STUDIES ON THE CONTROL OF ANTIBODY SYNTHESIS

Interaction of Antigenic Competition and Suppression of Antibody Formation by Passive Antibody on the Immune Response*

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Naturally occurring antigens of biologic importance (e.g., bacteria, viruses, bacterial toxins) in general contain numerous antigenic determinants. An understanding of the control of antibody formation to such antigens thus requires a knowledge of the behavior of multiple antigenic determinants when presented simultaneously. It is well known that the simultaneous presentation of two antigens may result in a decrease in the amount of antibody formed to one or both antigens. Previous studies on this phenomenon, generally referred to as "antigenic competition," have been extensively reviewed by Adler (1, 2). The mechanism of antigenic competition is still unknown.

It is well established that passively administered antibody can cause a specific suppression of active antibody synthesis (3–8). It has been postulated by Uhr (3, 4) that the suppression of active antibody synthesis by circulating antibody plays an important role in the control of antibody formation. While not definitively proved, it is generally assumed that the suppressive effect of passive antibody is the result of its ability to bind antigen and thus prevent it from stimulating active antibody synthesis.

The studies reported here are aimed at gaining greater insight into the mechanisms involved in antigenic competition and suppression and how these factors might be connected with the control of antibody synthesis in systems involving multiple antigenic determinants. Antibody formation against two haptenic determinants located either on the same or on different carrier molecules was studied. The effect of passive antibody against one determinant upon antibody synthesis to each haptenic determinant was observed. In the system studied the degree of antigenic competition was the same whether the two haptens were present on the same or on different carrier molecules. It was further found that

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passive antibody to one hapten resulted in suppression of antibody formation against that determinant without affecting the antibody production against the second determinant, provided the two determinants were on the same carrier molecule. When the two determinants were on different carrier molecules, suppression of synthesis of one antibody with passive antiserum resulted in increased formation of antibody against the second hapten.

Materials and Methods

Animals.—New Zealand rabbits weighing from 2 to 2.5 kg were used throughout.

Antigens.—Dinitrophenylated (DNP) proteins were prepared as Eisen et al. described (9–11), by reacting the protein with 2,4-dinitrobenzene sulfonic acid at room temperature under alkaline conditions. The product was purified by repeated acid precipitation followed by extensive dialysis.

Arsenilic acid derivatives (R-Azo) of proteins were prepared as follows. 0.26 g of *p*-arsenilic acid, 0.02 g of KBr, and 1.5 ml of 2 N HCl were dissolved in 34 ml of distilled water and held in an ice-salt bath. 0.085 g of $NaNO_2$ dissolved in 2.5 ml of cold distilled water was added to the solution of arsenilic acid with stirring over a 5 min period. The resulting solution was kept cold and immediately added, dropwise, over a 15 min period with continuous stirring, to a solution of 2.5 g of protein in 250 ml of 0.1 M borate buffer, pH 8.0. The reaction was carried out in the cold and the pH was maintained between 9.0 and 9.5 by addition of 1 N NaOH. After dialysis against phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4) the derivatized protein was precipitated by acidification of the solution to pH 3.5 with 5 N acetic acid. The precipitate was collected by centrifugation and dissolved in a 1:1 mixture of ethanol and PBS (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4). Acid precipitation and resolution were repeated four times and the final product was dissolved and dialyzed against multiple changes of PBS.

When doubly conjugated proteins were prepared, DNP groups were first coupled to the protein followed by coupling of R-Azo groups.

The concentrations of hapten-conjugated proteins were determined by drying known volumes to constant weight at 95–100°C. The "dry weights" so obtained were corrected for the weight of buffer present. The degree of DNP substitution was estimated spectrally by assuming that all hapten groups were coupled to ε-amino groups of lysine and by using the molar absorbancy of free ε-DNP-L-lysine [17,530 at 360 mμ (12)]. The degree of substitution of R-Azo derivatives was very roughly estimated (13) from their absorbancy in alkali at 500 mμ, assuming all R-Azo groups were coupled to tyrosine residues. The values reported for the degree of substitution are admittedly only rough estimates. However, since one preparation of each antigen was used throughout and none of the conclusions arrived at required accurate knowledge of the degree of substitution, the use of more critical methods for estimating the extent of substitution was not deemed necessary.

