

TRANSFER OF RESPONSIVENESS TO HAPTEN CONJUGATES OF  
POLY-L-LYSINE AND OF A COPOLYMER OF L-GLUTAMIC  
ACID AND L-LYSINE TO LETHALLY IRRADIATED NON-  
RESPONDER GUINEA PIGS BY BONE MARROW OR  
LYMPH NODE AND SPLEEN CELLS FROM  
RESPONDER GUINEA PIGS

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The immune response of guinea pigs to poly-L-lysine (PLL)<sup>1</sup> and hapten conjugates of this polymer is predicated upon the presence of an autosomal dominant gene which is referred to as the "PLL gene" (1-3). This same gene also determines the immune response of guinea pigs to a copolymer of L-lysine and L-glutamic acid (GL) and its hapten conjugates, although no cross-reactivity could be detected between anti-GL and anti-2,4-dinitrophenyl PLL (DNP-PLL) antibodies (4). Thus, a varying percentage of random-bred Hartley strain guinea pigs, depending upon their origin, and all strain 2 guinea pigs immunized with DNP-PLL, GL, or DNP-GL in complete Freund's adjuvant produce high levels of specific antibodies and also develop strong delayed hypersensitivity reactions when challenged with the immunizing antigen intradermally (4). These guinea pigs which possess the PLL gene are referred to as "responder" animals. "Nonresponders" lacking the PLL gene include a varying percentage of random-bred Hartley strain guinea pigs and all strain 13 guinea pigs.

The manner in which the PLL gene exercises its control of the immune response to these antigens is not known but appears to involve the recognition of a critical number of lysyl residues which characterize the structure of both PLL and GL and their hapten conjugates (5). In recent years, observations dealing with the control of specific immune responses have been extended to several other immune systems in

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<sup>1</sup> *Abbreviations used in this paper:* DNP, 2,4-dinitrophenyl; DNP-GL, 2,4-dinitrophenyl copolymer of lysine and glutamic acid; DNP-PLL, 2,4-dinitrophenyl poly-L-lysine; EACA,  $\epsilon$ -aminocaproic acid; GL, copolymer of lysine and glutamic acid; (H, G)-A-L, synthetic polypeptide containing random sequences of L-histidine and L-glutamic acid on a backbone of D,L-alanine and L-lysine; HVL, half value layer; MEM, Eagle's minimum essential medium; (Phe G)-A-L, synthetic polypeptide containing random sequences of L, phenylalanine and L-glutamic acid; PLL, poly-L-lysine; POPOP, 1,4-bis[2-(5-phenyloxazolyl)] benzene; PPO, 2,5-diphenyloxazole; (T, G)-A-L, synthetic polypeptide containing random sequences of L-tyrosine and L-lysine.

mice (6-8), rabbits (9), and rats (10, 11). Thus, the immunogenicity for mice of branched synthetic polypeptides containing random sequences of L-tyrosine, or L-histidine, or L-phenylalanine, and L-glutamic acid on a backbone of D,L-alanine and L-lysine ([T, G)-A-L, (H, G)-A-L, (PheG)-A-L] has been shown to be largely determined either by single dominant genes at a single locus, Ir-1, or by three closely linked genes (12). The immune response of mice to a random copolymer of L-glutamic acid and L-lysine containing 5% L-alanine is also under the control of another dominant gene (8). In addition, the ability to recognize porcine lactic dehydrogenase isoenzymes as antigens has been reported to be under simple genetic control in rats and rabbits (10). The discovery within a short time of several specific systems under genetic control indicates that genes affecting specific immune responses are not rare. However, these observations did not further our understanding of the process controlled by these genes. In order to understand the mode of action of genes concerned with specific immune responses, it is essential to identify the cell population and the cell type where the process controlled by the genes is expressed and to recognize how these cells are concerned with specific immune responses. One general method of achieving this goal is to transfer various types of cell populations from genetic responder animals into animals lacking the genes necessary for the response. Tyan et al. (13) have reported the successful transfer of the ability to respond to (T, G)-A-L to irradiated non-responder C<sub>3</sub>H parental recipients with adult unfractionated spleen cells or with fetal liver cells from (C57Bl × C<sub>3</sub>H) F<sub>1</sub> responder mice.

The present study is concerned with the transfer of the process controlled by the PLL gene to genetic nonresponder guinea pigs with cell populations derived from responder animals. In a first series of experiments, the capacity to form an immune response to DNP-PLL has been transferred successfully to lethally irradiated nonresponder Hartley strain guinea pigs with bone marrow transplants from allogeneic Hartley strain responder animals. However, in the course of these experiments many of the cell transfer recipients developed a severe graft versus host reaction which contributed to the high mortality observed in these animals. Moreover, lymph node cell populations containing large numbers of immunocompetent cells could not be used in these transfer experiments because of unavoidable homologous disease.

