THE SPECIFICITY OF ALLERGIC REACTIONS

VI. Unresponsiveness to Simple Chemicals

By JOHN E. COE, M.D., and S. B. SALVIN, Ph.D.

(From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana)

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Immunologic unresponsiveness to simple chemicals was first induced by Sulzberger with neoarsphenamine as antigen (1). Herein, intravenous administration of neoarsphenamine into guinea pigs before inoculation of a sensitizing dose of the same antigen produced a decrease in the amount of hypersensitivity. Because this unresponsiveness was not readily reproduced, tolerance to simple chemicals was not investigated intensively until unresponsiveness was reported in guinea pigs after "gastric feeding" of contact haptens (2–8). Feeding of a contact hapten such as picryl chloride (PiCl)[1] or dinitrochlorobenzene (DCB) to adult guinea pigs resulted in resistance to development of contact hypersensitivity or of circulating antibodies when the animals were subsequently inoculated with the contact hapten. The unresponsiveness was specific and long lasting. When guinea pigs, rendered unresponsive after having been fed 50 mg of PiCl in 3 mg doses, were inoculated with the picryl complex, picryl–guinea pig serum (Pi·GPS), presumably a weak antigen, the animals were resistant to the formation of circulating antibodies as measured by systemic anaphylaxis (3) When, however, the unresponsive guinea pigs were inoculated with picryl–bovine gamma globulin (Pi·BGG), presumably a stronger antigen, antibody specific to the picryl group was formed at a rate faster than that of the controls, *i.e.*, specific antibody appeared 2 days earlier than in the controls (5). Specific antipicryl antibody transferred passively to tolerant animals had a disappearance rate similar to that occurring in controls. This antibody sensitized the tolerant recipient so that early skin reactions developed after intradermal administration of specific hapten-protein conjugate, but did not overtly alter the unresponsiveness to the contact hapten (contact hypersensitivity) (4, 6). The passive transfer of cells from PiCl-sensitized, non-tolerant donors to "PiCl-fed" recipients resulted in the development of contact hypersensitivity (4). Cells from tolerant donors which had been inoculated with a sensitizing dose of PiCl did not passively induce contact hypersensitivity in

[1] HEA, hen egg albumin; HSA, human serum albumin; BSA, bovine serum albumin; BGG, bovine gamma globulin; GPS, guinea pig serum; GPSKIN, guinea pig skin; PiCl, picryl chloride (1-chloro-2,4, 6-trinitrobenzene); DCB, 1-chloro-2,4-dinitrobenzene; DFB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl; Pi, picryl; GPPX, guinea pig protein X; GPPY, guinea pig protein Y; PCA, passive cutaneous anaphylaxis.

normal recipients (6). When C^{14} PiCl was fed to guinea pigs for induction of tolerance, most of the radioactive material was excreted as picric acid and the quantity remaining in the tissues was inadequate to produce autoradiographs (8). A true "loading" of tissues by the hapten, therefore, did not seem to result from the gastric feedings, and only trace amounts of chemical actually effected the unresponsiveness. Small amounts of PiCl introduced directly into the mesenteric vein were recently demonstrated to have induced tolerance (9).

In this study, experiments have been performed wherein the dinitrophenyl system is used to define this type of tolerance more completely and to study the relationship of this type of unresponsiveness to other forms of tolerance and to other possible phases of antibody production. Emphasis has been placed on the specificity of this tolerance and on the relationship of hapten-protein conjugates to this hapten-induced tolerance.

Materials and Methods

Animals.—Guinea pigs of the Hartley strain weighing 350 to 400 gm at the start of the experiments were used for "gastric feeding" and for sensitization. White or albino guinea pigs weighing 300 to 400 gm were used for passive cutaneous anaphylaxis (PCA).

Antigens.—Five times recrystallized hen egg albumin (HEA) was obtained from K & K Laboratories, Inc., Jamaica, New York. Bovine gamma globulin (BGG) from Armour Pharmaceutical Co., Kankakee, Illinois, was used. Normal human serum albumin (HSA) from Cutter Laboratories, Berkeley, was used. Picryl chloride (PiCl), 1-chloro-2,4-dinitrobenzene (DCB), and 1-fluoro-2,4-dinitrobenzene (DFB) were obtained from Eastman Kodak Laboratories, Rochester, New York.

Conjugates.—DFB or PiCl was conjugated to HEA, BGG, HSA, GPS (guinea pig serum), or GPSKIN (guinea pig skin) according to previously described methods (10, 11). When the contact hapten combines with a protein, amino acid residues of lysine, etc., react with the halogenated carbon of the benzene ring and release the halogen. The result of DCB or DFB conjugation, therefore, is a dinitrophenyl compound, and conjugates will be termed DNP·GPS, DNP·HEA, etc.

Gastric Feeding.—Particular care must be taken that chemical contamination of the gingiva does not occur during the feeding procedure. Glass tubes, 7 mm long, inside diameter 3 mm, were inserted well into the guinea pig's pharynx. A heat-blunted piece of intra medic (Clay-Adams, Inc., New York) polyethylene tubing (I.D. 0.062 inch) was carefully inserted into the tube and pushed through into the stomach, where an appropriate amount (usually 0.3 ml) of corn oil solution was delivered by an attached syringe. The feeding schedule was that suggested by Chase (12): 3 feedings a week (usually Tuesday, Wednesday, and Thursday) for 3 weeks, followed by 2 weeks of rest before sensitization. The chemical to be fed (DCB) was dissolved in corn oil so that 0.3 ml delivered the appropriate amount of chemical (usually 3 mg). The control guinea pigs received 0.3 ml corn oil without antigen, with the same technique and schedule. Guinea pigs whose lips were soiled by DCB in corn oil during the feeding procedure were discarded.

Sensitization.—Protein antigens and their conjugates were dissolved in 1 per cent normal guinea pig serum in 0.85 per cent saline. The chemical haptens were taken from a freshly prepared acetone solution and diluted in sterile saline. The antigens were emulsified with equal volumes of Freund's adjuvant (Difco Laboratories, Inc., Detroit) and 0.5 ml of the water-in-oil emulsion was introduced into the 4 foot-pads. The guinea pigs were sensitized

with 5 μg of the native protein or 15 μg of its conjugate, and/or 50 μg (usually) of the contact hapten. In some experiments, 2 mg of mycobacteria per guinea pig were incorporated into the sensitizing emulsion.