The following compounds were prepared (subscripts refer to estimated numbers of groups per molecule of protein assuming a molecular weight of 150,000 for bovine and rabbit gamma globulin and 330,000 for bovine fibrinogen): dinitrophenylated bovine fibrinogen (DNP_{150}-BF), dinitrophenylated bovine gamma globulin (DNP_{60}-BGG and DNP_{21}-BGG), dinitrophenylated rabbit gamma globulin (DNP_{28}-RGG), arsenilate-azo-rabbit gamma globulin (R-Azo_{12}-RGG), arsenilate-azo-bovine gamma globulin (R-Azo_{15}-BGG), rabbit gamma globulin substituted with both arsenilate and DNP groups (R-Azo_{15}-DNP_{27}-RGG), and bovine gamma globulin substituted with both arsenilate and DNP groups (R-Azo_{15}-DNP_{21}-BGG).

Quantitative precipitin reactions were carried out on serum from animals immunized with either R-Azo_{15}-DNP_{27}-RGG or a mixture of DNP_{28}-RGG and R-Azo_{12}-RGG. In each case

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R-Azo_{15}-DNP_{21}-BGG, DNP_{21}-BGG, and R-Azo_{15}-BGG were used as precipitating antigens. The amount of antibody precipitated by R-Azo_{15}-DNP_{21}-BGG was always equal to the sum of the amounts of antibody precipitated with DNP_{21}-BGG and with R-Azo_{15}-BGG, whether the precipitation reactions with DNP_{21}-BGG and with R-Azo_{15}-BGG were carried out on separate aliquots of serum or sequentially on the same serum sample. It should be noted further that, by spectral analysis, the doubly coupled antigen (R-Azo_{15}-DNP_{27}-RGG) was completely precipitable by either purified anti-DNP antibody or purified anti-arsenilate antibody under conditions of antibody excess.

Immunization Procedures.—Rabbits were immunized by a single injection of 5 mg of antigen emulsified in complete Freund adjuvant. The antigens used for immunization were DNP_{28}-RGG, R-Azo_{12}-RGG, and R-Azo_{15}-DNP_{27}-RGG (RGG coupled with both DNP and R-Azo haptens). The antigen was administered in a total volume of 2.5 ml divided equally among the four foot pads and the back of the neck. Animals were bled 13 and 20 days after antigen injection. When animals were to be immunized with two haptens coupled to different carrier molecules, the two antigens were mixed before emulsification and 5 mg of each antigen was administered.

When passive antibody was to be administered, it was, in all cases, given intravenously 1 day prior to injection of antigen. Antiserum used for suppression was pooled antiserum obtained from groups of 5–15 rabbits bled either 4 wk after immunization with DNP_{60}-BGG, or 3 wk after immunization with R-Azo_{12}-RGG, or 6 wk after immunization with R-Azo_{15}-BGG. Details are indicated in legends to the appropriate tables.

Precipitation Reactions.—Antibody measurements were in all cases carried out by quantitative precipitation reactions (9, 14), using R-Azo_{15}-BGG and DNP_{150}-BF as precipitating antigens. Reaction mixtures were incubated at 37°C for 1 hr and were then held at 4°C for 24 hr before assay. Washed specific precipitates were dissolved in 0.02 M sodium lauryl sulfate (recrystallized from 95% ethanol before use) and assayed spectrally. Antibody concentrations were determined from the 278 mμ absorbancy of the dissolved specific precipitates after correction for the absorbancy contributed by the antigen. This was calculated from the absorbancy of the dissolved specific precipitates at 360 mμ in the case of DNP_{150}-BF and 400 mμ in the case of R-Azo_{15}-BGG. $E_{1\,cm}^{1\%}$ at 278 mμ for rabbit antibody was taken as 14.0. Complete precipitin curves using at least five different amounts of antigen were determined for each serum sample. The results reported are the amounts of antibody per milliliter of serum precipitated at the peak of the precipitin curves. To permit more accurate analysis, for the precipitation reactions we chose antigens which characteristically exhibit long, flat, equivalence zones with little inhibition in antibody excess (15).