Use was therefore made in a second series of experiments of the difference between the two inbred strains of guinea pigs, 2 and 13, in their ability to respond to PLL antigens. As would be expected for a process controlled by a dominant genetic factor, all (2 × 13) F<sub>1</sub> guinea pigs are able to respond to hapten-PLL conjugates.<sup>2</sup> Since (2 × 13) F<sub>1</sub> cells carry all the histocompatibility antigens present in both parental strains, the (2 × 13) F<sub>1</sub> immunocompetent cell population cannot recognize parental strains. On the other hand, lethally irradiated recipient strain 13 guinea pigs cannot readily reject transferred (2 × 13) F<sub>1</sub> cells. Strain 13 guinea pigs can be lethally irradiated and protected with

<sup>2</sup> Green, I., and B. Benacerraf. Unpublished observations.

syngeneic bone marrow graft while they are simultaneously immunologically reconstituted with ( $2 \times 13$ ) F<sub>1</sub> lymph node and spleen cells. In this system, a study can be made of the ability of ( $2 \times 13$ ) F<sub>1</sub> bone marrow or lymph node and spleen cell populations to transfer to lethally irradiated nonresponder strain 13 parental guinea pigs the capacity to respond to antigens, the immunogenicity of which is determined by the PLL gene, without interference from graft versus host reactions. The transfer of the capacity to respond to DNP-PLL was investigated, but, in addition, the transfer of responsiveness to a DNP conjugate of a copolymer of L-glutamic and L-lysine (DNP-GL), the immunogenicity of which is also controlled by the PLL gene, was studied. The use of DNP-GL in these experiments presents certain advantages because of its negligible net electric charge. When electrostatically complexed with immunogenic carriers, positively charged DNP-PLL can behave as a hapten and can elicit the formation of anti-DNP antibodies in nonresponder guinea pigs (14). Moreover, it has recently been shown that mycobacterial adjuvants, if used in very large amounts, can also act as immunogenic carriers and that nonresponder strain 13 guinea pigs immunized with positively charged DNP-PLL and 10 mg of mycobacteria/ml of adjuvant may produce small, but significant, levels of anti-DNP antibodies, in the absence of any evidence of cellular immunity (15). In contrast, repeated studies (15, 16) have demonstrated that nonresponder Hartley or strain 13 guinea pigs immunized with DNP-GL and the small amount of mycobacteria generally used in conventional Freund's adjuvant fail to produce detectable serum levels of anti-DNP antibodies. Thus, when DNP-GL and small amounts of mycobacteria are used as immunizing antigens, a successful transfer of the process controlled by the PLL gene to nonresponder guinea pigs can be unequivocally demonstrated by the development of delayed sensitivity to DNP-GL and/or by the formation of anti-DNP antibodies. In these experiments the ability to respond to DNP-PLL and to DNP-GL has been successfully transferred to lethally irradiated strain 13 guinea pigs with ( $2 \times 13$ ) F<sub>1</sub> bone marrow or lymph node and spleen cell populations.

#### Materials and Methods

*Polypeptides.*—Two poly-L-lysine HBr (PLL) preparations with average molecular weights of 90,000 and 110,000 respectively, and a copolymer of L-glutamic acid and L-lysine (GL) with an average molecular weight of 115,000 and a 60:40 molar ratio of glutamic acid to lysine, were used. These polypeptides were purchased from Pilot Chemicals Inc., Watertown, Mass.

The polypeptides were reacted with 2,4-dinitrofluorobenzene as described (4), and the following conjugates were prepared: DNP<sub>21</sub>-PLL, DNP<sub>62</sub>-PLL, and DNP<sub>33</sub>-GL. The subscripts refer to the average number of DNP groups per molecule.

*Other Reagents.*—Complete Freund's adjuvant was purchased from Difco Laboratories, Inc., Detroit, Mich. and contained 0.5 mg/ml *Mycobacterium butyricum* (Difco complete adjuvant) or was prepared by adding killed *Mycobacterium tuberculosis* to incomplete Freund's adjuvant in a final concentration of 2 mg/ml (*M. tuberculosis* adjuvant).

Eagle's minimum essential medium (MEM), penicillin, and glutamine were purchased from Grand Island Biological Co., Grand Island, N.Y.

Hydroxide of Hyamine 10X, PPO (2,5-diphenyloxazole), and dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene were all purchased from Packard Instrument Co., Inc., Downers Grove, Ill.

*Immunization Procedure to Determine Responder Status of Hartley Guinea Pigs.*—Random-bred Hartley strain guinea pigs weighing 300–400 g were purchased from Camm Research Laboratories, Inc., Wayne, N.J. These animals were immunized with a total of 100  $\mu$ g of GL in Difco complete adjuvant. 0.1 ml of emulsion containing 50  $\mu$ g of the copolymer was injected into the left front and hind footpads; the right footpads were saved for subsequent immunizations. 10 days after immunization, all animals were skin tested with 50  $\mu$ g of GL and 14 days after immunization with 10  $\mu$ g of GL. Animals showing consistently severe delayed hyper-

TABLE I  
*Effect of Allogeneic Bone Marrow Transplant on Mortality from Irradiation (750 r) of Random-Bred Hartley Strain Guinea Pigs*

No. of animals	Bone marrow transplant (Mean no. of cells transferred and range)	Mortality	Survival	
			median	range
22	none	22/22* 100	8	5–10
95	7.95 $\times 10^8$ (2.8 $\times 10^8$ –18 $\times 10^8$ )	75/95 79	19	6–51

\* Of these 75 animals, 7 died from acute radiation sickness, 7 from marrow failure, and 19 from characterized graft versus host reaction. The cause of death of the remaining animals was not ascertained.

sensitivity reactions were classified as "responder" guinea pigs and those with consistently negative skin tests were classified as "nonresponder" guinea pigs.

