CARRIER FUNCTION IN ANTI-HAPTEN ANTIBODY RESPONSES

IV. EXPERIMENTAL CONDITIONS FOR THE INDUCTION OF HAPTEN-SPECIFIC TOLERANCE OR FOR THE STIMULATION OF ANTI-HAPTEN ANAMNESTIC RESPONSES BY "NONIMMUNOGENIC" HAPTEN-POLYPEPTIDE CONJUGATES

BY DAVID H. KATZ, M.D., JOSEPH M. DAVIE, M.D., WILLIAM E. PAUL, M.D., AND BARUJ BENACERRAF, M.D.

(*From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014, and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115*)

There is now a considerable body of evidence demonstrating that two types of specific lymphoid cells are involved in the antibody response to a variety of antigens (1–10). One of these cells appears to be derived largely from the bone marrow cell population, to be the direct precursor of antibody-secreting cells, and to possess membrane-associated antigen-binding receptors, immunoglobulin in nature and similar (or identical) in specificity with the antibody produced (3, 11–13 and footnote 1). The activation of these antibody-forming cell precursors (AFCP)[2] either requires or is markedly enhanced by the activity of another specific cell type which appears to neither secrete detectable immunoglobulin nor to differentiate into an immunoglobulin-secreting cell (2, 3, 14). This latter cell type has been referred to as a "helper" cell and has been shown in the mouse to be derived from thymus cells and to bear the isoantigen θ (15, 16). Its activity, although undefined in mechanism, has been demonstrated to be resistant to X-irradiation (16a) and to account for the "carrier-specificity" of anamnestic antibody responses to hapten-carrier conjugates (8, 15, 17).

Recent studies in the mouse have demonstrated that specific immunologic tolerance is relatively easy to induce in the helper cell population and that such a state of unresponsiveness may also be induced in bone marrow lymphocytes, although in this population induction of tolerance is more difficult to achieve and the duration of un-

---

[1] Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on ^{125}I-DNP-GPA binding lymphocytes of normal guinea pigs. *J. Exp. Med.* In press.

[2] ABC, antigen-binding cells; AFCP, antibody-forming cell precursors; BCG, bovine gamma globulin; CFA, complete Freund's adjuvant; D-GL, copolymer of D-glutamic acid and D-lysine; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; GPA, guinea pig albumin; GVH, graft-*versus*-host; L-GL, copolymer of L-glutamic acid and L-lysine; OVA, ovalbumin; PFC, indirect plaque-forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenyl.

responsiveness is relatively short (18). Whether tolerance can be induced in mature bone marrow-derived cells and whether such induction requires the participation of helper cells has not been established.

In the studies presented in this paper, we have attempted to approach these issues by utilizing a system involving responses to hapten-carrier conjugates in guinea pigs. In this system, we have equated the hapten-specific antibody-secreting cell precursors with the bone marrow-derived AFCP of the mouse; further, carrier-specific cooperating cells have been assumed to be analogous operationally to the thymus-derived helper cells (7, 8). We now report that previous administration of 2,4-dinitrophenyl (DNP) conjugates of "nonimmunogenic" amino acid copolymers exerts a profound suppressive effect on the capacity of guinea pigs to display anti-DNP antibody responses to immunization with the DNP conjugate of a strong antigen, DNP-ovalbumin (OVA). Furthermore, this suppression has also been achieved in guinea pigs which have been primed with DNP-OVA before administration of such nonimmunogenic conjugates. Moreover, the hapten-specific unresponsiveness induced has been shown to be central in nature, in that a substantial diminution in numbers of anti-DNP antibody-secreting and of DNP-specific antigen-binding cells is observed.

In contrast, administration of DNP conjugates of a nonimmunogenic copolymer will be shown to exert a very different effect in DNP-OVA–primed guinea pigs undergoing graft-*versus*-host (GVH) reactions. As was previously demonstrated, the required stimulation of carrier-specific cells for the elicitation of anti-hapten secondary responses can be obviated by an appropriately timed GVH reaction; thus, induction of GVH reactions in DNP-OVA–primed guinea pigs permits the elicitation of marked secondary anti-DNP antibody responses to a DNP conjugate of an unrelated immunogenic carrier protein, bovine gamma globulin (BGG) (19). Similarly, the nonimmunogenic DNP copolymer employed here, highly tolerogenic under normal circumstances of administration, elicits striking secondary anti-DNP antibody responses in primed hosts undergoing a GVH reaction.

Materials and Methods

Proteins and Chemical Reagents.—The copolymers of D-glutamic acid and D-lysine (D-GL), and L-glutamic acid and L-lysine (L-GL) were obtained from Pilot Chemicals, Inc., Watertown, Mass. Average molecular weights were respectively 50,000 and 115,000, and both isomers had a ratio of G:L of 60:40. 2,4,6-trinitrobenzenesulfonic acid (TNP) was purchased from Eastman Organic Chemicals, Rochester, N. Y. All other proteins and chemical reagents used were, in general, identical with those described in the previous papers in this series (7, 8, 19).

Hapten-Carrier Conjugates.—The following 2,4-dinitrophenyl conjugates were prepared as previously described (20): DNP_8-OVA, DNP_{23}-BGG, DNP_{23}-GPA, and DNP_{50}-L-GL. DNP_{53}-D-GL was prepared by reacting D-GL with 1-fluoro-2,4-dinitrobenzene (DNFB) under the following conditions: the D-GL was dissolved in water and the pH was gently raised

to 6.5 with 1 N NaOH. An equal weight of DNFB in p-dioxane was added dropwise over a period of 90 min; the pH of the reaction mixture was slowly increased during this time by the addition of 1 N NaOH until a final pH of 8.0 was reached. After 24 hr at room temperature, 500 mg of glycyl-glycine was added and the reaction mixture was extensively dialyzed. Subscripts refer to the average number of moles of DNP per mole of carrier.

Immunizations.—Adult inbred strain 2 and strain 13 guinea pigs weighing 200–400 g were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. In general, primary immunization with hapten-carrier conjugates was carried out with 1.0 mg of conjugate in saline, intraperitoneally, on 3 successive days. Secondary immunization with hapten-carrier conjugates consisted of 1.0 mg in saline administered as a 200 μg intradermal dose followed 4 hr later by an 800 μg intraperitoneal dose. In the case of strain 13 guinea pigs, diphenhydromine hydrochloride (Benadryl, Parke, Davis & Co., Detroit, Mich.), 5.0 mg/kg, was administered intramuscularly 1 hr before the intraperitoneal dose of hapten-carrier conjugate as a prophylactic measure against anaphylaxis. Animals were bled just before the secondary challenge and at various intervals thereafter; antibody determinations were performed as described below. In certain experiments (see Results), immunization of guinea pigs with carrier proteins was carried out by administering, in the footpads, 50 μg of protein emulsified in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich.). For cell transfer experiments, donor guinea pigs were immunized in the footpads with saline emulsified in CFA.

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

Antibody Measurements.—

Measurement of anti-DNP antibodies: Serum anti-DNP antibody levels were determined by a modified Farr assay (21) using ^{3}H-DNP-ϵ-amino-N-caproic acid (22). Using standard curves as described previously (7), percentage of binding was converted into amount of anti-DNP antibody in micrograms per milliliter.

Measurement of anti-OVA antibodies: Serum anti-OVA antibody levels were determined by the Farr technique (21) using OVA-^{125}I prepared as described previously (8, 23). Antigen-binding capacities were determined on the basis of the serum dilution required to bind 33% of a sample of OVA-^{125}I (4 μg/ml).

Determination of Hapten-Specific Antigen-Binding Cells.—Cells in peripheral blood and spleen, bearing DNP-specific membrane-associated immunoglobulin, were enumerated by a radioautographic technique recently described in detail elsewhere (24). Briefly, lymphocytes were isolated from spleen or heparinized peripheral blood as described previously (24) and suspended in Eagle's minimum essential medium (10–50 $\times$ 10^6 cells/ml) containing heparin (50 units/ml) and sodium azide (1 mg/ml). After cooling the cells to 4°C, 40 ng of DNP$_{23}$-GPA-^{125}I (specific activity 19 μCi/μg) was added and the mixture was incubated for 30 min. Unbound antigen was removed by washing the cells four times through gradients of fetal bovine serum; the cells were smeared on serum-dipped slides, air dried, and fixed for 30 min in methanol-acetic acid-water (89:1:10). Slides were then dipped in Nuclear Track Emulsion (NTB-2, Eastman Kodak Co., Rochester, N. Y.) that had been diluted 1:2 in water and heated to 50°C. After appropriate exposure periods (2 days) the radioautographs were developed using standard methods, and lymphocytes were examined for silver grains.

