INDUCED UNRESPONSIVENESS TO SIMPLE ALLERGENIC CHEMICALS

II. Independence of Delayed-Type Hypersensitivity and Formation of Circulating Antibody*

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Special types of prior experience with allergenic compounds were reported in earlier publications to abolish or diminish a guinea pig's ability to develop delayed-type hypersensitivity or to form circulating antibodies specific for the allergen experienced (1–8). The immunological unresponsiveness of allergen-fed animals was found to persist for periods in excess of 10 months and even second attempts to induce contact hypersensitivity by intracutaneous injections of hapten were found unsuccessful (9–12).

To explore further the extent of the induced immunological tolerance, guinea pigs fed picryl chloride were exposed to more intense stimuli than those that had been used heretofore, not only by incorporating hapten-conjugates (picrylated proteins) into adjuvants but also by using hapten conjugated to protein of different animal origin. In this way, maximum opportunities to respond to the hapten were afforded the animals. As reported preliminarily (13, 10, 11, 14), the formation of hapten-specific antibody proved to be relatively easy to induce, providing the hapten carrier was altered from homologous to heterologous protein, a fact confirmed by Coe and Salvin (15) recently in somewhat parallel investigations with a dinitrophenyl system. However, unresponsiveness could be modified only to a slight extent with regard to development of contact-type hypersensitivity. The two responses, antibody formation and contact hypersensitivity, consequently appear to involve independent mechanisms.

The present paper shows further that hapten-fed animals are unable to develop contact-type hypersensitivity even after they are made to form hapten-specific circulating antibodies of both anaphylactic and precipitating varieties. Conversely, normal animals first made to produce hapten-specific circulating antibody subsequently can be made to develop contact-type hypersensitivity.

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Materials and Methods

Induction of Unresponsiveness.—All guinea pigs, except those noted, were fed picryl chloride in the manner already described; *i.e.*, 0.3 ml of 1 per cent corn oil solution fed 15 days over a 35 day interval (see reference 11). The animals were used in experiments 18 to 109 days (usually 30 days) after the final feeding.

Sensitization Techniques and Contact Testing.—To induce contact hypersensitivity toward picryl chloride, the "intracutaneous" and "combination" methods were used (16). Whichever the method used, normal animals were treated in parallel to provide "positive controls."

By the intracutaneous method the animals experienced hapten through 15 injections of 2.5 μg (in 0.1 ml saline-ethanol solution) spaced over 3 weeks.

With the more intensive combination method they received hapten-protein conjugate intramuscularly (picrylated guinea pig erythrocyte stromata, 0.75 mg, emulsified through aquaphor with 0.75 mg dried human tubercle bacilli in paraffin oil; 1 ml divided among 5 sites) followed several days later by cutaneously applied picryl chloride (2 drops of 1 per cent solution in corn oil on three occasions usually 7 days apart).

Contact tests to determine the degree of hypersensitivity induced were made 1 to 3 weeks after attempting sensitization by applying single drops of 3 or 4 concentrations of picryl chloride in olive oil (1, $\frac{1}{3}$, $\frac{1}{10}$, $\frac{1}{30}$, $\frac{1}{150}$, and $\frac{1}{450}$ per cent) to separate skin sites. All sites were compared 18 to 24 and 42 to 50 hours later to those on normal control animals included with each experiment to measure non-specific irritations.

Immunization Techniques.—To elicit circulating antibody formation animals were injected with picrylated guinea pig serum proteins (PGPS, 5 mg intraperitoneally on 5 occasions over $2\frac{1}{2}$ weeks), or picrylated bovine gamma globulin (PBGG, 5 mg intraperitoneally on 2 occasions, 6 days apart), or PBGG absorbed to aluminum hydroxide (10 mg on 7.0 mg intraperitoneally once). To each 10 ml of 1 per cent PBGG there were added 4.25 ml 10 per cent potassium alum, followed immediately by addition of NaOH in predetermined amount sufficient to neutralize and form nascent alumina.

Detection of Circulating Antibody.—Sera taken from animals at intervals after immunization were examined for hapten-specific circulating antibody by one of three methods. The animals themselves were often tested for anaphylaxis in order to detect residual antibody.