*Irradiation.*—Hartley strain nonresponder recipient guinea pigs received 750 R total body irradiation 24–48 hr before bone marrow transplantation. Animals were irradiated with a 200 kv machine delivering 38.6 R/min at a target distance of 45.5 cm using 1.0 mm aluminum and 0.05 mm copper filters (HVL, half value layer, 1.3 mm Cu; F-0.94). In animals not protected with bone marrow cells, this dose proved lethal (see Table I), as had been shown previously (17).

Strain 13 recipient guinea pigs were given 700 R with two X-ray tubes at a distance of 54 cm with a 250 kv machine. Conditions of irradiation were as follows: Voltage 200 kv, current 15 ma, filter 0.25 mm Cu and 0.55 mm Al. HVL = 0.91 mm Cu. Dose rate 139 R/min. This dose of irradiation is known to be uniformly lethal for strain 13 guinea pigs (18). All irradiated guinea pigs that did not receive a bone marrow transplant died within 12 days.

*Bone Marrow, Lymph Node, and Spleen Cell Transplants.*—Hartley guinea pigs were immunized with GL in Difco complete adjuvant to determine their responder status as described above. Strain 13 or (2  $\times$  13) F<sub>1</sub> guinea pigs, to be used as donors of bone marrow or of lymph node and spleen cells, were injected with complete Freund's adjuvant containing 0.5 mg/ml *M. butyricum* as a 1:1 emulsion prepared with physiological saline; 0.1 ml of the emulsion was injected into each footpad. This was done in order to stimulate the bone marrow and the lymphoid tissues and to increase cell yields. 10–14 days later the animals were exsanguinated and the cell suspensions were prepared for transfer.

Bone marrow donor animals were anesthetized with ether and exsanguinated from the

heart. Both tibiae, femora, and humeri were sterilely removed, the bone ends were snapped off with hemostats, and the contents of the marrow cavity were dislodged with a fine needle and flushed into Eagle's minimum medium containing 1% guinea pig serum (MEM) with a Pasteur pipette. The clumps of marrow were dissociated into single cell suspensions by means of gently, and repeatedly, sucking the aggregates into Pasteur pipettes and discharging the contents back into the medium. The suspensions were then filtered through two layers of gauze, centrifuged at 700 rpm for 5 min, and resuspended in 2.5-3.0 ml of medium. All the bone marrow cells obtained from one donor were injected intravenously into a single recipient. The number of cells transferred are shown in the tables. The time interval between exsanguination and suspension of cells in medium rarely exceeded 10 min, and the cells were always injected into the recipient within 30-45 min of removal from the donor. Some irradiated animals, chosen at random, were injected with MEM only.

Lymph node and spleen cell suspensions were prepared as follows: inguinal, axillary, and popliteal nodes and the spleen were removed sterilely. The lymph nodes and spleen were then teased apart with sharp instruments in 60 mm plastic Petri dishes containing MEM, using sterile techniques. The stroma discarded, the teased cells were then gently transferred with Pasteur pipettes to 12 ml conical centrifuge tubes to allow cell aggregates to settle spontaneously. The isolated cells in the supernatant were transferred again to 12 ml centrifuge tubes and spun at 700 rpm for 7 min. The cells were then resuspended in 3-4 ml of MEM and slowly injected intravenously into irradiated strain 13 recipients. A portion of the cells was also removed for counting. This technique yielded cell suspensions from both lymph nodes and spleens containing a large proportion of lymphoid cells; very few cells were identifiable as macrophages, as judged by uptake of neutral red into phagocytic vacuoles when exposed to 1:20,000 concentration of the dye. In the experiments where the proportion of cells taking up neutral red, and therefore identified as macrophages, were counted, they ranged from 0.25 to 4% in the lymph node cell populations and from 1 to 8% in the spleen cell populations. A recipient animal received the lymph node and spleen cells from one donor guinea pig.

The effects of radiation and of bone marrow and lymph node cell transplants on survival are shown in Tables I and II. All irradiated guinea pigs not protected with bone marrow died within 5-10 days. 95 irradiated nonresponder Hartley guinea pigs received bone marrow transplants. Seven of these animals died of acute radiation sickness; seven more died of obvious graft failure with severe persisting pancytopenia. 19 guinea pigs died within 16-28 days (median 21 days) carrying all the stigmata of severe graft versus host reactions with wasting, extensive desquamating dermatitis, moderate adenopathy, and diarrhea. Several of these guinea pigs also showed a most interesting change in their blood leukocytes: striking elevations in the number of blood basophil polymorphonuclear leukocytes, in conjunction with severe graft versus host reactions, were observed. One animal, with no basophils prior to irradiation and bone marrow transfer, attained a count of  $2500/\text{mm}^3$  just prior to death, while in several others the changes were less marked, but nevertheless highly significant. It was not established whether these basophils were of host or donor origin. The cause of death of the remaining 44 guinea pigs was not precisely ascertained, although many of these animals also showed some evidence of graft versus host disease and others appeared to be suffering from pulmonary infections (Table I). Thus, 75 of 95 irradiated animals died; 20 guinea pigs survived, and of these, 19 were in sufficiently good health to be included in the definitive experiments of the transfer of the ability to respond to DNP-PLL. The overall mortality was much lower when syngeneic or hemisyngeneic bone marrow transplants were used. Only 9 of 34 irradiated strain 13 animals restored with strain 13 or  $(2 \times 13)$   $F_1$  bone marrow died. Syngeneic strain 13 bone marrow gave significantly better protection than  $(2 \times 13)$   $F_1$  hybrid bone marrow. These data are presented in Table II. The animals that died displayed none of the clinical signs of graft versus host reaction. The surviving animals were in excellent condition at the time of immunization.

