A STUDY OF THE PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY TO DNP-POLY-L-LYSINE AND DNP-GL IN RESPONDER AND NONRESPONDER GUINEA PIGS*

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An essential step in the immune response of Hartley strain guinea pigs to poly-L-lysine (PLL) and to hapten conjugates of this homopolymer (H-PLL) is under the control of a dominant autosomal gene which will be designated as the PLL gene. This gene is also required for the recognition of the antigenicity of copolymers of L-lysine and L-glutamic acid (GL) (1–3). The exact process controlled by the PLL gene in the immune response to these synthetic antigens has not been determined but is clearly a reaction specific for a configuration determined by a sufficient number of repeated adjacent lysyl residues which characterize these synthetic polymers and their conjugates. Hapten conjugates of oligo-L-lysines must contain a minimum of 7–8 lysyl residues in order to be recognized as antigens by genetic responder guinea pigs (4,5).

Guinea pigs lacking the PLL gene and incapable of an immune response against 2,4-dinitrophenyl-poly-L-lysine (DNP-PLL) can nevertheless recognize this molecule as a hapten. Thus, following immunization with electrostatic aggregates of DNP-PLL and foreign albumins, such nonresponder animals produce large amounts of anti-DNP-PLL antibodies with apparently identical specificity, as judged by available techniques, for the PLL molecule as that found in antibodies produced by genetic responders immunized with DNP-PLL alone (6).

There is, however, a very significant difference in the immune response to DNP-PLL of these two types of animals. Genetic responders immunized with DNP-PLL in complete Freund's adjuvant show delayed hypersensitivity reactions to DNP-PLL and their lymph node cells in vitro respond to DNP-PLL with an increase in DNA synthesis.[1] Such reactions are characteristic of the response of actively sensitized cells to antigen in vivo and in vitro. In contrast, guinea pigs lacking the PLL gene im-

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[1] Green, I., W. E. Paul, and B. Benacerraf. 1967. Hapten carrier relationships in the DNP-PLL· foreign albumin complex system: Induction of tolerance and stimulation of cells in vitro. *J. Exptl. Med.* In press.

munized with complexes of DNP-PLL and bovine serum albumin or ovalbumin (OVA) do not show either of these reactions to DNP-PLL. These experiments demonstrate that an identical, although not precisely defined step, controlled by the PLL gene is required for recognition of the immunogenicity of H-PLL conjugates and for the specific response of actively sensitized cells to this antigen in vivo and in vitro.

Similar conclusions were reached by Schlossman et al. (4,5) concerning the specificity requirements for the induction of the primary immune response and for the elicitation of delayed hypersensitivity reactions with DNP-oligo-L-lysines as antigens. Conjugates with the same minimum number of 7–8 lysyl residues are essential both for immunization of responder guinea pigs and for the elicitation of delayed hypersensitivity reactions in these animals.

In order to more precisely define the nature of the process controlled by the PLL gene, a study was made of the ability of lymph node cells from genetic responder guinea pigs immunized with DNP-PLL or DNP-GL to passively transfer delayed hypersensitivity to guinea pigs with or without the PLL gene. In addition, the lymph node cells from nonresponder guinea pigs immunized with DNP-PLL·OVA complexes were transferred to genetic responder and nonresponder animals and the recipients were tested for the presence of delayed hypersensitivity to DNP-PLL. In these experiments delayed hypersensitivity to DNP-PLL or to DNP-GL could be transferred successfully only with sensitized cells from genetic responder guinea pigs and, in most cases, only to those recipient guinea pigs which possessed the PLL gene.

Materials and Methods

Polypeptides and Proteins.—Poly-L-lysine (PLL) hydrobromide with average molecular weight of 90,000 and a copolymer of 60% glutamic acid and 40% lysine (GL) with an average molecular weight of 115,000 were obtained from Pilot Chemical Co., Watertown, Mass. Ovalbumin (OVA) was obtained from Pentex, Inc., Kankakee, Ill.