1. For passive cutaneous anaphylaxis, each undiluted or saline-diluted serum sample was inoculated (0.1 ml) into the dorsal skin of at least 2 albino guinea pigs (220 to 280 gm). Reactions were developed 17 hours later by intrajugular injection of antigen (5 mg picrylated casein in 1 ml saline) combined with 0.5 per cent Evans blue dye Warner-Chilcott Laboratories, Morris Plains, New Jersey; about 0.025 ml per 10 gm body weight) and read after 30 minutes.

2. Hemagglutination was performed in the manner already described (12). Indeed, for these tests the tanned sheep erythrocytes were coated with the same picrylated guinea pig serum preparation used in the work cited.

3. Precipitation: The tests for precipitating antibody were done by mixing 1 drop of undiluted sera with one drop of each of 4 saline dilutions of hapten-conjugate (picrylated chicken serum albumin 2.0, 0.5, 0.1, and 0.05 mg/ml). The antigen-antibody mixtures were allowed to flow into capillary tubes (0.7 to 1.0 mm bore), followed by 10 to 12 mm^3 of air; the tubes were inserted vertically into plasticene (17), incubated 2 hours at room temperature and placed in the refrigerator. Observations for precipitates were made at intervals up to 10 days.

Active Anaphylaxis.—Two to 3 weeks after animals had been test-bled for antibody determinations they were inoculated *via* the jugular vein with picrylated casein in 1 ml doses that ranged from 15.0 mg down to 0.5 mg in order to determine the degree of anaphylactic sensitivity.

Preparation of Picryl Conjugates.—The conjugates used were coupled as described before

(16, 18), and the degrees of coupling (as moles "picryl" per gm carrier protein) were a picrylated guinea pig serum, 0.113; picrylated chicken serum albumin, 0.24; picr gamma globulin, averaging 0.066; picryl casein, 0.274; and picrylated guinea pig about 0.165.

RESULTS

Hapten-fed guinea pigs were examined for the extent of induced unrespo first toward developing delayed-type hypersensitivity. Along with control some were exposed to the intracutaneous method of stimulation with ha others to the more intensive combination method. Thus, 26 days after the l chloride feeding (0.3 ml of 1 per cent solution in corn oil fed 15 times over hapten-fed and control animals received either 15 intradermal injections of

TABLE I

Intensity of Delayed Contact Hypersensitivity Induced in Picryl Chloride–Fed (F) Control (C) Guinea Pigs Following Attempted Sensitization with Picryl Chlori by Two Methods

Sensitization method	Dermal response to picryl chloride					
	Neg.-trace	Least concentration for hypersensitive response*				
	1 per cent	1 per cent	0.3 per cent	0.1 per cent	0.02 per cent	0.0067 per cent
Intracutaneous...	F, F, F, F, F, F	F, F	C	C, C, C, C	C, C, C	
Combination.....	F	F	F, F, F, F, F, F			C, C, C, C

* Concentration to which the animals responded in contact tests with a conflu tion equal to or greater than very faint pink.

picryl chloride over 19 days or one intramuscular injection of a water-in-oil e of picrylated guinea pig erythrocyte stromata and tubercle bacilli (1.0 ml among 5 sites) followed by dermal applications of single drops of 1 per cer chloride in corn oil on days 14, 21, and 28. All animals were titrated for react the 35th day following the initial injection.

The intensity of the delayed contact hypersensitivity induced in each as judged by the least amount of hapten to which each would respond, is in Table I. Most apparent is the fact that control animals exposed to the nation method developed considerably greater sensitivity than did animals exposed to the intracutaneous procedure; all 8 of the former res to the most dilute solutions of picryl chloride (0.0067 to 0.0022 per cent) as the latter showed contact reactions only to more concentrated sc (0.3 to 0.02 per cent). Also obvious is the conversion of hapten-fed guin to low grade, yet definite, reactivity when exposed to the combination

zation procedure (7 out of 8 reacted when tested with 1 to 0.3 per cent picryl chloride). This contrasted with the lack of conversion of picryl chloride–fed animals treated with the less intense intracutaneous method (6 of 8 failed to react to 1 per cent picryl chloride and 2 responded to 1 per cent). Hapten-fed animals inoculated intracutaneously with the hapten were 10 to 50 times less sensitive than were similarly treated control animals and a 50- to 100-fold difference was seen between the two groups of animals sensitized by the combination method. Indeed, hapten-fed animals exposed to the combination method were unable to arrive to the same degree of sensitivity developed by control animals sensitized by the less intense intracutaneous method. Nevertheless, the point of importance is that immunological responsiveness could be restored by intensive haptenic stimuli, though the restoration was far from complete and the animals became stabilized at what appeared to be a shallow level of sensitivity.