*Immunization and Testing Procedures.*—White blood counts and differentials were done on Hartley strain survivors. After recovery from irradiation and cell transfers, transplant recipients with an absolute lymphocyte count of 800/mm<sup>3</sup> of blood or higher were immunized. Hartley recipient guinea pigs were injected with 100 µg/ml of DNP-PLL in complete Freund's adjuvant containing 2 mg/ml of *M. tuberculosis* at times varying from 12 to 29 days after bone marrow transplant. 0.1 ml of emulsion was injected into the right front and hind footpads. Strain 13 recipient animals were immunized either with 100 γ DNP-PLL in adjuvants containing 2 mg/ml *M. tuberculosis* H37 Rv or with 100 γ DNP-GL in adjuvants containing 0.5 mg/ml *M. butyricum* distributed in the four footpads. After the time intervals shown on the respective tables, the animals were skin tested for delayed hypersensitivity with 10 µg DNP-PLL or 50 µg of DNP-GL in 0.1 ml of saline. These concentrations of antigen caused only minimal irritation in unimmunized animals.

TABLE II  
*Relative Ability of Strain 13 and (Strain 2 × Strain 13) F<sub>1</sub> Bone Marrow Cells to Protect Lethally Irradiated (700 r) Strain 13 Guinea Pigs*

Origin of bone marrow cells	No. of recipient animals	Mortality at 2 months	
			%
(2 × 13)F <sub>1</sub>	14	9/14	43
Strain 13	20	3/20	15
Summary of all experiments	34	9/34	26

All animals were then bled and their sera collected for antibody determination. In addition, in the Hartley recipient animals the lymph nodes draining the sites of injection of antigen (DNP-PLL) were collected sterilely and the ability of their cells to proliferate in response to the immunizing antigen as manifested by increased DNA synthesis was investigated. The technique used has been described in detail previously (19).

Levels of anti-DNP antibody were measured by a modification of the Farr technique (15). The binding of <sup>3</sup>H-2,4-dinitrophenyl-ε-aminocaproic acid (<sup>3</sup>H-DNP-EACA) by the globulin fraction of the antisera was measured. <sup>3</sup>H-DNP-EACA was prepared as described in (14). The test was performed as follows: 0.1 ml of 10<sup>-8</sup> M <sup>3</sup>H DNP-EACA in PBS was added to 0.1 ml of serum in 10 × 75 mm glass test tubes. This mixture was incubated for 1 hr at 4°C, then 0.4 ml of 66% saturated ammonium sulfate solution was added. After mixing, the tubes were incubated again for 30 min at 4°C. The samples were then spun at 2500 rpm in an International PR-2 centrifuge. 0.2 ml of the supernatant was carefully withdrawn without disturbing the precipitate and added to a vial containing 10 ml of scintillation fluid. This was composed of: 2365 ml toluene, 500 ml Beckman Bio-Solv BBS3, and 100 ml Liquifluor, New England Nuclear Corp., Boston, Mass. The vials were then counted in a liquid scintillation counter. A normal guinea pig serum as well as a serum containing anti-DNP antibody was included in each assay. Control samples were prepared in which 0.1 ml PBS replaced the test sera. The per cent binding was calculated as follows:

$$\text{per cent binding} = 1 - \frac{\text{Counts in unknown sera}}{\text{Counts in control samples}} \times 100$$

The binding value for normal nonimmune sera ranged from 0 to 7%. To relate the per cent binding to the actual amounts of anti-DNP antibody in milligrams per milliliter, standard

curves were constructed using individual strain 2 antisera from guinea pigs immunized with DNP-PLL or DNP-GL and bled 26 days after immunization (Fig. 1). The binding values for  $^3\text{H}$  DNP-EACA of serial dilutions of these sera were plotted against the respective anti-DNP antibody concentrations of these dilutions. The anti-DNP antibody concentration of undiluted sera was measured by precipitin analysis with DNP-bovine fibrinogen as described (15).

