CARRIER FUNCTION IN ANTI-HAPTEN IMMUNE RESPONSES

I. Enhancement of Primary and Secondary Anti-Hapten Antibody Responses by Carrier Preimmunization

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The introduction of defined haptenic determinants into immunogenic carriers by Landsteiner (1) has provided a powerful tool for the analysis of specific interactions between antigens and specific cells in the immune response. Considerable evidence has been obtained demonstrating that cellular immune reactions to hapten-protein conjugates (delayed sensitivity [2–6], stimulation of DNA synthesis by antigen [7, 8], and hapten-specific secondary responses [9–11]) display a significant although variable degree of carrier specificity. Such carrier specificity of hapten-specific cellular reactions was initially interpreted to reflect the partial specificity of the antigen-binding receptors of specific cells for the carrier molecule (12–16), paralleling the demonstrated carrier specificity of anti-hapten humoral antibodies (16–18).

This interpretation of carrier function, however, is not able to explain several essential characteristics of hapten-specific immune responses:

(*a*) The magnitude of the carrier specificity of cellular immune reactions cannot be easily explained by the relatively modest contribution, in energetic terms, of the carrier molecule to the specificity of most anti-hapten humoral antibodies (16–18).

(*b*) Hapten conjugates of immunogenic molecules are required to elicit strong anti-hapten antibody responses; nonimmunogenic substances serve only poorly, or not at all, as carriers for haptens (19–21).

(*c*) The induction of immunological unresponsiveness to the carrier molecule results in the partial or total suppression of the responses to haptens on the tolerated proteins (22–27).

If the specificity of serum antibody accurately expresses the specificity of the antigen-binding receptors present on the precursors of antibody forming cells, then these findings suggest the operation of an additional recognition mechanism for the carrier molecule. This interpretation would be strengthened if cooperation between carrier-specific and hapten-specific cells were found to be essential for the development of anti-hapten immune responses. According to this view, cells capable of reacting with carrier molecules should interact with the antigen before anti-hapten antibody producing cells could be stimulated by hapten-protein conjugates.

Mitchison (28) and Rajewsky et al. (29), have recently demonstrated such cooperation between carrier-specific cells and hapten-specific cells in anti-hapten secondary

responses. Making use of the observation of Ovary and Benacerraf (9) that hapten-specific anamnestic responses display marked carrier specificity, Mitchison (28) showed that the injection of spleen cells obtained from mice immunized to bovine serum albumin (BSA) together with spleen cells from mice immunized with 4-hydroxy-3-iodo-5-nitrophenyl-ovalbumin (NIP-OVA) into irradiated syngeneic recipient mice enabled NIP-BSA to elicit strong anti-hapten secondary responses and to bypass the carrier specificity of the primary NIP-OVA immunization. Similarly Rajewsky et al. (29) reported that supplemental immunization with human gamma globulin (HGG) enabled rabbits primed with a *p*-azophenylsulfonic acid conjugate of BSA (Sulf-BSA) to form an anti-hapten secondary response to Sulf-HGG.

The present studies confirm and extend the earlier observations of cell cooperation between carrier-specific and hapten-specific cells in anti-hapten antibody synthesis. The current paper demonstrates that 2,4-dinitrophenyl (DNP) bovine gamma globulin (BGG) elicits hapten-specific secondary responses in rabbits or guinea pigs given primary immunization with DNP-ovalbumin (OVA) if those animals have also been immunized, in an appropriate way, with an optimal dose of unconjugated BGG. In addition, we show that: (*a*) Preimmunization with BGG markedly augments the primary anti-hapten antibody response to DNP-BGG in both rabbits and guinea pigs. (*b*) Transfer of anti-BGG sera into DNP-OVA immunized animals does not prepare them for an anti-DNP response to DNP-BGG. (*c*) The immunoglobulin class and the affinity of the anti-hapten antibody is characteristic of the mode and time of immunization with the hapten-carrier complex and not with that of the supplemental carrier.

Materials and Methods

Proteins and Chemical Reagents.—Hen ovalbumin (OVA), 5 × recrystallized and bovine gamma globulin (BGG) were obtained from Pentex Biochemicals, Kankakee, Ill. Bovine fibrinogen (BF) was obtained from Armour Pharmaceutical Co., Kankakee, Ill.

1-fluoro-2,4-dinitrobenzene (DNFB), *p*-dioxane, and chloramine-T were purchased from Eastman Organic Chemicals, Rochester, N.Y. 2,4-dinitrophenol (DNP-OH) was obtained from Fisher Scientific Company, Springfield, N.J. and was recrystallized from water before use. Epsilon-amino-*N*-caproic acid (EACA) and streptomycin sulfate were purchased from Mann Research Labs, Inc., N.Y., and disodium ethylenedinitrilo tetra-acetate (EDTA) from Matheson Scientific, Inc., Cincinnati, Ohio. α-tertiary-butyloxycarbonyl-L-lysine acetate (α-t-boc-lysine) was obtained from Cyclo Chemical Corp., Los Angeles, Calif. Sodium lauryl sulfate was recrystallized from 95% ethanol before use.

Preparation of Dinitrophenylated (DNP) Proteins.—2,4-dinitrophenylated (DNP) proteins were prepared by the reaction, at alkaline pH, of DNFB with proteins as previously described (5). DNP-conjugated proteins were extensively dialyzed in the cold; their nitrogen content was determined by micro-Kjeldahl analysis, and their DNP content was calculated from their absorbancy at 360 mμ (ϵ at 360 mμ for ϵ-DNP-L-lysine = 17,400 (30). The following DNP-proteins were prepared: DNP_7-OVA, DNP_{28}-BGG and DNP_{109}-BF. Subscripts refer to the average number of moles of DNP/mole of protein.

Preparation of Radioactive Compounds.—

Tritiated DNP-EACA: 1-fluoro-2,4-dinitrobenzene-^{3}H (^{3}H-DNFB) was obtained from Amersham/Searle, Chicago, Ill. (specific activity = 10 c/mM). ^{3}H-DNFB was reacted with a 100-fold molar excess of EACA dissolved in 0.1 ml of 5% Na_2CO_3. After being stirred at ambient temperature for 2 hr, the reaction mixture was applied to analytical thin layer chromatographic plates composed of silica gel. The chromatograms were developed with *n*-butanol and the portion of silica containing ^{3}H-DNP-EACA, as determined from the migration of nonradioactive DNP-EACA, was scraped from the plates. The product was eluted from the silica gel with phosphate buffered saline (PBS; 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.6).

Tritiated ε-DNP-L-lysine: ^{3}H-DNFB was reacted with α-t-boc-lysine under the same conditions as described above for EACA. After being stirred for several hours at ambient temperature, 0.1 ml of 12 N HCl was added to remove the t-boc group. 1 hr later, the mixture was chromatographed on silica gel using *n*-butanol:acetic acid:water (4:1:1) as the developing solvent. The final steps in isolating the ^{3}H-ε-DNP-L-lysine were identical to those described for ^{3}H-ε-DNP-EACA.