Determination of Hapten-Specific Antibody-Producing Cells.—The frequency of cells in spleens of immunized guinea pigs secreting anti-DNP antibodies was determined by a modification of the hemolytic plaque technique (25–27). For this assay sheep erythrocytes (SRBC) were conjugated with TNP as described by Rittenberg and Pratt (28). Washed dissociated spleen cell suspensions from individual guinea pigs were assayed for IgG antibody-producing cells (indirect plaque-forming cells [PFC]) using TNP-RBC as indicator and rabbit anti-

guinea pig γ_2-globulin as facilitating antiserum. It was determined that in the guinea pig spleens the background against SRBC is less than 2 PFC/10^6 cells.

Cell Transfers.—Donor guinea pigs were immunized in the footpads with saline in CFA. 3 wk later the animals were sacrificed and axillary, occipital, inguinal, and popliteal lymph nodes and spleens were removed. Single cell suspensions in Eagle's minimum essential medium were prepared and washed. 200 $\times$ 10^6 nucleated cells were transferred intravenously to recipient guinea pigs.

Statistical Analysis.—Serum antibody values were logarithmically transformed and means and standard errors calculated. Results from appropriate groups were compared by Student's t test. In those cases where no specific antigen binding could be detected in the serum, a value of 0.10 μg/ml was arbitrarily assigned in order to permit logarithmic transformation of the data.

RESULTS

Delineation of the Nonimmunogenic Nature of the DNP Copolymers Employed

DNP conjugates of the copolymer of L-glutamic acid and L-lysine belong to a class of substances to which strain 13 guinea pigs are genetically incapable of responding immunologically by virtue of the absence of the autosomal dominant PLL gene in this strain (29, 30). Although the precise role of the product of the PLL gene is unknown, it appears to exert its function primarily in cells mediating cellular immune responses and in helper cells (8, 31). Thus, the DNP-L-GL conjugate is operationally nonimmunogenic in strain 13 guinea pigs.

In general, poly-D-amino acids have been shown to be either nonimmunogenic (32) or very poor immunogens (33). In order to substantiate the nonimmunogenic nature of the DNP conjugate of the copolymer D-glutamic acid and D-lysine in both strain 13 and strain 2 guinea pigs, the following studies were performed.

To create the most favorable conditions for eliciting responses to very weak immunogens, strain 13 guinea pigs were primed with DNP-OVA (1.0 mg intraperitoneally in saline for 3 successive days) and 1 wk later were given supplemental immunization with 50 μg of free carrier (either BGG or D-GL) in CFA. A control group received no supplemental carrier immunization. 3 wk later, secondary challenge was carried out with DNP conjugated to the carrier used for supplemental immunization (DNP-BGG or DNP-D-GL) at a dose of 1.0 mg. As shown in Fig. 1, the guinea pigs which received 50 μg of BGG in CFA as supplemental carrier immunization followed by secondary immunization with DNP-BGG displayed anti-DNP antibody responses of considerable magnitude by days 4 and 7 after administration of DNP-BGG. In sharp contrast, guinea pigs receiving D-GL or CFA alone for supplemental immunization did not have the capacity to mount anamnestic responses to DNP-D-GL or DNP-BGG, respectively. In fact, the animals challenged with DNP-D-GL displayed sharp declines in anti-DNP antibody levels, which is consistent with
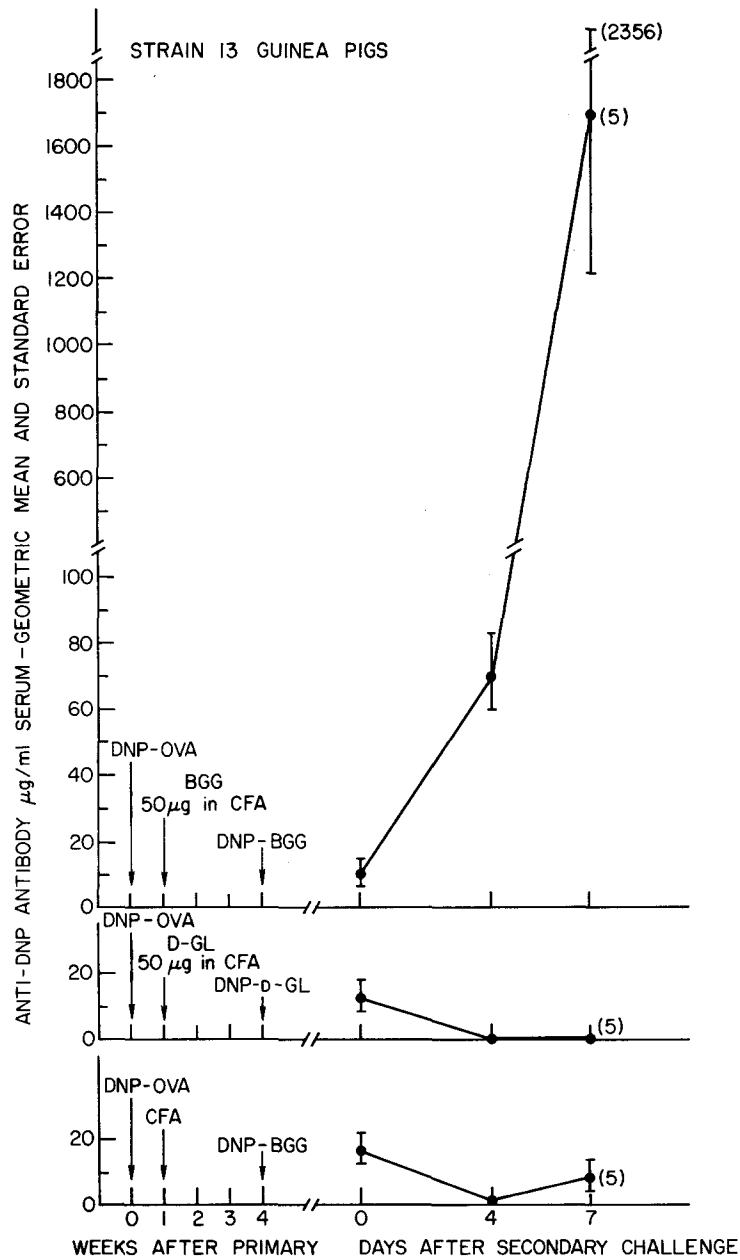
FIG. 1. Failure to elicit hapten-specific antibody responses in strain 13 guinea pigs with DNP-D-GL. Strain 13 guinea pigs received a primary immunization with 3.0 mg of DNP-OVA, administered intraperitoneally in saline at week 0. 1 wk later supplemental immunization with 50 μg of either BGG or D-GL emulsified in CFA or saline in CFA was carried out. 4 wk after primary immunization, the animals were challenged with 1.0 mg of either DNP-BGG or DNP-D-GL in saline. Serum anti-DNP antibody concentrations just before challenge and on days 4 and 7 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups.

our previously reported observations in strain 13 guinea pigs similarly challenged with DNP-L-GL (8).

A second experiment was carried out with strain 2 guinea pigs in order to determine the extent to which racemization from the D to L form of the copolymer might have occurred as a result of conjugation with DNFB. Strain 2 guinea pigs were immunized with either 100 µg of DNP-L-GL in CFA, 100 µg of DNP-D-GL in CFA, or 1.0 mg of DNP-D-GL in CFA. Animals were bled at 14 and 21 days after immunization and serum anti-DNP antibody levels were determined. As shown in Table I, there were considerable anti-DNP antibody responses after immunization with DNP-L-GL, whereas no detectable antibody was made to DNP-D-GL even when 1.0 mg was used as the immunizing dose. Thus, we were unable to demonstrate any immunogenicity for guinea pigs on the part of the DNP-D-GL employed for these studies within the limits of the methods used and apparently no appreciable degree of racemization has occurred.

TABLE I

Failure to Elicit Hapten-Specific Antibody Responses in Strain 2 Guinea Pigs after Immunization with a DNP Conjugate of the Copolymer of D-Glutamic Acid and D-Lysine

Group	Primary immunization	No. of guinea pigs	Anti-DNP antibody-ABC-33*	
			Day 14	Day 21
A	DNP$_{50}$-L-GL 100 µg in CFA	4	100	471
B	DNP$_{53}$-D-GL 100 µg in CFA	4	<1.0	<1.0
C	DNP$_{53}$-D-GL 1.0 mg in CFA	4	<1.0	<1.0

* ABC-33 is expressed as the dilution of antiserum at which 33% binding of the ligand, 10^{-8} M DNP-^{3}H-epsilon-amino-N-caproic acid, occurred. The geometric mean is presented.