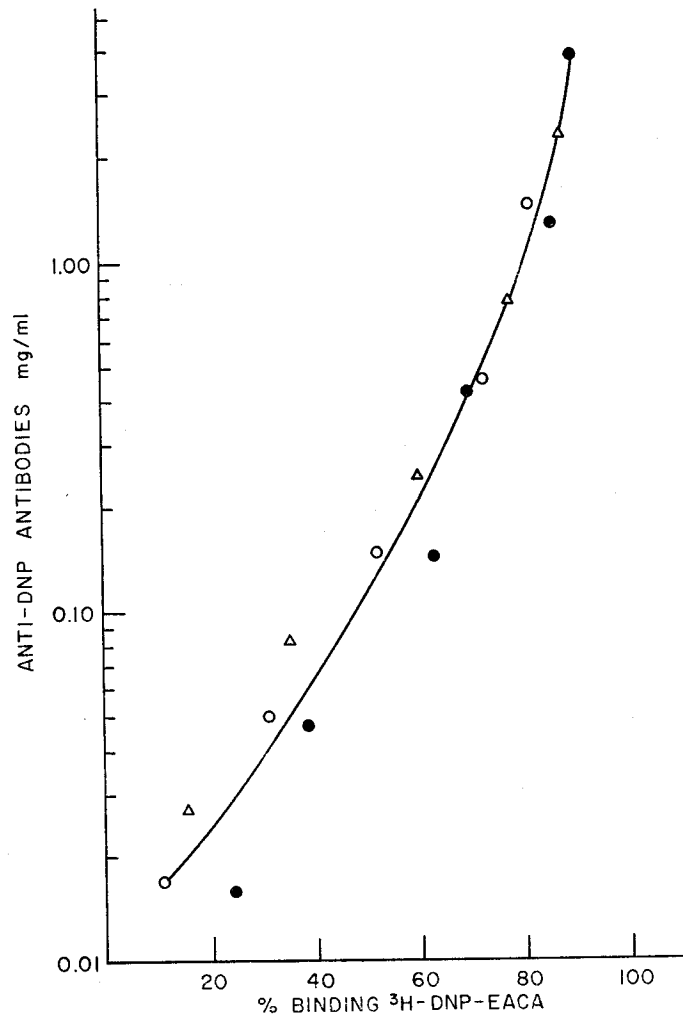


FIG. 1. Relationship between per cent of binding of  $10^{-8}\text{M}$   $^3\text{H}$  DNP-EACA by serial dilutions of strain 2 guinea pig anti-DNP-GL 26 days sera and the concentrations of anti-DNP antibodies, measured by quantitative precipitin analysis. Values obtained with three different sera are presented.

## RESULTS

*Transfer of Responsiveness to DNP-PLL to Lethally Irradiated Hartley strain Nonresponder Guinea Pigs by Bone Marrow Transplants from Allogeneic Responder Hartley Guinea Pigs.—*

Bone marrow cells from responder animals conferred upon irradiated non-responder recipients (previously unable to make an immune response to GL) the ability to form an immune response to DNP-PLL (Table III). 13 of 14 non-responder Hartley guinea pigs carrying responder bone marrow grafts developed delayed hypersensitivity reactions when challenged with 10  $\mu$ g of DNP-PLL after sensitization with this antigen. The lymph node cells from 9 of 12 animals tested were also capable of responding to optimal concentrations of antigen in vitro, with increased thymidine incorporation. 10 of 14 animals produced significant levels of antibody. Thus, 12 of 14 animals displayed immune responsiveness to DNP-PLL by at least two of the three parameters studied, while the remaining two animals (nos. 98, 100) had definite responses by one parameter each. In contrast, none of the nonresponder Hartley guinea pigs similarly grafted with nonresponder marrows were capable of mounting an immune response to DNP-PLL by any of the parameters studied (Table III). Of five animals, none developed delayed sensitivity to DNP-PLL after immunization with this antigen in complete Freund's adjuvant, nor produced measurable levels of anti-DNP antibody; their lymph node cells exposed to DNP-PLL in vitro were not stimulated to proliferate. It should be noted that both the number of bone marrow cells transferred and the levels of blood lymphocytes on day of immunization are within the same range in the experimental and control groups.

Some of the delayed hypersensitivity reactions of the immunized irradiated nonresponder recipients of responder marrow grafts were similar to those observed in immunized nonirradiated responder animals, but most reactions were less intense. The in vitro responses of lymph node cells from such animals did not regularly reach the levels previously observed in responder guinea pigs (20). The lymph nodes of irradiated animals were small and may not have been ideal sources of immunocompetent cells. Similarly, 4 of 14 transplanted animals did not produce significant titers of anti-DNP antibodies and the others did not attain the levels regularly seen in normal responder guinea pigs (4, 14, 15). The average serum concentration of anti-DNP antibodies observed in these animals, corresponding to 56.3% binding of  $^3\text{H}$  DNP-EACA, was 0.17 mg/ml, as extrapolated from the binding curve obtained with calibrated strain 2 anti-DNP-PLL sera.

*Transfer of Responsiveness to DNP-PLL and to DNP-GL to Lethally Irradiated Strain 13 Guinea Pigs by Bone Marrow or Lymph Node and Spleen Transplants from (2  $\times$  13) F<sub>1</sub> Animals.—*

All three lethally irradiated strain 13 guinea pigs reconstituted with strain 13



TABLE III  
 Transfer of the Ability to Respond to DNP-PLL\* to Lethally Irradiated Hartley Strain  
 Nonresponder Guinea Pigs with Bone Marrow Transplants from  
 Hartley Strain Guinea Pigs with the PLL Gene

Recipient nonresponder guinea pig No.	Responder bone marrow cells $\times 10^{-8}$ transferred	Day of immuniza- tion after transfer	Lympho- cytes/mm <sup>3</sup> blood on day of im- munization	Immune response		
				Delayed sen- sitivity to 10 $\mu$ g DNP- PLL reaction diameters	Stimulation of lymph node cells in vitro†	Anti-DNP antibody per cent bind- ing <sup>3</sup> H DNP- EACA§
2 (experimental)	18.0	15	6000	10 $\times$ 15	2.9	0
47	7.6	14	2600	8 $\times$ 8	NR	59
64	6.4	29	5300	9 $\times$ 9	NR	77
73	5.5	18	2200	10 $\times$ 10	3.7	2
77	10.0	17	3300	12 $\times$ 12	3.1	7
83	6.1	27	2500	15 $\times$ 15	4.3	80
85	4.6	27	2100	20 $\times$ 18	4.2	66
87	5.8	15	1700	12 $\times$ 12	Neg	10
91	10.8	14	1400	12 $\times$ 10	Neg	79
98	5.6	13	1300	8 $\times$ 8	Neg	5
100	4.8	24	3000	Neg	2.1	25
102	5.1	24	1900	12 $\times$ 10	9.6	82
103	8.9	24	2200	15 $\times$ 15	2.1	34
104	6.8	24	3000	15 $\times$ 15	7.7	51
Pos/total				13/14	9/12	10/14
Mean value	7.57		2750		4.4	56.3
		Nonrespon- der bone marrow cells $\times 10^{-8}$ transferred				
7 (controls)	10.5	21	1500	Neg	Neg	8
26	12.6	22	4400	Neg	Neg	6
30	11.8	21	2800	Neg	Neg	0
107	5.5	13	800	Neg	Neg	3
118	6.8	12	1100	Neg	Neg	7
Pos/total				0/5	0/5	0/5
Mean value	9.44		2120			

