Studies on Antigenicity. The Effect of Succinylation of ε-amino Groups on Antigenicity of Benzylpenicilloyl-Poly-L-Lysine Conjugates in Random-Bred and in Strain 2 Guinea Pigs.* (29471)

BERNARD B. LEVINE
Department of Medicine, New York University School of Medicine, New York City

Lightly coupled hapten-poly-L-lysine conjugates have been demonstrated to be antigenic in about 25% of random bred Hartley strain guinea pigs(1,2) and in 100% of the highly inbred strain 2 guinea pigs(3). The present work describes the effect upon the antigenicity of lightly coupled benzylpenicilloyl-poly-L-lysine (BPO-PLL) conjugates in random bred and strain 2 guinea pigs of exhaustive succinylation of their free amino groups with succinic anhydride. The effect of heavy coupling of the BPO-PLL conjugates upon their antigenicity was also studied. It was found that exhaustive succinylation of the lightly coupled BPO-PLL conjugates resulted in a complete loss of their antigenicity without greatly affecting their abilities to bind anti-BPO antibodies. The heavily coupled BPO-PLL conjugate was also found to be nonantigenic. These results are discussed with regard to the metabolizability of these conjugates.

Materials and methods. Crystalline potassium benzylpenicillin (KPG) was a gift of Bristol Laboratories, Syracuse, N. Y. Poly-L-lysine · HBr (PLL_{525}) with an average degree of polymerization of 525 lysine units (calculated from intrinsic viscosity measurements, manufacturer's analyses) was purchased from Pilot Laboratories, Watertown, Mass. Benzylpenicilloyl - poly - L - $lysine_{525}$ ($BPO\text{-}PLL_{525}$) conjugates were prepared by reacting KPG with PLL_{525} in aqueous solution, 25°, with the pH maintained at 11.5 ± 0.1 in a pH stat for 90 minutes. The mole ratios of the reactants (KPG/ε-amine) used are listed in Table I. Aliquots of each of the conjugates were acylated by reaction with succinic anhydride (2.5 molar equivalents per mole of amine) in aqueous solution in a pH stat, first at pH 11.5, and twice again at pH 9.5-10.0. Between each acylation reaction, the conjugate solution was transferred to a clean beaker and the pH-stat electrodes were washed thoroughly, in order to prevent contamination of the succinylated conjugates with trace (but immunologically significant) quantities of the unsuccinylated conjugate. Conjugates were purified by five 12 hour dialyses against 2 M NaCl, 0.005 M Tris, pH 8.2, followed by 2 dialyses against 0.005 phosphate buffer, pH 8.2. Dialyses against 2 M NaCl were necessary to remove completely the side product, sodium benzylpenicilloate, from unsuccinylated conjugates. Conjugate solutions were analyzed for PLL concentration by duplicate microKjeldahls(4) corrected for the nitrogen contribution of BPO groups, and for BPO concentration by duplicate penamaldate analyses(5). Average numbers of BPO groups per mole conjugate were calculated from BPO and PLL concentrations; mean deviations, ± 5%. Free ε-amino group concentration was determined by duplicate formol titrations, pH_i, 8.0; pH_f, 9.2 with corrections for formol blanks(6), mean deviations; ±5%. p-Nitrobenzenesulfonyl$_{17}$-PLL_{316} was prepared by reacting PLL_{316} with 0.075 molar equivalents of p-nitrobenzenesulfonyl chloride (Eastman Kodak, recrystallized) in aqueous solution, pH 9.0, 25°, one hour in a pH stat. The conjugate was purified by prolonged dialysis.

Immunizations and testing. Random bred Hartley strain guinea pigs were obtained from Dierolf Farms, Boyertown, Pa. Strain 2 guinea pigs were obtained from the National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Md., through the courtesy of Dr. M. Brandriss. Guinea pigs of either sex weighing 400-500 g were immunized. 0.4 ml of an emulsion of equal parts 0.15 M NaCl and Difco Complete Freunds adjuvant containing conjugate (100 μg or 1.0 μg of PLL base) was injected into the hind foot pads on *day 1.* On *day 14,* animals were

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TABLE I. Benzylpenicilloyl-Poly-L-Lysine (BPO-PLL) Conjugates Studied for Antigenicity.

