HAPTEN CARRIER RELATIONSHIPS IN THE DNP-PLL·FOREIGN ALBUMIN COMPLEX SYSTEM: INDUCTION OF TOLERANCE AND STIMULATION OF CELLS IN VITRO*

By IRA GREEN,‡ M.D., WILLIAM E. PAUL,§ M.D., and BARUJ BENACERRAF, M.D.

(From the Department of Pathology, New York University School of Medicine, New York 10016)

(Received for publication 7 August 1967)

The ability of guinea pigs to recognize poly-L-lysine (PLL), and hapten conjugates of PLL (H-PLL) as antigens is under the control of a single autosomal dominant gene which we will refer to as the PLL gene (1–3). Responder animals carrying this gene form specific antihapten antibodies and display delayed hypersensitivity reactions after immunization with H-PLL conjugates such as 2,4-dinitrophenyl-PLL (DNP-PLL); guinea pigs lacking the PLL gene are incapable of an immune response to DNP-PLL but can nevertheless recognize this conjugate as a hapten. Thus, immunization with DNP-PLL complexed with a negatively charged foreign albumin elicits the formation of high levels of anti–DNP-PLL antibodies in genetic nonresponder guinea pigs (4). Two interesting observations were made concerning the response of these guinea pigs: (*a*) only an antigenic foreign albumin could act as a carrier for DNP-PLL and stimulate the production of high concentrations of anti–DNP-PLL antibodies in guinea pigs lacking the PLL gene; guinea pig albumin was only marginally effective in this capacity; and (*b*) nonresponder guinea pigs immunized with DNP-PLL complexed with ovalbumin or bovine serum albumin did not show delayed hypersensitivity reactions to DNP-PLL in spite of the production of high levels of anti–DNP-PLL antibodies. This finding demonstrates the haptenic behavior of DNP-PLL in nonresponder guinea pigs as it has been well established that haptens are incapable of eliciting delayed hypersensitivity reactions (5–10).

The process controlled by the PLL gene in the immune response to hapten-PLL conjugates is unknown. Knowledge of the mechanism which allows an immunogenic carrier to elicit an immune response in a genetic nonresponder to the DNP-PLL hapten which it bears should eventually lead to a more complete understanding of the requirements for antigenicity and should also clarify the function of the PLL gene product. This system offers several distinct advantages over other, more complex, antigenic systems because first, the hapten PLL conjugate can behave either as a complete antigen or as a

* This study was supported by United States Public Health Service Grant AI-2094 and by the Health Research Council of the City of New York under Contract I-138.

‡ Recipient of a United States Public Health Service Special Fellowship 2-F3-CA-16,257, from the National Cancer Institute.

§ Supported by a United States Public Health Service Training Grant (5TI-GM-466).

hapten (4); second, the specificities of the antibodies directed against the hapten and carrier can be more precisely analyzed; and third, the electrostatic interaction between the hapten and carrier would be expected to produce a minimal amount of distortion of the carrier molecule. A further analysis of the hapten-protein carrier relationship in nonresponder guinea pigs immunized with DNP-PLL complexed with foreign albumins was therefore undertaken. To study in more detail the requirement for an antigenic carrier molecule, the effect of inducing tolerance to the foreign albumin used to form electrostatic complexes with DNP-PLL on the ability of these complexes to elicit the formation of anti-DNP-PLL antibodies by nonresponder guinea pigs was investigated. In addition, the ability of DNP-PLL to specifically stimulate DNA synthesis in vitro by lymph node cells from responder and nonresponder animals immunized with DNP-PLL or with DNP-PLL·albumin complexes was examined in order to correlate the presence of delayed hypersensitivity reactions to DNP-PLL with the capacity of the lymphoid cells of these animals to be stimulated by DNP-PLL.

Material and Methods

Polypeptides and Proteins.—Poly-L-lysine (PLL) hydrobromide with average molecular weight of 90,000, and a copolymer of L-glutamic acid and L-lysine (GL) with a molecular weight of 110,000 were obtained from Pilot Chemical Co., Watertown, Mass. Bovine serum albumin (BSA) was purchased from Armour Pharmaceutical Co., Chicago, Ill. Five times recrystallized hen ovalbumin (OVA) was obtained from Pentex, Inc., Kankakee, Ill. Guinea pig albumin (GPA) was prepared by electrophoresis on starch block of guinea pig serum (11).