Other Reagents.—Complete Freund's adjuvant was obtained from Difco Laboratories, Inc., Detroit, Mich. 1-fluoro-2,4-dinitrobenzene (DNFB) was purchased from Eastman Organic Chemicals, Rochester, N.Y. Tissue culture medium 199 (TC 199) was obtained from Microbiological Associates, Bethesda, Md.

Preparation of Conjugates.—2,4-dinitrophenyl (DNP) conjugates were prepared by the reaction of PLL or GL with DNFB in *p*-dioxane under alkaline conditions as previously described (1). Polypeptide and protein concentrations were determined by micro-Kjeldahl nitrogen analyses. 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 ($\epsilon = 17{,}400$) (7).

The following conjugates were prepared: DNP_{20}-PLL_{431}, DNP_{35}-PLL_{431} and DNP_{24}-GL. The subscripts refer respectively to the average number of groups of hapten and the average number of amino acid residues in the molecule.

Preparation of DNP-PLL· OVA Complexes.—Ovalbumin at a concentration of 5 mg/ml in water was added to an equal volume of DNP-PLL at a concentration of 5 mg/ml in water; 0.1 M NaOH was added dropwise until a precipitate formed.

Immunization of Donor Animals.—Random-bred Hartley strain guinea pigs weighing 300–400 g were purchased from Camm Research Inc., Wayne, N. J. These animals were immu-