The onset of contact sensitivity observed in normal and hapten-fed animals treated by the combination method was examined in a further experiment. Here the secondary haptenic stimulus usually provided by 3 successive paintings of 1 per cent picryl chloride to the skin was replaced by consecutive measurements of dermal hypersensitivity using dilutions of the hapten. Sensitization was started 109 days after the final picryl chloride feeding by intramuscular injection of water-in-oil emulsion containing killed tubercle bacilli and picrylated guinea pig erythrocyte stromata. The animals were first tested 34 days after the start of sensitization; subsequent tests were made at 1-week intervals. The degree of sensitivity developed was determined by the response to the least of 3 amounts of hapten applied to separate sites 24 to 48 hours earlier. As sensitivity rose, the concentrations applied were diminished.

Though a wide disparity between the hapten-fed and control groups was seen at each test, all animals appeared to develop contact sensitivity to picryl chloride in a stepwise manner (Table II). The difference between the groups is apparent from the outset (day 34); whereas the hapten-fed animals responded minimally or not at all (2 negative at 1 per cent, 1 positive at $\frac{1}{3}$ per cent), the control animals showed clearly that they had been stimulated by the intramuscular injection (3 positive at $\frac{1}{10}$ per cent, 1 at $\frac{1}{3}$ per cent). Thereafter, although sensitivity was seen to rise in some animals with each subsequent test, it appeared to do so at a slower pace. Thus, at the second test, a 5-fold increase in sensitivity was seen among the majority of the control animals, at the third a 3- to 9-fold difference and at the last test only a 0- to 3-fold difference was apparent. The hapten-fed animals were unable to do as well and remained almost unaffected from the time of the second test (day 41).

Next, hapten-fed animals were examined for the degree of unresponsiveness toward the ability to form circulating antibodies specific for the hapten. Together with normal animals, one group received injections of picrylated homologous proteins (5 mg of

picrylated guinea pig serum intraperitoneally on 5 occasions over 2½ weeks), while another group received a picrylated heterologous protein (5 mg of picrylated bovine gamma globulin intraperitoneally on 2 occasions 6 days apart). Sera taken 14 to 29 days after the initial antigen injection were tested for antibody by passive cutaneous anaphylaxis, precipitation, and hemagglutination. The animals themselves were tested for systemic anaphylactic shock at 31 to 49 days by intravenous injections of varied doses of picrylated casein, from 0.5 to 15.0 mg in order to ascertain the degree of anaphylactic sensitization.

Responses to picrylated guinea pig serum were essentially as reported before (2, 3) that is, none of the 6 hapten-fed animals possessed sera able to elicit

TABLE II

*Acquisition of Delayed-Type Allergy to Picryl Chloride in Control (C) and Picryl Chloride-Fed (F) Guinea Pigs during Attempted Sensitization by the Combination Method**

Guinea pig	Degree of contact sensitivity at day			
	34‡	41	48	55
1F	0	1		1
2F	0	1		3
3F	3	10		10
1C	10	50	150	150
2C	10	50	150	450
3C	3	50	450	450
4C	10	50	450	450

* The sensitivity is shown as the reciprocal of the smallest hapten concentration evoking a reaction, *i.e.* $\frac{1}{10}$ per cent = 10. The highest concentration used was 1 per cent.