Other Reagents.—Freund's complete adjuvant was obtained from Difco Laboratories, Inc., Detroit, Mich. 1-fluoro-2,4-dinitrobenzene (DNFB) was obtained from Eastman Organic Chemicals, Rochester, N.Y. ^{3}H-thymidine was purchased from New England Nuclear Corp., Boston, Mass. Eagles' Minimal Essential Medium (MEM) was obtained from Grand Island Biological Company, Grand Island, N.Y.

Preparation of Conjugates.—DNP-PLL and DNP-GL were prepared by the reaction of PLL or of GL with DNFB in *p*-dioxane under alkaline conditions as previously described (1). Polypeptide concentrations were determined by micro-Kjeldahl nitrogen measurements. The degree of DNP substitution was calculated from the absorbancy at 360 mμ on the basis of the molar extinction coefficient of free ε-DNP-L-lysine (ϵ = 17,400) (12). A single $DNP_{20}PLL_{431}$ conjugate was used for this study. The subscripts refer respectively to the average number of groups of hapten and the average number of lysyl residues in the molecule.

Preparation of DNP-PLL·Albumin Complexes.—OVA or BSA (at concentration of 5 mg/ml in 0.075 M NaCl) was added to an equal volume of a solution of 5 mg/ml of DNP-PLL in 0.075 M NaCl. 0.1 N NaOH was added dropwise until a precipitate formed.

Basic Immunization Procedure.—Hartley guinea pigs weighing 300–400 g were purchased from Camm Research Laboratories, Wayne, N.J. The animals were immunized in the four foot-pads with 0.1 mg of DNP-PLL in 0.4 ml of emulsion containing equal volumes of the antigen and of complete Freund's adjuvant. Booster injections of 10 μg of DNP-PLL were given intradermally at 7–8 days. When DNP-PLL·albumin complexes were used to immunize animals, 0.2 mg of the complexes in 0.4 ml of emulsion containing equal volumes of the antigen in saline and complete Freund's adjuvant was given in the four foot-pads.

Immune Responses to DNP-PLL and to DNP-PLL·Albumin Complexes.—The intensity of

delayed hypersensitivity reactions to DNP-PLL was evaluated 24 hr after intradermal injection of 10 μg of DNP-PLL in 0.1 ml of 0.15 NaCl. The presence of serum antibodies against DNP-PLL, BSA, or OVA was revealed by double diffusion analysis in agar gel against DNP_{41} GPA, BSA, or OVA at a concentration of 0.25 mg/ml. To detect low levels of anti-DNP antibodies, equilibrium dialysis of the globulin fraction of serum against ^{3}H-DNP-ϵ-amino caproic acid was performed as previously described (4). To detect low levels of anti-BSA antibodies, passive hemagglutination of BSA-tanned sheep erythrocytes was performed according to the method of Stavitsky (13).

Production of Tolerance to BSA.—40 guinea pigs were rendered tolerant to BSA by the method of Salvin (14). They were given 10 mg of cyclophosphamide intraperitoneally daily for 8 days. On the 3rd day of the cyclophosphamide administration, the animals were injected intraperitoneally with 50 mg of sterile bovine serum albumin. 40 days after this procedure, the 11 surviving animals (group I) were immunized with 0.2 mg of DNP-PLL·BSA incorporated in complete Freund's adjuvant. At the same time, a group of untreated animals (group II) were immunized with DNP-PLL·BSA in an identical fashion. 10 days after immunization all animals were boosted with 10 μg of DNP-PLL and with 100 μg of DNP-PLL·BSA complex. Genetic responder animals were identified as those displaying delayed hypersensitivity reactions to DNP-PLL (4). The animals were bled on the 18–20th day after immunization.

Stimulation of DNA Synthesis of Sensitized Lymph Node Cells in Vitro.—The effect of DNP-PLL and DNP-PLL complexed to OVA or BSA on the incorporation of ^{3}H-thymidine into DNA by lymph node cells from individual responder and nonresponder guinea pigs immunized with DNP-PLL or DNP-PLL complexed to OVA or to BSA was investigated. Animals were immunized as described under basic immunization procedures.