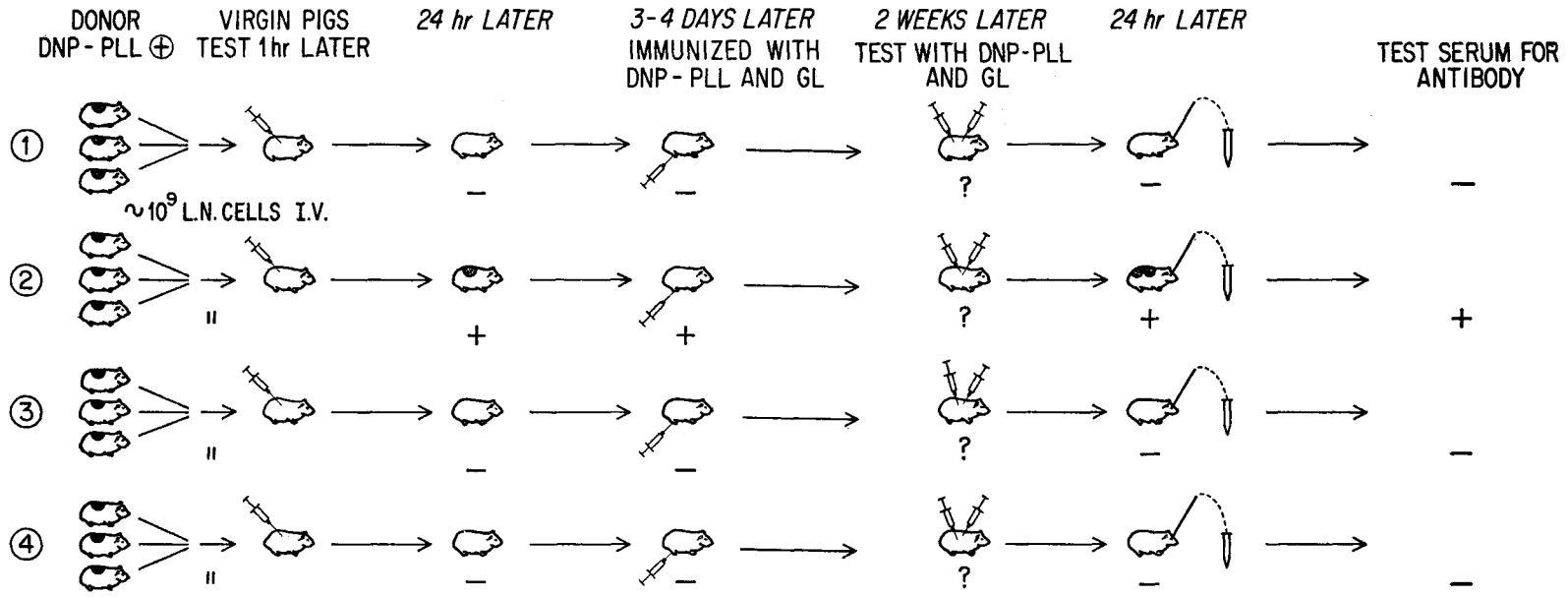

FIG. 1. The lymph node (L.N.) cells from three positive donor animals were given to each recipient; the recipient animals were tested 1 hr later . On the next day the presence or absence of successful transfer of delayed hypersensitivity was evaluated. The genetic status of the recipient animals was then determined from the results of subsequent active immunization. In the example shown, only animal 2 received a successful passive transfer of delayed sensitivity and this animal was later shown to be the only genetic responder. The experiments in groups II and III were performed in an analogous fashion.

nized in the four footpads with either 100 μg of DNP-PLL or DNP-GL in 0.4 ml of an emulsion containing Freund's adjuvant or with 200 μg of DNP-PLL·OVA complex in 0.4 ml of an emulsion containing Freund's adjuvant. The animals immunized with DNP-PLL or DNP-GL were skin tested at 8 days with 10 μg of the immunizing antigen. The animals immunized with DNP-PLL·OVA complex were skin tested at 8 days with 10 μg of DNP-PLL and with 20 μg DNP-PLL·OVA complex, Delayed hypersensitivity reactions were evaluated at 24 hr. Genetic responder animals were identified by their delayed hypersensitivity reaction to DNP-PLL (6).

Recipient Animals.—Random-bred normal Hartley guinea pigs weighing 300–400 g from Camm Research Inc. were used as recipient animals. About 30% of these animals carry the PLL gene. Also two recipient Hartley strain guinea pigs which were the offspring of genetic responder guinea pigs were kindly provided by Dr. B. B. Levine.

Method of Transfer.—A diagram of the experimental design is shown in Fig. 1. Genetic responder or genetic nonresponder donor guinea pigs were selected on the basis of their delayed hypersensitivity reactions to DNP-PLL or to DNP-GL. The animals were killed and all accessible peripheral lymph nodes were removed, dissected free of fat, teased apart in TC 199, and filtered through gauze. The cell suspensions prepared in this manner were then centrifuged at 700 rpm for 7 min at room temperature. The supernatant media was discarded and the cell button was resuspended in 2 ml of fresh TC 199. The cells of three donors were given intravenously to one recipient animal. Approximately 10^9 cells were transferred to each recipient guinea pig. Each recipient animal was individually and distinctly marked. The recipient animals were skin tested with 10 μg of DNP-PLL or 50 μg of DNP-GL 1 hr after transfer. In those animals receiving cells from donors immunized with DNP-PLL·OVA, skin tests with 10 or 50 μg of OVA were also performed. The skin reactions of the recipient animals were read 24 hr later. Since 10 μg of DNP-PLL produced a small inflammatory skin reaction (4–5 mm in diameter) in normal guinea pigs, control nonimmunized guinea pigs were injected with the test antigen in each experiment. The reactions in the recipient animals were compared with the reactions of untreated animals and scored as either positive or negative on the basis of the severity and size of induration and erythema.