\* Guinea pigs were immunized with 0.1 mg DNP-PLL in complete adjuvant containing 2 mg of *M. tuberculosis* H37 Rv.

† This number is the ratio

Counts in DNA from experimental cultures with 0.01 mg/ml DNP-PLL

Counts in DNA from control cultures without antigen

Values > 2 are considered significant.

§ Per cent binding of 0.1 ml  $10^{-8}$  M <sup>3</sup>H DNP-EACA by globulin fraction of 0.1 ml of serum.

Values > 10% are outside range of nonspecific binding and are considered significant.

|| NR, no result; contaminated cultures.

bone marrow and ( $2 \times 13$ )  $F_1$  lymph node and spleen cells made a specific immune response to DNP-PLL. All of these guinea pigs showed moderately strong delayed sensitivity reactions to DNP-PLL, and two of the animals also produced high serum levels of anti-DNP antibodies (1.0 and 1.1 mg/ml). Both lethally irradiated strain 13 guinea pigs given ( $2 \times 13$ )  $F_1$  bone marrow produced moderate levels of anti-DNP antibodies, and one of these animals also developed delayed hypersensitivity to DNP-PLL (Table IV).

Table V presents the immune responses of the guinea pigs immunized with DNP-GL. 7 of 10 lethally irradiated strain 13 animals restored with strain 13 bone marrow and ( $2 \times 13$ )  $F_1$  lymph node and spleen cells made an immune

TABLE IV

*Transfer of the Ability to Respond to DNP-PLL\* to Lethally Irradiated Strain 13 Recipients by Lymph Node and Spleen Cells or by Bone Marrow Cells from (Strain 2  $\times$  Strain 13)  $F_1$  Guinea Pigs*

Strain 13 recipient guinea pig	Donor cells transferred $\times 10^{-8}$			Immune response		
	Strain 13 bone marrow	( $2 \times 13$ ) $F_1$ bone marrow	( $2 \times 13$ ) $F_1$ lymph node + spleen	Delayed sensitivity to 10 $\mu$ g DNP-PLL	Anti-DNP per cent binding $^3H$ DNP-EACA $\ddagger$	Serum anti-bodies $\S$ mg/ml $\parallel$
No.						
1	3.62	—	2.84	++	90.5	1.1
3	3.43	—	1.87	+++	0	—
5	6.07	—	3.44	++	87.7	1.0
2	—	6.00	—	Neg	69.8	0.62
6	—	5.17	—	++	67.0	0.60

\* Recipient strain 13 guinea pigs were immunized with 0.1 mg DNP-PLL in complete Freund's adjuvant containing 2 mg/ml *M. tuberculosis* H37 Rv, 15 days after cell transfer.

$\ddagger$  Per cent binding of 0.1 ml  $10^{-8}$  M DNP-EACA by globulin fraction of 0.1 ml of serum.

$\S$  Serum anti-DNP antibodies 18 days after immunization.

$\parallel$  Measured by quantitative precipitin analysis with DNP-bovine fibrinogen.

response to DNP-GL, as shown by the formation of anti-DNP antibodies or the development of specific delayed hypersensitivity. The three animals that failed to respond received the lowest number of lymph node and spleen cells (less than  $3 \times 10^8$  cells).

However, only one of six lethally irradiated strain 13 guinea pigs given ( $2 \times 13$ )  $F_1$  bone marrow cells made a strong immune response to DNP-GL. This animal developed both delayed hypersensitivity and a significant serum level of anti-DNP antibodies. Another animal had a very low serum concentration of anti-DNP antibodies. None of the four control lethally irradiated strain 13 guinea pigs restored with only strain 13 bone marrow made an immune response to DNP-GL.

A summary and analysis of the results of the transfer of the ability to respond

to DNP-PLL or DNP-GL to strain 13 guinea pigs with (2 × 13) F<sub>1</sub> cells is shown in Table VI. It is apparent that lymph node and spleen cells are

TABLE V  
Transfer of the Ability to Respond to DNP-GL\* to Lethally Irradiated Strain 13 Recipients by Lymph Node and Spleen Cells or by Bone Marrow Cells from (Strain 2 × Strain 13)F<sub>1</sub> Guinea Pigs