Specific Suppression of Anti-Hapten Antibody Production in Guinea Pigs as a Result of Administration of DNP Conjugates of Nonimmunogenic Carrier Molecules

(a) *In the Elicitation of Augmented Hapten-Specific Primary Responses.*—Two groups of strain 13 guinea pigs received a series of injections of 1.0 mg of DNP-D-GL or DNP-L-GL intraperitoneally in saline daily for 3 successive days. A third group received saline injections during this period. 2 wk later, all guinea pigs were immunized with 50 µg of OVA in CFA in the footpads. This was followed 14 days thereafter (day 0) by primary immunization with 1.0 mg of DNP-OVA in saline (200 µg intradermally and 4 hr later 800 µg intraperitoneally). All animals were bled on days 0, 7, 14, and 21 and determinations of serum anti-DNP antibody levels and of numbers of antigen-binding cells (ABC) in peripheral blood were made. A secondary challenge with DNP-OVA was carried out on day 14. As shown in Table II, guinea pigs which had received

pretreatment with saline alone manifested typical augmented primary anti-DNP responses to DNP-OVA as a result of the preimmunization with unconjugated OVA in CFA. Furthermore, these animals displayed very marked secondary anti-DNP antibody responses after the final challenge with DNP-OVA administered on day 14. In contrast, the synthesis of anti-DNP antibodies in response to DNP-OVA was markedly suppressed in guinea pigs which had received pretreatment with either DNP-D-GL or DNP-L-GL. This was true both for the augmented primary responses to DNP-OVA after carrier preimmunization (OVA in CFA) and for anamnestic responses to the DNP-OVA challenge administered on day 14. Furthermore, before initial DNP-OVA immunization (day 0), the guinea pigs pretreated with either DNP copolymer had significantly fewer DNP-GPA-^{125}I ABC than did nonpretreated control animals. This suggests that the unresponsive state involves a reduction in precursors of anti-DNP antibody-forming cells, which the ABC are believed to represent.[3] At day 21, the number of ABC was still diminished in the pretreated groups as compared with the controls; the degree of depression was considerably less than the difference in serum anti-DNP binding activity at the same time. This apparent discrepancy may be due to at least two factors: (a) ABC, as precursors of antibody-forming cells, would be expected to recover from the tolerant state before appreciable increases in serum antibody levels would be observed. (b) The antibody assay used is dependent on both amount and affinity of antibody. If the pretreated groups had begun to recover from the unresponsive state, they would most likely be secreting antibodies of relatively low affinity.

(b) *In the Elicitation of Hapten-Specific Secondary Responses.*—Strain 13 guinea pigs were given a 3 day primary immunization course consisting of 1.0 mg daily of DNP-OVA intraperitoneally in saline. 2 wk later, two groups of guinea pigs received an intervening series of injections of either DNP-D-GL or DNP-L-GL. This consisted of 1.0 mg of hapten-copolymer conjugate in saline (200 μg intradermally followed 4 hr later by 800 μg intraperitoneally) administered daily for 3 successive days. A third group received no intervening treatment. 2 wk later (day 0), all animals received a secondary immunization with 1.0 mg of DNP-OVA. Animals were bled on day 0 before secondary immunization and 7 days later (day 7) for determinations of serum anti-DNP and anti-OVA antibodies. The results are summarized in Table III and presented graphically in Fig. 2. Guinea pigs which received no intervening treatment displayed normal levels of anti-DNP antibodies 4 wk after primary immunization (day 0) and developed very brisk anamnestic anti-DNP responses by day 7 after secondary challenge. On the other hand, guinea pigs which had

³ Davie, J. M., A. S. Rosenthal, and W. E. Paul. 1971. Receptors on immunocompetent cells. III. Specificity and nature of receptors on ^{125}I-DNP-GPA antigen-binding cells of immunized guinea pigs. *J. Exp. Med.* In press.

received intervening injections of DNP-D-GL or DNP-L-GL had essentially no detectable anti-DNP antibody on day 0 and were incapable of mounting anamnestic anti-DNP responses. That this suppressive effect was hapten specific and not reflective of a general depression of immune responsiveness is evidenced by the capacity of each guinea pig to mount primary and secondary

TABLE II

Specific Suppression of Anti-Hapten Antibody Production in Strain 13 Guinea Pigs after Administration of DNP Conjugates of the Copolymers of either D-Glutamic Acid and D-Lysine or L-Glutamic Acid and L-Lysine

| Group | Protocol* | | Anti-DNP antibody (μg/ml)‡ | | | | Total ABC/10^6 peripheral blood cells‖ | |
	Pretreatment	No. of strain 13 recipients	Day 0	Day 7	Day 14§	Day 21	Day 0	Day 21
A	None	5	<0.10	12.3	297.9	4858.2	136.0	11,554.0
B	DNP$_{53}$-D-GL	5	<0.10	<0.10	<0.10	0.58	57.0	630.0
C	DNP$_{50}$-L-GL	5	<0.10	<0.10	<0.10	3.6	48.0	4,388.0

* Strain 13 guinea pigs received a 3-day series of injections of either DNP$_{53}$-D-GL, DNP$_{50}$-L-GL, or saline. 2 wk later all guinea pigs were immunized with 50 μg of OVA in CFA. This was followed 2 wk thereafter by primary immunization with DNP$_8$-OVA (day 0). A final challenge with DNP$_8$-OVA was carried out on day 14.

‡ The data are expressed as geometric means. A comparison of the geometric mean anti-DNP antibody concentrations on day 7 and day 21 gave the following results: comparison of group A with groups B and C on both day 7 and day 21 yielded P values of <0.001 in each case.

§ Final challenge with DNP$_8$-OVA was administered on day 14 after each guinea pig was bled.

‖ Heparinized peripheral blood from individual guinea pigs was studied for ABC. The data presented represent the geometric mean numbers of ABC containing six or more grains per cell. A comparison of the geometric mean number of ABC on day 0 and day 21 gave the following results: on day 0, comparison of group A with groups B and C yielded P values of $0.02 > P > 0.01$ and $0.05 > P > 0.025$, respectively; comparison of group B with group C yielded a P value of $0.70 > P > 0.60$. On day 21, comparison of group A with groups B and C and similarly, group B with group C, yielded P values of <0.001 in each case.

anti-carrier (OVA) antibody responses irrespective of whether or not it had been subjected to intervening treatment with DNP copolymers.

(c) *Effect of Administration of DNP-D-GL on Frequency of Antigen-Binding Cells.*—Two groups of strain 13 guinea pigs were given a 3 day primary immunization course of 1.0 mg daily of DNP-OVA intraperitoneally in saline. 2 wk later, guinea pigs in one of these DNP-OVA–primed groups and a group of nonprimed animals were given a series of injections of 1.0 mg of DNP-D-GL intraperitoneally in saline for 3 successive days. 2 wk later all animals were sacrificed and the frequency of DNP-specific ABC in their mesenteric lymph

TABLE III

Specific Suppression of Anti-DNP Antibody Responses in DNP-OVA–Primed Strain 13 Guinea Pigs as a Result of an Intervening Treatment with DNP Conjugates of the Copolymers of D-Glutamic Acid and D-Lysine or of L-Glutamic Acid and L-Lysine

Group	Protocol*		Anti-DNP antibody (μg/ml)‡			Anti-OVA antibody-ABC-33 (μg/ml)‡		
	Intervening treatment	No. of animals	Day 0	Day 7	Boost	Day 0	Day 7	Boost
A	None	5	25.8	986.8	961.0	4.9	139.8	134.9
B	DNP$_{53}$-D-GL	5	<0.10	0.12	0.02	1.3	42.9	41.6
C	DNP$_{50}$-L-GL	5	<0.10	<0.10	0	12.5	193.7	181.2

* Strain 13 guinea pigs were given a 3 day primary immunization course with DNP$_8$-OVA. 2 wk later groups of guinea pigs received an intervening series of injections for 3 days of either DNP$_{53}$-D-GL, DNP$_{50}$-L-GL, or saline. 2 wk later all animals were boosted with DNP$_8$-OVA (day 0).