In this manner, it was established whether a successful transfer of delayed hypersensitivity had been effected in the recipient animals without knowledge of whether the animals carried the PLL gene. The genetic status of the recipient animals was subsequently determined by a test of their ability to be actively immunized by DNP-PLL and by GL. Thus, 4 days after the passive transfer of cells sensitive to DNP-PLL the recipients were immunized with 100 μg of DNP-PLL and 100 μg of GL in complete adjuvant. After the passive transfer of cells senstive to DNP-GL the recipient animals were immunized only with DNP-PLL. The capacity to form an active immune response to these antigens was tested by the presence of delayed hypersensitivity and of serum antibodies. Anti-DNP antibodies were tested for by gel diffusion analysis with DNP guinea pig albumin or by equilibrium dialysis with ^{3}H-DNP-ε-aminocaproic acid as previously described (6). Anti-GL antibodies were assayed by passive cutaneous anaphylaxis (8). It should be noted that no error could be expected to occur in determining the "responder status" as a result of the activity of the transferred sensitive cells because these cells would be expected to have been rejected by the time the actual immune response is established (12 days). In addition, delayed hypersensitivity reactions to GL and to DNP-PLL are not cross-reactive; the same is true of delayed hypersensitivity reactions to DNP-GL and to DNP-PLL (1).

Recipient animals classified as genetic responders showed both delayed hypersensitivity reactions to the immunizing antigens and specific antibodies in their sera. Genetic nonresponder animals displayed neither of these responses.

RESULTS

Sensitized cells from genetic responder guinea pigs immunized with DNP-PLL or with DNP-PLL·OVA (group I) transferred delayed hypersensitivity to DNP–PLL to 14 of 37 recipient guinea pigs (Table I). Of the 14 recipient animals with successful transfers, 12 were shown to possess the PLL gene and

TABLE I

Success or Failure of Transfer of Delayed Hypersensitivity to DNP-PLL and to DNP-GL into Recipient Guinea Pigs with and without the PLL Gene

Group	Genetic status of donor animals*	Immunological specificity transferred	No. of recipient animals	Positive transfer to genetic responder animals	Positive transfer to genetic nonresponder animals
I	PLL gene positive	DNP-PLL	37‡	12/12§	2/25
II	PLL gene positive	DNP-GL	21	6/7	1/14
III	PLL gene negative	DNP-PLL	21‖	0/6	0/15

* The lymph node cells of three immunized donor animals were used for each recipient. Approximately 10^9 cells were injected per animal.

‡ 19 of these 37 animals received cells from guinea pigs immunized with DNP-PLL·OVA complexes. Seven of these 19 animals were responders and in all seven, delayed sensitivity to OVA was transferred together with delayed hypersensitivity to DNP-PLL. The remaining twelve animals were nonresponder guinea pigs. Eight of these showed a transfer of delayed sensitivity to OVA in the absence of transfer of delayed sensitivity to DNP-PLL.

§ The numerator refers to the number of animals in which a successful transfer was accomplished and the denominator refers to the number of animals of that genetic type indicated in the column above.

‖ 15 of these 21 animals received cells from guinea pigs immunized with DNP-PLL·OVA. All of these 15 animals had a positive transfer of delayed hypersensitivity to OVA in the absence of a transfer of delayed reaction to DNP-PLL; five of these 15 recipient guinea pigs were responder animals.

two were nonresponder guinea pigs. All of the 23 animals which failed to receive the transfer were shown to be genetic nonresponders. Thus all recipient animals with the PLL gene and only two of the 25 genetic nonresponder animals manifested positive transfer of delayed hypersensitivity to DNP-PLL.

Similar results were obtained in transfer experiments of delayed hypersensitivity to DNP-GL from responder guinea pigs (group II). Seven out of 21 recipient animals showed successful transfers; of these positive animals, six were shown to have the PLL gene and one was a nonresponder guinea pig. Of the 14 animals which failed to receive the transfer, 13 were later demonstrated to be genetic nonresponders. Thus six of seven genetic responders and one of 14 genetic nonresponder guinea pigs showed a successful transfer of delayed hypersensitivity to DNP-GL (Table I).