‡ The start of attempted sensitization is considered as day 0.

passive cutaneous anaphylaxis reactions even when tested undiluted, whereas the sera of all 7 control animals elicited such reactions when diluted 1:75 (Table III). Trace precipitins for the hapten (carried by chicken serum albumin) were seen in the sera from 1 hapten-fed and 2 control animals. When finally tested for systemic anaphylaxis, none of the 6 hapten-fed animals exhibited any but minimal signs of shock (to 5 to 15 mg picrylated casein) and all showed elevated rectal temperatures (+1.1–2.0°C). On the other hand, all control animals but 1 developed shock (to 0.5 to 15 mg picrylated casein) and, of the 2 that did not die, 1 showed a decreased rectal temperature (−0.3°C) while the other (which received only 0.5 mg picrylated casein) showed an increased temperature (+1.3°C). Animals that were used to test the toxicity of the picrylated casein also showed elevated temperatures (+1.5–2.2°C) when given the highest dose, 15 mg intravenously. Thus, whereas the majority of hapten-fed and control animals injected with picrylated guinea pig serum did not produce significant

amounts of hapten-specific precipitins, only the hapten-fed animals failed to synthesize antibody of the anaphylactic type (both systemic and PCA).

Yet, when the haptenic stimulus was one in which hapten is coupled to a foreign protein such as BGG, the abilities of hapten-fed and control guinea pigs to form hapten-specific circulating antibody were found to be indistinguishable. Thus, the sera of all hapten-fed and control animals were able to elicit PCA reactions when diluted 1:75 and all sera, when diluted 1:50, demonstrated hemagglutination of erythrocytes coated with picrylated guinea pig serum. In addition, only 1 hapten-fed and 2 control animals showed precipitins in their

TABLE III

Detection of Picryl-Specific Antibody in Picryl Chloride-Fed and Control Guinea Pigs Injected with Picrylated Conjugates

Initial treatment	Conjugate injected*	Antibody detected by‡			
		PCA§	Hemagglutination‖	Precipitation	Anaphylaxis¶
PCl-fed	PGPS	0/6		1?/6	0/6; 0 dead
None	PGPS	7 7		2?/7	6/7; 5 dead
PCl-fed	PBGG	5/5	5/5	1/5	5/5; 4 dead
None	PBGG	7/7	7/7	2/7	5/7; 4 dead

* PGPS, picrylated guinea pig serum; PBGG, picrylated bovine gamma globulins.

‡ The number positive over the total number is shown.

§ Sera were tested for PCA in a dilution of 1:75; those listed as negative were tested also undiluted.

‖ Sera diluted 1:2000 to 1:8000 agglutinated cells coated with picrylated guinea pig serum proteins.

¶ PCl-fed animals injected with PGPS received 5 to 15 mg of picrylated casein intravenously; all others except 1 received 0.5 to 5 mg. The exception received 15 mg.

sera directed to the picryl group on picrylated chicken serum albumin and these appeared only after several days of incubation. Though it is not shown in Table III, antibody for the carrier protein, BGG, were also found in the sera of all animals producing antibody. Finally, when injected intravenously with picrylated casein (0.5–2.0 mg) all 5 hapten-fed animals and 5 of 7 control animals exhibited anaphylactic shock; 4 animals of each group died within 10 minutes and those that recovered from shock all showed depressed rectal temperatures (−0.1–1.0°C). Thus, it is apparent that the ability to form hapten-specific circulating antibody mediating passive cutaneous and systemic anaphylaxis as well as hemagglutination is restored to hapten-fed guinea pigs when the stimulus is provided by hapten coupled to highly antigenic heterologous protein.

To investigate the restoration of immunological responsiveness still further, hapten-fed animals along with controls were given a stronger haptenic stimulus in the form of a single intraperitoneal injection of picrylated bovine gamma globulin adsorbed to an adjuvant, aluminum hydroxide, 10 mg protein adsorbed to 7 mg Al $(OH)_3$, 40 days after the last hapten feeding. Sera taken at 8 and 15 days following this injection were examined for hapten-specific antibody by the precipitation test using picrylated chicken serum albumin as antigen.