Conjugates*	Mole ratios of reactants ($KPG/\epsilon NH_2$) used for preparation†	Avg No. per mole conjugate†	
		BPO groups	Free amino groups
BPO_{24}–PLL_{525}	.125	24	475
BPO_{21}–$PLL_{525}S$	.125	21	<15
BPO_{140}–PLL_{525}	1.0	140	340
BPO_{120}–$PLL_{525}S$	1.0	120	<15
BPO_{265}–PLL_{525}	10.0	265	250
BPO_{225}–$PLL_{525}S$	10.0	235	<15

* Subscripts refer to average numbers of BPO haptenic groups, and of lysine residues per mole conjugate. S refers to succinylated conjugates.

† See *Methods* section. Average molecular weight of PLL_{525} base, 67,000.

skin tested with 10 μg of the conjugate in 0.1 ml saline injected intradermally into the dorsum. Test sites were observed at 3 hours for Arthus reactions and at 24 hours for delayed reactions. In most experiments, the animals were boosted on *day 15* by intradermal injection of 50 μg of the conjugate. They were bled from the retro-orbital space on the *22nd day,* and skin tested again on the 23rd day. In some cases, animals were tested for anaphylactic sensitization by intravenous injection of 500 μg of BPO_{120}-$PLL_{525}S$ (day 26). Guinea pigs were immunized again with 100 μg p-nitrobenzenesulfonyl$_{17}$-PLL_{316} ($Nisyl_{17}$-PLL_{316}) in complete adjuvant in the front foot pads on *day 30,* bled on *day 43,* and skin tested with $Nisyl_{17}PLL_{316}$ on *day 44.*

Serum antibody assays. PCA was done on sera diluted 1/10 with 0.15 M NaCl by the method described by Ovary(7) using 500 μg of BPO_{120}-$PLL_{525}S$ or 500 μg of $Nisyl_{24}$-guinea pig albumin as antigen. Passive hemagglutination was done on sera diluted 1/10, which had been inactivated at 56° for 30 minutes and absorbed twice with sheep red blood cells (SRBC). SRBC were sensitized by reaction of a 5% suspension in an aqueous solution of 0.08 M sodium barbital and 0.07 M KPG, pH 9.5, 37°, 1 hour. Sensitized SRBC (0.05 ml of 1% suspension) were mixed with 0.2 ml of the serum dilution and centrifuged at 1000/RPM for 1 minute. Positive tests, (macroscopic clumping) were specific for the BPO haptenic group, as complete, specific inhibition of agglutination by the univalent hapten BPO-aminocaproate(5) at 2×10^{-3} M concentration was observed.

Results. Benzylpenicilloyl-poly-L-lysine (BPO-PLL) conjugates tested for antigenicity. The average numbers of BPO groups and of free ε-amino groups per mole conjugate are listed in Table I for the conjugates used in this study. The succinylated conjugates had a minimum of 97% of their free amino groups coupled. Approximately 7% of the BPO groups of BPO-PLL conjugates underwent acylation of the thiozolidine N_3 secondary amine during the succinylation procedure, as determined by penamaldate analyses. BPO-PLL conjugates contained approximately 2% of their BPO groups as the benzylpenamaldoyl isomer as determined from measurements of their optical densities at λ max 285 mμ(8). BPO groups were present almost entirely as α-diastereoisomers as determined from optical rotation measurements (5). A maximum of only 50-60% of the ε-amino groups of PLL could be coupled with BPO groups probably because of steric interference from the bulky BPO groups.