Radioiodinated proteins: Proteins were iodinated in the cold by the chloramine-T method (31, 32) using carrier-free ^{125}NaI (New England Nuclear Corp., Boston, Mass.).

Immunizations.—Adult, inbred strain 2 guinea pigs weighing 250–400 g and adult New Zealand white rabbits weighing 2.5–4.0 kg were obtained from the Animal Production Section, National Institutes of Health, Bethesda, Md. Guinea pigs received *primary* immunization with 1.0 mg DNP_7-OVA, in PBS, intraperitoneally on 3 successive days. 1 wk later, they received a *supplemental* immunization in the footpads, with either 1 or 50 μg of BGG emulsified in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich.) or with saline emulsified in CFA. 4 wk after initial immunization, the guinea pigs received a *secondary* immunization with 1.0 mg of DNP_7-OVA or DNP_{28}-BGG in PBS. This was administered as a 200 μg intradermal dose followed 4 hr later by an 800 μg intraperitoneal dose. Animals were bled just prior to the boost (day 0) and 4 (day 4) and 7 (day 7) days later, and antibody determinations were performed as described below.

The basic protocol for rabbit immunization was similar. Rabbits received a primary immunization consisting of 3 mg of DNP_7-OVA injected intravenously daily for 3 days. 1 wk later, a supplemental immunization with either 1 or 50 μg of BGG, as CFA emulsion, was administered subcutaneously. 5 wk after the primary immunization, 5 mg of DNP_7-OVA or of DNP_{28}-BGG in PBS was injected intraperitoneally as a secondary immunization. Animals were bled just prior to the booster injection and 4 and 7 days later.

Several modifications of this general protocol were employed; these are described in the Results section of the paper.

Preparation of Anti-BGG Antisera.—Rabbit and guinea pig anti-BGG antisera were prepared in the following manner: Rabbits were immunized with 500 μg of BGG emulsified in CFA and bled 2 wk later. The antiserum obtained from this first bleeding will hereafter be referred to as "early" anti-BGG antiserum. 7 wk after the primary immunization, all rabbits were boosted intraperitoneally with 5.0 mg of BGG in saline and were bled 1 1/2 and 3 wk after this secondary immunization. Sera from these last two bleedings were pooled and are hereafter referred to as "late" anti-BGG antisera. Guinea pigs were bled 3 wk after a single immunization with 50 μg of BGG in CFA. Sera from 17 guinea pigs were pooled and the anti-BGG antibody content determined by quantitative precipitin analysis. The anti-BGG antibody content of early and late antisera from individual rabbits was similarly determined.

Antibody Measurements.—

Quantitative precipitin analyses: Precipitin analyses were carried out as described by Eisen et al. (12), using DNP_{109}-BF to measure anti-DNP antibodies. After incubation at 37°C for

1 hr, the reaction mixtures were held at 4°C for 48 hr before analysis. Washed specific precipitates were dissolved in 0.02 M sodium lauryl sulfate and absorbancies at 278 and 360 mμ were determined. After correcting for the antigen present as indicated by the 360 mμ absorbancy, the amount of anti-DNP antibody precipitated was calculated from the 278 mμ absorbancy taking the extinction coefficients ($E^{1\%}_{1\,cm}$) for rabbit and guinea pig anti-DNP antibodies at 278 mμ as 13.5 and 13.2, respectively (33, 34).

Measurement of anti-DNP antibodies: Although high levels of anti-DNP antibody are satisfactorily determined by quantitative precipitin analyses, this technique is not suitable for measuring low levels of anti-DNP antibody because of the significant degree of nonspecific precipitation observed with highly conjugated DNP-proteins and normal sera (35). A recently described (35) modification of the Farr technique (36) was used to determine anti-DNP antibody levels. To 0.1 ml of antiserum (undiluted and serially diluted 10-fold to 1:1000 in 20% normal homologous serum) was added 0.1 ml of 1×10^{-8} M ^{3}H-DNP-EACA. After 1 hr at 4°C, two volumes of 75% saturated ammonium sulfate (SAS) was added. (66% SAS was used for undiluted guinea pig antiserum.) The samples were mixed and held at 4°C for 30 min and then centrifuged at 2500 rpm for 30 min. A 0.2 ml aliquot of each supernatant was added to 10 ml of a scintillation solvent consisting of 25 parts toluene, 5 parts Bio-Solv-3 (Beckman Instruments, Inc., Fullerton, Calif.) and 1 part Liquifluor (New England Nuclear Corp., Boston, Mass.); radioactivity was measured in a liquid scintillation spectrometer. Control samples contained PBS rather than antiserum. The percentage of binding of the ligand by each antiserum and its dilutions was calculated. Nonspecific binding of the ligand by normal nonimmune rabbit and guinea pig sera was usually less than 10%. For each experiment, binding curves were constructed with selected rabbit and guinea pig anti-DNP antisera, which had been analyzed by the quantitative precipitin technique. Using these curves, the percentage of binding of all other antisera in each experiment were converted into amounts of anti-DNP antibody in micrograms per milliliter. This conversion is valid if the unknown and standard antibodies have similar affinities. In each case, the standard antisera were chosen with the expectation that their affinity would be similar to that of the antisera being analyzed. The geometric means and standard errors of anti-DNP antibody concentrations were calculated.

Measurement of anti-BGG antibodies: Passive hemagglutination with BGG adsorbed tanned sheep erythrocytes was carried out as described by Boyden (37) and Stavitsky (38). Antisera to be tested and normal sera used for diluents were decomplemented at 56°C for 1 hr and absorbed with washed sheep erythrocytes. The specificity of the reactions was verified by appropriate controls and by specific inhibition of hemagglutination by BGG. All tests were performed with microtiter equipment (Cooke Engineering Co., Alexandria, Va.). The geometric mean of the reciprocal of the titers is presented.

Quantitative determination of precipitating anti-BGG antibody was performed with ^{125}I-BGG as follows: 0.2 ml of antiserum was reacted with 0.1 ml of ^{125}I-BGG in varying concentrations ranging from 10 to 3200 μg/ml. The mixtures were kept at 37°C for 1 hr and then at 4°C for 24 hr. The samples were centrifuged at 2500 rpm for 30 min in the cold, and the radioactivity of an aliquot of each supernatant was measured in a NaI crystal scintillation counter. Controls consisted of normal homologous serum mixed with each of the ^{125}I-BGG concentrations used. The per cent and amount of antigen precipitated at each antigen concentration by 0.2 ml of antiserum were calculated. Curves were constructed by plotting per cent antigen precipitated against antigen concentration. From these curves, the antigen concentration at which 50% of added antigen was precipitated was determined (P_{50}).