‡ The data are expressed as geometric means. Boost represents the increase in mean antibody levels from day 0 to day 7. A comparison of the geometric mean anti-DNP antibody concentrations on day 0 and day 7 gave the following results: comparison of group A with groups B and C on both day 0 and day 7 yielded P values of <0.001 in each case.
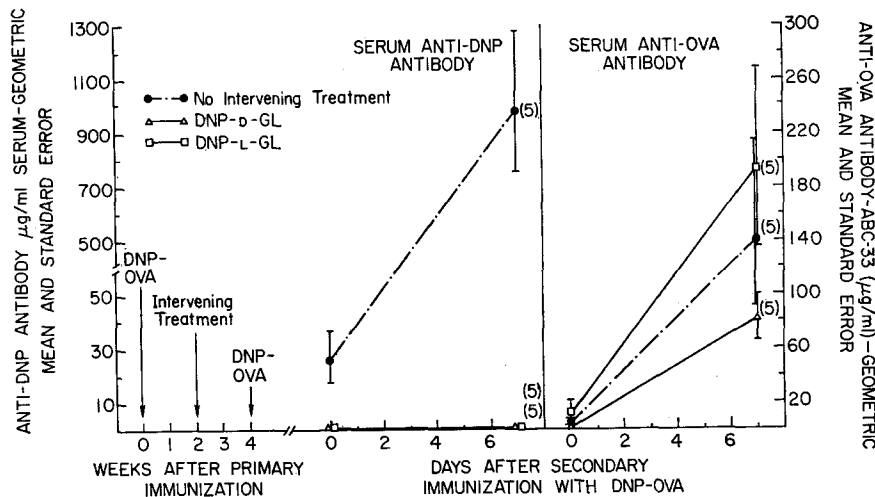

Fig. 2. DNP-specific tolerance induced in DNP-OVA–primed guinea pigs as a result of an intervening treatment with DNP-D-GL or DNP-L-GL. Strain 13 guinea pigs received a primary immunization with 3.0 mg of DNP-OVA administered intraperitoneally in saline at week 0. 2 wk later, an intervening treatment with 3.0 mg of either DNP-D-GL or DNP-L-GL in saline or saline alone was carried out. 4 wk after primary immunization, the animals were challenged with 1.0 mg of DNP-OVA in saline. Serum anti-DNP and anti-OVA antibody concentrations just before challenge and on day 7 are illustrated. The numbers in parentheses refer to the number of animals in the given groups.

nodes and in the mesenteric lymphs nodes of normal control guinea pigs was determined. The geometric mean numbers of ABC observed in the DNP-OVA–primed guinea pigs which received no intervening DNP-D-GL treatment and in the virgin controls were 1332 and 818 per 10^6 cells, respectively. In sharp contrast, the frequency of ABC in the guinea pigs treated with DNP-D-GL 2 wk after primary immunization with DNP-OVA and in the nonprimed guinea pigs treated with DNP-D-GL was 104 and 34 per 10^6 cells, respectively. Such striking reductions in frequency of observed DNP-specific ABC after administration of DNP-D-GL support the concept that the tolerant state resulting from such treatment involves diminution in the number of effective precursors of DNP-specific antibody-forming cells.

Stimulation of Anti-Hapten Antibody Production by a Nonimmunogenic DNP Copolymer after the Transfer of Immunocompetent Strain 2 Cells into Primed Strain 13 Recipients ("Allogeneic Effect")

In our previous report (19) we described a phenomenon (allogeneic effect) in which, as a result of induction of a graft-*versus*-host reaction, primed immunocompetent cells could be stimulated to produce large quantities of specific anti-hapten and anti-carrier antibodies even in the absence of further antigenic challenge. Moreover, when secondary antigenic challenge was administered at an optimal time after allogeneic cell transfer (6 days) the anti-hapten responses were greatly heightened even in cases where the secondary challenge was made with a hapten conjugate of a carrier antigenically unrelated to that used for primary immunization. Thus, it appeared that the stimulatory properties of the allogeneic effect obviated the necessity for carrier specificity in the elicitation of hapten-specific antibody responses. The present experiment was designed to test the possibility of eliciting hapten-specific secondary responses, through this allogeneic effect, when challenge is made with a hapten conjugate of a nonimmunogenic carrier molecule.

550 × 10^6 lymph node and spleen cells from strain 2 guinea pigs, which had been immunized 3 wk previously with CFA, were transferred intravenously into DNP-OVA–primed strain 13 recipients. 6 days later, two groups of recipients were challenged with DNP-BGG or DNP-D-GL; a third group received no secondary challenge. As shown in Table IV and Fig. 3, the allogeneic effect results in a significant increase in serum anti-DNP antibody concentrations between day 6 and day 13 after transfer in the absence of any additional antigenic stimulation. Even more striking, however, is the heightened stimulation of anti-DNP antibody production after the administration of DNP-heterologous carrier conjugates, regardless of whether the carrier molecule is highly immunogenic (BGG) or nonimmunogenic (D-GL). Determinations of indirect PFC/10^6 spleen cells corroborated the results of serum anti-DNP antibody levels in that there was a clear quantitative difference between

recipients which had been subjected to secondary challenge with either DNP-BGG or DNP-D-GL and those which had not; furthermore, the average PFC/10^6 in the spleens of guinea pigs challenged with DNP-D-GL was identical with that in the spleens of guinea pigs challenged with DNP-BGG. This suggests, perhaps, that the lower serum anti-DNP antibody levels of recipients challenged with DNP-D-GL compared to those challenged with DNP-BGG

TABLE IV

Elicitation of Antibody Responses to a Nonimmunogenic DNP-Carrier Conjugate in Strain 13 Guinea Pigs during the Course of the Allogeneic Effect after Transfer of Strain 2 Lymph Node and Spleen Cells

Group	Protocol*			Anti-DNP antibody (μg/ml)‡			Indirect PFC/10^6 spleen cells§
	No. of strain 2 donor cells transferred	Secondary immunization	No. of strain 13 recipients	Day after transfer			Day 17 after transfer
				Day 6	Day 13	Boost	
A	550 × 10^6	DNP$_{23}$-BGG	5	29.2	1084.7	1055.5	153
B	550 × 10^6	DNP$_{53}$-D-GL	5	11.3	672.8	661.5	153
C	550 × 10^6	None	5	23.8	276.5	252.7	47

* 550 × 10^6 lymph node and spleen cells from strain 2 guinea pig donors immunized with CFA were transferred to individual strain 13 recipients which had been immunized 3 wk earlier with DNP$_8$-OVA. Recipients were boosted 6 days after transfer with either DNP$_{23}$-BGG or DNP$_{53}$-D-GL.

‡ The data are expressed as geometric means. Boost represents the increase in mean antibody levels from the day of boosting (day 6 after transfer) to 7 days later (day 13). A comparison of the increases in geometric mean anti-DNP antibody concentrations from day 6 to day 13 after transfer gave the following results: comparison of group A with group B yielded a P value of $0.50 > P > 0.40$. Comparison of group A with group C and, similarly Group B with group C, yielded P values of $0.05 > P > 0.025$ in each case.

§ The recipients in each group were sacrificed on day 17 after transfer and individual spleens were assayed for anti-DNP PFC using TNP-conjugated SRBC as indicator. Facilitated plaques were developed with rabbit anti-guinea pig γ_2-globulin. The geometric mean number of PFC/10^6 spleen cells of each group is presented. A comparison of the geometric mean numbers of PFC gave the following results: comparison of group A with group C and, similarly group B with group C, yielded P values of $0.005 > P > 0.001$ in each case.

may reflect binding and subsequent clearance of antibody by an antigen molecule which is poorly metabolized.