RESULTS

The immune response of normal rabbits to R-Azo_{12}-RGG, DNP_{28}-RGG, mixtures of R-Azo_{12}-RGG and DNP_{28}-RGG, and to the doubly coupled antigen R-Azo_{15}-DNP_{27}-RGG is indicated in Table I. The data are expressed as milligrams of antibody per milliliter of serum as determined by quantitative precipitin reactions using DNP_{150}-BF or R-Azo_{15}-BGG as antigens. With RGG as carrier, animals would be expected to make little or no antibody specific for the carrier molecule itself and on testing of several samples, the amounts of antibody precipitable with DNP_{28}-RGG and with DNP_{150}-BF were essentially identical. Nevertheless, in order to ensure that only antihapten antibody was measured, precipitin reactions were carried out with the hapten attached to carriers different from those used for immunization. From the data reported in Table I, it is

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TABLE I

*Antibody Response of Normal Rabbits to 2,4-Dinitrophenyl and Arsenilate Haptenic Determinants**

Antigen	Rabbit No.	Day 13		Day 20	
		Anti-DNP	Anti-R-Azo	Anti-DNP	Anti-R-Azo
		mg/ml	*mg/ml*	*mg/ml*	*mg/ml*
R-Azo-RGG	1-78	—	0.76	—	0.86
	1-79	—	0.85	—	1.10
	1-80	—	0.55	—	0.66
	1-81	—	0.59	—	1.38
	1-82	—	0.74	—	0.50
Average		—	0.70	—	0.90
DNP-RGG	1-85	0.66	—	—	—
	1-86	0.94	—	0.94	—
	1-87	1.82	—	1.76	—
	1-88	0.54	—	0.61	—
	1-89	0.62	—	—	—
	1-90	0.63	—	—	—
	1-91	0.61	—	0.56	—
Average		0.83	—	0.97	—
R-Azo-RGG + DNP-RGG	45	0.18	0.09	0.32	0.04
	46	0.23	0.07	0.30	0.10
	47	0.58	0.18	0.55	0.12
	48	0.36	0.09	0.36	0.10
	49	0.21	0.13	0.12	0.04
Average		0.31	0.11	0.33	0.08
R-Azo-DNP-RGG	35	0.18	0.22	0.53	0.31
	36	0.34	0.13	0.55	0.23
	37	0.07	0.09	0.19	0.19
	38	0.16	0.13	0.52	0.21
	39	0.51	0.23	0.48	0.13
	1-06	0.10	0.03	0.15	0.02
	1-07	0.30	0.07	0.52	0.06
	1-08	0.10	0.00	0.21	0.00
	1-09	0.51	0.10	0.56	0.07
	1-10	0.58	0.18	0.73	0.06
	1-26	0.13	0.00	0.14	0.00
	1-27	0.31	0.07	0.19	0.04
	1-28	0.19	0.00	0.42	0.00
	1-29	0.46	0.08	0.79	0.04
	1-30	0.96	0.06	0.99	0.09
Average		0.33	0.09	0.46	0.10

* Animals immunized with the antigen or mixture of antigens indicated and bled at 13 and 20 days after immunization.