A detailed description of the tissue culture methods and of the extraction of the DNA is given in a companion paper (15) and therefore only a brief outline of the method will be described now. Sterile technique was used throughout. Lymph node cells were obtained from the peripheral lymph nodes of guinea pigs by teasing the nodes in MEM containing 15% pooled normal guinea pig serum and penicillin. 10×10^6 cells, suspended in the same medium, were placed in 12 × 75 mm plastic culture tubes (Falcon Plastics, Los Angeles, Calif.) and antigen was added. Total volume per tube was 1.5 ml. The cultures were incubated for 24 hr at 37°C in an atmosphere of 5% CO_2-95% air. 1 μc (2 μg) of ^{3}H-thymidine was then added and the cultures incubated for an additional 24 hr. The DNA was then extracted from the cells and hydrolyzed. The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Tubes were set up in duplicate, triplicate, or quadruplicate. The concentrations of test antigens added to the cultures are expressed as micrograms per milliliter and are shown in the respective tables. The results are expressed as the quotient of the ratio:

$$\frac{\text{Counts in tubes with a given antigen concentration}}{\text{Counts in tubes with no antigen}}$$

A value of this ratio greater than one indicates stimulation of DNA synthesis.

RESULTS

Effect of Tolerance to BSA on the Immune Response to DNP-PLL·BSA in Guinea Pigs Lacking the PLL Gene.—All normal guinea pigs immunized with DNP-PLL·BSA made anti-DNP antibodies, as shown by both gel diffusion analysis against DNP-GPA and equilibrium dialysis using ^{3}H-DNP-EACA. The nonresponder animals were identified as those not demonstrating delayed hypersensitivity reactions to DNP-PLL alone (4). All these guinea pigs also

produced anti-BSA antibodies, as demonstrated by gel diffusion analysis and by passive hemagglutination using BSA-coated sheep red blood cells (Table I, group II).

The 11 guinea pigs in which tolerance to BSA was induced were in excellent condition 40 days later when they were immunized with DNP-PLL·BSA in

TABLE I

Production of Anti-BSA Antibodies and Anti-DNP-PLL Antibodies in Genetic Nonresponder Guinea Pigs Rendered Tolerant to BSA and Immunized with DNP-PLL·BSA

Animals	Immunizing antigens	Anti-BSA antibodies		Anti-DNP-PLL Antibodies	
		Gel diffusion	Passive hemagglutination	Gel diffusion*	Equilibrium dialysis‡ (mμ hapten bound/ml serum) × 10^{10}
Group I Tolerant animals	DNP-PLL·BSA				
1	"	−	<1/20	−	422
2	"	−	<1/20	−	158
3	"	−	<1/20	−	374
4	"	−	<1/20	−	135
5	"	−	<1/20	−	1308
6	"	−	1/20	−	750
7	"	+	1/40	−	1500
8	"	+	1/40	+	1805
9	"	−	1/640	+	6960
Group II 12 control animals	DNP-PLL·BSA	+ (all 12 animals)	1/160 to 1/1280	+ (all 12 animals)	Mean 5900 Range 2900–8200
Group III 41 control animals	Complete adjuvant alone				Mean 240 ± 205

* With DNP-GPA 0.25 mg/ml.

‡ Equilibrium dialysis performed with ^{3}H-DNP-EACA at an initial concentration of 2×10^{-7} M. 2.5 μg of antibody can bind 1000×10^{-10} mM hapten when both sites are occupied, considering the mol wt for guinea pig antibodies to be 150,000.

complete adjuvant. Complete or partial tolerance to BSA was demonstrated in these animals. Two of these BSA-tolerant animals proved to have the PLL gene, as shown by their ability to develop delayed hypersensitivity to DNP-PLL. As expected, these two responder guinea pigs made normal amounts of anti-DNP-PLL antibodies, but no anti-BSA antibodies, indicating that the general ability of the animals to respond to antigens other than BSA, to which they had been made specifically tolerant, had not been affected by the treatment with cyclophosphamide.

