

DISCUSSION

It has been shown that the dose of antigen effects the kinetics of appearance of serum antibody, and the affinity of the antibody synthesized. Higher doses of antigen result in a more rapid appearance of detectable levels of serum antibody and significantly higher antibody levels early after immunization. However, with higher antigen doses peak levels of antibody appear early after immunization and antibody concentration plateau at levels below those reached after immunization with lower antigen doses. Thus a high antigen dose results in a relative depression of the antibody-forming system late after immunization. A very low antigen dose (50 μg) leads to a slow and weak immune response. The affinity of the antibody formed increases progressively after immunization. This increase in affinity is more rapid following low doses of antigen. Similar results have been reported previously by Eisen and Siskind (1). Comparable observations have also been made with respect to the response of guinea pigs to a haptenic determinant by Goidl et al.[2] and by Nussenzweig and Benacerraf

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[2] Goidl, E. A., W. E. Paul, G. W. Siskind, and B. Benacerraf. The effect of antigen dose

(20). It should be noted that at 13 days the affinities of the serum antibody formed following doses of antigen varying from 0.5 to 50 μg are essentially indistinguishable. Differences in affinity following different doses of antigen are reflected primarily in the subsequent rate of increase in affinity. Following 50 mg of antigen no increase in affinity was observed up to 27 days after immunization. Finally, it was observed that specific suppression of antibody formation by passive antibody results in a moderate increase in affinity of the residual antibody synthesized.

We have assumed that antigen (or "processed" antigen) acts in the immune response to select populations of precommitted lymphoid cells, stimulating them to proliferate and to synthesize antibody. This process is assumed to take place via an interaction with "antibody" molecules located presumably on the surface of lymphoid cells. This cell-associated antibody would presumably correspond to the antibody synthesized by that cell following interaction with antigen. On the basis of a competition of cells for antigen there is a selection of those cells bearing antibody molecules of highest affinity for the antigenic determinant. Evidence for the operation of such a thermodynamically driven selection system acting in the control of the immune response has been reported for several immune systems. Paul et al. have reported evidence for such a model in the selection of cells participating in the secondary immune response (3) and in the antigen-mediated stimulation of thymidine uptake in vitro (4). It has further been shown by Theis et al.[1] that induction of partial tolerance lowers the affinity of antibody produced suggesting that tolerance induction is also based on the interaction of antigen with antibody-like molecules: higher affinity cells being more readily rendered tolerant. Observations by Paul et al. (2) on the "termination" of tolerance by immunization with cross-reacting antigens are also consistent with a selection of populations of cells by antigen on the basis of the energetics of antigen-antibody interaction. The studies of Fazekas de St. Groth and Webster (5, 6) on "original antigenic sin" can also be interpreted as supporting the theory outlined above. It has been generally assumed that the suppression of antibody synthesis by passive antibody (7–13) is due to the binding of antigen by circulating antibody preventing it from stimulating antibody-forming cells. In terms of the theory outlined above passive antibody would be viewed as competing with cells for available antigen. As predicted by this theory we have found that high affinity antibody is more effective in suppression than is low affinity antibody (13).

The data which we have presented here is generally supportive of the above hypothesis. The increase in affinity with time and the greater increase in affinity with lower doses of antigen can both be understood in terms of increased selective pressure for antibody-forming cells of high affinity as antigen con-

centration decreases. The increase in affinity following passive antibody is fully consistent with the expectations of this hypothesis. Passive antibody competes with cells for antigen. "Low affinity cells" compete poorly for antigen as compared with cells bearing high affinity antibody molecules. Thus, "low affinity cells" are suppressed more readily than are "high affinity cells" and the average association constant of the antibody produced is consequently higher. This, it should be noted, is in contrast to results which we obtained in studying tolerance induction.[1] It appears that although "low affinity cells" are more readily suppressed, "high affinity cells" are more readily rendered tolerant. This points to a clear distinction in mechanism for these two different forms of depression of immunological reactivity.

Several points should be noted explicitly since they may require the introduction of some modifications to the simple theory outlined above. First of all, with 50 mg of antigen a depression of antibody concentration was observed following an initial rapid immune response. In comparison with the response to 0.5 mg of antigen, 50 mg clearly resulted in a depression of the immune response and markedly different kinetics of serum antibody formation. This might represent an induction of tolerance by high antigen concentration in "high affinity cells" resulting in a decrease in amount and affinity of the antibody synthesized. The possibility of tolerance induction functioning to control antibody affinity has also been postulated, by Goidl et al.[2] on the basis of observations of the response of guinea pigs to various doses of antigen. That tolerance may be involved in the variations seen in affinity was also suggested by Eisen (22). An additional possibility exists. The mechanism whereby a potential antibody-forming cell is stimulated either to merely divide or to differentiate and secrete antibody is not known. It appears probable that cell multiplication can occur, along with preparation for a secondary response, with relatively little antibody formation. For example we have observed (unpublished data) that animals immunized with 0.05 mg of antigen, while producing relatively weak primary responses, gave vigorous secondary responses upon boosting at 50 days. It is possible that differentiation for antibody formation requires either that the cell be exposed to multiple "hits" by antigen or that it binds multiple antigen molecules. A single "hit" or binding by few antigen molecules might result in proliferation without differentiation to antibody secretion. A large dose of antigen would thus favor differentiation of cells for antibody production, resulting in the rapid appearance of high antibody titers, but would tend to deplete the pool of proliferating cells and thus later limit the extent of the immune response. Lower doses of antigen, which would result in less immediate diversion of cells for antibody synthesis, would favor a more sustained and ultimately a greater immune response. Furthermore, with high antigen concentrations little selective pressure exists and the antibody formed would be expected to remain at relatively low affinity for a longer time. The relative