These experiments demonstrate that transfer of delayed hypersensitivity to antigens whose immunogenicity is controlled by the PLL gene is only achieved regularly in recipient guinea pigs who also possess the gene.

A third group of donor animals (group III), lacking the PLL gene, were immunized with DNP-PLL or with DNP-PLL·OVA complexes. These animals did not show delayed hypersensitivity reactions to DNP-PLL. When their lymph node cells were transferred to 21 guinea pigs, none of the recipients developed reactions to DNP-PLL, although 6 of these recipient guinea pigs were later shown to possess the PLL gene. Animals lacking the PLL gene can be induced to form anti–DNP-PLL antibodies by immunization with DNP-PLL·OVA (6). Such animals do not exhibit delayed hypersensitivity to DNP-PLL and, as shown in the present experiment, cannot passively transfer delayed reactions to DNP-PLL to animals possessing the PLL gene.

To investigate whether delayed reactions to other antigens, the immunogenicity of which is not controlled by the PLL gene, can be transferred between genetic responder and nonresponder animals, a study was made of the transfer of delayed hypersensitivity to ovalbumin in guinea pigs immunized with DNP-PLL·OVA complexes. 19 of the 37 animals in group I received cells from donor animals immunized with DNP-PLL·OVA. Seven of these recipients possessed the PLL gene and all showed a transfer of delayed hypersensitivity to ovalbumin. The remaining twelve animals lacked the PLL gene; transfer of delayed hypersensitivity to ovalbumin was successful in nine of these guinea pigs. 15 animals in group III received cells from nonresponder guinea pigs immunized with DNP-PLL·OVA. All showed a successful transfer of ovalbumin sensitivity. Five of the animals were genetic responders and 10 lacked the PLL gene. These experiments indicate that the transfer of delayed hypersensitivity to an unrelated antigen, ovalbumin, is easily achieved from guinea pigs lacking the PLL gene to guinea pigs with the PLL gene. This was also achieved in nine out of twelve instances of transfer from genetic responder to nonresponder guinea pigs. It should be noted that in eight of the nine cases, ovalbumin sensitivity was transferred in the absence of transfer of sensitivity to DNP-PLL. These findings indicate that the failure to transfer DNP-PLL sensitivity to these eight nonresponder recipients is not caused by an early destruction of the transferred donor cells.

DISCUSSION

An analysis of the passive transfer of delayed hypersensitivity to those synthetic antigens the immunogenicity of which is controlled by the PLL gene reveals that actively sensitized lymph node cells from responder animals fail, in most cases, to transfer delayed hypersensitivity reactions to guinea pigs which lack the PLL gene. It should be noted that these cell populations although incapable of mediating this transfer to genetic nonresponder animals are fully

capable of being stimulated by DNP-PLL in vitro as shown by an increase in DNA synthesis after exposure of these cells to this antigen.[1]

Delayed hypersensitivity reactions have been considered to be elicited by the reaction of sensitized cells with antigen in vivo. Several conclusions can be drawn from these experiments. (*a*) The transfer of delayed hypersensitivity reactions with actively sensitized cells requires the active participation of host mechanisms. (*b*) The hypothesis (9) that delayed hypersensitivity reactions are caused by the local reaction of very high-affinity circulating antibody with antigen is not compatible with these transfer experiments which indicate an essential and active process by the recipient animal. (*c*) The same immunological properties of antigens are necessary for the induction of the immune response and for the elicitation of delayed hypersensitivity reactions. It is well known that unreactive haptens or incomplete antigens are incapable of eliciting delayed hypersensitivity reactions (10, 11). This is further illustrated by the failure of DNP–PLL to elicit delayed reactions in nonresponder guinea pigs immunized with DNP-PLL·OVA (6), and by the failure of α-DNP-PLL$_6$ with only six lysyl residues to elicit delayed reactions in responder guinea pigs immunized with α-DNP-PLL$_8$ (4, 5). The present studies extend these earlier observations and indicate that with these antigens, the ability to form an immune response, to show delayed hypersensitivity reactions, and to be capable of expressing a passive transfer of delayed hypersensitivity involves the activity of the same genetically controlled mechanism.