The remaining nine animals in this group (group I) were genetic nonresponder guinea pigs; three of these nine guined pigs made significant amounts of anti-

DNP-PLL antibodies, but less than is usually observed in nontolerant control animals. These same animals produced small amounts of anti-BSA antibodies, indicating that complete tolerance to the carrier albumin had not been achieved. The remaining six animals produced trace or undetectable amounts of both

TABLE II

Effect of Antigen on the In Vitro Incorporation of ^{3}H-Thymidine into DNA by Lymph Node Cells from Genetic Responder Guinea Pigs Immunized with DNP-PLL, DNP-PLL·BSA, or DNP-GL

Animal No.	Immunizing antigen	No. days after immunization	Test antigens			
			DNP-PLL 10 μg/ml	DNP-PLL 1 μg/ml	DNP-PLL ·BSA 1 μg/ml	BSA 1 μg/ml
1*	Adjuvant + saline	10	1.39‡	0.97		
2	Adjuvant + saline	14	0.99			
3	Adjuvant + saline	14	1.8			
4	Adjuvant + saline	19	1.15	1.22		
5	Adjuvant + saline	19	0.70	1.04		
6	DNP-PLL	75	7.7	3.2		
7	DNP-PLL	75	10.5	4.5		
8	DNP-PLL	85		20.9		
9	DNP-PLL·BSA	15	4.78	1.75	6.84	1.6
10	DNP-PLL·BSA	15	6.6	4.4	6.84	1.56
			DNP-GL 100 μg/ml	DNP-GL 1 μg/ml	DNP-GL 0.01 μg/ml	
11	DNP-GL	13	6.78	5.64	2.56	
12	DNP-GL	19	7.65	7.30	3.72	

* Animals 1–5 are control guinea pigs of unknown status with regard to the PLL gene. Animals 6–12 are genetic responder guinea pigs.

‡ Relative incorporation of ^{3}H-thymidine into DNA obtained from: Counts in DNA from cultures with antigen/Counts in DNA from cultures without antigen. Values >1 indicate stimulation of DNA synthesis.

anti-BSA and anti-DNP antibodies. Thus, in BSA-tolerant genetic nonresponder guinea pigs immunized with DNP-PLL·BSA a correlation between their inability to make anti-BSA antibodies and their inability to make anti-DNP-PLL antibodies was demonstrated. This experiment illustrates that when complete tolerance to the carrier albumin is established, this molecule can no longer induce antibody synthesis against oppositely charged haptens complexed with it.

Stimulation by DNP-PLL of DNA Synthesis by Specifically Sentisitized Lymph Node Cells.—The effect of antigen added in vitro on DNA synthesis by lymph

node cells from guinea pigs immunized with adjuvant alone, with DNP-PLL, with DNP-GL alone, with DNP-PLL·OVA, or with DNP-PLL·BSA was investigated. The response of cells from control animals immunized only with adjuvant and from genetic responder animals immunized with DNP-PLL,

TABLE III

Effect of Antigen on the In Vitro Incorporation of ^{3}H-Thymidine into DNA by Lymph Node Cells of Genetic Nonresponder Animals Immunized with DNP-PLL, DNP-PLL·BSA, DNP-PLL·OVA, or DNP-GL

Animal no.	Immunizing antigen	No. days after immunization	DNP-PLL 10 μg/ml	DNP-PLL 1 μg/ ml	DNP-PLL·BSA or DNP-PLL·OVA 2 μg/ml	Ova 1 μg/ml
1	DNP-PLL	15	0.91*	.87		
2	DNP-PLL	15	0.56	1.05		
3	DNP-PLL	14	0.98	0.77		
4	DNP-PLL	14	0.40	1.4		
5	DNP-PLL·BSA	22		1.26	7.2	
6	DNP-PLL·BSA	22		1.22		
7	DNP-PLL·OVA	15	1.47	1.15	6.8	3.0
8	DNP-PLL·OVA	15	0.57	1.06	10.2	2.6
9	DNP-PLL·OVA	21	0.35	1.41	3.7	5.0
10	DNP-PLL·OVA	21	0.44	1.7	4.2	2.3
11	DNP-PLL·OVA	11		0.76	1.86	1.94
12	DNP-PLL·OVA	11		0.54	4.73	4.40
13	DNP-PLL·OVA	11		0.70	2.12	1.76
			DNP-GL 10 μg/ml	DNP-GL 1 μg/ml	DNP-GL 0.01 μg/ml	
14	DNP-GL	13	1.09	1.20	1.37	
15	DNP-GL	19	1.17	1.11	0.50	

* Relative incorporation of ^{3}H-thymidine into DNA obtained from: Counts in DNA from cultures with antigen/Counts in DNA from cultures without antigen. Values >1 indicate stimulation of DNA synthesis.