Failure of Allogeneic Effect in Nonprimed Guinea Pigs to Modify Hapten-Specific Immunosuppression by Nonimmunogenic DNP Copolymers

(a) *Induction of Allogeneic Effect 1 Day after Administration of DNP-D-GL or DNP-L-GL.*—The preceding study demonstrated clearly that, when given at the optimal time after induction of the allogeneic effect, DNP-D-GL is capable of evoking hapten-specific anamnestic responses in DNP-OVA–primed

guinea pigs. This sharply contrasts with the results in the first experiments in this paper, where, in the absence of allogeneic cell transfer, it was shown that administration of DNP-D-GL caused marked suppression of anti-DNP responses in both primed and nonprimed guinea pigs. Thus, studies were made to determine the effect of inducing GVH reactions in nonprimed guinea pigs
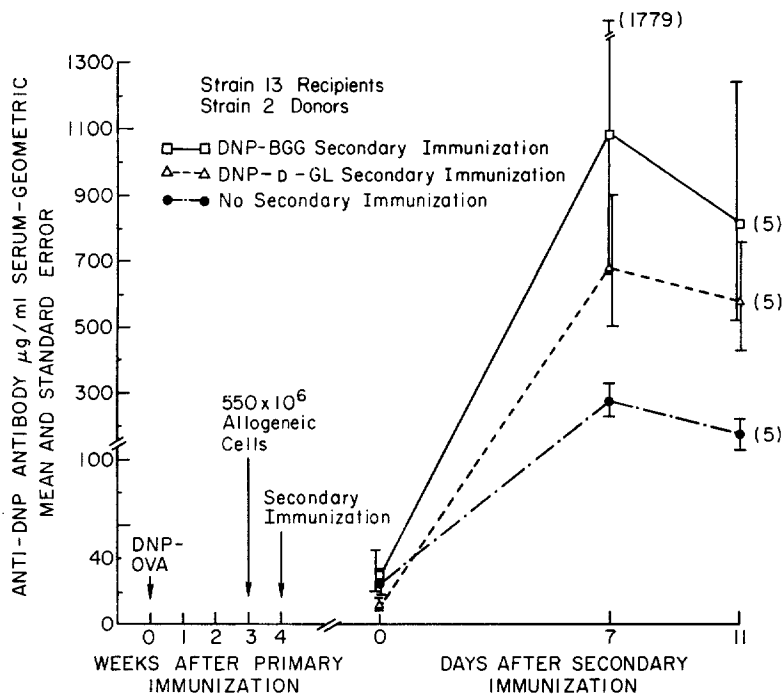

FIG. 3. Stimulation of anti-DNP antibody production as a result of transfer of immunocompetent strain 2 cells into primed strain 13 recipients. The effect of secondary antigenic challenge with DNP-BGG and DNP-D-GL. 550 × 10^6 strain 2 lymph node and spleen cells were injected intravenously into strain 13 recipients primed 3 wk earlier with DNP-OVA. Recipients were either not boosted or boosted with 1.0 mg of DNP-BGG or DNP-D-GL in saline 6 days after transfer. Serum anti-DNP antibody concentrations just before secondary immunization and on days 7 and 11 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups.

either before or after administration of these nonimmunogenic DNP copolymers. Strain 13 and strain 2 guinea pigs received 1.0 mg of DNP-D-GL or DNP-L-GL intraperitoneally in saline for 3 successive days. 24 hr after the last dose, the treated guinea pigs and normal strain 13 control guinea pigs received 200 × 10^6 lymph node and spleen cells from CFA-immunized strain 2 donors. Thus, strain 13 guinea pigs were recipients of allogeneic cells while the strain 2 guinea pigs were recipients of syngeneic cells. 2 or 3 wk after cell transfer,

all guinea pigs were immunized with 50 μg of OVA in CFA in the footpads. This was followed 2 wk later (day 0) by immunization with 1.0 mg of DNP-OVA in saline (200 μg intradermally and in 4 hr 800 μg intraperitoneally). A final challenge with DNP-OVA was made on day 14 or day 15. Results of one study are presented in Table V and Fig. 4. Thus, control strain 13 recipients of allogeneic cells displayed typical augmented primary anti-DNP responses to

TABLE V

Specific Suppression of Anti-Hapten Antibody Production in Strain 13 and Strain 2 Guinea Pigs after Administration of a DNP conjugate of the Nonimmunogenic Copolymer of D-Glutamic Acid and D-Lysine and Failure to Modify the Tolerance with the Allogeneic Effect

Group	Protocol*		Anti-DNP antibody (μg/ml)‡				Anti-OVA antibody-ABC-33 (μg/ml)‡			Indirect PFC/ 10⁶ spleen cells§	Total ABC/ 10⁶ spleen cells§
	Pretreatment	No. and strain of guinea pigs	Day 0	Day 7	Day 11	Day 22	Day 0	Day 11	Day 22	Day 25	Day 25
A	None	5 Strain 13	<0.10	5.5	16.1	3129.1	1125	6398	8707	142	21,500
B	DNP₅₃-D-GL	5 Strain 13	<0.10	<0.10	<0.10	0.26	1394	7884	9677	<1	3,200
C	DNP₅₃-D-GL	5 Strain 2	<0.10	<0.10	<0.10	0.49	866	5830	7531	12	2,100

* 200 x 10⁶ lymph node and spleen cells from strain 2 guinea pig donors were transferred to allogeneic strain 13 and syngeneic strain 2 recipients which had completed a 3-day series of injections of DNP₅₃-D-GL or normal saline 24 hr before transfer. 3 wk later, all recipients were immunized with 50 μg of OVA in CFA followed 2 wk thereafter by immunization with DNP₈-OVA (day 0). A final challenge with DNP₈-OVA was made on day 15.

‡ The data are expressed as geometric means. A comparison of the geometric mean anti-DNP antibody concentrations on day 7 and day 22 gave the following results: comparison of group A with groups B and C on both day 7 and day 22 yielded P values of <0.001 in each case.

§ All guinea pigs were sacrificed on day 25 and individual spleens were assayed for anti-DNP PFC and ABC. Facilitated plaques were developed with rabbit anti-guinea pig γ₂-globulin using TNP-conjugated SRBC as indicator. ABC were determined by radioautography using DNP-GPA-¹²⁵I as antigen. The data are expressed as geometric means. A comparison of the geometric mean numbers of PFC gave the following results: comparison of group A with groups B and C yielded P values of <0.001 in each case; comparison of group B with group C yielded a P value of 0.025 > P > 0.020. A comparison of the geometric mean numbers of ABC yielded the following results: comparison of group A with groups B and C yielded P values of 0.005 > P > 0.001 and P < 0.001, respectively; comparison of group B with group C yielded a P value of 0.40 > P > 0.30.

DNP-OVA as a result of carrier preimmunization, and brisk anamnestic responses to the final DNP-OVA challenge administered on day 15. In contrast, all guinea pigs which had initially received DNP-D-GL failed to mount either augmented primary or appropriate anamnestic antibody responses to DNP-OVA. This was true of both the strain 13 recipients of allogeneic cells and the strain 2 recipients of syngeneic cells. Again, the anti-OVA antibody responses were normal pointing out the hapten-specific nature of the unresponsiveness. The significantly depressed levels of anti-DNP antibody-forming cells and DNP-specific antigen-binding cells in spleens of guinea pigs treated with DNP-D-GL argues strongly for a central mechanism of tolerance.

The data from a second study is summarized in Table VI. All guinea pigs in this experiment were strain 13 recipients of allogeneic cells. Again, control guinea pigs manifested augmented primary and anamnestic anti-DNP antibody responses to DNP-OVA, whereas guinea pigs previously treated, in this case with either DNP-D-GL or DNP-L-GL, were markedly suppressed in their ability to respond.

(b) *Induction of Allogeneic Effect 6 Days before Administration of DNP-D-GL or DNP-L-GL.*—200 × 10⁶ lymph node and spleen cells from strain 2 guinea
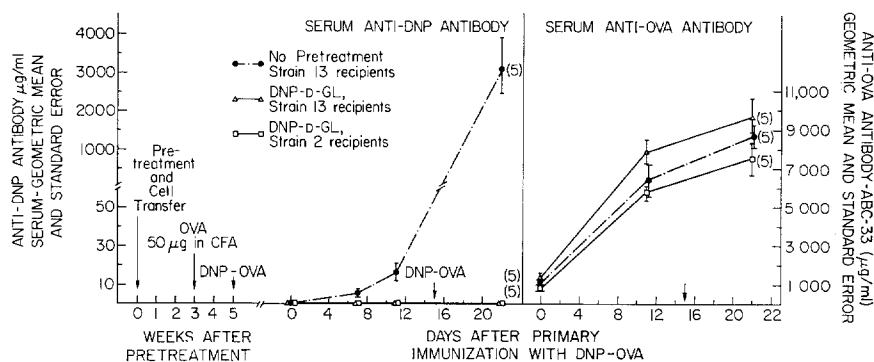

