

CARRIER FUNCTION IN ANTI-HAPTEN IMMUNE RESPONSES

II. SPECIFIC PROPERTIES OF CARRIER CELLS CAPABLE OF ENHANCING ANTI-HAPTEN ANTIBODY RESPONSES

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A marked enhancement of anti-hapten antibody synthesis in response to immunization with a hapten-protein conjugate occurs as a result of pre-immunization of animals with unmodified carrier. This is true both for primary and secondary anti-hapten responses and is most strikingly demonstrated when an animal which has received a primary immunization with a hapten conjugate of a given carrier is boosted with the same hapten conjugated to a different carrier, to which the animal has been preimmunized (1-6).

This phenomenon indicates the existence of independent recognition units for hapten and for carrier. Cooperative interaction between these units may be essential to the production of an anti-hapten antibody response, or may amplify a modest immune response which occurs in the absence of carrier-specific recognition.

An understanding of the nature of the carrier recognition unit and of the range of substances which it recognizes and acts upon is required in order to construct a coherent picture of cooperative interactions in the immune response. Studies presented both by Mitchison and Rajewsky, et al. (4, 5) and those reported in the first paper of this series (6) show that humoral antibody is not the carrier recognition unit.

In the adoptive secondary antibody response in mice, hapten-carrier cooperative interactions appear to be mediated by hapten-specific and carrier-specific lymphoid cells (4, 7). In the present communication we show that live lymphoid cells from strain 2 guinea pigs immunized to bovine gamma globulin (BGG) enable syngeneic animals immunized with 2,4-dinitrophenyl ovalbumin (DNP-OVA) to form a secondary anti-DNP response to DNP-BGG. Thus, in both species the carrier recognition unit appears to be associated with a lymphoid cell.

Several relevant questions may then be asked concerning the significance of this phenomenon and the mechanism by which carrier-specific cells enhance anti-hapten antibody response. One suggested function of the carrier-specific cell is the concentration and presentation of antigen (4, 7). Such cells would bind multideterminant antigens by one of their determinants and simply present other determinants to appropriate hapten-specific cells in certain anatomical locations. However, the possibility must also be considered that

the carrier-specific cell or its product exerts a more sophisticated and complex function which is concerned, in part, with the recognition of certain classes of molecules, such as proteins or polypeptides, as immunogens. A study was therefore made of the kinds of substances with which carrier function is associated and of the immunological specificity of this phenomenon.

The results will show that:

(a) An antigen such as a copolymer of L-glutamic acid and L-lysine (GL) to which a given strain of guinea pig is genetically unable to accord a complete immune response does not function as a carrier in that strain; on the other sensitization to a simple carrier, poly-L-lysine (PLL), does prepare "responder" guinea pigs for an augmented secondary response to a hapten conjugate of PLL.

(b) Attempts to sensitize animals to haptens by immunization with hapten conjugates of foreign proteins fail to prepare for enhanced primary or secondary responses to other determinants associated with that hapten on a different carrier. These findings indicate that hapten alone cannot function specifically as carrier for other haptens or, in other words, that haptens generally do not constitute determinants capable of being recognized by cells concerned with carrier function or, alternatively, of stimulating such cells to perform this function. However, haptens may contribute to such determinants; hapten conjugates of an autologous protein, such as DNP-guinea pig albumin, may stimulate the formation of carrier-specific cells capable of enhancing the antibody response to other haptens presented on the same molecule.

(c) The capacity of carrier preimmunization to augment secondary responsiveness to hapten-carrier conjugates is abolished by specific immunologic tolerance to that carrier.

Materials and Methods

Proteins and Chemical Reagents.—Guinea pig albumin (GPA) was obtained from Pentex Biochemicals, Kankakee, Ill. Glucose oxidase (GO), from *Aspergillus niger*, was purchased from Sigma Chemical Co., St. Louis, Mo. Poly-L-lysine hydrobromide (PLL), poly-D-lysine hydrobromide (PDL) and the copolymer of L-glutamic acid and L-lysine (GL) were obtained from Pilot Chemicals, Inc., Watertown, Mass. Average molecular weights were respectively 90,000, 140,000 and 115,000, and the GL had a ratio of G:L of 60:40. 5-dimethylaminonaphthalene sulfonyl chloride (DANSYL-Cl) was purchased from Calbiochem., Los Angeles, Calif. and sulfanilic acid was obtained from Fisher Scientific Company, Springfield, N.J. All other proteins and chemical reagents have been described in the preceding paper (6).

Hapten-carrier conjugates.—The following 2,4-dinitrophenyl conjugates were prepared as previously described (6): DNP₇-OVA, DNP₂₈-BGG, DNP₂₄-GO, DNP₂₇-GO, DNP₃₀-GPA, DNP₃₅-PLL, DNP₇₅-PDL and DNP₂₀-GL. Subscripts refer to the average number of moles of DNP per mole of carrier.

p-azophenylsulfonic acid conjugates of BGG and GPA (Sulf-BGG and Sulf-GPA) were prepared as described by Campbell et al (8). The degree of substitution, calculated on the basis of absorbancy at 460 m μ in 0.1 N NaOH ($\epsilon = 9800$) (9) and micro-Kjeldahl nitrogen analysis, was 29 and 30 moles of *p*-azophenylsulfonic acid per mole of BGG and GPA, respectively.