DNP-GL, or DNP-PLL·BSA is presented in Table II. The results are expressed as the amount of ^{3}H-thymidine incorporation into DNA by cell cultures exposed to a given antigen concentration divided by the incorporation of ^{3}H-thymidine into DNA by cultures derived from the same guinea pig but not exposed to antigen. A value greater than 1.0 for this ratio is evidence of stimulation of DNA synthesis by the antigen. DNP-PLL at concentrations of 1 or 10 μg/ml did not appreciably stimulate DNA synthesis by control cell cultures from

animals immunized with adjuvant alone. The highest value obtained was only 1.8 and most values were considerably lower. A significant stimulation of DNA synthesis by 1 or 10 μg/ml of DNP-PLL was observed with all lymph node cell cultures from genetic responder animals immunized with DNP-PLL or DNP-PLL·BSA. A similar degree of stimulation was also observed with DNP-GL in the case of cells from responder guinea pigs immunized with this antigen.

In contrast to these responses, lymph node cells from nonresponder guinea pigs which had been immunized with DNP-PLL or DNP-GL did not show stimulation of DNA synthesis by these antigens in tissue culture (Table III). When cells from nonresponder guinea pigs immunized with DNP-PLL·BSA or DNP-PLL·OVA were used, identical results were obtained; DNP-PLL caused no stimulation of DNA synthesis by these cell populations, in spite of the synthesis of anti–DNP-PLL antibodies by the animals from which these cells were derived. However, the lymph node cells from these guinea pigs responded typically with increased DNA synthesis to the immunizing complexes, DNP-PLL·OVA or DNP-PLL·BSA, or to the carrier albumins used for immunization, OVA or BSA. No consistent difference could be observed between the magnitude of the response of the cells to DNP-PLL·foreign albumin aggregates and the response to the albumin alone, although in a few animals the response to DNP-PLL·OVA was greater than to OVA. The extent to which such differences could be due to the physical state of the two preparations was not determined. Thus, a complete correlation was demonstrated in nonresponder guinea pigs immunized with DNP-PLL·OVA or DNP-PLL·BSA between the absence of delayed hypersensitivity reaction to DNP-PLL and the absence of stimulation of in vitro DNA synthesis of lymph node cell cultures in response to DNP-PLL.

DISCUSSION

The results of these experiments confirm the behavior of DNP-PLL as a hapten in guinea pigs lacking the PLL gene. Immunization of such animals with complexes of DNP-PLL with a foreign albumin induces the synthesis of antibodies against DNP-PLL; but in spite of this antibody response, delayed hypersensitivity to DNP-PLL cannot be elicited and lymph node cells capable of responding in vitro to DNP-PLL with an increase in DNA synthesis are not detected. In contrast, delayed hypersensitivity to the foreign carrier albumin is observed and the lymph node cells of these guinea pigs show an increase in incorporation of ^{3}H-thymidine into DNA in response to the foreign carrier albumin in vitro.

The data presented also demonstrate that the foreign albumin must be capable of eliciting an immune response in order to induce antibody formation to DNP-PLL complexed with it in animals genetically incapable of responding to DNP-PLL alone. Thus, prior establishment of tolerance to BSA abolished

the production of anti-DNP-PLL antibodies in these guinea pigs. This last experiment is in apparent contradiction with the observation that hapten-protein immunization of rabbits tolerant to the carrier protein may lead to the production of antihapten antibodies (16–19). However, these two situations are not completely analogous because it has been shown that the usual covalent conjugation procedures alter the protein carrier molecule sufficiently to produce new antigenic configurations; such configurations may not necessarily encompass the hapten (20–23). The simple electrostatic aggregation of BSA with DNP-PLL probably does not modify the structure of the BSA molecule to the same extent as does covalent conjugation and therefore such new antigenic determinants would not be expected to appear.

How can the findings that tolerance to the carrier albumin renders the animals unresponsive to the hapten which it bears clarify the nature of the defect in genetic nonresponder animals and thereby the process governed by the PLL gene? Two general mechanisms for the initiation of the immune response can be considered in relationship to the activity of the PLL gene. According to the first hypothesis, the ability of specific cells to be stimulated by antigen depends solely upon the binding by the cells of antigen; this interaction is a thermodynamically driven reaction of antigen with a cell-associated antibody whose specificity is identical with that of the antibody to be produced by the cell or its progeny. With antigen concentrations achieved under normal conditions of immunization the binding energy of the antibody for the antigen must be quite high for the cells to be stimulated. If this simple interaction is not only necessary but also sufficient to stimulate specific cells, the PLL gene would have to be concerned with the immunological specificity of the immunoglobulins and would indeed control the capacity of the guinea pig to synthesize molecules with structural complementarity for stretches of lysine residues.