Skin Tests.—Guinea pigs were skin tested on the sides, (*a*) intradermally with 0.1 ml of a 50 μg per ml solution of protein or protein conjugate, (*b*) percutaneously with 0.05 ml of a freshly prepared 4 to 1 acetone–corn oil or, usually, 4 to 1 acetone–olive oil solution containing 5 mg of contact hapten per ml. Initially, both DCB and DFB were employed as contact reactants, but because of the uniformly stronger reactions of the DFB in animals sensitized with either DCB or DFB, DFB was used as the standard DNP skin-testing antigen. The intradermal reactions were measured for the areas of induration at 4 and 24 hours and graded as follows: 1+ = 10 to 14 mm, 2+ = 15 to 19 mm, 3+ = 20 to 24 mm, 4+ = 25 mm or greater. The contact sites were treated with a depilatory at 20 to 24 hours and read 30 to 60 minutes thereafter under uniform artificial light. The strength of the contact reaction was graded according to the redness, elevation, edema, or hemorrhagic appearance of the contact site, so that +W = definite confluent pinkness, 1+ = definite redness and slight elevation, 2+ = marked erythema and edema with usually a hemorrhagic appearance. A normal guinea pig was tested percutaneously at the same time as the experimental animals to provide a control for comparison of reactions.

Antibody Determination.—Guinea pigs were bled by intracardiac puncture before skin testing and the presence of antibody was determined by the PCA reaction (13). In this procedure, 0.1 ml of a test serum was injected intradermally on the side of a normal guinea pig which 3 to 4 hours later was injected intravenously with 1 ml of a 0.5 per cent Evans blue solution containing 350 μg antigen protein. The areas of blue skin were examined 30 minutes later and the results recorded. Sera without detectable antibody were usually retested. Antibody titers were determined by the hemagglutination technique (14) with DNP·HSA and Pi·HSA as antigens.

RESULTS

A. The Induction of Unresponsiveness by Feeding of DCB and the Effect of Incorporating Mycobacteria in the Sensitizing Emulsion.—

Seventy guinea pigs were "gastric fed" 27 mg of DCB in corn oil over a 3 week period, rested for 2 weeks, and then divided into 5 groups, which were inoculated in the foot-pads with either 50 μg DFB, 50 μg PiCl, 15 μg DNP·GPSKIN, 15 μg DNP·HEA, or 15 μg DNP·BGG emulsified in Freund's adjuvant with (complete) or without (incomplete) 2 mg mycobacterium. Ninety control animals were fed a similar regimen of corn oil without hapten and sensitized with the same antigens. At various intervals thereafter, 6, 8, 14, or 20 days, paired groups of experimental and control animals were bled for antibody determination and then skin-tested (Table I).

Guinea pigs fed DCB and inoculated with DFB in incomplete adjuvant definitely failed to develop contact hypersensitivity to the DNP group. When these "fed" animals were inoculated with DFB in complete adjuvant, the unresponsiveness was less apparent in that approximately the same number of guinea pigs responded in the "DCB-fed" and control groups, although the reactions in the DCB-fed group were weaker. In the controls sensitized with DFB, addition of mycobacteria to the inoculum enhanced the contact hypersensitivity to the DNP group and the cross-reactivity with the picryl group. When guinea pigs fed DCB were sensitized with PiCl, with or without myco-

TABLE I

*Reactions of DCB-Fed and Control Guinea Pigs Inoculated with Haptens or Conjugates with or without Mycobacteria**

Gastric feeding	Antigen inoculated in incomplete adjuvant‡	No. of guinea pigs	Contact reactions to		Antibody (by PCA)		Antigen inoculated in complete adjuvant§	No. of guinea pigs	Contact reactions to		Antibody (by PCA)		Delayed skin reactions to HEA
			DFB	PiCl	DNP·GPS	Pi·GPS			DFB	PiCl	DNP·GPS	Pi·GPS	
DCB-fed‖	DFB (50 μg)	7	1¶	0	0**	0	DFB (50 μg)	9	8	1	0	0	
Control-fed‡‡	DFB (50 μg)	9	8	0	0	0	DFB (50 μg)	11	11	5	1	1	
DCB-fed	PiCl (50 μg)	6	0	6	0	0	PiCl (50 μg)	8	2	8	0	0	
Control-fed	PiCl (50 μg)	9	0	8	0	0	PiCl (50 μg)	10	2	9	0	0	
DCB-fed	DNP·GPSKIN (15 μg)	7	0	—	2	—	DNP·GPSKIN (15 μg)	9	2	—	2	0	
Control-fed	DNP·GPSKIN (15 μg)	9	3	—	2	—	DNP·GPSKIN (15 μg)	11	5	—	1	0	
DCB-fed	DNP·BGG (15 μg)	7	0	—	4	—	DNP·BGG (15 μg)	8	0	—	0	0	
Control-fed	DNP·BGG (15 μg)	9	0	—	5	—	DNP·BGG (15 μg)	11	0	—	1	1	
DCB-fed							DNP·HEA (15 μg)	9	0	—	3	2	9
Control-fed							DNP·HEA (15 μg)	11	0	—	3	0	11

—, not done.

* Tested 6, 8, 14, or 20 days after inoculation.

‡ Antigen in Freund's adjuvant without mycobacteria.

§ Antigen in Freund's adjuvant with 2 mg mycobacteria per guinea pig.

‖ Guinea pigs gastric-fed DCB–corn oil solution.

¶ Number of guinea pigs with positive reactions.

** Number of sera with antibody detected by PCA (13).

‡‡ Guinea pigs gastric-fed corn oil without hapten.

bacterium, they developed contact hypersensitivity to the picryl group equal to that of the control animals which had been sensitized with PiCl. In the DCB-fed and control groups which had both been sensitized with PiCl in complete adjuvant, the amount of cross-reactivity to percutaneous application of DFB was the same. In the DCB-fed and control groups inoculated with DFB in complete adjuvant, however, the animals fed DCB and inoculated with DFB had less cross-reactivity to PiCl than did the controls.

The DCB-fed and control groups that had been sensitized with DNP·BGG and DNP·HEA did not have contact reactions to DFB, and antibodies to DNP were produced in both DCB-fed and control groups with equal frequency. No anti-BGG or HEA antibodies were detected. When sensitized with DNP·GPSKIN, the DCB-fed animals, in contrast to the controls, did not develop appreciable contact reactivity to the DNP group. The control and DCB-fed groups sensitized with DNP·HEA in complete adjuvant demonstrated approximately the same delayed hypersensitivity to HEA, as measured by skin test.

B. Effect of Feeding of DCB on the Formation of DNP-Specific Antibody in Guinea Pigs Inoculated with Hapten or Hapten-Protein Conjugates.—

Guinea pigs were gastric-fed with 27 mg DCB in corn oil during a 3 week period and rested for 2 weeks. They were then divided into 5 groups of 10 guinea pigs each, and with 5 corresponding control groups of 10 guinea pigs each, which had been fed corn oil without specific antigen, were inoculated in the foot-pads to one of the following: 50 μg DFB, 50 μg PiCl, 15 μg DNP·GPS, 15 μg DNP·HEA, or 15 μg DNP·BGG in Freund's adjuvant without mycobacteria. Starting on the 3rd day after inoculation, 2 or 3 animals from each group were bled every day until day 34 and the sera tested by PCA for antibody with DNP, picryl, HEA, or BGG specificities.