DANSYL conjugates were prepared by the reaction, at alkaline pH, of DANSYL-Cl with the carriers. Weight ratios of DANSYL-Cl:carrier of 0.20 to 0.40 were employed; the reactions were carried out at room temperature and were allowed to proceed overnight. Conjugates were extensively dialyzed against phosphate buffered saline (PBS: 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.6). Degree of substitution was calculated from absorbancy at 330 m μ ($\epsilon = 4570$ for ϵ -DANSYL-LYSINE) (10) and nitrogen content. The following conjugates were prepared: DANSYL₃₉-GPA, DANSYL₁₂-PLL, DANSYL₂₈-PLL and DANSYL₈₁-PDL.

p-azophenylsulfonic acid conjugates of DNP and DANSYL proteins were prepared in a manner similar to the preparation of Sulf-proteins. Sulf₁₆-DNP₂₇-GO, Sulf₉₂-DNP₂₄-GO, Sulf₂₁-DNP₃₀-GPA and Sulf₁₆-DANSYL₃₉-GPA were prepared.

DNP conjugates of DANSYL-polypeptides were prepared as described above. DNP₂₃-DANSYL₁₂-PLL and DNP₁₇₄-DANSYL₈₁-PDL were synthesized.

Preparation of radioactive compounds.—OVA was labeled with ¹²⁵I by the reaction of 5 mg of OVA in 4.0 ml 0.1 M sodium carbonate, pH 9.5, with 2 mc of ¹²⁵NaI in 0.15 ml of a solution of I₂ in KI (I₂, 1.23 mg/ml; KI, 1.57 mg/ml) (11). The reaction was allowed to proceed for 1 hr at 0°C; ¹²⁵I-OVA was extensively dialyzed against PBS. All other radioactive compounds were prepared as described in the preceding paper (6).

Immunizations.—Strain 2 and strain 13 inbred guinea pigs and New Zealand White rabbits were immunized according to the general protocol described in the preceding paper (6) and outlined in the results section of this communication.

Antibody measurements.—Serum concentrations of anti-Sulf antibodies were assayed by passive hemagglutination with sulfanil-azo-sheep erythrocytes prepared by the method of Ingraham (12). The classical passive hemagglutination procedure was followed (13, 14). Antisera to be tested and normal sera used for diluents were decemplemented at 56°C for 30–60 min and absorbed with washed sheep erythrocytes. The specificity of the reactions was verified by appropriate controls and by specific inhibition of hemagglutination with sulfanil-azochloroacetyl tyrosine. All tests were performed with microtiter equipment (Cooke Engineering Co., Alexandria, Va.). The geometric mean of the reciprocal of the titers is presented.

Anti-OVA antibody concentration was estimated by the Farr technique (15): 0.1 ml of antisera was mixed with 0.1 ml of a solution of ¹²⁵I-labeled OVA (10 μ g/ml). After incubation at 37°C for 1 hr, 0.4 ml of cold 66% saturated ammonium sulfate was added. After 30 min at 4°C, the radioactivity of the total sample was measured. After centrifugation at 2500 rpm for 20 min, 0.2 ml of each supernate was removed and radioactivity determined in a NaI crystal scintillation counter. The percentage of binding of ¹²⁵I-OVA by each antiserum was calculated as follows:

$$\% \text{ binding} = 100 - \frac{\text{radioactivity of 0.2 ml supernate}}{\text{Total radioactivity} / 3} (100)$$

The serum concentrations of anti-DNP antibodies and anti-BGG antibodies were measured as described in the preceding paper (6).

Statistical Analyses.—Serum antibody values were logarithmically transformed and means and standard errors calculated. Results from groups were compared by the Student's *t* test.

RESULTS

Ability of BGG-specific lymphoid cells to prepare DNP-OVA immunized guinea pigs for a secondary response to DNP-BGG.—Strain 2 donor guinea pigs were immunized in the footpads with either 50 μ g of BGG emulsified in complete Freund's adjuvant (CFA) or with saline in CFA. 3 wk later, the animals were sacrificed, and axillary, occipital, inguinal, and popliteal lymph nodes and

spleen were removed. Single cell suspensions, in minimum essential medium (Eagle), were prepared, washed, and transferred to recipients; a varying number of nucleated cells were injected intravenously into strain 2 guinea pig recipients which had been immunized 3 wk earlier with 3 daily doses of 1.0 mg of DNP₇-OVA administered intraperitoneally in saline.

Recipients were boosted either 1 or 6 days after cell transfer with 1.0 mg of DNP₂₈-BGG in saline (200 μ g intradermally followed by 800 μ g intraperi-

TABLE I
*Enhancement of Hapten-Specific Anamnestic Responses in Guinea Pigs Following Passive Transfer of Carrier-Specific Syngeneic Cells**

Group	Protocol†		Number of animals	Anti-DNP antibody (μ g/ml)§			Boost
	Specificity of cells transferred	Interval (days)		Day 0	Day 7	Day 11	
A	BGG	1	5	4.6	5.2	46.8	42.2
B	CFA	1	5	5.0	0.90	11.8	6.8
C	BGG	6	5	0.70	49.3	N.D.¶	48.6
D	CFA	6	5	1.4	0.74	N.D.	0

* 1 to 1.6×10^9 lymph node and spleen cells obtained from donors 3 wk after immunization with 50 μ g of BGG in CFA or saline in CFA.

† All recipients received DNP₇-OVA for primary immunization 3 wks prior to cell transfer and were boosted with DNP₂₈-BGG. Interval refers to the time between cell transfer and secondary immunization.