P_{50} is expressed as micrograms of BGG added per milliliter of antiserum.

Purification of Anti-DNP Antibodies.—Antibodies specific for the DNP-determinant were prepared by the method of Farah et al. (39). Specific precipitates were formed by the reaction of antisera and DNP-BF, at equivalence, in the presence of 0.01 M EDTA. Anti-DNP anti-

bodies were eluted with 0.1 M DNP-OH. DNP-BF was precipitated with streptomycin sulfate (35 mg/ml) and DNP-OH was removed by extensive dialysis.

Preparation of Guinea Pig γ_2-Globulin and Anti-Guinea Pig Globulin Antisera.—Guinea pig γ_2-globulin was separated from the globulin fraction of pooled normal guinea pig serum by ion-exchange chromatography using DEAE-cellulose equilibrated with 0.01 M phosphate buffer, pH 8.0 (40). Purity of the γ_2-fraction was confirmed by immunoelectrophoresis (41). Rabbit antisera specifically directed against guinea pig γ_1-globulin and pepsin-digested fragments of guinea pig γ_2-globulin, $F(ab)_2'$, were obtained as previously described (42). Anti-γ_1 antiserum was repeatedly absorbed with purified γ_2-globulin until no further precipitation could be observed. After absorption with γ_2-globulin, the anti-γ_1 antiserum precipitated less than 2% of ^{125}I-labeled γ_2-globulin in contrast to unabsorbed anti-γ_1 which precipitated 27–30%. Anti-γ_2 antiserum absorbed with $F(ab)_2'$ was the gift of Dr. Victor Nussenzweig and precipitated 96% of purified ^{125}I-γ_2-globulin.

Measurements of Antibody-Binding Affinities.—Average association constants were calculated for the binding of anti-DNP antibodies to ^{3}H-DNP-L-lysine from data obtained by an ammonium sulfate precipitation technique (43) and were termed K_F. In order to determine the total concentration of antigen-binding sites present, 20 μl of undiluted serum were mixed with 10 μl of ^{3}H-ϵ-DNP-L-lysine. The final concentration of the latter ranged from 0.167×10^{-5} M to 5.337×10^{-5} M. The total site concentration was determined from a plot of bound hapten versus free hapten, after correction for nonspecific precipitation. K_F's for primary response anti-DNP antibodies were also calculated from this data. To determine K_F for secondary response anti-DNP antibodies, an antibody concentration of 1×10^{-7} M and ^{3}H-ϵ-DNP-L-lysine concentrations ranging from 0.5 to 8×10^{-8} M were used in the binding assay. Utilizing the expression:

$$B/F = K_F(Ab_o - B)$$

where B is concentration of bound hapten, F is concentration of free hapten, and Ab_o is the total antibody site concentration, K_F was calculated from plots of B/F versus B.

RESULTS

Enhancement of Haplen-Specific Anamnestic Responses by Carrier Preimmunization.—Guinea pigs and rabbits were immunized according to the general protocols described in the methods section and outlined in Tables I and II. Table I and Fig. 1 present the results obtained in strain 2 guinea pigs; Table II and Fig. 2 present similar data for rabbits.

When DNP is presented on the same carrier molecule (OVA) for both primary and secondary immunizations, a clear secondary response is obtained in guinea pigs and rabbits (Tables I and II, Group A). On the other hand, secondary immunization with DNP on a different carrier (BGG) does not elicit an anti-DNP response in guinea pigs, and evokes a small, but significant, increase in anti-DNP antibodies in rabbits (Tables I and II, Group B). (Similar observations of secondary anti-hapten responses in rabbits immunized with heterologous carrier conjugates have been reported previously [10, 44, 45]).

However, when a supplemental immunization with unconjugated BGG in CFA is given prior to secondary immunization with DNP_{28}-BGG, a markedly enhanced secondary anti-DNP response is obtained in both species (Tables I

and II, Groups C and D). The magnitude of the response is determined by the amount of BGG used for supplemental immunization. Thus, in guinea pigs a dose of 50 μg is significantly more effective than is 1 μg, whereas in rabbits

TABLE I

Enhancement of Hapten-Specific Secondary Responses in Guinea Pigs by Preimmunization with the Carrier Molecule

Group	Immunization protocol			Number of animals	Anti-DNP antibody (μg/ml)*				Anti-BGG antibody titer*		
	Primary	Supplement	Secondary		Day 0	Day 4	Day 7	Boost‡	Day 0	Day 7	Boost‡
A	DNP_7-OVA	CFA	DNP_7-OVA	10	3.2	9.0	73.4	70.2	0	0	0
B	DNP_7-OVA	CFA	DNP_{28}-BGG	7	14.7	6.2	14.1	−0.6	0	0	0
C	DNP_7-OVA	BGG-CFA (1 μg)	DNP_{28}-BGG	4	3.9	3.9	143.0	139.1	91	609	518
D	DNP_7-OVA	BGG-CFA (50 μg)	DNP_{28}-BGG	9	4.2	26.7	626.0	621.8	4798	20.838	16,040
E	DNP_7-OVA	OVA-CFA (50 μg)	DNP_7-OVA	5	0.82	31.2	603.4	602.6	N.D.§	N.D.	

* The data are expressed as geometric means.
‡ Represents the increase in mean antibody levels or titers from day 0 to day 7.
§ N.D., not determined.

TABLE II

Enhancement of Hapten-Specific Secondary Responses in Rabbits by Preimmunization with the Carrier Molecule

Group	Immunization protocol			Number of animals	Anti-DNP antibody (μg/ml)*				Anti-BGG antibody titer*		
	Primary	Supplement	Secondary		Day 0	Day 4	Day 7	Boost‡	Day 0	Day 7	Boost‡
A	DNP_7-OVA	BGG-CFA (50 μg)	DNP_7-OVA	15	1.9	43.6	108.5	106.6	979	2842	1863
B	DNP_7-OVA	CFA	DNP_{28}-BGG	15	3.2	4.5	12.1	8.9	0	48	48
C	DNP_7-OVA	BGG-CFA (1 μg)	DNP_{28}-BGG	8	3.4	40.9	276.9	273.5	76	1884	1808
D	DNP_7-OVA	BGG-CFA (50 μg)	DNP_{28}-BGG	8	4.1	25.8	69.7	65.6	1878	13,883	12,005
E	DNP_7-OVA	BGG-Alum (9 mg)	DNP_{28}-BGG	4	1.1	6.1	9.3	8.2	64	861	797
F	DNP_7-OVA	BGG-Saline (15 mg)	DNP_{28}-BGG	4	1.2	20.6	43.3	42.1	N.D.§	N.D.	
G	DNP_7-OVA	Saline	DNP_{28}-BGG	5	2.4	2.1	14.8	12.4	N.D.	N.D.	