Effect of succinylation of BPO-PLL conjugates on their antigenicity in random bred guinea pigs. Groups of 20 random bred guinea pigs were immunized with the BPO-PLL conjugates listed in Table I. Table II shows

TABLE II. Antigenicity of BPO-PLL Conjugates in Random Bred Guinea Pigs.

Immunizing conjugates (100 μg in adjuvant)	No. of animals		
	Immunized	Developing an immune response*	Developing an immune response to $Nisyl_{17}$–PLL_{316}†
BPO_{24}–PLL_{525}	18	4	4
BPO_{21}–$PLL_{525}S$	20	0	6
BPO_{140}–PLL_{525}	20	3	3
BPO_{120}–$PLL_{525}S$	17	0	5
BPO_{265}–PLL_{525}	20	0	4
BPO_{235}–$PLL_{525}S$	18	0	4

* Responders exhibited Arthus and delayed skin reaction and their sera contained anti-BPO antibodies detectable by PCA (titres, 1/50 to 1/200) and by passive hemagglutination (titres, 1/160 to 1/640). Non-responders showed no evidence of an immune response by these tests and by challenge for systemic anaphylaxis (see text).

† Animals were reimmunized with p-nitrobenzenesulfonyl$_{17}$–PLL_{316} and tested 14 days later. Responders showed Arthus and delayed skin reactions and serum anti-Nisyl antibodies.

that the comparatively lightly coupled conjugates were capable of inducing immune responses in approximately 20% of the animals immunized; (for $BPO_{24}PLL_{525}$, 4/18; $BPO_{140}PLL_{525}$, 3/20). The responders showed both Arthus and delayed skin reactivity to the immunizing conjugate, (see Table IV for results with individual animals) and their sera contained serum anti-BPO antibodies detectable by PCA and by passive hemagglutination. On reimmunization of the 38 animals with $Nisyl_{17}$-PLL_{316}, only the same 7 animals developed an immune response to that conjugate (Table II). This indicates that all the guinea pigs which could respond immunologically to hapten-PLL conjugates responded to the lightly coupled BPO-PLL conjugates (cf 2).

In contrast, the exhaustively succinylated conjugates (BPO_{21}-$PLL_{525}S$ and BPO_{120}-$PLL_{525}S$), were incapable of inducing immune responses. Table II shows that groups of 20 and 17 guinea pigs, respectively, failed to show positive skin tests to these conjugates both after the primary immunization and after a booster dose. These 37 animals did not demonstrate serum anti-BPO antibodies by PCA or by passive hemagglutination, nor did they demonstrate anaphylactic symptoms after intravenous injection of 500 μg of BPO_{120}-$PLL_{525}S$ (a dose sufficient to cause immediate anaphylactic death of 2 sensitive animals). These 37 animals were then reimmunized with 100 μg of $Nisyl_{17}$-PLL_{316} in adjuvants and tested with the conjugate 14 days later. Six of the 20 and 5 of the 17 developed immune responses to $Nisyl_{17}PLL_{316}$ demonstrating that these animals were capable of responding immunologically to hapten-PLL conjugates (cf 2).

Effect of extent of conjugation on antigenicity of BPO-PLL conjugates in random bred guinea pigs. Table II shows that none of 20 guinea pigs immunized with the maximally coupled conjugate, BPO_{265}-PLL_{525} developed an immune response to this conjugate. They failed to exhibit Arthus or delayed skin reactivity, serum anti-BPO antibodies, and anaphylactic symptoms after intravenous injection of 500 μg of $BPO_{120}PLL_{525}S$. The succinylated conjugate, BPO_{235}-$PLL_{525}S$ also failed to induce an immune response in 18 guinea pigs when tested in an identical manner. Table II shows that 4/20 and 4/18 animals from these groups were capable of developing an immune response to $Nisyl_{17}$-PLL_{316}.

TABLE III. Antigenicity of BPO-PLL Conjugates in Strain 2 Guinea Pigs.