Surprisingly, tests with sera taken on the 8th day (Table IV) revealed hapten-specific precipitins among more of the hapten-fed than normal animals, 5 of 7 as contrasted with 2 out of 6. This difference between the groups was no longer detectable by the 15th day. In contrast to sera drawn from animals

TABLE IV

Earlier Detection of Picryl-Specific Antibody in Picryl Chloride-Fed Guinea Pigs Injected with Picrylated Bovine Gamma Globulin (PBGG) and Alumina Adjuvant

Initial treatment	Serum taken post PBGG injection	Precipitins specific for the picryl group on chicken serum albumin			
		2.0 mg/ml	0.5 mg/ml	0.1 mg/ml	0.02 mg/ml
	days				
PCl-fed	8	0/3*	4/7	5/7	5/7
None	8	0/2	1/6	2/6	1/6
PCl-fed	15	5/7	6/7	7/7	1/7
None	15	3/6	5/6	5/6	0/6

* The number of animals having precipitating antibody over the total tested is given. Four concentrations of antigen, as shown, were tested with equal volumes of undiluted serum.

given PBGG without alumina, precipitation often commenced within 1 hour or less. Thus, not only were hapten-fed guinea pigs injected with highly antigenic hapten-conjugate able to form circulating precipitating antibody for the hapten but they were found to do so earlier than control animals. It may be added that this clear difference between the fed and control groups was not seen in a later experiment, with a preparation of PBGG averaging 5.7 moles per mole BGG. The reason for the discrepancy between the two experiments remains unclear. Apparently the conditions for stimulating the animals are critical.

The next experiment was undertaken to determine whether a hapten-fed animal with restored ability to form precipitating hapten-specific antibody would then be able to develop hapten-specific delayed hypersensitivity. One group of hapten-fed guinea pigs was exposed to the intracutaneous method of sensitization before, and another group along with control animals after, receiving picrylated bovine gamma globulin adsorbed to aluminum hydroxide as adjuvant. Sera taken 15 days

after PBGG, were tested for hapten-specific circulating antibody and the animals, themselves, were examined for contact hypersensitivity.

None of 4 picryl chloride–fed animals subjected to attempted sensitization by the intracutaneous method developed picryl-specific contact sensitivity (group A, Table V) and all continued unable to show sensitivity though precipitating antibody for the picryl group was formed by 3 of the animals. In addition, 7 of 7 hapten-fed animals injected with PBGG and alum developed picryl-specific precipitins (group B), yet when later subjected to the intracutaneous method of sensitization 6 of these failed to develop picryl-specific delayed hypersensitivity. This contrasted markedly with a control group of

TABLE V

Production of Picryl-Specific Antibody by Picryl Chloride–Fed Guinea Pigs without Incitement of Picryl-Specific Delayed Hypersensitivity

Group	Initial treatment	Method of attempted sensitization*	Contact sensitivity to 1 per cent PCl	Immunization attempted‡	Anti-picryl precipitins detected‡	Method of attempted sensitization*	Contact sensitivity to PCl	
							1/3 per cent	1 per cent
A§	PCl-fed	i.d.	0/4	PBGG + Alumina	3/4	—	—	0/4
B	PCl-fed	—	—	PBGG + Alumina	7 7	i.d.	1/7	1/7
C	None	—	—	PBGG + Alumina	6/7	i.d.	5/7	5/7
D	None	—	—	—	—	i.d.	5/7	6/7

* A shortened series of 4 daily injections of 2.5 μg picryl chloride, made intradermally (i.d.), was given.

‡ Method as in Table IV.

§ Animals of this group were fed on various abbreviated schedules: 1 received a 2 per cent solution 4 times in 1 week, the others were fed 1 per cent solution, 1 on 4 occasions in 2 weeks and the remainder on 9 occasions in 3 weeks.

animals (group C), 5 of 7 of which became hypersensitive *via* the intracutaneous method even after circulating antibody had been formed by 6. The number that acquired contact hypersensitivity is not unlike the 6 of 7 in control group D that did also. Thus, hapten-fed animals were found incapable of responding to the hapten with delayed contact reactions no matter whether the attempt to induce the hypersensitivity came before or after formation of circulating antibody.

DISCUSSION

Termination of unresponsiveness has been investigated in several areas. In the instance of unresponsiveness to protein antigens (*cf.* reference 19), injections of altered antigens (20–24) or antigens related to but not identical with that used for inducing tolerance (25–28) have been employed. Homograft toler-

ance has been abolished by sublethal irradiation (29) and by the injection of normal (isologous) lymphoid cells (30, 31). In the case of unresponsiveness to chemical allergens, attempts to terminate delayed-type hypersensitivity and hapten-specific antibody have been studied separately, as here reported in detail (*cf.* references 13, 10, 11, 14, 32).