* The data are expressed as geometric means.
‡ Represents the increase in mean antibody levels or titers from day 0 to day 7.
§ N.D., not determined.

1 μg appears to be the optimal dose. Moreover, supplemental immunization is most effective when BGG is injected in CFA; alum-precipitated BGG administered intravenously (3.0 mg for 3 successive days) did not significantly affect the secondary anti-DNP response of rabbits to DNP_{28}-BGG (Table II, Group

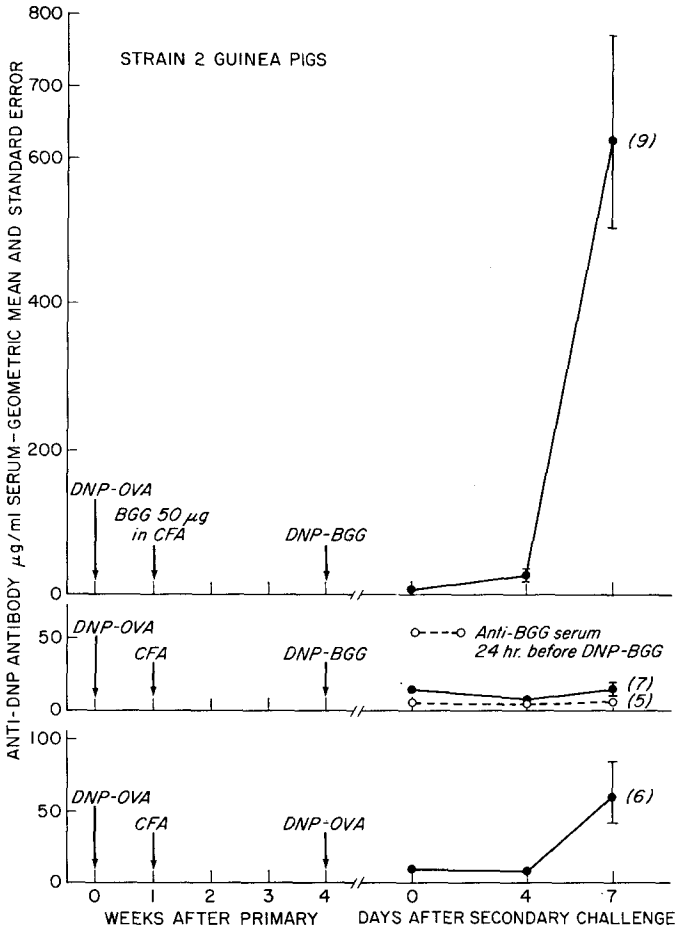

FIG. 1. Enhancement of hapten-specific anamnestic responses by carrier preimmunization in guinea pigs.

Primary immunization with 3.0 mg of DNP_7-OVA, administered intraperitoneally in saline, was performed at week 0. 1 wk later supplemental immunization with either 50 μg of BGG emulsified in CFA or with a saline-CFA emulsion was carried out. 4 wk after primary immunization, the animals were challenged with 1.0 mg of either DNP_{28}-BGG or DNP_7-OVA in saline. Serum anti-DNP antibody concentrations just prior to challenge and on days 4 and 7 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups. The lowermost panel illustrates the normal secondary response of DNP-OVA-primed animals to DNP-OVA challenge; the middle panel shows the absence of a secondary response to DNP-BGG in DNP-OVA-primed animals, and furthermore demonstrates the failure of transfused anti-BGG serum to stimulate a response. The uppermost panel presents the enhancement of the secondary response to DNP-BGG in DNP-OVA-primed animals which have been supplementally immunized with BGG.

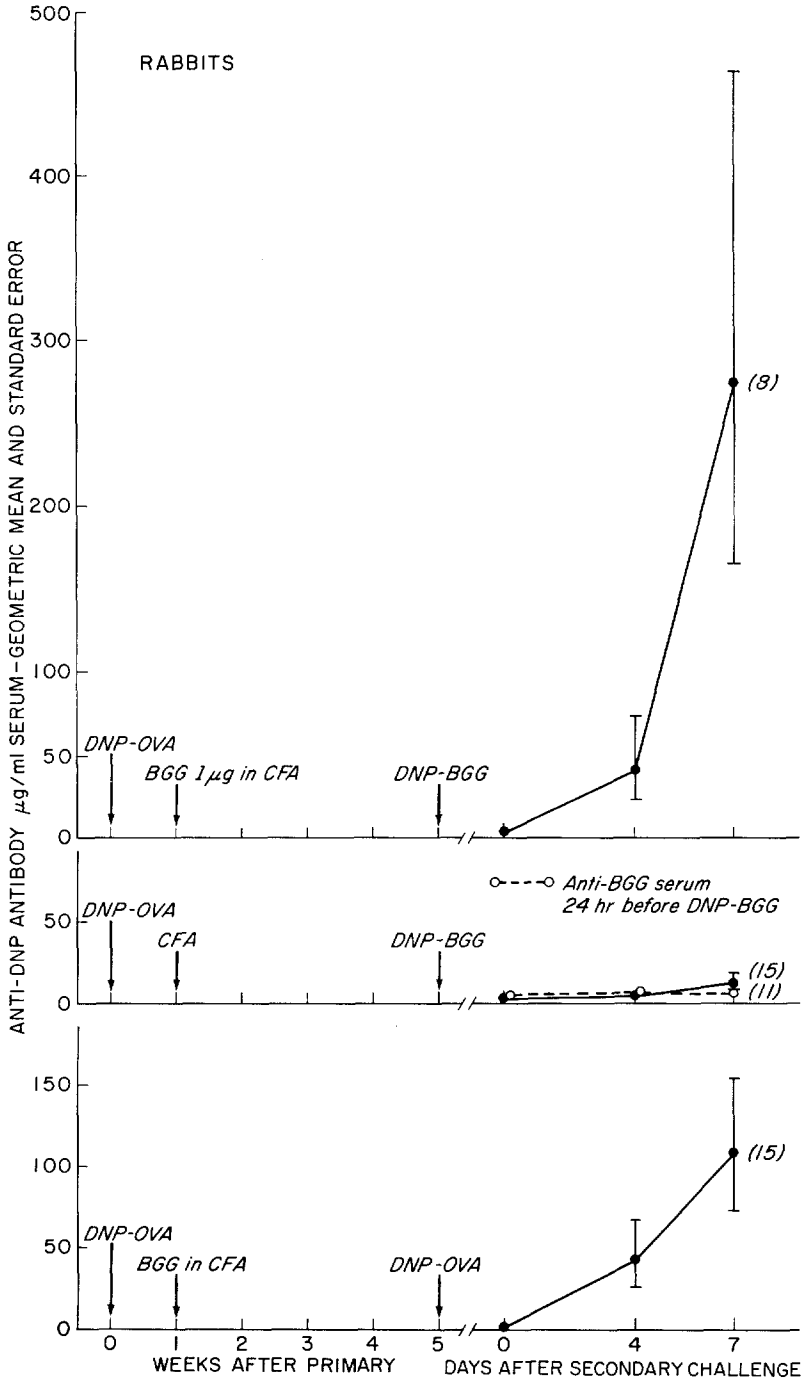
RABBITS
ANTI-DNP ANTIBODY μg/ml SERUM—GEOMETRIC MEAN AND STANDARD ERROR
500
400
300
200
100
50
0
DNP-OVA
BGG 1μg in CFA
DNP-BGG
(8)
DNP-OVA
CFA
DNP-BGG
Anti-BGG serum
24 hr before DNP-BGG
(15)
(11)
150
100
50
0
DNP-OVA
BGG in CFA
DNP-OVA
(15)
0 1 2 3 4 5
WEEKS AFTER PRIMARY
0 4 7
DAYS AFTER SECONDARY CHALLENGE