§ The data are expressed as geometric means. A comparison of the geometric mean anti-DNP serum antibody concentrations of groups A and B, on day 11, yielded a p value of $0.1 > p > 0.05$. A comparison of the geometric means of groups C and D, on day 7, yielded a p value of < 0.01 .

|| Represents the increase in mean antibody levels from day 0 to day 11 (Groups A and B) or day 7 (Groups C and D).

¶ N.D., not determined.

toneally 4 hr later). Animals were bled just prior to secondary immunization (day 0) and 4, 7, and 11 days later.

The results of these experiments are presented in Tables I and II and Fig. 1. Guinea pigs which received 1.0 – 1.6×10^9 cells from BGG-sensitized donors displayed secondary anti-DNP responses to immunization with DNP₂₈-BGG (Table I, Groups A and C); a secondary anti-BGG response was also observed in these animals. When secondary immunization was performed 1 day after cell transfer, the secondary anti-DNP response was not apparent until day 11 (Table I, Group A). However, guinea pigs which were boosted 6 days after transfer manifested clear secondary responses by day 7 after challenge (Table I, Group C and Fig. 1). In contrast, guinea pigs which received cells from donors sensitized to CFA only, did not manifest secondary responses to either DNP or BGG (Table I, Groups B and D and Fig. 1).

TABLE II
*Enhancement of Hapten-Specific Anamnestic Responses in Guinea Pigs Following Passive Transfer of Carrier Specific Syngeneic Cells**

Group	Protocol†	Number of cells transferred	Number of animals	Anti-DNP Antibody ($\mu\text{g/ml}$)§		
				Day 0	Day 7	Boost
A	BGG	1000×10^6	3	1.2	57.1	55.9
B	BGG	200×10^6	3	2.0	29.4	27.4
C	BGG	50×10^6	4	1.0	13.6	12.6
D	CFA	200×10^6	4	0.99	0.62	-0.37

* Lymph node and spleen cells obtained from donors 3 wk after immunization with 50 μg of BGG in CFA or saline in CFA.

† All recipients received DNP₇-OVA for primary immunization 3 wk prior to cell transfer and were boosted with DNP₂₈-BGG 6 days after cell transfer.

§ The data are expressed as geometric means. A comparison of the geometric mean serum anti-DNP antibody concentrations, on day 7, of group D with those of groups A, B, and C yielded a p value of <0.01 in each case. Furthermore, a similar comparison for groups A and C yielded a p value of <0.05.

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

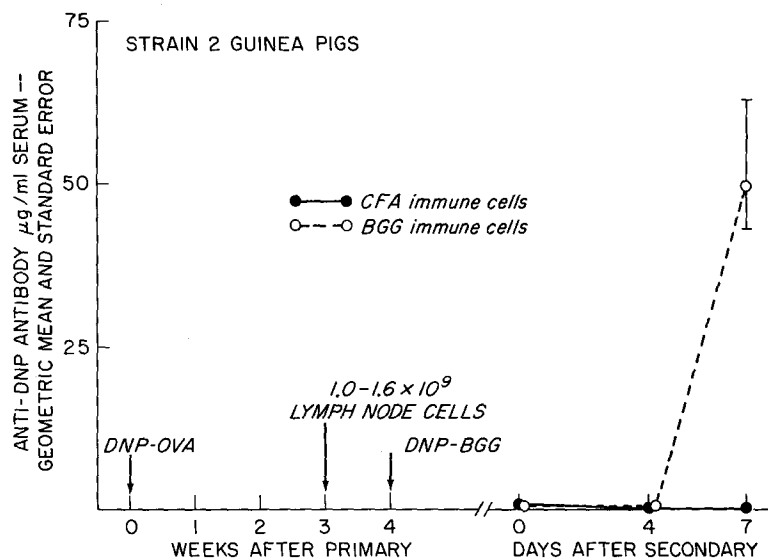


FIG. 1. Ability of BGG-specific lymphoid cells to prepare DNP-OVA immunized guinea pigs for an enhanced response to DNP-BGG. Primary immunization of recipients was performed at week 0 with 3.0 mg of DNP₇-OVA administered intraperitoneally in saline. 3 wk later they were transfused with $1.0-1.6 \times 10^9$ lymphoid cells from syngeneic donors which had been immunized with either 50 μg of BGG in CFA or saline in CFA 3 wk earlier. 6 days after cell transfer, the recipients were boosted with 1.0 mg of DNP₂₈-BGG in saline. Serum anti-DNP antibody concentration just prior to challenge and on days 4 and 7 are illustrated.

The number of BGG-sensitized cells required to transfer this response was shown to be quite modest. Thus, as shown in Table II, as few as 50×10^6 cells from BGG-sensitized donors transferred the capacity to obtain a secondary anti-DNP response to DNP₂₈-BGG when boosting was performed 6 days after transfer (Group C). The magnitude of the anti-DNP secondary response seemed to be related to the log of the number of cells transferred in the range used in this experiment. Secondary anti-BGG responses were detectable only in the group receiving 1000×10^6 BGG-sensitized cells (Group A).

Requirement for immunogenicity to mediate carrier function.—An autosomal dominant gene (PLL gene) exists in guinea pigs which controls the immune response to PLL, GL, and their haptenic derivatives as well to several other compounds (16). Strain 2 guinea pigs possess the PLL gene, whereas strain 13 guinea pigs lack this gene. Thus, strain 13 guinea pigs fail to display an immune response to GL.