In animals made unresponsive by prior hapten-feeding, attempts to reinstate delayed-type hypersensitivity failed when sensitization was attempted *via* the intracutaneous method, but exposure to hapten *via* the combination method succeeded in inducing a low level of contact hypersensitiveness (Table I). Here, a 50- to 100-fold greater sensitivity was seen in the control animals over that of hapten-fed animals, because by this technique the reactivity of normal animals was increased manyfold whereas hapten-fed animals were brought only to a measurably reactive, but stationary, level. Allergic conversion induced to this extent in hapten-fed animals was, however, significantly greater than with other methods of attempted sensitization. Had the combination method of inducing sensitization not been used, the degree of sensitivity developed by normal and hapten-fed animals would have appeared somewhat similar (*i.e.* tests at day 34, Table II). Coe and Salvin (15) have confirmed the fact that injection of hapten-fed guinea pigs with a sensitizing emulsion containing hapten or hapten-conjugate and mycobacteria lessens the tolerance ordinarily seen upon intradermal sensitization with hapten. Unfortunately, they could not make as discriminating a differentiation as is possible when the combination method of sensitizing, involving successive contact reactions with the simple chemical following injection of a hapten-conjugate in Freund-type adjuvant, is used.

During sensitization of control animals by the combination method, it was apparent that the intramuscular injection of the hapten-stromata conjugate in adjuvant did not alone bring the animals to the highest level of reactivity. The subsequent dermal applications of hapten made at weekly intervals boosted markedly and uniquely the hypersensitive response (Table II). Whether the particular levels of sensitivity to which the animals were brought by each test represented plateaus or were only points detected during a continually ascending sensitivity may be answered by other experiments. In addition, it would be of some interest to know whether higher levels of sensitivity would be attained by animals exposed to the intracutaneous method if they were subsequently exposed to the combination method of sensitization.

Guinea pigs fed picryl chloride were found not to form hapten-specific antibodies following injections of hapten coupled *in vitro* to guinea pig serum proteins, although normal guinea pigs did so. The antibodies primarily sought were those that mediate passive cutaneous and systemic anaphylaxis. Table III confirms the earlier reports (2–4).

The ability to form hapten-specific antibody was restored successfully when

the stimulus consisted of hapten coupled to a heterologous protein (picrylated bovine gamma globulin, PBGG). Anaphylactic and hemagglutinating antibodies, for example, were as readily demonstrable in hapten-fed guinea pigs as in normal animals (Table III).

Recent investigations have revealed two classes of guinea pig antibodies possessing different functions. White, Jenkins, and Wilkinson (33) pointed to the presence chiefly of 7S β-globulin in guinea pigs injected with ovalbumin only, and the finding of large amounts of an additional antibody, 7S gamma globulin, when ovalbumin was injected in Freund's complete adjuvant. Benacerraf and coworkers (34–36) have studied these antibodies in detail, designating them 7S γ_1 and 7S γ_2 respectively. The 7S γ_1 class represents the antibodies responsible for systemic and passive cutaneous anaphylaxis, and these antibodies predominate in the guinea pig unless Freund's adjuvant is used. Precipitins are represented by the 7S γ_2 class.