Strain 13 recipient guinea pig	Donor cells transferred × 10 <sup>-8</sup>			Immune response		
	Strain 13 bone marrow	(2 × 13) F <sub>1</sub> bone marrow	(2 × 13) F <sub>1</sub> lymph node + spleen	Delayed sensitivity to 50 $\mu$ g DNP-GL	Anti-DNP antibodies† per cent binding <sup>3</sup> H DNP-EACA§	Quantity
No.				diameters (mm)		mg/ml
10	3.70	—	2.46	Neg¶	52	0.13
13	12.00	—	3.12	10 × 8	60	0.21
16	9.50	—	5.65	20 × 15	78	0.70
19	4.68	—	2.24	Neg	3	—
24	9.80	—	4.80	7 × 6	4	—
30	9.40	—	9.24	8 × 8	72	0.50
34	4.70	—	11.30	7 × 7	67	0.32
38	8.20	—	8.10	7 × 7	46	0.09
40	7.80	—	1.25	Neg	0	—
42	9.60	—	2.49	Neg	0	—
11	—	14.00	—	Neg	0	—
17	—	7.00	—	Neg	0	—
27	—	2.64	—	Neg	6	—
28	—	12.00	—	30 × 20	26	0.03
29	—	10.50	—	Neg	9	0.01
31	—	12.00	—	Neg	0	—
12 (control)	3.70	—	—	Neg	3	—
15 "	9.10	—	—	Neg	2	—
18 "	6.24	—	—	Neg	0	—
21 "	6.24	—	—	Neg	6	—

\* Recipient strain 13 guinea pigs were immunized with 0.1 mg DNP-GL in complete Freund's adjuvant containing 0.5 mg/ml *M. butyricum* 14–16 days after cell transfer.

† Serum anti-DNP antibodies 25 days after immunization.

§ Per cent binding of 0.1 ml 10<sup>-8</sup> M DNP-EACA by globulin fraction of 0.1 ml of serum.

|| Values extrapolated from curve obtained with calibrated anti-DNP-GL sera by plotting per cent binding versus antibody concentration at various dilutions (Fig. 1).

¶ This animal was ill at time of skin testing.

more effective than bone marrow cells for this purpose. In the former situation 10 of 13 animals made an immune response of one type or another, whereas in the latter case only 4 of 8 animals made an immune response. However, in every case where the transfer of the ability to respond to DNP-PLL or to DNP-GL

was successful, the responses observed were not as intense as are regularly produced by normal strain 2 responder guinea pigs immunized with these antigens.

## DISCUSSION

The inability of genetically unresponsive guinea pigs to respond to DNP-PLL and to DNP-GL can be overcome by the transfer of cells from genetic responder animals.

The ability to respond to DNP-PLL was transferred to lethally irradiated nonresponder Hartley strain guinea pigs with allogeneic bone marrow cells from guinea pigs with the PLL gene. The PLL responder status of the animals was established in these experiments from the response to previous immunization with GL, a copolymer, the immunogenicity of which is also controlled by the

TABLE VI  
*Analysis of the Transfers of the Ability to Respond to DNP-PLL or to DNP-GL to Lethally Irradiated Strain 13 Guinea Pigs with Cells from (Strain 2 × Strain 13)F<sub>1</sub> Guinea Pigs*

Origin of donor cells	No. strain 13 recipients	Number of guinea pigs displaying immune response to DNP-PLL or to DNP-GL		
		Delayed sensitivity	Anti- DNP antibody	Delayed sensitivity or anti-DNP antibody
Strain 13 bone marrow (control)	4	0	0	0
(2 × 13) F <sub>1</sub> bone marrow	8	2	4	4
(2 × 13) F <sub>1</sub> lymph node + spleen	13	9	8	10

PLL gene (4). Similarly, the ability to respond to DNP-PLL and to DNP-GL was transferred to lethally irradiated strain 13 guinea pigs which normally do not respond to these antigens with (2 × 13) F<sub>1</sub> bone marrow cells.

The immune response elicited by DNP-PLL in most irradiated nonresponder recipients after the transfer of bone marrow cells from animals with the PLL gene was characterized by delayed hypersensitivity and other evidence of cellular immunity to DNP-PLL, as well as by the synthesis of significant levels of anti-DNP antibodies. Although positively charged DNP-PLL can behave as a hapten in nonresponder guinea pigs, and elicit the formation of anti-DNP antibodies when associated with a variety of immunogenic carriers (14, 15), DNP-PLL has never been able to elicit delayed sensitivity in either Hartley strain or strain 13 nonresponder guinea pigs, nor has it been able to stimulate the proliferation of their lymph node cells in vitro (14, 15, 20). These experiments therefore establish conclusively that the ability to recognize hapten-PLL conjugates as antigens can be transferred to guinea pigs lacking the PLL gene, and that the process controlled by this gene finds expression in bone marrow derived cells.

These studies also demonstrate that the inability of nonresponder guinea pigs to recognize PLL antigens is not due to nonspecific nonimmunological mechanisms. The bone marrow is known to contain precursors of many cell types with diverse functions. In addition to hematopoietic cells without immunological roles, bone marrow also gives rise to macrophages (21, 22) and lymphocytes and is thought by some to be the source of all immunocompetent cells (23, 24).

However, in a second series of experiments, the ability to respond to DNP-PLL and to DNP-GL was also transferred to lethally irradiated strain 13 guinea pigs with lymph node and spleen cell populations from ( $2 \times 13$ ) F<sub>1</sub> responder guinea pigs. ( $2 \times 13$ ) F<sub>1</sub> lymph node and spleen cells were generally more successful than ( $2 \times 13$ ) F<sub>1</sub> bone marrow in conferring responsiveness to PLL antigens to strain 13 guinea pigs. This finding suggests that the essential cells in which the PLL gene is expressed may be a lymphocyte of one kind or another, rather than a monocyte or macrophage. Bone marrow cell populations are known to contain a larger number of monocyte precursors than do lymph node cell populations (25). The number of macrophages in the lymph node and spleen cell populations transferred can be considered to average less than 4%. However, a definitive identification of the precise cell types responsible for the transfer of the ability to respond to PLL antigens must await the result of transfer experiments with purified cell populations. These studies are now in progress. It would also be of considerable interest to ascertain whether the cells which produced anti-DNP antibodies or which mediated cellular hypersensitivity reactions against PLL antigens in lethally irradiated strain 13 guinea pigs immunologically restored with ( $2 \times 13$ ) F<sub>1</sub> cells, are of donor or host origin. Unfortunately, the unavailability of suitable allotype markers on guinea pig immunoglobulins did not permit this study to be made. It is possible, however, that sera directed respectively against strain 2 and strain 13 histocompatibility antigens may be produced and used for this purpose.