In order to determine whether the capacity to mediate carrier function requires that a compound be immunogenic by conventional standards both strain 2 and strain 13 guinea pigs, which had been immunized with DNP₇-OVA 1 wk earlier, were immunized with 100 μ g of GL emulsified in CFA. 3 wk later a secondary immunization with 1.0 mg of DNP₂₀-GL in saline was performed. The strain 2 guinea pigs displayed the expected secondary anti-DNP response, whereas the strain 13 animals did not manifest any response, and their anti-DNP antibody concentration fell markedly (Fig. 2). As a control, a group of strain 13 guinea pigs, which had been sensitized to DNP₇-OVA, were immunized with 50 μ g of BGG in CFA and boosted with 1.0 mg of DNP₂₈-BGG. These animals showed marked secondary anti-DNP responses, as expected.

Thus, the locus of action of the product of the PLL gene appears to involve a step in the immune response required for the expression of carrier function.

Specific immunologic tolerance and carrier function.—In order to determine if the carrier function for an antigen could be abrogated by the establishment of tolerance to that substance, a group of rabbits were rendered specifically tolerant to BGG in the following manner. Adult rabbits received two intravenous injections weekly of aggregate-free BGG which was obtained by centrifugation at 105,000 g for 90 min. The top two-thirds of the supernatant was used (17, 18). The doses were 1.0 mg in the 1st wk, 10 mg in the 2nd wk, and a single 100 mg dose in the 3rd wk. 1 wk after the final dose, tolerant and control rabbits were immunized intravenously with 3.0 mg of DNP₇-OVA in saline on 3 successive days, followed 1 wk later by supplemental immunization with 1 μ g of BGG emulsified in CFA or with saline in CFA. 4 wk later, they received secondary immunization intraperitoneally with 5.0 mg of either DNP₇-OVA or DNP₂₈-BGG in saline. The rabbits in the tolerant groups were only partially tolerant since their anti-BGG responses were significantly, but not totally, depressed.

The protocols and results of these experiments are presented in Table III. Tolerant rabbits which received supplemental immunization with 1 μg of BGG in CFA displayed a mean secondary anti-DNP response of 8.4 $\mu\text{g}/\text{ml}$ (Group A), whereas nontolerant animals treated in the same way showed a mean net increase of 273.5 $\mu\text{g}/\text{ml}$ (Group B). Indeed, the response of tolerant animals which received the 1 μg BGG supplemental immunization was essen-

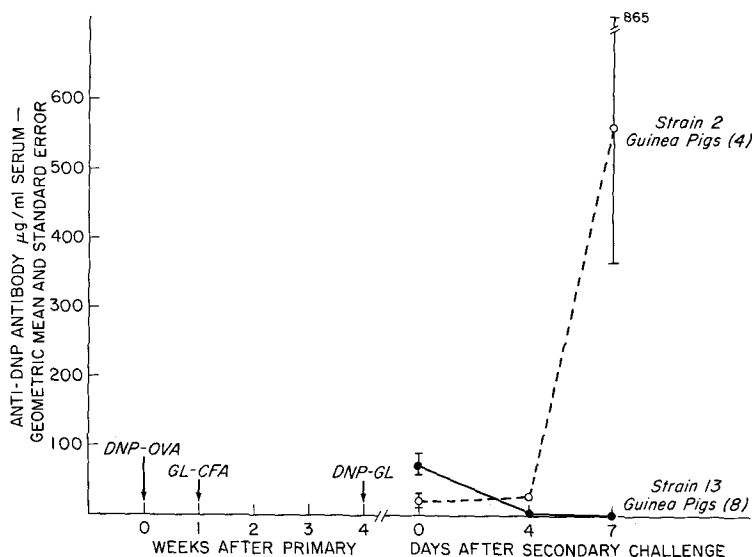


FIG. 2. Requirement for immunogenicity to mediate carrier function. Strain 13 and strain 2 guinea pigs received a primary immunization with 3.0 mg of DNP₇-OVA, administered intraperitoneally in saline at week 0. 1 wk later supplemental immunization with 100 μg of GL emulsified in CFA was carried out. 4 wk after primary immunization, the animals were challenged with 1.0 mg of DNP₂₀-GL in saline. Serum anti-DNP antibody concentration 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.

tially equivalent to tolerant or nontolerant rabbits which had received only CFA supplemental immunization. (Groups C and D, respectively.)

The response of BGG-tolerant animals to a secondary immunization with DNP₇-OVA (Group E) was reduced only slightly when compared to nontolerant rabbits (Group F), and this difference was not statistically significant. Thus, specific immunologic tolerance to an antigen prevents an animal from recognizing that antigen as a carrier.

Failure of immunization to haptenic determinants to prepare for enhanced secondary responses.—In the experiments thus far described by this and the preceding paper (6), it has been shown that presensitization to carrier molecules prepares for an enhanced anti-hapten antibody response to hapten-carrier

conjugates. In order to determine whether carrier function requires simply preimmunization to determinants associated with the boosting hapten on the same molecule, a series of experiments were undertaken in which animals were preimmunized with a given hapten-carrier conjugate and then the antibody response to other determinants associated with the same hapten on another carrier was evaluated.