It is apparent in Table III that hapten-fed guinea pigs do not synthesize anaphylactic-type antibodies when they are stimulated by hapten-homologous protein conjugate (PGPS). Stimulation by means of soluble PBGG gives rise to anaphylactic antibodies, but seldom leads to the formation of precipitins and when these are found the amount is low. Precipitins appeared readily, however, when the antigenic stimulus was increased by absorbing PBGG to alumina (Table IV). This technique evidently leads to the production of 7S γ_2 antibodies just as Freund's adjuvant does. Both hapten-fed and normal guinea pigs came to form precipitating antibody, the majority of hapten-fed animals forming them somewhat earlier than did control animals (Table IV). At 15 days, however, the two groups of animals were alike. It must be stated that this differentiation with respect to earlier appearance of antibody in the fed group was not found in a later experiment, perhaps owing to the physical properties of the stimulating complex. An earlier production of antibody by hapten-fed animals would help in understanding the mechanism of unresponsiveness by establishing that immunologically competent cells have had experience with the hapten for which they are tolerant, and support would perhaps be offered for the concept of Dorner and Uhr that, preceding unresponsiveness, an actual stimulation of the immunological apparatus occurs (37). The observation of Frey, Geleick, and deWeck, that guinea pigs fully sensitized to 2:4 dinitrochlorobenzene can later be made tolerant by administering intravenously very large amounts of the relatively non-toxic compound 2:4 dinitrobenzenesulfonate (38), might represent an up-scaled instance of this principle. Another observation related to the topic of the tolerant animal's experience with antigen prior to test is that of Garvey, Eitzman, and Smith (39) who report a slight, selective accumulation of the antigen S^{35}-labeled sulfanilic acid-azoalbumin, in the nuclear fraction of liver homogenates of unresponsive rabbits.

Coe and Salvin (15), in an elaborate study, reported that the tolerance of

guinea pigs fed dinitrochlorobenzene was abolished by injecting into the footpad dinitrophenyl-hen egg albumin emulsified in incomplete Freund's adjuvant. Hapten-specific anaphylactic antibody was formed, but no earlier than in control guinea pigs. While these workers allowed only a brief time to prepare the skin for PCA reactions, in our experience such antibody can remain undetected when only 4 hours is allowed to prepare sites for such reactions (*cf.* references 40, 41). In our search for precipitating antibody, the experiment shown in Table IV showed clearly an earlier production of antibody in hapten-fed guinea pigs than in normal animals. Yet a later experiment with another conjugate did not show this difference. More work is needed to learn whether preparation of the particular stimulating hapten-protein complex is crucial for the outcome.

Seemingly, hapten must arrive at the centers it will depress before these receive an allergenic or antigenic stimulus that is adequate to excite normal, full responses. Feeding (2), intravenous injection (7, 11, 38), or topical application (11) can be effective routes, or hapten may be applied while the immunological apparatus is held in check by metabolic antagonists such as methotrexate (42) or cyclophosphamide (43).

The mechanism is unknown by which hapten absorbed from the gut comes to control the immunological apparatus with respect to both its manifestations of responsiveness, namely, contact-type hypersensitivity and formation of circulating antibody. It is worthy of note that the control exerted over the immunological apparatus occasionally can be found to be partial. For example, some animals continue to show low-grade contact-type reactivity that is not increased by further stimuli. Also, animals that have been rendered unresponsive with regard to contact hypersensitivity, *e.g. vis à vis* phthalyl chloride, have been found to exhibit a continuing limited degree of Arthus reactivity, though minor indeed in comparison with normal guinea pigs similarly stimulated (11).

When attempts were made to abolish unresponsiveness by use of the "combination method," a low level of contact-type hypersensitivity resulted. Probably this is due to the well known proliferation of monocytic cells that is stimulated by Freund-type adjuvant. The newly-acquired contact hypersensitivity may be attributable either to minimal response by all of the "once tolerant" cells or to an adequate response by a limited number of cells that may have escaped the full effects of the fed hapten. Yet the animals continue to respond only partially and fail to attain normal status towards haptenic stimulation. Fefer and Nossal (29), who have observed the abolition of homograft tolerance by sublethal x-irradiation, propose the view that irradiation selectively favors a sensitized residual population of cells that had remained unaffected by the neonatal injection. But in this instance, also, it is not known whether rejection comes about by cells that have attained only a fractional degree of competence.

A point still undetermined is the degree to which unresponsiveness to delayed contact-type hypersensitivity would be altered if tolerant animals were given complete Freund's adjuvant containing hapten coupled to heterologous protein.