In guinea pigs sensitized with DNP conjugates prepared *in vitro*, antibody to DNP appeared in the DCB-fed animals at approximately the same time as in the controls, namely, day 7 to 9 with DNP·GPS as sensitizing antigen, day 7 to 9 with DNP·HEA, and day 9 with DNP·BGG (Table II). Both DCB-fed and control guinea pigs sensitized with DNP·HEA had antibody which reacted with the DNP group, as well as with the picryl group. DCB-fed and control groups sensitized with DNP·BGG showed a similar antibody response, with few sera containing detectable cross-reacting anti-picryl antibody. The DCB-fed and control groups sensitized with DNP·GPS showed a moderate antibody response with frequent picryl cross-reactivity similar to the DNP·HEA sensitized groups.

Sera obtained on day 42 from the DNP·HEA and DNP·BGG groups and on day 46 from the DNP·GPS groups were examined in paired hemagglutination tests in which DNP·HSA and Pi·HSA were used as antigens (Table III). Two points are evident: (*a*) The DNP·GPS preparation in the control animals induced higher antibody titers than the DNP·BGG conjugate. (*b*) The DCB-

fed and control groups which were sensitized with DNP·BGG or DNP·HEA produced similar titers of antibody. However, the DCB-fed group sensitized with DNP·GPS produced titers lower than its control group. When these sera from the groups sensitized with DNP·GPS were tested for antibody by PCA, all the controls had anti-DNP antibody which cross-reacted with the picryl group. The DCB-fed group, however, although having anti-DNP antibody, had less cross-reacting anti-picryl antibody.

TABLE II

Appearance of Antibody of DNP and Picryl Specificity in DCB-Fed and Control Guinea Pigs after Sensitization with DNP·GPS, DNP·HEA, or DNP·BGG

Gastric feeding	15 μg antigen inoculated (in incomplete adjuvant)	Antigen (for PCA)	Time after sensitization, days								
			6	7	8	9	10	11	12	13	14
DCB-fed	DNP·GPS	DNP·GPS	0/2*	0/3	1/2	3/3	2/2	3/3	2/2	3/3	2/2
		Pi·GPS	0/2	0/3	1/2	1/3	1/2	3/3	1/2	3/3	1/2
Control-fed	DNP·GPS	DNP·GPS	0/2	1/3	2/2	3/3	2/2	3/3	2/2	3/3	2/2
		Pi·GPS	0/2	0/3	1/2	3/3	2/2	3/3	2/2	3/3	2/2
DCB-fed	DNP·HEA	DNP·GPS	0/2	1/3	1/2	3/3	2/2	3/3	2/2	3/3	2/2
		Pi·GPS	0/2	0/3	1/2	3/3	2/2	3/3	2/2	3/3	2/2
Control-fed	DNP·HEA	DNP·GPS	0/2	1/3	2/2	3/3	2/2	3/3	2/2	3/3	2/2
		Pi·GPS	0/2	0/3	2/2	3/3	2/2	2/3	2/2	3/3	2/2
DCB-fed	DNP·BGG	DNP·GPS	0/2	0/3	0/2	3/3	2/2	3/3	2/2	2/3	2/2
		Pi·GPS	0/2	0/3	0/2	0/3	0/2	0/3	0/2	0/3	0/2
Control-fed	DNP·BGG	DNP·GPS	0/2	0/3	0/2	2/3	2/2	3/3	2/2	3/3	2/2
		Pi·GPS	0/2	0/3	0/2	0/3	0/2	3/3	0/2	0/3	0/2

* Numerator indicates number of animals with antibody detected by PCA; denominator indicates number of animals tested.

Antibody to HEA or BGG was not detected in groups sensitized to DNP·HEA or DNP·BGG, respectively. In animals inoculated with the haptens DFB and PiCl, antibodies with DNP or picryl specificity were not detected up to the 34th day postinoculation in PCA tests with DNP·GPS or Pi·GPS as antigens.

The guinea pigs which had been sensitized previously with DNP·HEA or DNP·BGG and had circulating anti-DNP antibody (Tables II and III) were inoculated in the foot-pads on day 52 with 50 μg DFB in incomplete adjuvant (Table IV). Ten days afterward, the animals were again bled and tested for contact hypersensitivity to DFB and PiCl. The groups fed DCB were still

resistant to sensitization with the hapten DFB, for only 2 weak reactions of contact hypersensitivity could be elicited, even though circulating antibodies specific to DNP were present.

Those DCB-fed and control groups which had been inoculated initially with DFB and PiCl were bled and skin tested percutaneously on the 47th day with DFB and PiCl (Table V). The animals fed DCB and inoculated with DFB

TABLE III

Antibody Titer by Tanned Cell-Hemagglutination Test (14) of Sera from DCB-Fed and Control Guinea Pigs, Bled 42 to 46 Days after Sensitization with DNP·GPS, DNP·HEA, or DNP·BGG

15 μg antigen (inoculated in incomplete adjuvant)	Gastric feeding	No. of guinea pigs	Hemagglutinating antigen	Hemagglutination titer							
				<20	20	40	80	160	320	640	1280
DNP·GPS	DCB-fed	10	DNP·HSA			1*	2	4‡	2	1	
			Pi·HSA	4	2	4					
	Control-fed	9	DNP·HSA					1	2	5‡	1
			Pi·HSA	5			2	1	1		
DNP·HEA	DCB-fed	9	DNP·HSA					2	2	5‡	
			Pi·HSA	4		1	1	3			
	Control-fed	10	DNP·HSA					1	2	5‡	2
			Pi·HSA	4	2		3				
DNP·BGG	DCB-fed	10	DNP·HSA			1	3	3‡	2	1	
			Pi·HSA	4	2	1	3				
	Control-fed	10	DNP·HSA				1	4‡	4	1	
			Pi·HSA	4	2	3	1				

* Number of guinea pigs with detectable hemagglutinins at the indicated titer.

‡ Indicates group with median anti-DNP titer.

in incomplete adjuvant were still unresponsive to the DNP group. However, the animals fed DCB and sensitized with PiCl became hypersensitive to PiCl to a degree similar to the "non-fed" guinea pigs. Animals in the group originally inoculated with DFB were then reinoculated in the foot-pads with 50 μg DFB in incomplete adjuvant, and the group initially sensitized with PiCl reinoculated with 50 μg PiCl in incomplete adjuvant. The percutaneous application of heterologous hapten in the foregoing tests for contact hypersensitivity acted as the sensitizing experience for the heterologous hapten. Ten days later, the guinea pigs were bled and tested percutaneously with haptens at sites different from those used in the previous contact test.

The reinjection of DFB into animals initially inoculated with DFB increased the number of reactors in the animals fed DCB from 2 of 10 to 5 of 10, and increased the intensity of the contact responses in the already 100 per cent reacting control group. Weak hypersensitivity to PiCl was observed in both groups. Antibodies with DNP and picryl specificities were present in the control group, although antibodies to the DNP and picryl groups could not be detected by PCA test in the group fed DCB.