(a) *Lack of enhancement of the primary response to protein determinants:* Strain 2 guinea pigs were immunized with either 50 μ g of DNP₂₈-BGG in CFA or with saline in CFA. 3 wk later, primary immunization with either DNP₇-OVA or Sulf₂₉-BGG (200 μ g intradermally followed in 4 hr by 800 μ g intraperitoneally in saline) was carried out. The protocols and data are summarized in

TABLE III

Effect of Tolerance to the Secondary Carrier on Hapten-Specific Anamnestic Responses in Rabbits

Group	Immunization protocol			Number of animals	Anti-DNP antibody (μ g/ml)*		Boost
	Primary	Supplement	Secondary		Day 0	Day 7	
A, tolerant	DNP ₇ -OVA	BGG-CFA (1 μ g)	DNP ₂₈ -BGG	6	1.2	9.6	8.4
B, nontolerant	DNP ₇ -OVA	BGG-CFA (1 μ g)	DNP ₂₈ -BGG	8	3.4	276.9	273.5
C, tolerant	DNP ₇ -OVA	CFA	DNP ₂₈ -BGG	7	1.8	14.0	12.2
D, nontolerant	DNP ₇ -OVA	CFA	DNP ₂₈ -BGG	15	3.2	12.1	8.9
E, tolerant	DNP ₇ -OVA	CFA	DNP ₇ -OVA	5	1.8	68.8	67.0
F, nontolerant	DNP ₇ -OVA	CFA	DNP ₇ -OVA	10	1.9	136.3	134.4

* The data are expressed as geometric means. A comparison of the geometric mean serum anti-DNP antibody concentrations, on day 7, of groups A and B revealed a p value of <0.01. Comparison of the means of groups A and C, A and D, and E and F revealed no significant differences (p values, 0.6, > 0.9, and 0.7 respectively).

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

Table IV. As can be seen, the experiment was designed so that one group (Group B) received the same hapten for preimmunization and primary immunization, whereas another group (Group D) received the same carrier in both immunizations.

As shown in Table IV, the existence of a state of immunity to DNP₂₈-BGG did not heighten the anti-OVA antibody response to DNP₇-OVA (Group B). On the other hand, sensitivity to DNP₂₈-BGG enhanced significantly the primary anti-Sulf response to Sulf₂₉-BGG (Group D) as compared to the controls (Group C). Thus, under these conditions immunization with DNP₂₈-BGG results in the clear expression of carrier function by the BGG molecule but not by DNP determinants.

(b) *Lack of enhancement of the secondary response to a second hapten:* New Zealand white rabbits were immunized intravenously with 3 daily doses of 3.0 mg of DNP₇-OVA in saline. 1 wk later, they received supplemental immunization with 50 μ g of either Sulf₂₉-BGG in CFA or of GO in CFA. 4 wk

later, a secondary immunization of 5.0 mg of either DNP₂₇-Sulf₁₆-GO, DNP₂₈-BGG, Sulf₂₉-BGG, or DNP₂₇-GO in saline was administered intraperitoneally.

The protocols and data are presented in Table V. Rabbits which received

TABLE IV
Effect of Hapten Preimmunization on Subsequent Anti-Carrier Response in Guinea Pigs

Group	Immunization protocol		Number of animals	Anti-OVA (%binding)*		Anti-Sulf titer‡	
	Preimmunization	Primary		Day 0	Day 7	Day 0	Day 7
A	CFA	DNP ₇ -OVA	4	13	10	—	—
B	DNP ₂₈ -BGG-CFA (50 µg)	DNP ₇ -OVA	5	8	11	—	—
C	CFA	Sulf ₂₉ -BGG	5	—	—	0	0
D	DNP ₂₈ -BGG-CFA (50 µg)	Sulf ₂₉ -BGG	5	—	—	0	42

* The data are expressed as arithmetic means. In a normal secondary response to DNP₇-OVA, a 1:500 dilution of antiserum was found to bind 89% of ¹²⁵I-OVA in this assay.

‡ The data are expressed as arithmetic means.

TABLE V
Lack of Enhancement of DNP-Specific Secondary Responses to DNP-Sulf-GO in Rabbits Preimmunized to Sulf-BGG

Group	Immunization protocol*		Number of animals	Anti-DNP antibody (µg/ml)‡		
	Supplement	Secondary		Day 0	Day 7	Boost§
A	Sulf ₂₉ -BGG-CFA	DNP ₂₇ -Sulf ₁₆ -GO	8	0.9	2.9	2.0
B	Sulf ₂₉ -BGG-CFA	DNP ₂₇ -GO	4	2.8	8.6	5.8
C	Sulf ₂₉ -BGG-CFA	DNP ₂₈ -BGG	5	1.7	2681	2679
D	GO-CFA	DNP ₂₇ -GO	5	0.7	1713	1712

* All rabbits were immunized intravenously with 9 mg of DNP₇-OVA, in saline, distributed in 3 daily doses. 1 wk later they received a supplemental immunization with 1 µg of the indicated compound emulsified in CFA. 4 wk later they were boosted with 5 mg of the indicated conjugate, intraperitoneally.

‡ The data are expressed as geometric means. A comparison of the geometric mean serum anti-DNP antibody concentrations, at day 7, of groups A and C revealed a p value of <0.01; the p value for the comparison of groups A and B yield a p value of: 0.3 > p > 0.2.