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clear that a marked degree of antigenic competition exists between the DNP and the R-Azo haptenic determinants. Presentation of the two haptens simultaneously results in approximately a 60% decrease in anti-DNP antibody synthesis and a 90% decrease in anti–R-Azo antibody synthesis. Thus, in this

TABLE II
*Effect of Anti-DNP Antibody upon the Response of Rabbits to Dinitrophenyl and Arsenilate Haptenic Determinants**

Antigen	Rabbit No.	Day 13		Day 20	
		Anti-DNP	Anti-R-Azo	Anti-DNP	Anti-R-Azo
		mg/ml	*mg/ml*	*mg/ml*	*mg/ml*
R-Azo-DNP-RGG	40	0.01	0.00	0.04	0.03
	41	0.03	0.34	0.06	0.08
	42	0.05	0.00	0.37	0.50
	43	0.07	0.22	0.15	0.22
	44	0.02	0.04	0.01	0.13
	1-11	0.10	0.03	0.05	0.02
	1-12	0.11	0.16	0.06	0.06
	1-13	0.19	0.37	0.57	0.04
	1-14	0.12	0.03	0.01	0.02
	1-21	0.05	0.14	0.05	0.05
	1-22	0.04	0.12	0.09	0.12
	1-23	0.00	0.06	0.05	0.04
	1-24	0.07	0.16	0.11	0.07
	1-25	0.05	0.07	0.04	0.05
Average		0.07	0.12	0.12	0.10
R-Azo-RGG + DNP-RGG	50	0.09	0.31	0.23	0.20
	51	0.06	0.16	0.18	0.19
	52	0.02	0.25	0.02	0.23
	53	0.09	0.25	0.21	0.19
	54	0.06	0.26	0.23	0.24
Average		0.06	0.25	0.17	0.21

* 20.4 mg anti-DNP antibody given intravenously on day before immunization with the antigen indicated.

system, competition results in a decrease in antibody synthesis against both determinants with the anti–R-Azo response being depressed to a significantly greater extent than the anti–DNP response. Furthermore, the sum of the amounts of anti–DNP and anti–R-Azo antibody formed when both haptens are presented simultaneously is significantly less than ($P < 0.05$ by Student's t test) the amount of antibody formed against either hapten presented alone.

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Furthermore, the degree of depression of antibody synthesis is identical whether the two haptens are presented on the same carrier molecule (R-Azo_{15}-DNP_{27}-RGG) or on separate carrier molecules (a mixture of DNP_{28}-RGG and R-Azo_{12}-RGG).

TABLE III
*Effect of Anti-Arsenilate Antibody upon the Response of Rabbits to the Dinitrophenyl and the Arsenilate Haptenic Determinants**

Antigen	Rabbit No.	Day 13		Day 20	
		Anti-DNP	Anti-R-Azo	Anti-DNP	Anti-R-Azo
		mg/ml	*mg/ml*	*mg/ml*	*mg/ml*
R-Azo-RGG	2-79	0.14	0.00	0.16	0.00
+	2-80	0.43	0.00	1.12	0.00
DNP-RGG	2-81	0.64	0.02	0.90	0.01
	2-82	0.58	0.00	0.70	0.03
	2-83	0.42	0.00	0.68	0.00
Average		0.44	<0.01	0.71	0.01
R-Azo-DNP-RGG	2-07	0.07	0.04	0.28	0.03
	2-08	0.08	0.05	0.17	0.00
	2-09	0.52	0.05	1.06	0.04
	2-10	0.15	0.05	0.92	0.03
	2-58	0.26	0.03	0.71	0.02
	2-59	0.66	0.03	0.89	0.00
	2-60	0.41	0.08	0.44	0.03
	2-61	0.05	0.08	0.29	0.00
	2-62	0.16	0.04	0.23	0.02
Average		0.26	0.05	0.56	0.02

* 20 mg of anti-R-Azo antibody given intravenously 1 day before immunization with the antigen indicated. Rabbits 2-07-2-10 received purified anti-R-Azo-BGG. Rabbits 2-58-2-62 and 2-79-2-83 received anti-R-Azo-RGG antiserum.