FIG. 2

E). Nevertheless, rabbits which received soluble BGG intraperitoneally (5.0 mg on 3 successive days) for supplemental immunization manifested an enhanced anti-DNP response (Table II, Group F) comparable in magnitude to that of rabbits given 50 μg of BGG in CFA (Table II, Group D) and significantly greater than the controls (Table II, Group G). Some correlation was noted between the anti-hapten and anti-carrier secondary responses to DNP_{28}-BGG in both species.

The data presented demonstrate clearly that anti-hapten secondary responses to a hapten conjugate of a heterologous carrier can be considerably enhanced by supplemental preimmunization with such a carrier molecule. This phenomenon is best seen when the serum anti-hapten antibody concentration of the primed animals is low at the time of secondary challenge, as occurs with primary immunization without adjuvants, and when the supplemental preimmunization of the second carrier is administered with adjuvant. Under these circumstances the anti-DNP response of DNP_7-OVA primed animals supplementally immunized with BGG in CFA is consideraby greater to DNP_{28}-BGG than is the normal secondary to DNP_7-OVA (Tables I and II).

However, using the same type of experimental protocol cooperative effects between carrier and hapten immunizations can be demonstrated without resorting to the use of different carrier proteins for primary and secondary challenge. Thus, as shown in Table I (Group E) the anti-DNP response to DNP_7-OVA of guinea pigs primed with DNP_7-OVA in saline was markedly enhanced by supplemental preimmunization with 50 μg of OVA in CFA, administered 1 wk after primary immunization. This response was essentially equivalent to that of animals immunized with DNP_7-OVA, supplementally immunized with 50 μg of BGG in CFA and boosted with DNP_{28}-BGG (Table I, Group D).

Enhancement of Hapten-Specific Primary Responses by Carrier Preimmunization.—Guinea pigs and rabbits were preimmunized with BGG or OVA either

FIG. 2. Enhancement of hapten-specific anamnestic responses by carrier preimmunization in rabbits.

Primary immunization with 9.0 mg of DNP_7-OVA, administered intravenously in saline, was performed at week 0. 1 wk later supplemental immunization with either 1 μg of BGG emulsified in CFA or with a saline-CFA emulsion was carried out. 5 wk after primary immunization the animals were challenged with 5.0 mg of either DNP_{28}-BGG or DNP_7-OVA in saline. Serum anti-DNP antibody concentrations just prior to challenge and on days 4 and 7 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups. The lowermost panel illustrates the normal secondary response of DNP-OVA-primed animals to DNP-OVA challenge; the middle panel shows the meager secondary response to DNP-BGG in DNP-OVA-primed animals, and furthermore demonstrates the failure of transfused anti-BGG serum to augment this response. The uppermost panel presents the enhancement of the secondary response to DNP-BGG in DNP-OVA-primed rabbits which have been supplementally immunized with BGG.

in saline or emulsified in CFA. Guinea pigs received primary immunization with 1.0 mg of DNP_{28}-BGG or DNP_7-OVA in saline 3 wk later. Rabbits received primary immunization with an intraperitoneal dose of 5.0 mg of DNP_{28}-BGG in saline 4 wk later. Control animals were preimmunized with saline emulsified in CFA. The protocols are outlined in Tables III and IV.

The guinea pig experiments are summarized in Table III and presented graphically in Fig. 3. The normal primary anti-DNP response to a single dose of antigen in saline intraperitoneally is very meager in guinea pigs (Groups A

TABLE III

Enhancement of Hapten-Specific Primary Responses in Guinea Pigs by Preimmunization with the Carrier Molecule

Group	Immunization protocol		Number of animals	Anti-DNP antibody (μg/ml)*		Anti-BGG antibody titers*		
	Carrier preimmunization	Primary‡		Day 4	Day 7	Day 0	Day 7	Boost§
A	CFA	DNP_{28}-BGG	10	0.10	0.22	0	0	0
B	BGG-CFA (1 μg)	DNP_{28}-BGG	4	0.13	22.1	362	2048	1686
C	BGG-CFA (50 μg)	DNP_{28}-BGG	9	0.17	139.8	3797	56,780	52,983
D	BGG-Saline‖ (500 μg)	DNP_{28}-BGG	5	0.10	0.22	N.D.¶	N.D.	
E	CFA	DNP_7-OVA	5	0.40	0.26	N.D.	N.D.	
F	OVA-CFA (50 μg)	DNP_7-OVA	5	1.14	41.6	N.D.	N.D.	

* The data are expressed as geometric means.

‡ 0.2 mg intradermally followed by 0.8 mg intraperitoneally in saline.

§ Represents the increase in mean antibody levels from day 0 to day 7.

‖ Administered intradermally.

¶ N.D., not determined.

and E). In contrast, guinea pigs preimmunized with BGG or OVA in CFA and then injected with the DNP-homologous protein conjugate develop considerably greater primary anti-DNP responses (Groups B, C, and F). As in the preceding experiments, 50 μg rather than 1 μg of BGG in CFA was the most effective dose in guinea pigs. A correlation between anti-DNP and anti-BGG responses was again noted. No enhancement of the anti-DNP primary response was observed when guinea pigs were preimmunized intradermally with BGG in saline (Group D).

The rabbit experiments are shown in Table IV. The normal rabbit primary anti-DNP response to a single dose of DNP_{28}-BGG in saline is greater than that of guinea pigs (Group A). Preimmunization with 50 μg of native BGG in

CFA did not enhance significantly the primary anti-DNP response (Group C). However, when the optimal dose of the carrier molecule, 1 μg of BGG in CFA, was used for preimmunization, the primary anti-DNP response to DNP_{28}-BGG was clearly enhanced (Group B).

Effect of Passively Transferred Anti-Carrier Antibody on Hapten-Specific Primary and Secondary Responses.—As shown in the preceding experiments, supplemental immunization of guinea pigs and rabbits with BGG in CFA produced large amounts of circulating anti-BGG antibodies prior to immunization with DNP_{28}–BGG. This raises the question of the role that circulating anti-carrier antibodies may play in the enhanced anti-hapten antibody responses. This problem was investigated in the following experiments using the same antigen dose and route of administration as in the previous experiments.