The reinoculation with PiCl of animals initially sensitized with PiCl increased the contact hypersensitivity to PiCl equally in both DCB-fed and control groups. The first percutaneous tests with DFB (and DCB in this instance) produced distinct hypersensitivity in the control group, but only 2 weak reactions in the group fed DCB. Prior sensitization with PiCl, therefore, did not interfere with unresponsiveness to the DNP group. The amount of sensitization to the DNP group which can be induced by contact testing with DFB and DCB in DCB-fed and control animals without prior sensitization with PiCl is noted (bottom of Table V).

TABLE IV

Response of DCB-Fed and Control Guinea Pigs Inoculated First with DNP·HEA or DNP·BGG and 52 Days Later with DFB

Gastric feeding	First inoculation with 15 μg in incomplete adjuvant	No. of guinea pigs	Second inoculation with 50 μg in incomplete adjuvant	Results of			
				Contact reactions to		PCA tests with	
				DFB	PiCl	DNP·GPS	Pi·GPS
DCB-fed	DNP·HEA	9	DFB	1*	0	9‡	7
Control-fed	DNP·HEA	9	DFB	8	0	9	6
DCB-fed	DNP·BGG	10	DFB	1	0	9	4
Control-fed	DNP·BGG	10	DFB	8	1	9	4

* Number of guinea pigs with positive reactions.

‡ Number of sera with antibody detected by PCA.

Animals inoculated with DFB and PiCl were then reinoculated in the footpads with 50 μg DFB in incomplete adjuvant and tested 20 days later for contact hypersensitivity to the DNP group. Stronger contact reactions were noted in the controls and in those DCB-fed animals which had previously reacted. Additional animals with contact hypersensitivity, however, were not detected in the DCB-fed group. Unresponsiveness to DNP was still evident in DCB-fed animals which had been strongly sensitized with PiCl in incomplete adjuvant.

C. Relationship between the Quantity of DCB-Fed and the Degree of Unresponsiveness Induced.—

Sixty-seven guinea pigs were divided into 3 groups and fed with 1 of 3 different DCB-corn oil preparations (1.0 mg, 10.0 mg, or 100.0 mg DCB per ml corn oil), so that the standard feeding schedule (0.3 ml 3 times a week for 3 weeks) would result in a total of 2.7, 27.0, or 270.0 mg of DCB fed to each of the respective groups. A control group of 29 guinea pigs was fed a similar volume of corn oil without hapten. After the usual 2 week rest period, each of the

TABLE V

Results of Repeated Inoculations with DFB and PiCl in DCB-Fed and Control Animals

Gastric feeding	50 μg antigen inoculated (in incomplete adjuvant)	No. of guinea pigs	Results on day 47				Boosted with 50 μg (in incomplete adjuvant) on day 52	Results on day 62				Boosted with 50 μg (in incomplete adjuvant) on day 76	Contact reactions to DFB on day 96
			Contact reactions to		PCA tests with			Contact reactions to		PCA tests with			
			DFB	PiCl	DNP·GPS	Pi·GPS		DFB	PiCl	DNP·GPS	Pi·GPS		
DCB-fed	DFB	10	2*	0	0‡	0	DFB	5	1	0	0	DFB	4/9§
Control-fed	DFB	10	10	0	1	1	DFB	10	2	8	7	DFB	8/8
DCB-fed	PiCl	10	0	6	0	0	PiCl	2	8	3	3	DFB	2/9
Control-fed	PiCl	10	0	4	0	0	PiCl	9	8	1	2	DFB	6/7
DCB-fed	—	10	0	—	—	—		3‖					
Control-fed	—	8	0	—	—	—		6					

* Number of guinea pigs with positive reactions.

‡ Number of guinea pigs with antibody detected by PCA.

§ Numerator indicates number of animals with contact reactions; denominator indicates total number tested.

‖ Sensitized to DFB by DFB and DCB contact skin test 10 days previously.

above groups was further divided into 3 groups and inoculated in the foot-pads with either 50 μg DFB, 50 μg PiCl, or 15 μg DNP·HEA in Freund's adjuvant. In an attempt to induce different levels of sensitization, half of each group received the antigen in incomplete adjuvant and the other half in complete adjuvant. Twenty-six days later, the animals were bled and tested with DFB and PiCl for contact hypersensitivity (Table VI).

The following points are noted:

1. A good correlation exists between the quantity of DCB gastric fed and the degree of unresponsiveness to DNP subsequently noted in the experimental animal.

TABLE VI

Relationship Between the Amount of DCB Fed and the Subsequent Degree of Unresponsiveness to DFB, PiCl, and DNP·HEA

Gastric feeding (amount)	Antigen inoculated (in Freund's adjuvant)	No. of guinea pigs	Contact reactions to		Antibody (by PCA test)		Delayed skin reactions to HEA
			DFB	PiCl	DNP ·GPS	Pi· GPS	
DCB-fed							
2.7 mg	DFB + TB*	4	4‡	0	0§	0	
	DFB − TB 50 μg	3	2	0	0	0	
27.0 mg	DFB + TB 50 μg	4	3	1	0	0	
	DFB − TB 50 μg	4	1	0	0	0	
270.0 mg	DFB + TB 50 μg	4	0	0	0	0	
	DFB − TB 50 μg	4	0	0	0	0	
Control-fed	DFB + TB 50 μg	5	5	3	0	0	
	DFB − TB 50 μg	5	5	3	0	0	
DCB-fed							
2.7 mg	PiCl + TB 50 μg	4	0	3	1	2	
	PiCl − TB 50 μg	4	0	2	2	2	
27.0 mg	PiCl + TB 50 μg	4	0	4	0	1	
	PiCl − TB 50 μg	3	0	2	0	2	
270.0 mg	PiCl + TB 50 μg	4	0	4	0	1	
	PiCl − TB 50 μg	4	0	2	0	3	
Control-fed	PiCl + TB 50 μg	5	0	5	1	1	
	PiCl − TB 50 μg	4	0	2	1	1	
DCB-fed							
2.7 mg	DNP·HEA + TB 15 μg	4	0	0	1	0	3‡ [3 = 3+]‖
	DNP·HEA − TB 15 μg	3	0	0	3	3	1 [3+]

TABLE VI—*Continued*

Gastric feeding (amount)	Antigen inoculated (in Freund's adjuvant)	No. of guinea pigs	Contact reactions to		Antibody (by PCA test)		Delayed skin reactions to HEA
			DFB	PiCl	DNP·GPS	Pi·GPS	
27.0 mg	DNP·HEA + TB 15 μg	4	0	0	0	0	4 [3 = 2+, 1 = 1+]
	DNP·HEA − TB 15 μg	4	0	0	4	4	3 [3 = 1+]
270.0 mg	DNP·HEA + TB 15 μg	3	0	0	0	1	2 [2 = 3+]
	DNP·HEA − TB 15 μg	3	0	0	3	2	1 [1 = 1+]
Control-fed	DNP·HEA + TB 15 μg	5	0	0	2	0	4 [1 = 3+, 2 = 2+, 1 = 1+]
	DNP·HEA − TB 15 μg	5	0	0	4	4	2 [1 = 2+, 1 = 1+]

* 2 mg mycobacterium (Tb)/animal.