Fig. 4. Failure of allogeneic effect to modify DNP-specific tolerance induced by the administration of DNP-D-GL or DNP-L-GL 1 day before cell transfer. At wk 0, strain 13 and strain 2 guinea pigs were injected intraperitoneally with 3.0 mg of either DNP-D-GL or DNP-L-GL in saline, or saline alone; 24 hr later all guinea pigs received 200 × 10⁶ lymph node and spleen cells from strain 2 donors. 2 or 3 wk after cell transfer, all recipients were immunized with 50 μg of OVA emulsified in CFA. This was followed 2 wk thereafter by primary immunization with 1.0 mg of DNP-OVA in saline (day 0). A final challenge with 1.0 mg of DNP-OVA in saline was carried out on day 14 or day 15. Serum anti-DNP and anti-OVA antibody concentrations just before primary DNP-OVA immunization and on days 7, 11, and 22 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups.

pig donors were transferred intravenously to nonimmune strain 13 guinea pig recipients. These animals were injected 6 days later with 1.0 mg of DNP-BGG, DNP-D-GL, or DNP-L-GL intraperitoneally in saline for 3 successive days. A control group of recipients received no pretreatment. 2 wk later, all recipients were immunized with 50 μg of OVA in CFA. This was followed 2 wk thereafter (day 0) by immunization with 1.0 mg of DNP-OVA in saline (200 μg intradermally and 4 hr later 800 μg intraperitoneally). A final challenge with DNP-OVA was made on day 14. The results are summarized in Table VII and presented graphically in Fig. 5. Those recipients not given the tolerizing injections or injected with DNP-BGG were capable of mounting augmented primary and anamnestic anti-DNP antibody responses to DNP-OVA. On the other

hand, those recipients which had been treated with either DNP-D-GL or DNP-L-GL were rendered tolerant to the DNP determinant even though they had received allogeneic immunocompetent cells previously. Thus, induction of graft-*versus*-host reactions in nonprimed guinea pigs fails to abrogate the strong tolerogenic effects of these nonimmunogenic DNP copolymers regardless of

TABLE VI

Specific Suppression of Anti-Hapten Antibody Production in Strain 13 Guinea Pigs after Administration of DNP Conjugates of the Copolymers of either D-Glutamic Acid and D-Lysine or L-Glutamic Acid and L-Lysine and Failure to Modify the Suppression with the Allogeneic Effect

Group	Protocol*			Anti-DNP antibody (μg/ml)‡			
	Pretreatment	Allogeneic transfer	No. of strain 13 recipients	Day 0	Day 7	Day 14§	Day 21
A	None	200×10^6 strain 2 cells	5	<0.10	3.8	161.1	2978.4
B	DNP_{53}-D-GL	200×10^6 strain 2 cells	5	<0.10	<0.10	<0.10	0.60
C	DNP_{50}-L-GL	200×10^6 strain 2 cells	5	<0.10	<0.10	0.22	7.4

* 200×10^6 lymph node and spleen cells from strain 2 guinea pig donors were transferred to allogeneic strain 13 guinea pig recipients which had received a 3-day series of injections of DNP_{53}-D-GL, DNP_{50}-L-GL, or normal saline 24 hr before transfer. 2 wk later, all guinea pigs were immunized with 50 μg of OVA in CFA. This was followed 2 wk thereafter by immunization with DNP_8-OVA (day 0). A final challenge with DNP_8-OVA was made on day 14.

‡ The data are expressed as geometric means. A comparison of the geometric mean anti-DNP antibody concentrations on day 7 and day 21 yielded the following results: on day 7, comparison of group A with groups B and C yielded P values of <0.001 in each case. On day 21, comparison of group A with groups B and C yielded P values of $P < 0.001$ and $0.005 > P > 0.001$, respectively; comparison of groups B and C yielded a P value of 0.01 $> P > 0.005$.

§ Final challenge with DNP_8-OVA was administered on day 14 after each guinea pig was bled.

whether allogeneic cell transfer is carried out before or shortly after administration of these compounds.

DISCUSSION

Two sharply contrasting effects on immunocompetent cells may be brought about by the administration of nonimmunogenic DNP-carrier conjugates: (a) the induction of a state of DNP-specific unresponsiveness after administration of these substances to nonprimed guinea pigs and to guinea pigs which have

been previously primed with an immunogenic conjugate, DNP-OVA. (*b*) The elicitation by a nonimmunogenic DNP copolymer of secondary anti-hapten antibody responses in primed guinea pigs undergoing an appropriately timed graft-*versus*-host reaction.

For the purposes of this discussion, we will define "hapten-specific tolerance" as the specific suppression of anti-hapten antibody responses to conjugates of

TABLE VII

Failure of Previously Induced Allogeneic Effect to Prevent Suppression of Anti-DNP Responses in Strain 13 Guinea Pigs Subsequently Administered DNP conjugates of the Copolymers of D-Glutamic Acid and D-Lysine or of L-Glutamic Acid and L-Lysine

Group	Protocol* Pretreatment	No. of strain 13 recipients	Anti-DNP antibody (μg/ml)‡				
			Day 0	Day 7	Day 14§	Day 21	Boost
A	None	5	<0.10	0.49	109.9	3170.8	3170.7
B	DNP_{23}-BGG	5	0.14	6.7	83.0	950.0	949.9
C	DNP_{53}-D-GL	5	<0.10	<0.10	<0.10	0.49	0.39
D	DNP_{50}-L-GL	4	<0.10	0.12	0.25	1.77	1.67

* 200 × 10^6 lymph node and spleen cells from strain 2 guinea pig donors were transferred to nonimmune allogeneic strain 13 guinea pig recipients. 6 days after allogeneic transfer, groups of recipients were given a 3-day series of injections of either DNP_{23}-BGG, DNP_{53}-D-GL, DNP_{50}-L-GL, or normal saline. 2 wk later, all recipients were immunized with 50 μg of OVA in CFA. This was followed 2 wk thereafter by immunization with DNP_8-OVA (day 0). A final challenge with DNP_8-OVA was carried out on day 14.

‡ The data are expressed as geometric means. Boost represents the increase in mean antibody levels from day 0 to day 21. A comparison of the geometric mean anti-DNP antibody concentrations on day 14 and day 21 gave the following results: on day 14, a comparison of group A with group B yielded a P value of 0.80 > P > 0.70; comparison of group A with groups C and D and, similarly, group B with groups C and D yielded P values of <0.001 in each case. On day 21, comparison of group A with group B yielded a P value of 0.20 > P > 0.10; comparison of group A with groups C and D and, similarly, group B with groups C and D yielded P values of <0.001 in each case.

§ Final challenge with DNP_8-OVA was administered on day 14 after each guinea pig was bled.

a given hapten resulting from the prior administration of the haptenic determinant alone or of the hapten conjugated to an unrelated carrier. The induction of tolerance to hapten-carrier conjugates has been described by several investigators (34–36). In general, impaired anti-hapten antibody responses were restricted to the hapten-carrier conjugates used for tolerance induction. The tolerant animals displayed either no suppression or only a transient decrease of anti-hapten responses when challenged with the hapten coupled to a carrier unrelated to that used in the paralysis-inducing regimen. Such experiments (34–36), therefore, cannot be considered examples of hapten-specific tolerance.
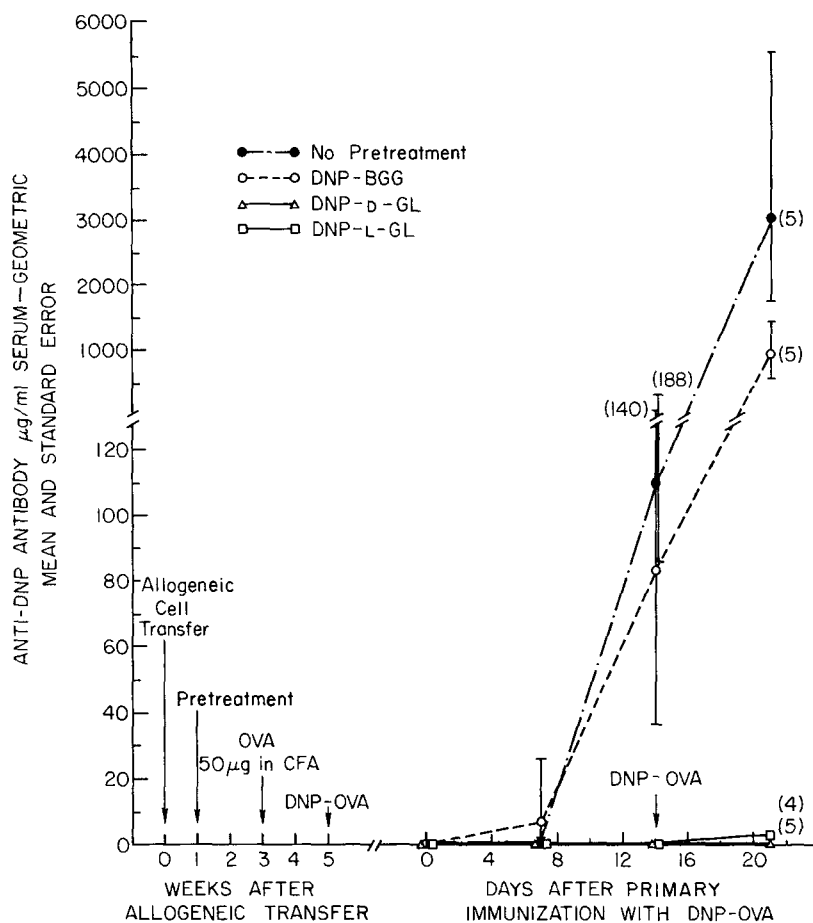
Fig. 5. Failure of allogeneic effect to modify DNP-specific tolerance induced by the administration of DNP-D-GL or DNP-L-GL 6 days after allogeneic cell transfer. 200 × 10⁶ lymph node and spleen cells from strain 2 donors were injected intravenously into nonimmune strain 13 recipients at week 0. 6 days later, the recipients were injected intraperitoneally with 3.0 mg of either DNP-BGG, DNP-D-GL, or DNP-L-GL in saline, or saline alone. 2 wk later, all recipients were immunized with 50 μg of OVA emulsified in CFA. This was followed 2 wk thereafter by primary immunization with 1.0 mg of DNP-OVA in saline (day 0). A final challenge with 1.0 mg of DNP-OVA in saline was carried out on day 14. Serum anti-DNP antibody concentrations just before primary DNP-OVA immunization and on days 7, 14, and 21 are illustrated. The numbers in parentheses refer to the numbers of animals in the given groups.