TABLE IV

Elicitation of Hapten-Specific Primary Responses in Rabbits

Group	Immunization protocol: Carrier preimmunization	Immunization protocol: Primary‡	Number of animals	Anti-DNP antibody (μg/ml)*: Day 4	Anti-DNP antibody (μg/ml)*: Day 7
A	CFA	DNP_{28}-BGG	5	0.16	1.2 (0.59– 2.4)
B	BGG-CFA (1 μg)	DNP_{28}-BGG	5	0.25	46.8 (29.4–74.4)
C	BGG-CFA (50 μg)	DNP_{28}-BGG	5	0.16	4.7 (2.3– 9.6)

* The data are expressed as geometric means; the range encompassed by ±1 SE is shown within parentheses.

‡ 5 mg intraperitoneally in saline.

The experimental protocols of the guinea pig experiments are shown in Table V. One group (Group A) was preimmunized with 50 μg of BGG in CFA, two groups (B and C) with saline in CFA. 3 wk later, Group C guinea pigs were injected intravenously with 10 ml of guinea pig anti-BGG serum (18.2 mg of antibody) divided in two doses given on 2 successive days. 24 hr after the second injection of anti-BGG serum, the standard primary immunization with DNP_{28}-BGG in saline was carried out. Each guinea pig in Groups A and B received 10 ml of normal guinea pig serum prior to primary immunization with DNP_{28}-BGG. Guinea pigs in Groups D and E which had been primed with DNP_7-OVA at the beginning of the experiment were treated in an identical manner as animals in Groups A and C, respectively.

As expected from the results of the preceding experiments, guinea pigs in Groups A and D manifested enhanced primary and secondary anti-DNP responses, respectively. Passively transferred anti-BGG serum, however, had no enhancing effect upon either a primary (Group C) or a secondary (Group

E) anti-DNP response in guinea pigs. These data are also presented graphically in Fig. 1 and 3.

The experimental protocols for the rabbit experiments are listed in Table VI. 1 wk after primary immunization with DNP_7-OVA, one group (Group A) was preimmunized with 1 μg of BGG in CFA, three groups (B, C, and D)

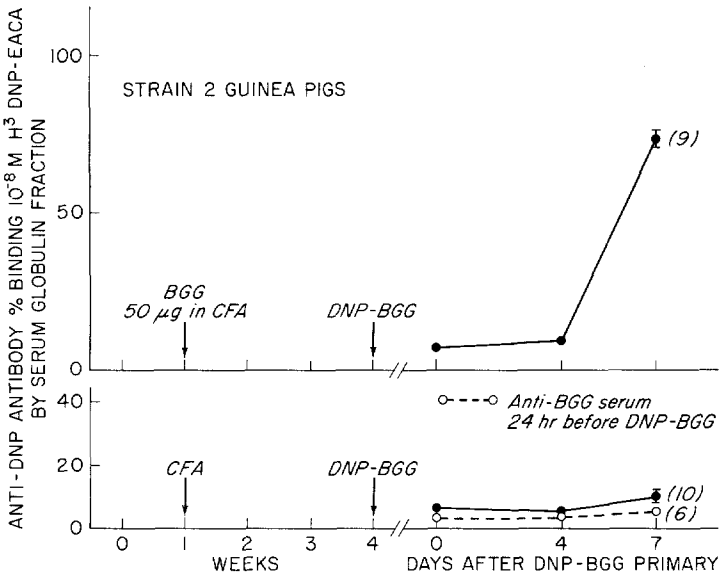

FIG. 3. Enhancement of hapten-specific primary responses by carrier preimmunization in guinea pigs.

Animals were preimmunized with either 50 μg of BGG emulsified in CFA or with a saline-CFA emulsion. 3 wk later, they received a primary immunization with 1.0 mg of DNP_{28}-BGG in saline. Serum anti-DNP binding activities just prior to primary immunization and on days 4 and 7 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups. The lower panel illustrates the meager primary anti-DNP response in non-preimmunized guinea pigs, and the failure of transfused anti-BGG serum to augment this response. The upper panel shows the enhanced primary anti-DNP response to DNP-BGG in guinea pigs preimmunized with BGG.

with saline in CFA. 4 wk later, each rabbit in Group C received 7.5 mg of early rabbit anti-BGG serum, and those in Group D received 70 mg of late anti-BGG serum intravenously in a single dose; each rabbit in Groups A and B received normal rabbit serum. 24 hr later, standard secondary immunization with DNP_{28}-BGG in saline was carried out. Rabbits in Group A displayed the expected enhanced secondary anti-DNP response and those in Group B showed the small secondary response usually seen to DNP-heterologous conjugates. As in guinea pigs, passively transferred anti-BGG serum had no enhancing effect

TABLE V

Effect of Passive Transfer of Anti-Carrier Antibody on Anti-Hapten Responses in Guinea Pigs

Group	Immunization protocol			Number of animals	Anti-DNP antibody (μg/ml)*				Anti-BGG antibody titer*		
	Primary	Supplement	Secondary		Day 0	Day 4	Day 7	Boost‡	Day 0	Day 7	Boost‡
A	—	50 μg BGG-CFA Normal serum‖	DNP_{28}-BGG§	5		0.26	211.8		2358	37,927	35,569
B	—	Normal serum	DNP_{28}-BGG§	5		0.10	0.13		<2	<2	0
C	—	Anti-BGG Serum (18.2 mg)	DNP_{28}-BGG§	6		0.10	0.10		161	57	−104
D	DNP_7-OVA	50 μg BGG-CFA Normal serum	DNP_{28}-BGG	5	4.7	41.3	717	712.3	3112	14,367	11,255
E	DNP_7-OVA	Anti-BGG Serum (18.2 mg)	DNP_{28}-BGG	5	3.2	2.0	3.9	0.7	388	21	−367

* The data are expressed as geometric means.

‡ Represents the increase in mean antibody levels or titers from day 0 to day 7.

§ This is primary immunization with DNP_{28}-BGG.

‖ Normal serum or anti-BGG serum were injected intravenously 48 and 24 hr before DNP_{28}-BGG challenge.

on the secondary anti–DNP responses of rabbits regardless of whether antiserum of presumably low (early) or high (late) avidity was employed (Groups C and D, respectively). In fact, a considerable depression of the anti–DNP response was observed, particularly with late anti–BGG antiserum.