The nature of the process governed by the PLL gene in inducing the immune response and in allowing the expression of delayed hypersensitivity to these synthetic antigens is unclear. The demonstration that the transfer of lymph node cells sensitive to DNP-PLL to animals lacking the PLL gene generally does not transfer delayed hypersensitivity to DNP-PLL suggests that some essential genetically controlled processing step on the antigen, specific for L-lysine sequences, must take place before sensitized cells capable of binding the antigenic determinants can be stimulated by such antigen. An identical processing step can be postulated to be also required for initiation of the immune response to antigens, the immunogenicity of which is controlled by the PLL gene. Such a processing step may take place in macrophages as suggested by the experiments of Fishman and Adler (12), Friedman et al. (13), and Askonas and Rhodes (14). The small number of macrophages of proper genetic type in passively administered donor lymph node cell suspensions may not be sufficient to perform this duty and thus no delayed reaction ensues.

The observation that nonresponder guinea pigs immunized with DNP-PLL·OVA complexes do not show delayed reactions to DNP-PLL in spite of the synthesis of anti–DNP-PLL antibodies, and are not able to transfer delayed reactions to DNP-PLL to guinea pigs possessing the PLL gene, requires an

explanation consistent with the above interpretation. The data presented indicate that no cells are produced which can be stimulated specifically by DNP-PLL in nonresponder guinea pigs immunized with DNP-PLL·OVA aggregates. This fact is also confirmed by the inability of DNP-PLL added in tissue culture to stimulate DNA synthesis by lymph node cells from these animals.[1] How can these phenomena be explained if the PLL gene controls a specific processing step on the antigen in the initiation of the immune response? One would need to postulate that the processing of the ovalbumin conveyor molecule in genetic nonresponder animals immunized with DNP-PLL·OVA, which is required to produce anti–DNP-PLL antibodies in such animals, cannot result in the development of cells capable of responding to DNP-PLL alone. In this sense no cell which can react specifically to DNP-PLL can be formed by nonresponder guinea pigs because the stimulation of such cells requires the proper processing of this antigen which nonresponder guinea pigs cannot perform.

Genetic nonresponder animals are capable of displaying adoptive delayed sensitivity to ovalbumin but not to DNP-PLL. It can be postulated therefore that in more complex antigens such as ovalbumin a variety of sites exist for the operation of genetically controlled processing steps of similar nature but different specificities. Thus, in the case of more complex antigens one rarely appreciates the presence or absence of a single processing gene.

Another possibility, not mutually exclusive with the first, is that the interaction between the donor cells and processed antigen is not the sole requirement in the expression of the delayed reaction but requires, in addition, the active participation at later steps of host mononuclear cells of proper genetic type. The importance of host mononuclear cells for the proper expression of passive transfer of delayed sensitivity has been recently emphasized by other authors (15, 16).

SUMMARY

A study of the passive transfer of delayed hypersensitivity to DNP-poly-L-lysine and to DNP-GL was performed in Hartley guinea pigs. Delayed hypersensitivity to DNP-PLL and DNP-GL could be transferred successfully only by means of sensitized cells from genetic responder guinea pigs and in most cases, only into those guinea pigs genetically capable of responding to PLL.

The inability to transfer delayed hypersensitivity to DNP-PLL or DNP-GL to genetic nonresponder guinea pigs is not the result of the early destruction of the transferred cells by an incompatible host, since it was shown that delayed hypersensitivity to ovalbumin could be successfully transferred from guinea pigs with the PLL gene into genetic nonresponder animals.

The requirement of active participation of specific and genetically controlled host mechanisms in the successful passive transfer of delayed sensitivity to DNP-PLL and DNP-GL has been demonstrated.

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