The results of specific suppression of anti-DNP antibody formation by passive antibody upon the immune response to the DNP and R-Azo haptens when they are presented simultaneously, either on the same or on separate carrier molecules, are presented in Table II. 20.4 mg of passive antibody was given intravenously 1 day prior to immunization. It can be seen (by comparison with Table I) that under both conditions passive anti-DNP antibody results in a marked and specific suppression of anti-DNP antibody synthesis. Approximately 80% suppression of anti-DNP antibody production was observed 13 days after immunization. When immunization was carried out with the doubly conjugated antigen R-Azo_{15}-DNP_{27}-RGG, suppression of anti-DNP antibody

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synthesis was not accompanied by any change in anti–R-Azo antibody synthesis. In contrast, when a mixture of DNP_{23}-RGG and R-Azo_{12}-RGG was employed, suppression of anti-DNP antibody formation with passive antibody resulted in a more than twofold increase in the amount of anti–R-Azo antibody produced. This increase was found to be statistically significant ($P < 0.01$ by Student's t test). Thus, suppression of antibody formation against one hapten resulted in a partial elimination of antigenic competition if, and only if, the competing antigenic determinants were on different molecules. When the two haptens were present on the same carrier molecule, suppression of antibody formation against one hapten had no effect upon antibody formation against the other.

Table III indicates the effect of suppressing anti–R-Azo antibody formation with passively administered anti–R-Azo antibody upon antibody formation against DNP and R-Azo haptens administered simultaneously (cf. Table I). The results obtained were completely compatible with the data discussed above (Table II) relating to suppression with anti–DNP antibody. Suppression of the response to one hapten resulted in an increased response to the second hapten when the two haptens were present on different carrier molecules and had no effect on the response to the second hapten when the two determinants were present on the same molecule.

DISCUSSION

The experiments reported indicate that, in the system studied, antigenic competition between haptenic determinants occurs to the same extent whether the two haptens are on the same or on separate carrier molecules. Competition resulted in decreased antibody production to both determinants. When determinants were presented simultaneously, the sum of the amounts of antibody formed against each determinant was significantly less than that formed to either determinant presented alone. It was further shown that suppression of antibody formation to one determinant by passive antibody partially eliminated the depressive effects of antigenic competition when the two haptens were located on separate carrier molecules but had no effect on antibody production to the second hapten when the two determinants were present on the same molecule.

Suppression of antibody formation by passive antibody is assumed to involve binding of antigen, but the detailed mechanism is still not understood. Two general possibilities exist: (*a*) the binding of antigenic determinants by antibody results in a rapid clearance and destruction of antigen molecules by phagocytic cells and thus prevents antigen molecules from reaching sites essential for the initiation of antibody synthesis, or (*b*) the binding of antigenic determinants prevents interaction of the determinant with some receptor site and thus prevents initiation of antibody formation. If the first possibility were correct, it would be

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expected that suppression of antibody formation against an antigen would be "molecule specific": that is, antibody against any determinant would result in opsonization and rapid clearance of the entire antigenic molecule with consequent suppression of antibody synthesis to all antigenic determinants present on the molecule. This hypothesis has been tested. It was found that with two determinants present on one molecule, suppression of antibody formation against one determinant clearly does not suppress antibody formation against the second determinant. We feel that these findings conclusively exclude the first hypothesis. Thus, simple phagocytosis and elimination of antigen prior to reaching antibody-forming sites cannot account for immunological suppression. The second possibility is consistent with all available data.

As we have discussed previously (16), the stimulation of immunocompetent cells to proliferate and produce antibody may be dependent upon the interaction of antigen with preformed "antibody" present on or in these cells. Such a "selection" theory presumes that cells precommitted to respond to a particular antigen are present in the animal prior to contact with antigen. Whether the "antigen" triggering such a cell is "native" or in some manner "processed" cannot be stated at present. Furthermore, the nature of the "antibody" associated with such precommitted cells is totally unknown. It is hypothesized that this cell-associated "antibody" has a binding site identical with that of the antibody molecules to be produced by the cell after interaction with antigen. Such a theory is consistent with the mechanism of suppression discussed above. Passively administered antibody competes with "cell-associated antibody" for available antigen molecules. Once bound to circulating antibody, the antigenic determinant is no longer available for binding to "cell-associated antibody" and thus is incapable of triggering cell proliferation and antibody synthesis. This theory would imply (*a*) that a second determinant present on the same antigen molecule should be relatively unaffected by the binding of antibody to one determinant, and (*b*) that the ability of antibody to suppress should be related to its binding affinity for the homologous haptenic determinant. The first prediction was tested and confirmed by the experiments reported in the present paper. Findings to be reported elsewhere are consistent with the second.[1]