‡ Number of guinea pigs with positive reactions.

§ Number of sera with antibody detected by PCA.

‖ Delayed skin reactions graded according to mm of induration at 24 hours with: 1+ = 10 to 14 mm; 2+ = 15 to 19 mm; 3+ = 20 to 24 mm; 4+ = 25 mm or greater.

2. The quantity of DCB fed did not alter the capacity of an animal to develop hypersensitivity to PiCl.

3. The incorporation of mycobacteria into the sensitizing inoculum augmented the response to percutaneous application of the hapten. Although the complete adjuvant also increased the delayed response to HEA in animals sensitized to DNP·HEA, the antibody response to DNP when measured by PCA at 26 days was found to be diminished. In the groups sensitized with DNP·HEA, the delayed hypersensitivity to HEA, as measured by skin testing, was not overtly inhibited by the feeding of DCB.

D. Effect of Prior Sensitization on the Induction of Unresponsiveness by Gastric Feeding.—

Sixty-four guinea pigs were divided into 4 groups, 3 of which were inoculated in the footpads with either 15 μg DNP·HEA, 50 μg DFB, or 50 μg PiCl, respectively, in incomplete adjuvant. The 4th group was not sensitized at this time with a specific antigen or hapten. Eleven days later, 3 ml of blood was obtained from each of the animals in the sensitized groups for antibody determination. Each group was then divided so that a total of 41 (*cf.* Table VII) were fed 27 mg of DCB in corn oil per animal over a 3 week period and the 23 remaining were

TABLE VII

Effect of Sensitization Prior to Gastric Feeding on Induction of Tolerance

Antigen inoculated (in incomplete adjuvant) before gastric feeding	Anti-DNP antibodies (by PCA test)	Gastric feeding	Antigen inoculated (50 μg in incomplete adjuvant) after gastric feeding	No. of guinea pigs	Contact reactions to	
					DFB	PiCl
0		DCB-fed	DFB	9	3* [+W]‡	—
0		Control-fed	DFB	9	9 [5 = 2+, 3 = 1+, 1 = +W]	—
DNP·HEA 15 μg	+13/13§	DCB-fed	DFB	13	4 [+W]	—
DNP·HEA 15 μg	+3/3	Control-fed	DFB	3	3 [1 = 2+, 2 = 1+]	—
PiCl 50 μg		DCB-fed	DFB	9	3 [1 = 1+, 2 = +W]	6 [1 = 1+, 5 = +W]
PiCl 50 μg		Control-fed	DFB	5	5 [1 = 2+, 3 = 1+, 1 = +W]	4 [1 = 1+, 3 = +W]
DFB 50 μg		DCB-fed	DFB	10	10 [3 = 2+, 5 = 1+, 2 = +W]	—
DFB 50 μg		Control-fed	DFB	6	6 [2 = 2+, 4 = 1+]	—

* Number of guinea pigs with positive reactions.

‡ Contact reactions graded with: +W = definite confluent pinkness, 1+ = definite redness and slight elevation, 2+ = marked edema and erythema, usually hemorrhagic appearance.

§ *Cf.* Table II for explanation.

fed corn oil without hapten. After a 2 week rest, 3 ml of blood was obtained from each of the guinea pigs, and all animals were subsequently inoculated in the foot-pads with 50 μg DFB in incomplete adjuvant. Fourteen and 15 days later, the animals were again bled and then tested percutaneously with DFB and PiCl for contact hypersensitivity (Table VII).

Gastric feeding of DCB induced unresponsiveness to sensitization with DFB, except for weak reactions, even when DNP-specific circulating antibodies from DNP·HEA sensitization were present prior to the period of gastric feeding. These DNP-specific antibodies were demonstrated at the onset of gastric feeding and were still present afterward at the time of DFB inoculation.

TABLE VIII

Effect of DCB-Feeding on Subsequent Sensitization with PiCl

Gastric feeding (amount)	Antigen inoculated	No. of guinea pigs	Contact reactions to		Anaphylactic deaths after 1.0 mg intravenously		Antibody by PCA test to	
			DFB	PiCl	DNP·GPS	Pi·GPS	DNP·GPS	Pi·GPS
DCB-fed 27 mg	PiCl (70 μg)	16	5* [+W]‡	16 [15 = 1+, 1 = +W]	8/12§ (+4 signs)‖	4/4	12/16¶	11/16
DCB-fed 270 mg	PiCl (70 μg)	17	4 [+W]	17 [1 = 2+, 14 = 1+, 2 = +W]	9/13 (+3 signs)	3/4 (0 signs)	9/17	10/17
Control-fed	PiCl (70 μg)	25	8 [+W]	25 [3 = 2+, 21 = 1+, 1 = +W]	13/17 (+3 signs)	5/8 (+2 signs)	13/17	9/17
DCB-fed 27 mg	DFB (120 μg)	6	2 [+W]	0	1/6 (0 signs)	—	0/6	0/6
DCB-fed 270 mg	DFB (120 μg)	8	1 [+W]	0	0/8 (0 signs)	—	0/8	0/8
Control-fed	DFB (120 μg)	12	12 [9 = 1+, 3 = +W]	8 [+W]	10/10	1/2 (+1 signs)	9/12	9/12

* Number of guinea pigs with positive reactions.

‡ Contact reactions graded with: +W = definite confluent pinkness; 1+ = definite redness and slight elevation, 2+ = marked edema and erythema, usually hemorrhagic appearance.

§ Numerator indicates number of animals which died of acute anaphylaxis after intravenous injection of 1.0 mg antigen; denominator indicates number of animals tested.

‖ ( ) indicates number of guinea pigs showing definite signs of nonfatal anaphylaxis.

¶ *Cf.* Table II for explanation.

This unresponsive state could not be produced if the animals had been sensitized with the hapten DFB before feeding. Such DFB-sensitized animals were normally responsive and did not exhibit any signs of desensitization. However, sensitization with the hapten PiCl before gastric feeding of DCB did not detectably reduce the ability to induce unresponsiveness to DNP.