These various observations may be reconciled and understood in terms of the two-cell theory of immune responsiveness. If the precursors of anti-hapten antibody-forming cells are equated with bone marrow-derived lymphocytes and carrier reactive helper cells with thymus-derived lymphocytes, the unre-

sponsiveness to challenge with the hapten-carrier used for tolerance induction may reflect tolerance in either or both cell populations. On the other hand, hapten-specific tolerance, as defined above, requires a state of specific unresponsiveness in the bone marrow-derived precursors of antibody-forming cells. Chiller et al. have recently demonstrated that in mice the rate of induction and duration of tolerance differ markedly in the thymus and bone marrow cell populations (18). In the latter, larger doses and a longer induction period are required and the duration is shorter. These differences account for the earlier difficulty in achieving hapten-specific tolerance.

In the present studies, however, a marked degree of DNP-specific tolerance was readily induced with moderate doses of DNP conjugates of nonimmunogenic copolymers of glutamic acid and lysine. This was reflected not only by dramatic reductions in serum anti-DNP antibody levels after subsequent challenge but also by a diminished frequency of DNP-specific antigen-binding cells and of anti-DNP antibody-secreting cells, which is evidence for a central tolerance mechanism. Further, after the tolerance-inducing regimens, the frequency of DNP-specific antigen-binding cells was lower than in nontreated controls, suggesting either a reduction in the number of specific precursors of anti-DNP antibody-forming cells or the prior saturation of the receptors on these cells with the DNP determinants of the tolerogen.

A possible explanation for the ease of tolerance induction with the DNP conjugates of nonimmunogenic copolymers and also for other non- or weakly immunogenic substances may be the lack of the simultaneous occurrence of immunization and tolerance induction when these substances are administered. If, as we suggest, the weak immunogenicity of a substance is primarily determined by (a) the absence or scarcity of effective thymus-derived helper cells specific for that substance, and (b) the relative inability of the material to activate bone marrow-derived cells without the cooperation of thymus-derived cells, then tolerance induction by these nonimmunogenic materials and their DNP conjugates may be interpreted to result from direct interaction in the appropriate dose range with specific precursors of antibody-forming cells without intervening helper cells. Indeed, it appears that the precursors of antibody-forming cells are susceptible to tolerance induction by the DNP copolymers we have employed even if the animal in which they reside has previously been primed with DNP-OVA.

Although the simplest explanation of the data is that no specific helper cells exist for these DNP copolymers, one could also explain these findings by envisaging the existence of nonimmunogen-specific helper cells which are always rendered tolerant by interaction with these types of substances. An additional alternative is to postulate the existence of two subpopulations of helper cells, one subserving the function of stimulating responses in the precursors of antibody-forming cells and the other providing a suppressive signal to such

precursors leading to tolerance induction in those cells. For nonimmunogens, one would postulate either a complete absence of the stimulatory helper cells or a marked excess of the suppressive type. Recent studies by Baker et al. in pneumococcal polysaccharide responses suggest the possibility that in mice thymus-derived cells exert a suppressive effect on anti-S_{III} antibody synthesis (37).

These concepts may explain the puzzling phenomenon described by some investigators studying the "termination" of tolerance to protein antigens by administration of modified or cross-reactive proteins (38–40). In those experiments, the simultaneous administration of even very small quantities of the tolerated protein prevented termination of the unresponsive state. It has been postulated that the mechanism in the termination of tolerance involves the presentation of the determinants of the tolerated protein to precursors of antibody-forming cells through the action of helper cells specific for the cross-reactive or altered protein (18). In this set of circumstances, injection of the tolerated protein simultaneous with the cross-reactive protein should lead to a potent suppressive effect on the precursors of antibody-forming cells either because of the absence of helper cells specific for the tolerated substance or because of the action of a suppressive type of thymus-derived cell.

In striking contrast with the tolerogenic properties of DNP conjugates of nonimmunogenic copolymers for normal animals discussed above, the elicitation of a GVH reaction in a DNP-OVA–primed recipient renders the animal refractory to tolerance induction by DNP-D-GL and permits this molecule to behave as an immunogen capable of stimulating a strong anti-DNP secondary response. The response obtained with DNP-D-GL is comparable to that observed with DNP-BGG under identical experimental conditions. We have previously suggested that the response to DNP-BGG under this set of circumstances may reflect a direct stimulation of precursors of antibody-forming cells by antigen when a simultaneous stimulation of such cells by a GVH reaction is occurring (19). The observation of McCullagh (41) that adult rats tolerant to SRBC nevertheless are stimulated to form an anti-sheep erythrocyte antibody response by administration of allogeneic immunocompetent cells and SRBC is analogous to the allogeneic effect described in this and our earlier study (19). Although the precise mechanism(s) by which the GVH reaction causes these effects is not known, the ability to obtain a response with DNP-D-GL strengthens the foregoing hypothesis in that helper cells for this nonimmunogenic compound most likely do not exist or are not functional. Our failure to obtain evidence of activation of precursors of specific antibody-forming cells with the nonimmunogenic DNP copolymers in nonprimed guinea pigs undergoing GVH reactions is unexplained. Nevertheless, it is consistent with our failure to obtain heightened responses to an immunogenic DNP

conjugate under the same circumstances. A more detailed consideration of this observation is presented in another publication.[4]

This study also raises the issue of the definition of an immunogen in terms of the two-cell concept of antibody formation. A molecule incapable of being bound by, or of stimulating, thymus-derived helper cells is expected to be endowed with only very weak immunogenicity, but to be an excellent tolerogen. Such a molecule should stimulate only very low antibody responses and little cellular proliferation by specific bone marrow cells in a narrow range of antigen concentrations. At higher concentrations tolerance will be induced. Thus, when used at tolerogenic levels or when relatively insensitive assays for antibodies are used, such a molecule will appear operationally nonimmunogenic. These compounds, however, may behave as excellent haptens when bound to carrier molecules capable of stimulating thymus-derived helper cells. The nonimmunogenic copolymers used in this study and the well-studied pneumococcal polysaccharides may be considered typical examples of such molecules which are nonimmunogenic for thymus-derived cells and yet may bind to bone marrow-derived precursors of antibody-secreting cells with resulting tolerance or production of small amounts of antibodies according to the experimental conditions.