According to this interpretation nonresponder guinea pigs would be incapable of synthesizing immunoglobulins which can bind DNP-PLL with sufficient energy to result in stimulation of sensitive cells but could form immunoglobulins which bind DNP-PLL·BSA with an affinity sufficient to perform this function. Tolerance to BSA would be regarded as eliminating those cells whose response to DNP-PLL·BSA is dependent upon the partial specificity contributed by the portion of the BSA which is postulated to be part of the antigenic determinant in DNP-PLL·BSA. The major difficulty with this hypothesis is the failure to demonstrate a significant degree of BSA specificity in the anti-DNP-PLL antibodies produced by nonresponder animals immunized with DNP-PLL·BSA. This lack of BSA specificity was determined by the absence of BSA binding by anti-DNP antibodies purified from the sera of nonresponder animals immunized with DNP-PLL·BSA,[1] and by the fact that in these animals the anti-DNP-PLL antibodies and the anti-BSA antibodies are produced in

[1] Paul, W. E. and I. Green. Unpublished observations.

separate cells (24). Indeed, the anti-DNP-PLL antibodies produced by responder and nonresponder guinea pigs have the same degree of PLL specificity as judged by fluorescence quenching titrations with model ligands (25, 4).

The alternative mechanism for the initiation of the immune response requires a specific processing step which is essential for the antigen to be able to stimulate sensitized cells. According to this hypothesis, the formation of an antigen-inducer complex under the control of the PLL gene would be required for the stimulation of specific cells in responder guinea pigs. The binding of antigen by the cells would still depend upon the binding affinity of a cellular antibody whose specificity would be identical with that of serum antibody, but no cellular response would result unless the antigen had been specifically processed as an initial step. Indeed, this initial step may be the process through which "immunogenicity" is recognized and determinants are selected. Complex molecules such as normal protein antigens might be expected to offer a number of different sites for the operation of genetically controlled processing steps whereas amino acids homopolymers, hapten-homopolymer conjugates, and copolymers might be expected to offer but a single processing site, thus allowing the genetic control of the recognition of immunogenicity to be clearly demonstrated.

SUMMARY

Genetic nonresponder guinea pigs made tolerant to BSA and then immunized with DNP-PLL·BSA failed to make anti-DNP-PLL antibodies. Thus, tolerance to a carrier protein renders animals unresponsive to the hapten which it bears.

The addition in vitro of DNP-PLL or DNP-GL to lymph node cell cultures derived from genetic responder animals immunized with these materials led to a significant stimulation of ^{3}H-thymidine incorporation into DNA. However, the addition of DNP-PLL or DNP-GL to lymph node cell cultures from nonresponder animals immunized with these materials failed to produce any stimulation of DNA synthesis. Furthermore, the addition of DNP-PLL to lymph node cell cultures from nonresponder animals immunized with DNP-PLL·BSA or DNP-PLL·OVA also failed to stimulate cell proliferation in spite of the fact that the lymph node cells of these animals were producing anti-DNP-PLL antibodies.

The above facts suggest that the function of the PLL gene product is to act at an early crucial step in the immune mechanism to form an antigen-inducer complex. The specificity of this early step may be of a simple order and different than that of the antibody which is later produced in the immune response.

BIBLIOGRAPHY

1. Kantor, F. S., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-polylysine and DNP copolymers of lysine and glutamic acid in guinea pigs. *J. Exptl. Med.* **117**:55.
2. Levine, B. B., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens.

III. The genetic control of the immune response to hapten-poly-L-lysine conjugates in guinea pigs. *J. Exptl. Med.* **118**:953.