E. Effect of DCB Gastric Feeding on the Immune Response to PiCl.—

Eighty-four guinea pigs were divided into 3 groups and gastric-fed in the usual way. The 1st group received a total of 27 mg DCB, with the feeding of 0.3 ml quantities of a 10 mg/ml DCB–corn oil solution 3 times a week for 3 weeks. The 2nd group received a total of 270 mg DCB in a similar feeding regimen with a 100 mg/ml DCB–corn oil solution. The 3rd group received corn oil without hapten. After a 2 week rest, each of the groups was further subdivided into 2 and the animals were inoculated with either PiCl or DFB. A schedule was

TABLE IX

Anamnestic Effect of HEA in DCB-Fed and Control Guinea Pigs after Primary Sensitization with DNP·HEA

Gastric feeding	First antigen inoculated (in incomplete adjuvant)	Second antigen inoculated (5 μg in incomplete adjuvant)	Time of appearance of antibody to HEA after secondary injection, days					
			5	6	7	8	9	10
DCB-fed	DNP·HEA 5 μg	HEA	0/3*	0/3	3/3	3/3	3/3	3/3
Control-fed	DNP·HEA 5 μg	HEA	0/3	0/3	3/3	3/3	3/3	3/3
Control-fed	Saline	HEA	0/3	0/3	0/3	1/3	3/3	3/3

* *Cf.* Table II for explanation.

adopted in an attempt to induce high titers of antibody, wherein a total of 45 μg of hapten in saline was injected into the foot-pads in divided doses on days 0, 4, 6, 8, and 10. On day 13, an additional 25 μg of hapten in incomplete adjuvant was injected into the foot-pads. On day 23, adequate PiCl sensitization was demonstrated, although inoculation with DFB had produced only infrequent DNP-specific antibodies in the controls. Accordingly, starting on day 27, the groups being inoculated with DFB were boosted in the foot-pads with an additional 10 μg DFB in saline daily for 5 days.

On days 29 and 31, animals in the PiCl-sensitized groups were bled and skin-tested with DFB and PiCl (Table VIII). After the 24 hour reading of the contact site, the guinea pigs were injected intravenously with 1 mg of DNP·GPS or Pi·GPS in 1.0 ml saline and signs of active anaphylaxis recorded. On day 37, the DFB-inoculated groups were bled and then tested percutaneously with DFB and PiCl. Twenty-four hours later, after the results of the contact tests had been recorded, the animals were also injected intravenously with 1.0 mg DNP·GPS or Pi·GPS in 1.0 ml saline and signs of anaphylaxis were noted (Table VIII).

When DCB-fed guinea pigs were sensitized with PiCl, the subsequent immune response appeared to be identical with that of control animals. Not only did a normal degree of hypersensitivity to the picryl group develop but also a cross-reactivity with the DNP group similar to that in controls was seen. This

cross-reactivity could be measured by contact hypersensitivity as well as by active systemic and passive cutaneous anaphylaxis. DNP-tolerant animals were unresponsive to inoculation with DFB and did not exhibit any cross-reactions with PiCl.

F. Effect of Gastric Feeding of DCB on the Secondary Response to HEA after Primary Inoculation of DNP·HEA.—

Ten guinea pigs which had been fed DCB and 10 control guinea pigs which had been fed corn oil alone were sensitized, after a 2 week rest, in the foot-pads with 15 μg DNP·HEA in incomplete adjuvant (Table IX). At the same time, another group was inoculated in the foot-pads with incomplete adjuvant without antigen. Eight days later, the 3 groups were injected in the foot-pads with 5 μg HEA in incomplete adjuvant. Groups of 3 animals were bled daily and the presence of anti-HEA antibody determined by PCA.

Both DCB-fed and control animals sensitized with DNP·HEA demonstrated a similar anamnestic response to a secondary injection of HEA.

DISCUSSION

Contact haptens such as DFB and PiCl have the common property of forming covalent bonds with amino acid residues, such as the ε-amino group of lysine. The capacity of these contact haptens to induce contact hypersensitivity can be directly correlated with the avidity with which the haptens combine with amino acid groups. Thus, DFB, which conjugates more rapidly with protein than DCB, is a more potent sensitizer than DCB, although the allergic responses elicited by the two haptens have the same specificity, that of the DNP group (15). The contact reaction is probably an expression of delayed hypersensitivity because the skin response to the simple chemical requires 24 hours for maturation and is morphologically similar to the delayed skin reaction. Contact hypersensitivity has not been related to the presence or absence of circulating antibody and can be passively transferred by cells, but not by serum. Contact hypersensitivity, like delayed hypersensitivity, is best induced by intracutaneous modes of sensitization and has been demonstrated in agammaglobulinemic patients (16, 17).

Although sensitization with the simple hapten DFB or PiCl results initially in contact hypersensitivity and later in specific circulating antibodies (8, 18), the capacity of *in vitro* prepared hapten conjugates to induce contact hypersensitivity is limited. Such conjugates, nevertheless, readily induce hapten-specific antibodies (15). Contact hypersensitivity to DNP conjugates has been induced by the inoculation of DNP–guinea pig red blood cell stromata conjugates in complete adjuvant (19) or by DNP-GPSKIN conjugates in incomplete adjuvant (11). The specificity of a hapten-protein conjugate during the period of delayed hypersensitivity has been demonstrated to be directed primarily toward the protein-carrier (20, 21). This type of specificity probably

exists in the contact hapten system (11). If, therefore, a conjugate prepared *in vitro* is to induce contact hypersensitivity, the conjugate must have protein determinants which are similar to those formed *in vivo* upon application of the hapten to the skin.

The results of this study suggest that when DCB is introduced to a guinea pig *via* an immunologically null path, as exemplified by the gastric route, a complex of DNP and a guinea pig somatic protein (DNP·GPPX) is formed *in vivo*, and the protein carrier (GPPX) of this *in vivo* complex (DNP·GPPX) has a predominant role in determining the specificity of the ensuing unresponsiveness to DNP. When the DCB-fed animal is subsequently inoculated with DFB or DCB as a hapten, a similar complex (DNP·GPPX) is formed to which the guinea pig is unresponsive, and neither contact hypersensitivity nor antibody is induced. Sensitization of the DCB-fed animal with an *in vitro* prepared conjugate containing heterologous protein and DNP groups induces hypersensitivity and circulating antibody in a normal manner because of the dissimilarity to the carrier protein of DNP·GPPX, which is the antigen responsible for the tolerance. Conjugates made *in vitro* with homologous proteins such as DNP·GPS or, especially, DNP·GPSKIN would be less antigenic in the DCB-fed animals because of determinants shared with the tolerance-inducing antigen, DNP·GPPX. Such unresponsiveness would be similar to the diminished response of bovine serum albumin (BSA)–tolerant rabbits when they were sensitized with HSA and other cross-reacting albumins (22). Presumably, the successful *in vitro* duplication of the naturally formed *in vivo* complex (DNP·GPPX) could be measured by the inability of this *in vitro* compound to induce the DCB-fed animal to develop contact hypersensitivity or antibody to the DNP group, whereas a control animal sensitized with DNP·GPPX would demonstrate hapten-specific contact hypersensitivity and circulating antibody similar to guinea pigs sensitized with the hapten DFB. Preliminary data with DNP·GPSKIN indicate that this conjugate does not fulfill these criteria completely in the DCB-fed animal, probably because of the heterogeneity of this conjugate; *i.e.*, although some DNP·GPPX is formed, in a quantity sufficient to induce contact hypersensitivity in normal guinea pigs sensitized with DNP·GPSKIN, other non-GPPX protein conjugates, recognized as "foreign," are present that can sensitize the DNP-tolerant animal, although not as strongly as in the normal animal. DNP·GPS seems to be less antigenic in the DCB-fed than in the control animal.