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

Sulf₂₉-BGG for supplemental immunization followed by boosting with DNP₂₇-GO (Group B) had a secondary anti-DNP response of 5.8 µg/ml, whereas rabbits boosted with DNP₂₈-BGG (Group C) made an anti-DNP secondary response of 2679 µg/ml. The experimental group, boosted with DNP₂₇-Sulf₁₆-GO (Group A), demonstrated a response of only 2.0 µg/ml, even less than the

group boosted with DNP₂₇-GO. As control, it was shown that: (a) the rabbits were indeed primarily immunized against the Sulf hapten, as a group of these animals challenged with Sulf₂₉-BGG showed an increase in anti-Sulf titer from 0.8 to 915; and (b) rabbits which received supplemental immunization with GO in CFA displayed marked secondary anti-DNP responses to DNP₂₇-GO (Group D). Thus, prior immunization to the *p*-azophenylsulfonic acid group

TABLE VI

Effect of Preimmunization to DNP Determinant on the Enhancement of Anti-Sulf Secondary Responses to DNP-Bearing Sulfanilazo Conjugates in Guinea Pigs

Group	Immunization protocol*			Number of animals	Anti-Sulf antibody titer†		Boost‡
	Primary	Supplement	Secondary		Day 0	Day 7	
A	Sulf ₂₉ -BGG	DNP ₃₀ -GPA-CFA	Sulf ₉₂ -DNP ₂₄ -GO	6	1.7	0	0
B	Sulf ₂₉ -BGG	DNP ₃₀ -GPA-CFA	Sulf ₂₁ -DNP ₃₀ -GPA	8	2.5	10.5	8.0
C	Sulf ₂₉ -BGG	DNP ₃₀ -GPA-CFA	Sulf ₁₆ -DANSYL ₃₉ -GPA	5	1.2	0.4	0
D	Sulf ₂₉ -BGG	DNP ₃₀ -GPA-CFA	Sulf ₃₀ -GPA	5	4.4	1.2	0

* Strain 2 guinea pigs were immunized intraperitoneally with 3 mg of Sulf₂₉-BGG in saline, distributed in 3 daily doses. 1 wk later they received a supplemental immunization with 50 μ g of DNP₃₀-GPA, emulsified in CFA and 3 wk later they were boosted with 1 m of the indicated conjugate (200 μ g intradermally followed by 800 μ g intraperitoneally 4 hr later).

† Reciprocal of highest dilution of serum yielding agglutination of Sulfanilazo-sheep erythrocytes. Data are arithmetic means. A comparison of the arithmetic mean serum anti-sulf antibody titers at day 7, of groups A and B revealed a p value of <0.05. Comparisons for groups B and C, and B and D showed a p value of 0.1 > p > 0.5 for both.

‡ Increase in mean titers between days 0 and 7.

did not prepare for augmented secondary anti-DNP antibody responses to DNP₂₇-Sulf₁₆-GO.

A similar lack of effectiveness of hapten presensitization to enhance secondary responsiveness to a different hapten was observed in strain 2 guinea pigs. Four groups of animals were immunized with 3 daily doses of 1.0 mg of Sulf₂₉-BGG administered intraperitoneally in saline. 1 wk later, they received a supplemental immunization with 50 μ g of DNP₃₀-GPA emulsified in CFA. 3 wk later, the guinea pigs were boosted with 1.0 mg of a Sulf-protein. The protocols and data are summarized in Table VI. Both Sulf₉₂-DNP₂₄-GO and Sulf₃₀-GPA failed to elicit detectable secondary anti-Sulf responses (Groups A and D, respectively). On the other hand, Sulf₂₁-DNP₃₀-GPA elicited a marked

increase in anti-Sulf antibody titer (Group B). This finding is of interest not only in that it again confirms the failure of hapten preimmunization to prepare for an enhanced secondary response but also that it demonstrates that the immune response accorded to a haptenic conjugate of an autologous protein (DNP-GPA) does prepare for an enhanced secondary response to a Sulf derivative of this hapten-protein conjugate (Sulf₂₁-DNP₃₀-GPA). The failure of Sulf₁₆-DANSYL₃₉-GPA to elicit a secondary anti-Sulf response suggests that the carrier-specific cells, indeed, are appreciating the presence of the DNP group and not simply a general alteration in GPA imposed by substituting

TABLE VII
Relative Effectiveness of the Dansyl Group and PLL in Enhancing Secondary Anti-DNP Responses in Dansyl-PLL Preimmunized Guinea Pigs

Group	Immunization protocol*			Number of Animals	Anti-DNP Antibody (μg/ml)†		
	Primary	Supplement	Secondary		Day 0	Day 7	Boost‡
A	DNP ₇ -OVA	DANSYL ₂₈ -PLL-CFA	DNP ₁₇₄ -DANSYL ₈₁ -PDL	5	1.8	0.6	-1.2
B	DNP ₇ -OVA	DANSYL ₂₈ -PLL-CFA	DNP ₃₅ -PLL	5	2.2	52.1	49.9
C	DNP ₇ -OVA	DANSYL ₂₈ -PLL-CFA	DNP ₂₃ -DANSYL ₁₂ -PLL	5	1.0	635	634
D	DNP ₇ -OVA	DANSYL ₂₈ -PLL-CFA	DNP ₇₉ -PDL	5	1.6	0.5	-1.1

* Strain 2 guinea pigs were immunized with 3.0 mg of DNP₇-OVA in saline, intraperitoneally, distributed in 3 daily doses. 1 wk later they received a supplemental immunization with 50 μg of DANSYL₂₈-PLL, emulsified in CFA, and 3 wk later they were boosted with 1 mg of the indicated conjugate (200 μg intradermally followed by 800 μg intraperitoneally 4 hr later).