3. Levine, B. B., and B. Benacerraf. 1964. Genetic control in guinea pigs of the immune response to conjugates of haptens and poly-L-lysine. *Science.* **147**:517.
4. Green, I., W. E. Paul, and B. Benacerraf. 1966. The behavior of hapten-poly-L-lysine conjugates as complete antigens in genetic responders and as haptens in nonresponder guinea pigs. *J. Exptl. Med.* **123**:859.
5. Eisen, H. N. Hypersensitivity to simple chemicals. 1959. *In* Cellular and Humoral Aspects of the Hypersensitive States. H. S. Lawrence, editor. Paul B. Hoeber, Inc., New York. 89.
6. DeWeck, A. L., K. Vogler, J. R. Frey, and H. Geleick. 1966. The induction of contact hypersensitivity to dinitrochlorobenzene (DNCB) in guinea pigs by dinitrophenyl-amino acids. *Intern. Arch. Allergy Appl. Immunol.* **29**:174.
7. Barker, S. A., C. N. D. Cruickshank, J. H. Morris, and S. R. Wood. 1962. The isolation of trichophytin glycopeptide and its structure in relation to the immediate and delayed reactions. *Immunology.* **5**:627.
8. Jankovic, B. D., and B. H. Waksman. 1962. Delayed hypersensitivity to purified blood group substances in the guinea pig. *J. Immunol.* **89**:598.
9. Borek, F., A. M. Silverstein, and P. G. H. Gell. 1963. Delayed hypersensitivity to hapten-protein conjugates. III. Saccharides as haptens. *Proc. Soc. Exptl. Biol. Med.* **114**:266.
10. Holborow, W. J., and C. Loewi. 1962. The immune response to blood group substances. *Immunology.* **5**:278.
11. Kunkel, H. J. 1954. Zone electrophoresis. *Methods Biochem. Analy.* **1**:141.
12. Carsten, M. E., and H. N. Eisen. 1953. The interaction of dinitrobenzene derivatives with bovine serum albumin. *J. Am. Chem. Soc.* **75**:4451.
13. Stavitsky, A. B. 1964. Haemagglutination and haemagglutination inhibition reactions with tannic acid and bis-deazotized benzidine-protein-conjugated erythrocytes. *In* Immunological Methods. F. J. Ackroyd, editor. F. A. Davis Company, Philadelphia. 363.
14. Salvin, S. B., and B. F. Smith. 1964. The specificity of allergic reactions. VII. Immunologic unresponsiveness, delayed hypersensitivity and circulating antibody to proteins and hapten-protein conjugates in adult guinea pigs. *J. Exptl. Med.* **119**:851.
15. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1968. Specificity of cellular immune responses. Antigen concentration dependence of stimulation of DNA synthesis in vitro by specifically sensitized cells, as an expression of the binding characteristics of cellular antibody. *J. Exptl. Med.* **127**:25.
16. Cinader, B., and J. M. Dubert. 1955. Acquired immune tolerance to human albumin and the response to subsequent injection to diazo human albumin. *Brit. J. Exptl. Pathol.* **36**:515.
17. Weigle, W. O. 1962. Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens. *J. Exptl. Med.* **116**:413.
18. Schecter, I., B. Bauminger, M. Sela, D. Nachtigal, and M. Feldman. 1964. Immune response to polypeptidyl-proteins in rabbits tolerant to the protein carriers. *Immunochemistry.* **1**:249.

19. Nachtigal, D., R. Eschel-Zussman, and M. Feldman. 1965. Restoration of specific immunological reactivity of tolerant rabbits by conjugated antigens. *Immunology.* **9**:543.
20. Haurowitx, F. 1942. Separation and determination of multiple antibodies. *J. Immunol.* **43**:331.
21. Eisen, H. N., M. E. Carsten, and S. Belman. 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction. *J. Immunol.* **73**:296.
22. St. Rose, J. E. M., and B. Cinader. 1967. The effect of tolerance on the specificity of the antibody response and on immunogenicity. *J. Exptl. Med.* **125**:1031.
23. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1967. A study of the "termination" of tolerance to BSA with DNP-BSA in rabbits: Relative affinities of the antibodies for the immunizing and paralysing antigens. *Immunology.* **13**:147.
24. Green, I., P. Vassalli, and B. Benacerraf. 1967. Cellular localization of anti-DNP-PLL and anti conveyor albumin antibodies in genetic nonresponder guinea pigs immunized with DNP-PLL albumin complexes. *J. Exptl. Med.* **125**:527.
25. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1966. Studies on the effect of the carrier molecule on antihapten antibody synthesis. II. Carrier specificity of anti-2,4-dinitrophenyl-poly-L-lysine antibodies. *J. Exptl. Med.* **125**:689.