Characterization of Anti-DNP Antibodies.—

Determinations of relative amounts of γ_1 and γ_2 anti–DNP antibodies: Two types of 7S immunoglobulins, γ_1 and γ_2, have been described in guinea pigs (46, 47). Their differences in physical, chemical, and biological properties have been recently reviewed (48). The relative proportions of γ_1 and γ_2 globulins produced by guinea pigs immunized with hapten-protein conjugates are influenced by several factors. Antibodies produced following immunization with

TABLE VI

Effect of Passive Transfer of Anti-Carrier Antibody on Anti-Hapten Responses in Rabbits

Group	Immunization protocol			Number of animals	Anti-DNP antibody (μg/ml)*				Anti-BGG antibody P_{50} (μg/ml)*		
	Primary	Supplement	Secondary		Day 0	Day 4	Day 7	Boost‡	Day 0	Day 7	Boost‡
A	DNP_7-OVA	1 μg BGG-CFA Normal Serum§	DNP_{28}-BGG	7	4.3	48.0	374.8	370.5	42‖	576	534
B	DNP_7-OVA	Normal Serum	DNP_{28}-BGG	6	2.4	2.8	27.4	25.0	<25	<25	0
C	DNP_7-OVA	Early Anti-BGG Serum (7.5 mg)	DNP_{28}-BGG	6	4.8	4.0	10.5	5.7	25	<25	−25
D	DNP_7-OVA	Late Anti-BGG Serum (70 mg)	DNP_{28}-BGG	5	4.6	4.1	5.2	0.6	180	50	−130

* Geometric means.

‡ Represents the increase in mean antibody levels from day 0 to day 7.

§ Normal serum or anti-BGG serum were injected intravenously 24 hr before DNP_{28}-BGG challenge.

‖ Amounts of BGG which when added to 1 ml of antiserum result in 50% precipitation.

antigens in saline belong predominantly to the γ_1 class, whereas antigens emulsified in CFA stimulate the production of predominantly γ_2 antibodies (47). Since in the present studies primary and secondary immunizations were carried out with soluble antigens in saline, and supplemental immunization with BGG in CFA, it was of interest to determine whether the use of adjuvant for the supplemental immunization changed the proportions of γ_1 and γ_2 anti-DNP antibodies produced following secondary challenge with DNP_{28}-BGG.

Purified ^{125}I-labeled anti-DNP antibodies were prepared from day 7 sera of guinea pigs given primary and secondary immunizations with DNP_7-OVA without supplemental carrier preimmunization (Group A, Table I), and of guinea pigs which received DNP_{28}-BGG for secondary immunization following supplemental preimmunization with BGG in CFA (Group D, Table I). 10 μl aliquots of ^{125}I-labeled antibody containing 1, 2, or 5 μg of protein were mixed with 25 μl each of three specific rabbit antisera: (*a*) anti-γ_1 globulin, (*b*) anti-

γ_2-globulin, and (*c*) anti-F(ab)$_2$'. Control tubes contained 25 μl of normal rabbit serum. The reaction mixtures were incubated overnight at 4°C, and then each whole sample (35 μl) was counted for total counts per minute (cpm). After centrifugation at 2500 rpm for 20 min, 10 μl of each supernate was removed and counted. The percentage of each antibody precipitated by each of the specific antisera was calculated in the following way:

$$\% \text{ precipitated} = 100 - \frac{\text{cpm of 10 } \mu\text{l supernate}}{\text{total cpm}/3.5} (100)$$

The results shown in Table VII demonstrate that both groups produced relatively larger amounts of γ_1 than of γ_2 antibodies. There is slightly more γ_2 immunoglobulin in the antibody of the group which received supplemental

TABLE VII

Relative Proportions of γ_1 and γ_2 Globulins in Guinea Pig Anti-DNP Antibodies

Group	Primary	Supplement	Secondary	Anti-DNP antibody (μg/ml) Day 7	Per cent γ_1-globulin	Per cent γ_2-globulin	Per cent precipitated by Anti-F(ab)$_2$' serum*
A	DNP_7-OVA	CFA	DNP_7-OVA	560	70	33	97
				285	64	33	98
D	DNP_7-OVA	BGG-CFA (50 μg)	DNP_{28}-BGG	650	50	46	95
				1020	58	37	98

* The percentage of antibody precipitated in the control tubes containing normal rabbit serum was less than 2% in all cases.

BGG immunization, but the small differences observed do not seem significant. It would appear, therefore, that supplemental preimmunization with free BGG in CFA did not affect significantly the proportions of γ_1 and γ_2 anti-DNP antibodies produced. Rather, this ratio seems to have been determined by the nature of primary immunization.

Affinity of anti-DNP antibodies for ^{3}H-DNP-L-lysine: The association constants of anti-DNP antibodies obtained after the secondary anti-DNP responses, in both rabbits and guinea pigs, were more than 10^7 L/M (Table VIII). This was noted both in the case of antibodies produced by animals which received primary and secondary immunizations with DNP_7-OVA and in those given primary immunization with DNP_7-OVA and secondary immunization with DNP_{28}-BGG after having received a supplemental immunization with BGG.

On the other hand, rabbits and guinea pigs given primary immunization with DNP_{28}-BGG, after BGG preimmunization, produced anti-DNP antibodies of relatively low affinity (0.14×10^7 or less). Thus, the affinity of the anti-

DNP antibody was affected by the time interval between the primary DNP-protein immunization and the bleeding, and not by the time between the supplemental BGG immunization and the bleeding.

TABLE VIII

Affinity of Primary and Secondary Response Anti-DNP Antibodies for ^{3}H-ε-DNP-L-Lysine

	Immunization protocol			Time after primary immunization	
	Primary	Supplement	Secondary		K_F* L/M × 10^7
				wk	
Guinea Pigs	DNP_7-OVA	CFA	DNP_7-OVA	5	1.8
					2.1
	DNP_7-OVA	BGG-CFA (50 μg)	DNP_{28}-BGG	5	21.7
					17.0
					7.2
					5.8
					3.5
					2.0
	—	BGG-CFA (50 μg)	DNP_{28}-BGG	1	0.13
					0.14
Rabbits	DNP_7-OVA	BGG-CFA (50 μg)	DNP_7-OVA	6	25.2
					43.2
	DNP_7-OVA	BGG-CFA (1 μg)	DNP_{28}-BGG	6	13.4
					16.2
	—	BGG-CFA (1 μg)	DNP_{28}-BGG	1	0.014
					0.020

* Average association constant for ^{3}H-DNP-L-Lysine of anti-DNP antibodies from individual animals.

DISCUSSION

The experiments reported here demonstrate that secondary anti-hapten antibody responses to hapten-conjugates of a protein not used in primary immunization are markedly enhanced by an interim immunization with the unconjugated protein. This observation confirms the findings of Mitchison (28) and of Rajewsky, et al. (29). Furthermore, preimmunization with carrier enhances the primary anti-hapten antibody response to a hapten-carrier complex in both rabbits and guinea pigs.