The ability to transfer immune reactivity to PLL antigens from nonimmunized donor responder animals to lethally irradiated nonresponder guinea pigs is at first glance at variance with previous observations from our laboratory. These previous studies had demonstrated that the classical passive transfer of delayed sensitivity to DNP-PLL and DNP-GL could, with rare exception, be successfully accomplished in random-bred Hartley strain guinea pigs only by transferring sensitized cells from immunized responder animals into other responder guinea pigs (26). Transfer of these cell populations into nonresponder guinea pigs failed, in the great majority of cases, to passively transfer delayed sensitization. This type of result has also been observed by Battisto et al. with another immune system (27).

There is as yet no satisfactory explanation for these differences. However, the two types of transfer experiments are very different and their results may still be reconciled. In the case of successful cell transfer into lethally irradiated ani-

mals, it appears that all the cell types necessary for an immune response to PLL antigens were transferred and also given a sufficient time to mature and proliferate in a favorable environment (at least 21 days). Thus, in the transfer of the ability to respond to PLL antigens, the nonresponder recipient animals had enough time to accumulate all the required donor cell types at the time of immunization. On the other hand, the difficulty in transferring delayed sensitivity passively from responder to nonresponder guinea pigs imply either that in this experimental situation the sensitized cells transferred were not capable to elicit the reaction by themselves and required an active participation by host cells with the same genetic makeup to elicit a specific reaction at the site of injection of the PLL antigens, or that the sensitized cells from Hartley strain responder guinea pigs found a less favorable environment in allogeneic nonresponder guinea pigs. Whatever the explanation for the failure to passively transfer delayed sensitivity in the PLL system from responder to nonresponder guinea pigs, the experiments presented in this study establish unequivocally that the ability to form an immune response to antigens whose immunogenicity is controlled by the PLL gene can be effectively transferred to lethally irradiated nonresponder guinea pigs with cell populations known to be concerned with immune responsiveness. The data presented are also compatible with the interpretation that the cell type or types responsible are some classes of lymphocytes present in the bone marrow and in larger numbers in lymph nodes and spleen. Furthermore, the transfer of the ability to respond to PLL antigens is comparable to the finding of Tyan et al. (13) on the successful transfer of responsiveness to (TG)-A-L from responder mice into nonresponder mice with spleen cells or fetal liver cells, and illustrates the similarities presented by these two immune systems where specific, though complex, immune responses have been shown to be under the control of dominant genetic factors.

#### SUMMARY

Hartley guinea pigs genetically unresponsive to hapten-PLL (poly-L-lysine) conjugates were lethally irradiated and given allogeneic bone marrow from Hartley responder animals. Many of the animals died of graft versus host disease before their response to 2,4-dinitrophenyl-PLL (DNP-PLL) could be measured. The immune response of the surviving recipient animals was evaluated by anti-DNP antibody production, development of delayed hypersensitivity to DNP-poly-L-lysine, as well as by lymph node cell stimulation *in vitro* by this antigen. 12 of 14 recipient animals thus treated made an immune response as measured by 2 of the 3 parameters.

Strain 13 guinea pigs, genetically unable to respond immunologically to DNP-PLL and to DNP-GL (2,4-dinitrophenyl-L-glutamic acid L-lysine copolymer) were lethally irradiated and given bone marrow from (2 × 13) F<sub>1</sub> responder animals or strain 13 bone marrow and (2 × 13) F<sub>1</sub> lymph node and spleen cells. A high proportion of the animals survived this procedure; no evidence

of graft versus host disease was observed. Three of three strain 13 animals irradiated and, given strain 13 bone marrow and ( $2 \times 13$ ) F<sub>1</sub> lymph node and spleen, and then immunized with DNP-PLL, made a specific immune response. 7 of 10 irradiated strain 13 animals given strain 13 bone marrow and ( $2 \times 13$ ) F<sub>1</sub> lymph node and spleen made an immune response to DNP-GL. However, only one of six irradiated strain 13 animals made a vigorous immune response to DNP-GL after reconstitution with ( $2 \times 13$ ) F<sub>1</sub> bone marrow alone.

The ability to transfer the immune response to PLL antigens from responder to nonresponder animals demonstrates unequivocally that the defect in the non-responder animals is immunological rather than due to some other type of non-immunological mechanism. The bone marrow contains all the immunological cells necessary for the expression of the PLL gene. However, the finding that ( $2 \times 13$ ) F<sub>1</sub> lymph node and spleen cells were more effective than ( $2 \times 13$ ) F<sub>1</sub> bone marrow cell populations (known to be a rich source of monocyte precursors) suggests that the cells in which the PLL gene function is expressed may be lymphocytes rather than monocytes and macrophages.

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