The ability to form hapten-specific circulating antibodies was apparently completely reestablished by stimulation with picrylated BGG rather than picrylated guinea pig proteins. The hypothesis invoked to explain the limited restoration of delayed-type hypersensitivity shown in Table I (*i.e.* adjuvant stimulation of the few cells not fully affected by hapten-feeding) might pertain as well to synthesis of circulating antibody. The explanation does not appear equally applicable, however, to both immune responses since hapten-fed animals, instead of lagging far behind controls as they do in showing delayed-type hypersensitivity, form circulating antibody as well as control animals and may, indeed, pace them. A reasoning that better fits these observations is that the tolerant cells of a hapten-fed animal were unable to reject the stimulus provided by the entirety of the hapten-heterologous protein complex, the complex providing a stronger stimulus to initiate antibody synthesis than the hapten-moiety to prevent it. Indeed it remains unknown whether tolerance becomes broken with respect to picrylated-self protein. However, in the instance presented by animals fed phthalic anhydride (11) partial antibody response does occur to phthalylated self-protein. It is evidently more difficult to suppress the synthesis of circulating antibody than to block the pathway that leads to contact-type delayed hypersensitivity.

Additional information was gained by further attempts to abolish immunological tolerance, for once that tolerant guinea pigs were found to form hapten-specific precipitins the question arose: Would they now respond with hapten-specific delayed-type hypersensitivity? The answer obtained was that regardless of whether the intracutaneous haptenic stimuli preceded or followed synthesis of precipitins, the animals remained unable to develop hapten-specific delayed-type sensitivity (Table V). Control animals by contrast responded with delayed-type hypersensitivity even after hapten-coupled bovine gamma globulin adsorbed to alumina had stimulated synthesis of hapten-specific circulating antibody, indicating that the presence of the latter in no way prevented the development of delayed hypersensitivity.

Turk and Humphrey (44) reported also that a group of guinea pigs, rendered immunologically unresponsive to human gamma globulin by early contact with the antigen, formed circulating antibody to human gamma globulin in Freund's adjuvant but not delayed hypersensitivity. In this instance, the antigenic stimuli were not carried on molecules different from those that had induced unresponsiveness and, still, both responses were not developed equally. Thus, though an explanation for the difference is lacking, once again it is seen that

unresponsiveness directed toward circulating antibody formation is more easily reversed than that directed to delayed hypersensitivity.

The important point, however, which must not be overlooked is that an animal's delayed hypersensitivity response can be completely circumvented and it can be directed to occur either before or after circulating antibody synthesis. Thus, the one immune response does not appear to be invariably linked to the other, instead, the available information suggests that they are separate responses even though, at times, both may be directed to the same antigenic stimulus.

SUMMARY

Normal guinea pigs fed chemical haptens develop a specific state of unresponsiveness, inhibiting subsequent development of dermal sensitization with the same hapten and modifying profoundly the synthesis of anaphylactic antibody in response to hapten conjugated to guinea pig proteins.

The degree of unresponsiveness has been tested by exposing hapten-fed animals to intense haptenic stimulation. Animals of groups that were demonstrably unresponsive to picryl chloride could be made to form hapten-specific antibody by injecting picrylated bovine gamma globulin. Specific anaphylactic-type antibodies, presumably 7S γ_1, were synthesized, and in animals given PBGG adsorbed to alumina there arose a measurable concentration of precipitating antibody, presumably 7S γ_2, perhaps slightly earlier than in similarly treated control animals.

Attempts to impose contact-type reactivity on such unresponsive animals met with limited success. Injection of picrylated guinea pig erythrocyte stromata in a complete Freund's adjuvant, with subsequent applications of picryl chloride to the dermis, led to definite contact sensitivity to 0.3 per cent picryl chloride, whereas parallel treatment of normal control animals induced sensitivity to 0.006 or 0.002 per cent. By this double method of stimulation, hapten-fed animals did not advance in sensitivity by reason of the secondary dermal applications of the simple chemical, whereas control animals developed increasingly higher sensitivity by these contacts in what appeared to be a stepwise manner.

Picryl chloride–fed guinea pigs injected intradermally with picryl chloride either after or before forming picryl-specific circulating antibody still remained unable to develop picryl-specific contact hypersensitivity.

Control animals synthesizing picryl-specific antibody subsequently responded to intradermal injection of picryl chloride with contact-type sensitivity.

Interpretations of these results are discussed and the view is presented that delayed-type hypersensitivity and circulating antibodies of the varieties measured here are formed independently of each other.

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