Additional examples of augmentation of a response to a given antigenic determinant as a result of a concomitant, or prior, immune response to another determinant on the same particle have been described. Schierman and McBride (26) noted enhanced primary antibody responses, in chickens, to weak erythrocyte isoantigens when highly immunogenic isoantigens were also present on the same erythrocyte. Salvin and Smith (3, 49) reported that guinea pigs immunized with protein antigens developed anti-hapten antibodies, of the γ_1 class,

at an accelerated rate after immunization with hapten conjugates of these proteins. Several other investigators have failed to obtain evidence of augmented primary responses to hapten as a consequence of carrier preimmunization (29,50–53). As has been shown in the current paper, however, the conditions of immunization are quite crucial to the demonstration of this phenomenon. Thus, carrier preimmunization is most effective when the protein is administered in complete Freund's adjuvant. Furthermore, attention to dose is quite important. 50 μg of BGG prepared guinea pigs for significantly greater anti-hapten responses than did 1 μg of BGG. In contrast, rabbits manifested a greater anti–hapten antibody response a as result of preimmunization with 1 μg of BGG than of 50 μg of BGG. In addition, primary immunization with hapten-protein conjugates without adjuvant permitted a most effective demonstration of the enhancing effect of carrier preimmunization.

This enhancing effect, in both primary and secondary anti-hapten responses, could not be obtained by the transfer into animals of serum antibody directed at carrier determinants. Anti–carrier antibodies obtained both early and late in the course of an immune response were ineffective in this regard. However, lymphoid cells from syngeneic animals immunized to carrier will transfer enhanced responsiveness to animals which themselves have not received supplemental carrier immunization. This has been demonstrated by the studies of Mitchison, with CBA mice (28), and by experiments reported in the second paper of this series, with strain 2 guinea pigs (54).

This group of findings strongly suggest that, in anti–hapten antibody responses, an interaction of carrier-specific cells with hapten-carrier conjugate is required for maximal stimulation of the precursors of anti–hapten antibody producing cells. This cellular cooperation may well have an analogy in the cooperation of antigen reactive cells and the precursors of plaque forming cells in antibody responses of mice to sheep erythrocytes. In the latter system, it has been shown that antigen reactive cells are derived from the thymus and do not, themselves, secrete antibody (55–58). Nevertheless, they are specific and they are required for the production of anti-sheep erythrocyte antibody by cells derived from the bone marrow. One may then reasonably ask whether, in the carrier-hapten cooperation, carrier-specific cells are thymus dependent antigen reactive cells and hapten-specific cells, bone marrow derived precursors of antibody-forming cells. (It should, of course, be noted that there would also be precursors of antibody-producing cells specific for determinants of the carrier molecules). Experiments are currently in progress in our laboratory to answer these questions. Preliminary results of Mitchison, et al. suggest, indeed, that carrier-specific cells in mice are thymus-dependent lymphocytes (59).

The nature of the interaction between carrier-specific cells and hapten-specific cells, in the development of an anti–hapten antibody response is not clear. Several possible mechanisms may be considered:

(*a*) The carrier cell acts as a device to concentrate antigen and to present it

to hapten-specific cells, perhaps in a specified anatomic compartment of the lymphoid system. This explanation has been suggested by Mitchison (28, 59).

(*b*) The stimulation of a hapten-specific cell requires an interaction not only with antigen but also with the carrier-specific cell. The latter may provide a signal of some sort which is crucial to the differentiation and/or proliferation of the hapten-specific cell.

(*c*) The carrier-specific cell secretes a special class of anti-carrier antibody which must interact with antigen in order that antigen may then stimulate the hapten-specific cell. This antibody appears in the serum in concentrations too low for the response to be transferred by serum.

(*d*) The carrier-specific cell processes the antigen; this processed antigen may then specifically stimulate hapten-specific cells.

A choice among these mechanisms will require detailed information regarding the properties of carriers and the functions of carrier-specific cells. The second paper of this series describes some of these properties and functions (54).

The data presented in the current study do demonstrate that both the immunoglobulin class and the affinity of anti–hapten antibody are determined by the mode and time of primary immunization with hapten-carrier conjugate and not by the conditions of the supplemental carrier immunization. Thus, a majority of the anti-DNP antibody molecules produced by guinea pigs which had been primed with DNP_7-OVA in saline, immunized with BGG in complete Freund's adjuvant, and challenged with DNP_{28}-BGG in saline are of the γ_1 immunoglobulin class whereas γ_2 antibodies predominate in the immune response to antigens administered in complete Freund's adjuvant (46–48). This result is analogous to allotype data of Mitchison et al. in that the class of the anti–hapten antibody is determined by the primary hapten-carrier immunization and not by the subsequent carrier immunization (59). Similarly, the affinity of the anti–hapten antibody for ^{3}H-ϵ-DNP-L-lysine is determined, as in conventional immunization, by the length of time after primary immunization with the hapten conjugate and is not affected by supplemental carrier immunization.

The phenomenon of carrier cell–hapten cell cooperation may have general relevance to immune responses and is very likely not limited to the experimental models reported here and by others. This is strongly suggested by the observation that guinea pigs given primary immunization with DNP_7-OVA and secondary immunization with this same conjugate show considerably enhanced anamnestic anti–hapten antibody responses if they have received an interim immunization with unconjugated OVA emulsified in complete Freund's adjuvant.

Indeed, one must consider whether the stimulation of hapten-specific precursors of antibody forming cells always requires the participation of carrier-

specific cells. According to this view, the modest hapten-specific secondary responses to DNP_{28}-BGG in rabbits primed with DNP_7-OVA but not receiving supplemental BGG immunization may be ascribed to the few BGG specific carrier cells present prior to BGG immunization or developing early in the course of a primary response.

SUMMARY

Preimmunization of either guinea pigs or rabbits to bovine gamma globulin (BGG) prepares the animals for markedly enhanced antibody responses to 2,4-dinitrophenyl-BGG (DNP-BGG). This phenomenon is observed both in the primary anti-DNP antibody response to DNP-BGG and in the secondary anti-DNP antibody response to DNP-BGG in animals primed with DNP-ovalbumin (DNP-OVA). The BGG preimmunization is most effective if the antigen is administered as a complete Freund's adjuvant emulsion; in rabbits, a dose of 1 μg of BGG is more effective than a dose of 50 μg, whereas the reverse is true in guinea pigs. Transfusion of homologous anti-BGG sera fails to replace active immunization with BGG in the preparation of animals for these enhanced anti-DNP antibody responses. Both the immunoglobulin class and the average association constant for ϵ-DNP-L-lysine of the anti-DNP antibody produced in these enhanced responses is determined by the mode and time of immunization with haptenic conjugates and is not appreciably influenced by the nature of the carrier preimmunization.

These studies indicate that the carrier specificity of hapten-specific anamnestic antibody responses is largely due to the interaction of two independent cell associated recognition units, one specialized for carrier and the other specific for haptenic determinants.

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