importance of the three factors which we have discussed above in determining the character of the immune response to high doses of antigen cannot be specified at this time. In addition, one should note that 13 days after immunization the affinity of the antibody present was the same over a 100-fold change in antigen dose (0.5–50 mg). Apparently relatively little selection occurred up to this time in immunization. In considering this finding it must be remembered that with respect to affinity there is presumably present in the animal a normal distribution of potential antibody-forming cells. Prior to contact with antigen the vast majority of cells potentially capable of reacting with the antigen would be of "average" affinity while only a few cells capable of synthesizing antibody of very high affinity would exist. For the cells capable of synthesizing antibody of high affinity to divide sufficiently to become a predominant part of the total population requires time. In addition, tolerance induction in high affinity cells might exist which would further tend to limit the initial affinity observed. It is thus not surprising that with the methods used the initial antibody formed is similar in affinity over a wide antigen dose range.

Finally, we must consider the observation that with a very low antigen dose (0.05 mg) affinity is somewhat lower than after 0.5 mg of antigen. 0.5 mg appears to be the optimal antigen dose not only with respect to amount of antibody synthesized but also with respect to the affinity of the antibody. This is somewhat surprising and the explanation is not immediately obvious. Induction of low dose tolerance, difficulties in efficient distribution of small amounts of antigen, depletion of antigen prematurely so as to have insufficient recruitment of antibody-forming cells from the pool of proliferating cells to give a fair representation of the true potential of the later stage in the immune response are only a few of the possibilities which must be considered. Once antigen stores are completely depleted, of course, no further selective pressure can exist. This might become a significant factor with very low antigen doses. Based upon equilibrium considerations alone one would expect that with a very small antigen dose only cells bearing antibody of very high affinity would capture antigen and be stimulated to produce antibody. It is reasonable to assume that equilibrium conditions frequently do not prevail in the in vivo situation. If stimulation of antibody-forming cells does not occur under strictly equilibrium conditions then statistical considerations would presumably play some role in determining the population of cells that responds to antigen. Although at equilibrium antigen would most likely be bound to "high affinity cells," if the system were not at equilibrium, and stimulation depended purely upon random hits between antigen molecules and potential antibody-forming cells then, that a cell belonging to the relatively large population of cells capable of forming antibody of "average" affinity might come into contact with antigen would be more likely than that a cell belonging to the very small group of "high affinity cells" would encounter antigen. Such statistical considerations,

based upon the assumption that prior to contact with antigen the vast majority of potentially responding cells are capable of synthesizing antibody of "average" affinity, might well account for the finding that early in immunization (13 days) no difference in affinity is observed after markedly different antigen doses, and might also account for the finding that with a very low antigen dose (0.05 mg) antibody affinity was lower than would be expected from purely thermodynamic considerations. Essentially one envisages two opposing factors: (a) equilibrium considerations which would tend to maximize antibody affinity; and (b) statistical factors which would tend towards production of "average" affinity antibody. If this were the case then the existence of an antigen dose leading to optimal antibody affinity might reasonably be expected.

It thus appears clear that antigen dose is important in controlling the character of the immune response. Selection of cell populations by virtue of the binding affinity, for antigen, of cell-associated antibody appears to be one mechanism involved in this control process. Induction of tolerance in high affinity cells by excessive doses of antigen may also play an important role in control of the immune response. It is also possible that antigen dose is involved in controlling the relationship between cell proliferation and differentiation for antibody formation. Similar considerations may be involved in Sterzl's observations that with a high dose of sheep red cells one obtains better primary responses but subsequent boosting gives a poorer secondary response as compared with a smaller primary dose (22, 23).

Uhr (7) suggested that circulating antibody may function as an important mechanism in controlling the immune response. By virtue of its interaction with antigen, circulating antibody serves to limit further antibody synthesis. It has also been suggested that the termination of 19S antibody synthesis might be mediated by the appearance of 7S antibody (10). We have previously suggested that in systems where multiple antigens are present, circulating antibody might serve to shift the specificity of the immune response (24). On the basis of the data presented here an additional factor in the control functions of circulating antibody may be suggested. It appears that low affinity antibody-producing cells are more readily suppressed than are high affinity antibody-producing cells. Circulating antibody would thus be expected to preferentially suppress function of low affinity antibody-forming cells and in this manner direct the immune response towards producing increasingly higher affinity antibody.

SUMMARY

The effect of antigen dose on the kinetics of circulating antibody synthesis and on antibody affinity was studied in a haptenic system. High doses of antigen resulted, early in immunization, in higher concentrations of antibody followed later in the immune response by decreased serum levels of antibody as compared with lower doses of antigen. The affinity of the initial antibody synthesized was very similar over a wide antigen dose range. Subsequently,

however, a rapid rise in affinity was seen in animals immunized with low doses of antigen, while relatively little change in affinity was seen in animals immunized with higher antigen doses. Suppression of active antibody formation by passive antiserum led to an increase in antibody affinity.

The results are discussed in terms of the mechanisms involved in the selection of a population of cells to participate in the immune response and the mechanisms whereby antigen dose and circulating antibody function to control antibody synthesis.

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