The experiments presented in this study and the considerations discussed above imply that the interaction of antigenic determinants and receptors of precursors of antibody-forming cells may lead to very different outcomes determined by the differences in form in which the antigen is presented or, particularly, by the state of the cell at the time of interaction. Thus, in non-primed and primed animals interaction in the adequate dose range of DNP on nonimmunogenic carriers with specific precursors of antibody-forming cells leads to an inactivation of those cells. On the other hand, the direct or indirect stimulation of precursors of antibody-forming cells by GVH reactions allows these cells to be stimulated by the nonimmunogenic DNP conjugate. This latter stimulatory outcome may well proceed by a mechanism akin to that which normally operates for the stimulation of these cells through the interaction of immunogenic conjugates and helper cells. This process might be mediated by products of stimulated thymus-derived cells destined to regulate the stimulation of antibody-forming cell precursors by antigen. More detailed analysis of this allogeneic effect may provide insight into the nature of collaborative interactions between two cell types and of the factors produced in the development of immune responses.

Finally, it is conceivable that the stimulation of precursors of antibody-forming cells by normally nonimmunogenic substances during GVH reactions

may well have significant pathogenetic implications. For example, host antigens which may be tolerated as a result of unresponsiveness in the helper cell population, might stimulate the development of autoantibodies, as is observed in various disease states, by virtue of their capacity to directly stimulate precursors of antibody-forming cells when the latter are subjected to potent stimuli similar to those observed in the GVH reaction.

SUMMARY

Administration of nonimmunogenic 2,4-dinitrophenyl (DNP) conjugates of copolymers of D or L-glutamic acid and lysine (GL) induces hapten-specific tolerance in nonimmune and DNP-ovalbumin–primed strain 13 guinea pigs. This tolerant state is evidenced by depressed anti-DNP antibody synthesis in response to challenge with DNP-ovalbumin and by a diminished frequency of DNP-specific antigen-binding cells and of anti-DNP antibody-secreting cells.

Such a nonimmunogenic compound (DNP-D-GL) will nevertheless elicit a DNP-specific anamnestic antibody response when administered at an appropriate time to DNP-ovalbumin–primed guinea pigs undergoing a graft-versus-host reaction. These experiments are discussed in terms of a two-cell theory of stimulation of antibody responses.

BIBLIOGRAPHY

1. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* **1**:3.
2. Claman, H. N., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells—A model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
3. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* **1**:43.
4. Schierman, L. W., and R. A. McBride. 1967. Adjuvant activity of erythrocyte isoantigens. *Science (Washington).* **156**:658.
5. Mitchison, N. A. 1969. Cell populations involved in the immune response. *In* Immunological Tolerance. M. Landy and W. Braun, editors. Academic Press, Inc., New York. 149.
6. Rajewsky, K., V. Schirrmacher, S. Nase, and N. K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J. Exp. Med.* **129**:1131.
7. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses. I. Enhancement of primary and secondary anti-hapten antibody responses by carrier preimmunization. *J. Exp. Med.* **132**:261.
8. Paul, W. E., D. H. Katz, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses. II. Specific properties of carrier cells capable of enhancing anti-hapten antibody responses. *J. Exp. Med.* **132**:283.

9. Taylor, R. B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1**:114.

10. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1970. Cellular sites of immunologic unresponsiveness. *Proc. Nat. Acad. Sci. U.S.A.* **65**:551.

11. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.

12. Warner, N. L., P. Byrt, and G. L. Ada. 1970. Blocking of the lymphocyte's antigen receptor site with anti-immunoglobulin sera *in vitro*. *Nature (London)*. **226**: 942.

13. Wigzell, H., and O. Mäkelä. 1970. Separation of normal and immune lymphoid cells by antigen-coated columns. Antigen-binding characteristics of membrane antibodies analyzed by hapten-protein antigens. *J. Exp. Med.* **132**:110.

14. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic duct lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* **59**:296.

15. Mitchison, N. A., K. Rajewsky, and R. B. Taylor. 1970. Cooperation of antigenic determinants and of cells in the induction of antibodies. *In* Developmental Aspects of Antibody Formation and Structure. J. Šterzl and H. Riha, editors. Academia, Publishing House of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia. 547.

16. Raff, M. C. 1970. Role of thymus-derived lymphocytes in the secondary humoral immune response in mice. *Nature (London)*. **226**:1257.

16a. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1970. Radioresistance of cooperative function of carrier-specific lymphocytes in anti-hapen antibody responses. *Science (Washington)*. **170**:462.

17. Ovary, Z., and B. Benacerraf. 1963. Immunological specificity of the secondary response with dinitrophenylated proteins. *Proc. Soc. Exp. Biol. Med.* **114**:72.

18. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science (Washington)*. **171**:813.

19. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1971. Carrier function in anti-hapten antibody responses. III. Stimulation of antibody synthesis and facilitation of hapten-specific secondary antibody responses by graft-*versus*-host reactions. *J. Exp. Med.* **133**:169.

20. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of the delayed and immediate hypersensitivity reactions. *J. Exp. Med.* **115**:1023.

21. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I* BSA and antibody. *J. Infec. Dis.* **103**:329.

22. Green, I., B. Benacerraf, and S. H. Stone. 1969. The effect of the amount of mycobacterial adjuvant on the immune response of strain 2, strain 13, and Hartley strain guinea pigs to DNP-PLL and DNP-GL. *J. Immunol.* **103**:403.

23. Biozzi, G., B. Benacerraf, C. Stiffel, B. N. Halpern, and D. Mouton. 1957. Influence de la quantité d'iode fixée sur les proteines sériques normales et modifiées par le chaleur sur la phagocytose de ces colloides par les cellules du S.R.E. *Ann. Inst. Pasteur (Paris)*. **92**:89.

24. Davie, J., W. E. Paul, R. G. Mage, and M. B. Goldman. 1971. Membrane-asso-

ciated immunoglobulin of rabbit peripheral blood lymphocytes: allelic exclusion at the *b* locus. *Proc. Nat. Acad. Sci. U.S.A.* **68**:430.

25. Mishell, R. I., and R. W. Dutton. 1966. Immunization of normal mouse spleen cell suspensions *in vitro. Science (Washington).* **153**:1004.

26. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Washington).* **140**:405.

27. Plotz, P. H., N. Talal, and R. Asofsky. 1968. Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin classes. *J. Immunol.* **100**:744.

28. Rittenberg, M. B., and K. L. Pratt. 1969. Antitrinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575.

29. Benacerraf, B., I. Green, and W. E. Paul. 1967. The immune response of guinea pigs to hapten-poly-L-lysine conjugates as an example of the genetic control of the recognition of antigenicity. *Cold Spring Harbor Symp. Quant. Biol.* **32**:569.

30. McDevitt, H. O., and B. Benacerraf. 1969. Genetic control of specific immune responses. *Advan. Immunol.* **11**:31.

31. Green, I., W. E. Paul, and B. Benacerraf. 1966. The behavior of hapten–poly-L-lysine conjugates as complete antigens in genetic responder and as haptens in nonresponder guinea pigs. *J. Exp. Med.* **123**:859.

32. Benacerraf, B., A. Ojeda, and P. H. Maurer. 1963. Studies on artificial antigens. II. The antigenicity in guinea pigs of arsanilic acid conjugates of copolymers of D- or L-α-amino acids. *J. Exp. Med.* **118**:945.

33. Janeway, C. A., and M. Sela. 1967. Synthetic antigens composed exclusively of L- or D-amino acids. I. Effect of optical configuration on the immunogenicity of synthetic polypeptides in mice. *Immunology.* **13**:29.

34. Cinader, B., and J. H. Pearce. 1958. The specificity of acquired immunological tolerance to azo proteins. *Brit. J. Exp. Pathol.* **39**:8.

35. Weigle, W. O. 1965. The immune response of rabbits tolerant to one protein conjugate following the injection of related protein conjugates. *J. Immunol.* **94**:177.

36. Brownstone, A., N. A. Mitchison, and R. Pitt-Rivers. 1966. Biological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology.* **10**:481.

37. Baker, P. J., P. W. Stashak, D. F. Amsbaugh, B. Prescott, and R. F. Barth. 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to type III pneumococcal polysaccharide. *J. Immunol.* **105**:1581.

38. Porter, R. J. 1962. Studies on termination of tolerance by heterologous antigen injection. *Fed. Proc.* **21**:28.

39. Weigle, W. O. 1964. Studies on the termination of acquired tolerance to serum protein antigens following injection of serologically related antigens. *Immunology.* **7**:239.

40. Humphrey, J. H. 1964. Immunological unresponsiveness to protein antigens in rabbits. II. The nature of the subsequent antibody response. *Immunology.* **7**:462.

41. McCullagh, P. J. 1970. The abrogation of sheep erythrocyte tolerance in rats by means of the transfer of allogeneic lymphocytes. *J. Exp. Med.* **132**:916.