The mechanism of antigen competition is still not understood. That the locus of competition is at the level of the antibody-forming cell itself is inconsistent with any selectional theory of antibody synthesis which presumes precommitted cells each of which is competent to produce only a single or a very small number of antibodies of different specificity. If we assume, as appears reasonable at present, a selectional theory of antibody synthesis, we are forced to conclude that antigenic competition occurs at some step in the antibody-

[1] Walker, J. G., and G. W. Siskind. Studies on the control of antibody synthesis. Effect of antibody affinity upon its ability to suppress antibody formation. Submitted for publication.

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forming mechanism prior to the antibody-synthesizing cell. It has been suggested by previous workers (17) that some variety of "processing" of antigen, perhaps in macrophages, is required prior to the initiation of antibody synthesis. Presumably, it would be at some point in this "processing" step that antigenic competition occurs. It is clear from the data presented that antigenic competition and suppression by passive antibody occur at different steps in the sequence of events leading to antibody synthesis. The step affected by passive antibody appears to involve specifically the antigenic determinant. In contrast, the step involved in antigenic competition appears to involve more of the antigen molecule than the specific determinant. It is difficult to visualize how the differences in behavior seen when the two determinants were on the same or on different molecules could be explained purely on the level of antibody specificity.

The finding reported that two haptenic determinants exhibit equivalent competition whether they are located on the same or on different carrier molecules may be purely fortuitous. This is suggested by the observation recently reported by Amkraut et al. (18) that, in their systems, competition occurred only when both haptens were on the same carrier molecule. Thus, the results obtained in such comparisons may be dependent upon a variety of variables, e.g., nature of the two haptens, nature of the carrier molecules, degree of substitution, dose of antigens used in immunization, etc.

Uhr has suggested that circulating antibody operates through a type of "feedback" mechanism in the control of antibody synthesis (3, 4). By combining with antigen, circulating antibody would function in limiting the immune response and prevent uneconomical hyperimmunization. Our studies suggest an additional regulatory function for circulating antibody in shifting the specificity of the immune response under certain circumstances. Most important, naturally occurring antigenic materials (e.g., bacteria) contain a variety of determinants located both on the same and on different molecules. When an infected animal is presented with a variety of antigens elaborated by any bacteria, antigenic competition would be expected to limit the response to some or all of these antigens. It is known that with certain bacteria, antibodies directed against some antigens can provide significant protection while antibodies against other antigenic determinants of these organisms are ineffective. If the initial antibodies formed by an infected host should have specificities ineffective in conferring protection, a mechanism to shift the immune response towards other antigenic determinants would be very advantageous. Under these circumstances the intial circulating antibody response would serve to suppress further antibody formation to these antigens and would, as demonstrated here, increase the antibody response to other antigens. A control mechanism which encouraged a shift of the immune response towards different antigens if an infection failed to be controlled by the initial immune response would, of course, be highly advantageous to the animal.

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SUMMARY

In the system studied, antigenic competition between two haptenic determinants was found to be of the same extent whether the haptens were on the same or on separate carrier molecules. Suppression of antibody formation to one determinant by administration of passive antibody partially eliminated the depressive effects of antigenic competition when the two haptens were located on separate carrier molecules but had no effect on antibody production to the second hapten when the two determinants were present on the same molecule. The results are discussed in terms of the mechanisms of suppression, antigenic competition, and control of antibody formation.

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