Immunizing conjugate*	Dose, μg	Fraction of animals: Developing an immune response†	Fraction of animals: Developing immune response to $Nisyl_{17}$-PLL_{316}‡
BPO_{24}-PLL_{525}	100	3/3	3/3
BPO_{140}-PLL_{525}	100	3/3	3/3
BPO_{120}-$PLL_{525}S$	100	0/3	3/3
BPO_{120}-$PLL_{525}S$	1.0	0/3	3/3
BPO_{265}-PLL_{525}	100	0/3	3/3
BPO_{265}-PLL_{525}	1.0	0/3	3/3

* See legend Table I for abbreviations.

† Responders exhibited Arthus and delayed skin reactions to the immunizing conjugates, and their sera contained anti-BPO antibodies by PCA (titres, 1/50 to 1/200) and by passive hemagglutination (titres, 1/160 to 1/640). Non-responders did not show skin reactions nor serum anti-BPO antibodies.

‡ Responders showed Arthus and delayed skin reactions to $Nisyl_{17}$-PLL_{316}, and serum anti-Nisyl antibodies by PCA.

Antigenicity of BPO-PLL in strain 2 guinea pigs. Table III shows that the 2 lightly coupled conjugates, $BPO_{24}PLL_{525}$ and BPO_{140}-PLL_{525}, were capable of inducing immune responses in all 3 of the strain 2 guinea pigs. In contrast immunizing doses of 100 μg of the succinylated conjugate, $BPO_{120}PLL_{525}S$, and of the heavily coupled conjugate, BPO_{265}-PLL_{525} were incapable of inducing immune responses in groups of 3 strain 2 guinea pigs. In view of the possibility that a dose of 100 μg of a poorly metabolizable conjugate might induce a state of immune paralysis, 2 groups of 3 animals were immunized with 1.0 μg dose of these conjugates. Table III shows that these animals also failed to develop an immune response to the 1.0 μg doses.

Allergic cross-reactions among the BPO-PLL conjugates. Table IV shows that guinea pigs sensitized with the lightly coupled BPO-PLL conjugate (BPO_{24}-PLL_{525} and $BPO_{140}PLL_{525}$) exhibited only slightly less

intense Arthus skin reactions to the heavily coupled and to the succinylated conjugates. In contrast, these animals did not show delayed allergic cross-reactions to the succinylated and to the heavily coupled conjugates. These patterns of allergic cross-reactivity may be interpreted as being due to the differences in structure of the hapten-carrier, *i.e.* carrier specificity. Delayed allergic reactions evoked by hapten conjugates show a marked requirement for carrier specificity(9, 10) whereas allergic reactions mediated by serum antibodies show this requirement to a considerably lesser degree(11).

Discussion. Considerable evidence indicates that macromolecular antigens must first be metabolized *in vivo* in order to induce an immune response(12-14,2). An initial step of the metabolic pathway would be the enzymatic fragmentation of the antigen(12-14). This view is based on findings that non-digestible macromolecular substances such as alkali racemized proteins(12), PVP(13), gum acacia(13), copolymers of D-amino acids (14), and hapten conjugates of poly-D-amino acids(15,16) are non-antigenic in several species. The present finding that the maximally coupled BPO_{265}-PLL_{525} conjugate is non-antigenic in guinea pigs may be considered to support this view. It appears plausible that the large number of bulky BPO groups present on the conjugate would interfere with the binding of catheptic enzymes to the PLL backbone. Studies on the digestibility of such conjugates by guinea pig spleen extracts are under way.

The present data also demonstrate that exhaustive succinylation of lightly coupled BPO-PLL conjugates completely destroys their antigenicity in guinea pigs, without greatly affecting their abilities to bind anti-BPO antibodies. In experiments to be described elsewhere(17) it was found that an exhaustively succinylated conjugate of fluorescein-PLL could be degraded to small fragments by saline extracts of guinea pig spleens. Thus, the non-antigenicity of the succinylated, lightly coupled BPO-PLL conjugates may not be due to an inability to undergo degradation *in vivo,* but it may be due to an inability of the degradation products to un-

TABLE IV. Allergic Cross-Reactions Among BPO–PLL Conjugates.