The foregoing hypothesis is supported by the studies of Cinader and Pearce (23) on the specificity of proteins and their azo derivatives in rabbits with acquired tolerance. The majority of rabbits made tolerant to human serum albumin (HSA) by neonatal injections were also unresponsive to a conjugate of sulfanilic acid diazotized to human serum albumin (DHSA). Similarly, newborn rabbits inoculated with DHSA and subsequently unresponsive to DHSA were

also unresponsive to HSA. When neonatal rabbits were given a conjugate of sulfanilic acid diazotized to bovine ribonuclease or of sulfanilic acid diazotized to rabbit serum, upon subsequent sensitization with DHSA, they developed an antibody response similar to the uninjected controls. The absence of a response to DHSA in HSA-tolerant rabbits has recently been confirmed (24). Thus, the role of the carrier protein in the specificity of a conjugate seems to be dominant not only in the phase of delayed hypersensitivity but also in the mechanism of acquired tolerance. This specificity could be expected if delayed hypersensitivity is an early step in the formation of circulating antibody (21).

Guinea pigs, fed PiCl and inoculated with picrylated conjugates of theoretically poor antigenicity, such as Pi·GPS, have been reported to form little, if any, detectable antibody in comparison with that of "non-fed" controls. On the other hand, when PiCl-fed guinea pigs were sensitized with the conjugate Pi·BGG, which was considered to be a stronger antigen, they developed an accelerated antibody response in comparison with the controls (5). When DCB-fed animals and their controls were sensitized with DNP·HEA, as described in this paper, antibodies to the DNP group appeared at a similar time, were of a similar titer, and had similar cross-reactivity with the picryl group. Although DNP·BGG was a weaker antigen than DNP·HEA, similar antibody responses appeared in the DCB-fed and control groups. Even though the conjugate of DNP·GPS used in this study produced in normal animals a stronger anti-DNP antibody response than DNP·BGG, a diminished response to DNP·GPS could be seen in the tolerant group in comparison to that of the control group. The conjugate, DNP·GPS, may, therefore, be a less effective antigen in the animal unresponsive to DNP not because of lower antigenicity *per se* but because of a closer antigenic relationship to the *in vivo* tolerance-inducing conjugate DNP·GPPX, formed after gastric feeding.

When DCB-fed guinea pigs are sensitized with DNP·HEA or DNP·BGG, they produce DNP-specific antibodies but are still unresponsive to sensitization with the contact hapten DFB. Such findings are similar to those with the PiCl system (7). The presence of circulating anti-DNP antibodies resulting from sensitization with DNP·HEA before the guinea pigs were gastric-fed DCB did not diminish the effectiveness of DCB feeding in inducing unresponsiveness to DNP. When guinea pigs were sensitized with the hapten DFB prior to feeding, neither unresponsiveness nor desensitization resulted. Prior sensitization with PiCl also prevented gastric feeding of PiCl from inducing unresponsiveness (7). Sensitization with an *in vitro* homologous protein conjugate that is sufficiently close antigenically to DNP·GPPX to be able to induce contact hypersensitivity should reduce the effectiveness of the subsequent feeding procedure to produce unresponsiveness to the specific hapten. In animals sensitized with the hapten DFB or in animals with contact hypersensitivity to the DNP group, the inductive phase (25) for DNP·GPPX theoreti-

cally would have been initiated, and the subsequent induction of tolerance, therefore, made difficult. In guinea pigs sensitized with DNP·HEA, an inductive phase actually specific to HEA was initiated because of the predominant role of the protein carrier on the cellular specificity of the conjugate. This sensitization with a conjugate which has a basic HEA specificity did not stimulate a GPPX-specific response, and left the animal still susceptible to the induction of unresponsiveness by gastric feeding. DCB-fed guinea pigs subsequently sensitized with DNP–heterologous protein conjugates and producing antibodies specific to DNP are still unresponsive in the DNP·GPPX phase, although this unresponsiveness can be shown only by resistance of the animals to contact sensitization.

Guinea pigs sensitized with microgram quantities of conjugates develop delayed hypersensitivity specific to the carrier protein and antibodies specific to the hapten (20, 21). When small amounts of a protein conjugate highly saturated with hapten are used for sensitization, antibodies directed to the carrier protein are not detected by passive cutaneous anaphylaxis or active systemic anaphylaxis, but reactions of delayed hypersensitivity may occur after intracutaneous injection of the carrier antigen. Upon subsequent exposure of the sensitized animal to the carrier protein, however, antibodies with specificity to the protein are produced at an anamnestic rate (26). The capacity of DNP·HEA to induce delayed hypersensitivity specific to HEA in DCB-fed and control animals was evaluated by skin tests with HEA and by an anamnestic response to secondary injection of HEA. Differences between the 2 groups in the delayed response to HEA could not be discerned.

DFB and PiCl cross-react in guinea pigs sensitized to one hapten and tested percutaneously with the other (27). In the antibody phase, cross-reactions between DNP and picryl-specific antibodies become even more evident (*cf.* Table VIII). In this study, interactions between PiCl and the system responsible for unresponsiveness to DNP could not be discerned. When DCB-fed animals were subsequently sensitized to PiCl, contact hypersensitivity to PiCl developed which was similar to that in the controls. When those animals hypersensitive to the picryl group were then sensitized with DFB, an unresponsive state specific to DNP was still noticeable. Sensitization with PiCl, therefore, did not overcome the unresponsiveness to DNP. Furthermore, sensitization with PiCl before induction of unresponsiveness to DNP did not diminish the effectiveness of the gastric feeding procedure to induce unresponsiveness to the DNP group. When DCB-fed guinea pigs were sensitized strongly with DFB in complete adjuvant, the unresponsiveness was less apparent upon percutaneous application of DFB, although the animals were still less reactive than controls. When the DCB-fed and control groups were strongly sensitized with DFB, cross-reactions to percutaneous testing with PiCl frequently occurred. The group fed DCB, however, cross-reacted less, at a degree commensurate

with its decreased contact reactivity to DFB (Tables I, VI, VIII). When DCB-fed and control animals were strongly sensitized to PiCl, an equal degree of contact reactivity to PiCl and of contact and antibody cross-reactivity to the DNP group was detected in both groups (Tables I, VIII). Sensitzation with DFB seems to produce cells hypersensitive only to the DNP conjugate and not to the picryl conjugate, although under excessive stimulation the specificity of some cells hypersensitive to DNP broadens and cross-reactions with picryl occur. In a PiCl-sensitized animal, cells hypersensitive only to the picryl conjugate are produced although with excessive stimulation the specificity for picryl broadens and cross-reactions with DNP occur which are independent of specific unresponsiveness to DNP.