† The data are expressed as geometric means. Comparisons of geometric mean serum anti-DNP antibody concentration, at day 7, for group A with the mean of group B and C, respectively, yielded p values of <0.01 in both instances. In addition, a comparison of the geometric means of groups B and C yielded a p value of <0.01.

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

many groups on the ε-amino functions of lysines. Thus, one has a situation in which the DNP group plays a necessary, but insufficient, role in antigen carrier cell interactions.

One might argue that the capacity of preimmunization with a protein to prepare for enhanced secondary responsiveness and the failure of preimmunization with hapten, conjugated to a protein not used again for immunization, could be explained by the great diversity of antigenic groups on proteins in contradistinction to the large number of similar haptenic-groups in hapten conjugates. In order to test this hypothesis, supplemental immunization with hapten conjugates of a very simple immunogen, poly-L-lysine, was resorted to, and the relative efficacy of the hapten (in this case DANSYL) and the carrier (PLL) in enhancing subsequent secondary responses were compared.

Four groups of strain 2 guinea pigs were immunized intraperitoneally with

3 daily doses of 1.0 mg of DNP₇-OVA in saline. Supplemental immunization with 50 μ g of DANSYL₂₃-PLL in CFA was carried out 1 wk later. 3 wk later, they were challenged with 1.0 mg of either DNP₃₅-PLL, DNP₇₉-PDL, DNP₂₃-DANSYL₁₂-PLL, or DNP₁₇₄-DANSYL₈₁-PDL. Thus, in the case of challenge with DNP₃₅-PLL, the effectiveness of carrier preimmunization was being evaluated, whereas the effectiveness of hapten (DANSYL) preimmunization was being evaluated in the case of the boost with DNP₁₇₄-DANSYL₈₁-PDL. Because PDL has physical properties similar to PLL, it was employed as a nonimmunogenic or weakly immunogenic carrier for the DNP and DANSYL haptens.

A summary of the protocols and data for these experiments is presented in Table VII. A marked secondary anti-DNP response was obtained to DNP₃₅-PLL (Group B), whereas no response was obtained to DNP₁₇₄-DANSYL₈₁-PDL (Group A). However, the secondary anti-DNP response to DNP₂₃-DANSYL₁₂-PLL (Group C) was considerably greater than that to DNP₃₅-PLL, again demonstrating the participation of hapten in antigen recognition by carrier cells. Finally, no response was obtained to DNP₇₉-PDL (Group D).

One should note that DNP₁₇₄-DANSYL₈₁-PDL did not elicit an anti-DNP response despite the fact that it was more heavily substituted with respect to both DNP and DANSYL groups than was the corresponding L-polymer, which might have been expected to favor its binding by hapten-specific cells.

DISCUSSION

Lymphoid cells from strain 2 guinea pigs immunized to BGG can transfer to syngeneic recipients primed with DNP-OVA the capacity to mount an enhanced secondary anti-DNP response to DNP-BGG. This effect was obtained with as few as 50×10^6 lymph node and spleen cells which suggests that "carrier-specific cells" are, relatively, quite abundant or very efficient. A similar conclusion has been reached by Mitchison in studies of adoptive secondary responses in mice (7). In our experiments, successful transfer of this response is obtained when secondary challenge is administered 1 day after transfer; however, the response is considerably better 6 days after transfer. It seems unlikely that this difference is due to proliferation of the transferred cells, as the recipients have not been irradiated and no antigenic stimulus was applied during this period. It is, perhaps, more probable that it reflects a need for the cells to reach some special anatomic location to perform this function. However, this explanation is not wholly satisfying either, and studies are currently in progress in an attempt to clarify the events during this period.

It has been known since the studies of Landsteiner that individual determinants by themselves do not elicit significant immune responses; association of these determinants with carrier molecules, however, leads to an antibody response which displays a significant degree of hapten specificity (19). The

role played by the carrier molecule (hereafter referred to as carrier function) may have two interpretations. On the one hand, the carrier molecule may simply be a vehicle for the association of several similar, or different determinants; this in turn, presumes that more than one determinant is required for the initiation of immune responses. Alternatively, the carrier molecule may be independently recognized by a certain cell class on the basis of properties independent of conventional antigenic determinants. This recognition would be regarded as required for subsequent stimulation of synthesis of antibodies directed against the determinants which the carrier bears. Indeed, it is already very likely that carrier function involves the activity of a different cell type than does the synthesis and secretion of humoral antibodies (7).

The results of the experiments presented in this paper may be considered in the light of these alternatives.

Several biologic characteristics of the carrier function have been demonstrated. Specific immunologic tolerance to BGG prevents immunization to BGG from enhancing a subsequent secondary anti-DNP response to DNP-BGG in rabbits. Thus, the cells mediating carrier function are capable of being rendered immunologically tolerant. This may well be analogous to the state of specific immunologic tolerance to bovine serum albumin induced in mouse thymus cells as reported by Taylor (20).

Certain characteristics of the functional specificity of carrier cells have also been explored. First, we have shown that guinea pigs genetically incapable of making a complete immune response to a copolymer of L-glutamic acid and L-lysine (GL) fail to develop an enhanced secondary anti-hapten response after supplemental immunization with GL. Indeed these animals, which had been primed with DNP-OVA, made no secondary response whatever to DNP-GL. This shows that carrier function is not exercised on nonimmunogens even though they may bear multiple haptenic determinants.