Sensitized with	Guinea pig strain	Allergic skin reactions*									
		BPO_{21}–$PLL_{625}S$		BPO_{24}–PLL_{625}		BPO_{120}–$PLL_{625}S$		BPO_{140}–PLL_{525}		BPO_{235}–$PLL_{625}S$		BPO_{265}–PLL_{525}	
		A	D	A	D	A	D	A	D	A	D	A	D
BPO_{24}–PLL_{625}	Strain 2	2+	Neg	3+	15 Necr	2+	Neg	3+	10 Necr	2+	Neg	2+	Neg
	Strain 2	2+	,,	3+	15 Necr	2+	,,	3+	10 Necr	2+	,,	2+	,,
	Random bred	2+	,,	3+	15 Necr	2+	,,	2+	10 Necr	3+	,,	2+	,,
	Random bred	Trace	,,	1+	12 Nodule	1+	,,	1+	9 Nodule	Tr	,,	Tr	,,
BPO_{140}–PLL_{625}	Strain 2	1+	,,	3+	12 Necr	1+	,,	2+	10 Necr	1+	,,	1+	,,
	Strain 2	1+	,,	2+	14 Necr	1+	,,	2+	10 Necr	1+	,,	1+	,,
	Random bred	1+	,,	1+	15 Necr	1+	,,	1+	10 Necr	1+	,,	1+	,,

* A = Arthus reaction: 1+ 15 mm edema, trace hemorrhage; 2+ 15 mm edema, 2-5 mm hemorrhage; 3+ 15 mm edema, > 6 mm hemorrhage.
D = Delayed reaction expressed as average diameter of reaction in mm. Necr = necrosis.
5 Controls (animals injected with adjuvant alone), BPO_{21}–$PLL_{625}S$ and BPO_{120}–$PLL_{625}S$ gave 1-3 mm papules. The other gave 2-5 mm pink edema at 3 hr, and 4-7 mm flat pink papule at 24 hr.

dergo subsequent necessary metabolic steps. It is unlikely that the non-antigenicity of the succinylated BPO-PLL conjugates is due to an inability of these conjugates to be phagocytized by macrophages present in immunological tissues. Both succinylated and non-succinylated H^3-tagged DNP-PLL conjugates appear in draining lymph node macrophages in comparable amounts following their injection into the foot-pad(18).

The known requirements of macromolecular substances for antigenicity are "foreignness" of structural configurations and *in vivo* degradability. These present results suggest an additional requirement, *ie.*, that degradation products of antigen must be capable of undergoing subsequent, as yet undefined, specific metabolic steps. In view of what is known of this system(2,3,17), one possibility is that such a step may be the coupling of antigenic fragments to RNA, which would require an activating enzyme specific for the lysine side chain.

Summary. The antigenicities of various benzylpenicilloyl-poly-L-lysine (BPO-PLL) conjugates were compared in random bred and in strain 2 guinea pigs. Lightly coupled BPO-PLL conjugates were antigenic. Lightly coupled BPO-PLL conjugates whose ε-amino groups had been exhaustively succinylated with succinic anhydride were non-antigenic. A heavily coupled BPO-PLL conjugate was non-antigenic. These findings are interpreted with regard to the metabolism of antigens.

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Virus-Induced Intranuclear Antigen in Cells Transformed by Papovavirus SV40.* (29472)

FRED RAPP, JANET S. BUTEL AND JOSEPH L. MELNICK
Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

Transformation of hamster cells by papovavirus SV40 is accompanied by induction of new cellular antigen(s). The specificity of this antigen can be measured by complement-fixation tests(1) as well as by *in vivo* immunity tests(2-5). Thus, SV40-transformed cells fail to produce tumors when introduced into hamsters previously inoculated with the virus; prior inoculation of other papovaviruses has not conferred significant protection against cells transformed by SV40. The

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