Picryl chloride also forms covalent bonds with amino acid residues. The resulting conjugate (Pi·GPPY), however, seems to have a different carrier specificity than the DNP conjugate (DNP·GPPX). Otherwise, a close relationship between DNP and picryl unresponsiveness would exist, as the response to both haptens would be controlled by a basic GPPX specificity. The formation *in vivo* by each hapten of a somatic protein conjugate of different specificity would help to explain the striking contact specificity of contact haptens, because in animals sensitized with hapten conjugates prepared *in vitro*, the specificity of the response during the phase of delayed hypersensitivity is mainly directed toward the carrier protein (20, 21); the hapten has relatively little influence and has a broadened specificity in comparison with the specificity during the antibody phase (28).

There is some evidence that the immune process is less specific than an evaluation of cross-reactions in the antibody phase would indicate. Thus, in the delayed phase of the immune response, the specificity seems broadened (20, 21, 28). In the tolerant animal, unresponsiveness to related heterologous antigens has been noted and seems greater than what would be expected from the small amount of cross-reaction at the antibody level (29, 30). In addition, viruses may have a greater cross-reactivity than that indicated by their serologic patterns (31).

On the other hand, various studies attest to the expected specificity of tolerance. In rabbits tolerant to Bence Jones protein and subsequently inoculated with the antigenically related (homologous) myeloma protein, antibodies were elicited which were specific for those determinants of the myeloma protein not shared with the Bence Jones protein (32). Similarly, in rabbits tolerant to chicken serum and subsequently inoculated with a cross-reacting turkey serum, antibodies were produced which were specifically directed toward the turkey serum and did not cross-react with chicken serum (33). All-or-none type responses, however, were elicited in BSA-tolerant rabbits (22): either no response occurred after inoculation with a very closely related antigen, such as sheep serum albumin, or a complete response, even to determinants shared with BSA,

occurred after sensitization with an antigen with less cross-reactivity, such as human serum albumin. These BSA-tolerant animals, however, produced much less anti-BSA antibody after HSA sensitization than did control animals.

In this study, tolerance to DNP after DCB feeding was found to be specific. Picryl chloride inoculation either before or after gastric feeding of DCB did not affect the DNP unresponsiveness. Unresponsiveness to the DNP group did not have a discernible effect on the capacity of PiCl to produce hypersensitivity to the picryl group and cross-reactivity with the DNP group. It seems paradoxical that two antigens so similar structurally and antigenically would be so completely independent when evaluated in the DCB-fed tolerant animal. This paradox can probably be explained, however, by the haptenic nature of these similar contact chemicals and the predominant role played by their dissimilar carrier proteins in the specificity of the immune reaction at the "cellular level." Therefore, because Pi·GPPY is different from the DNP·GPPX to which the animal is tolerant, the Pi·GPPY is able to effect a normal immune response even with the production of antibodies which cross-react with the DNP group. The ability of some contact reactions to DFB to appear at an apparently normal rate in DNP-tolerant animals sensitized to PiCl probably indicates that the contact hapten in addition to the GPPX does have some expression in the contact reaction, although GPPX and GPPY may have some small degree of cross-reactivity which cannot be discerned by other means.

The amount of DCB fed to a guinea pig is directly related to the degree of unresponsiveness, as measured by contact hypersensitivity to the DNP group. Because a DFB-sensitized guinea pig develops contact hypersensitivity to the DNP group before specific antibodies to the DNP group can be detected, the amount of DCB fed is probably also directly related to the inhibition of antibodies to the DNP group. This dose-degree relationship between the amount of hapten fed and the degree of contact unresponsiveness induced is probably similar to the dose-duration relationship of protein antigens in acquired tolerance (34, 35), since the contact reaction in the hapten-sensitized animal is an earlier and more sensitive immunologic reaction than specific antibody formation to the hapten. In this study, when DCB-fed guinea pigs responded to sensitization with DFB, the response was most frequently manifested by the appearance of contact hypersensitivity, although some of these animals would have antibodies.

The production of unresponsiveness to haptens by gastric feeding is unusual in that the tolerance is induced in adult animals without the need of a concomitant immunologic depressant, such as 6-MP (36), x-ray (37, 38), or cyclophosphoramide (39). The relative ease with which this unresponsiveness can be induced by gastric feeding may be partially explained on the following basis: (*a*) The hapten is administered *via* an immunologically inert path and thereby forms an *in vivo* conjugate with a body protein already recognized as "self"

(40). (*b*) Tolerance may be produced only with difficulty to antigens with a great heterogeneity of determinants (35, 37). Since a simple hapten as DNP or its conjugate DNP·GPPX would have a comparatively simple spectrum of determinants, the induction of tolerance would be facilitated.

SUMMARY

"Gastric feeding" of adult guinea pigs with dinitrochlorobenzene (DCB) resulted in a specific unresponsiveness to sensitization with the specific contact hapten. The more DCB gastric-fed to a guinea pig, the more complete the unresponsiveness to the hapten. When mycobacteria were incorporated into the sensitizing emulsion, the state of unresponsiveness to the dinitrophenyl (DNP) group was less apparent. When animals gastric-fed with DCB were later sensitized with an *in vitro* conjugate of the hapten combined with a heterologous protein such as dinitrophenyl–hen egg albumin (DNP·HEA), an immune response similar to that in the controls occurred both to the hapten and to the protein carrier. However, when the tolerant animals were sensitized with a conjugate containing a homologous protein carrier such as dinitrophenyl–guinea pig serum (DNP·GPS), they showed diminished immune responses in comparison with those in the non-tolerant controls. The presence of circulating anti-DNP antibodies from sensitization with DNP·HEA did not affect the unresponsiveness to the specific contact hapten, regardless of whether these antibodies are present before or after induction of tolerance. Sensitization with picryl chloride (PiCl) (a cross-reacting hapten), either before or after gastric feeding of DCB, did not affect the state of unresponsiveness to DNP. Similarly when the DNP-tolerant animal was sensitized with PiCl, the subsequent immune response was similar to that in the controls; cross-reactions with the DNP group both in the contact and circulating antibody phase occurred at a rate similar to that in the controls.

The foregoing relationships can be explained by presuming that, upon the gastric feeding of DCB, an *in vivo* conjugate is formed with a somatic protein, which determines the basic specificity of the tolerance. Acquired tolerance seems to manifest an immunologic specificity similar to that of delayed hypersensitivity, a relationship not unexpected if delayed hypersensitivity is an early phase of the immune response.

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