It was not unexpected, therefore, that preimmunization to haptenic determinants did not result in the expression of carrier function, at least in the experimental systems employed here. Indeed, neither the primary immune response to ovalbumin in guinea pigs nor the secondary immune response to the sulfanyl determinant in rabbits or to DNP in guinea pigs were enhanced by boosting with heterologous conjugates which contained a hapten to which the animal had been immunized. However, haptens may contribute to determinants recognized by cells which mediate carrier function. This is demonstrated by the findings that DANSYL-PLL is superior to PLL as a carrier in animals preimmunized with DANSYL-PLL, and that DNP-GPA is similarly superior to both GPA and DANSYL-GPA in DNP-GPA preimmunized guinea pigs. The objection may be raised to this interpretation that conjugation imparts an alteration in the protein or polypeptide which is crucial in immunologic terms. While it is undoubtedly true that the conformation of proteins is altered

as a result of conjugation (21–24), it does not seem likely that such new determinants should be appreciated by carrier-specific cells but the haptens themselves not so appreciated. Furthermore, if alterations are important, they must be rather specific, for in a DNP-GPA preimmunized guinea pig Sulf-DANSYL-GPA fails to enhance the secondary response to Sulf, whereas Sulf-DNP-GPA does. Thus, the cells would have to distinguish alterations induced by two haptens both of which are mainly substituted on ϵ -amino groups of lysine. Finally, the fact that a homopolymer of L-lysine (PLL) is a carrier suggests that antigenic complexity, in the sense of the possession of many antigenic determinants of different specificity, is not the crucial feature for expression of carrier function.

Indeed, the attempt to obtain enhanced secondary responses by hapten preimmunization was perhaps naive. If such effects could be easily obtained then one would never have expected carrier specificity of hapten-specific antibody responses to have been described in the first place. It should, nevertheless, be pointed out that Mitchison has reported marginal enhancement in such a hapten preimmunization system in mice (7, 25).

One may then consider why haptenic determinants fail to express carrier function, or do so only marginally, although they can participate in the specificity of the cells which mediate carrier function. Three general explanations may be entertained:

(a) Cells mediating carrier function, as a class, have antigen-sensitive receptors generally similar to those of the precursors of antibody forming cells; however, the receptors of the former cells differ from those of the latter in their range of specificity, inasmuch as they are specialized to recognize the determinants of native and of altered proteins and polypeptides.

(b) Cells mediating carrier function generally possess receptors of low affinity relative to the concentration conditions in their environment, and can bind and be activated by exposure to a total antigenic determinant and only rarely by the partial determinant which haptens constitute.

(c) Cells mediating carrier function are specialized either in the possession of an entirely different class of recognition units or in the normal binding of antigen followed by an additional operation which can only be performed on substrates of given structure.

Evidence presented here and previously (26) that carrier function is under a simple genetic control may be considered an argument against the notion that carrier function is simply mediated by multiple determinants on the same backbone. The failure of DNP-DANSYL-PDL to enhance anti-DNP secondary responses in DANSYL-PLL immunized animals is an important point against the latter view, especially considering that the high degree of substitution of both haptens should favor cooperative interactions with cellular receptors.

The nature of the interactions of antigens and carrier-specific cells appears

to be identical to those of antigens and sensitized cells in the elicitation of delayed hypersensitivity and its *in vitro* correlates.

As discussed in the previous paper and elsewhere (6, 7, 20), carrier function in the synthesis of anti-hapten antibody in response to hapten-carrier immunization may be analogous to the requirement for thymus dependent cells in the anti-sheep erythrocyte antibody synthesis by the bone marrow derived lymphoid cells of mice (27-30). These phenomena may well reflect an essential process in all immune responses.

SUMMARY

Transfer of live lymphoid cells from BGG-immunized strain 2 guinea pigs into syngeneic animals primed with DNP-OVA prepares the recipients for a markedly enhanced secondary anti-DNP antibody response upon challenge with DNP-BGG. This phenomenon has been demonstrated when the recipients were challenged 1 day after cell transfer, but it was considerably more striking when an interval of 6 days was allowed between transfer of cells and challenge with antigen.

As demonstrated in the preceding paper (6), BGG preimmunization enhanced anti-DNP antibody responses to challenge with DNP-BGG. An analysis of the characteristics of substances to which preimmunization was effective in enhancing subsequent anti-hapten responses was made. It was shown that preimmunization of strain 13 guinea pigs with a copolymer of glutamic acid and lysine (GL), to which these animals are genetically unable to accord an immune response, failed to prepare them for an enhanced anti-DNP response to DNP-GL. Tolerance to BGG specifically abrogated the capacity of subsequent BGG immunization to prepare DNP-OVA primed rabbits for an enhanced anti-DNP response to DNP-BGG.

Sensitization of animals to haptens by preimmunization with hapten-protein conjugates failed to prepare them for enhanced primary or secondary antibody responses to other determinants associated with that hapten on a different carrier.

These studies indicate that the enhancing effect of carrier preimmunization reflects a cooperative interaction between lymphoid cells specific for carrier and cells specialized for haptenic determinants. Furthermore, the capacity of a substance to behave as a carrier requires more than its possession of a variety of antigenic determinants.

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