IMMUNOLOGICAL UNRESPONSIVENESS TO SENSITIZATION WITH SIMPLE CHEMICAL COMPOUNDS

A Search for Antibody-Absorbing Depots of Allergen*

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Preceding communications (12, 13) revealed that simple allergenic chemicals administered to normal adult guinea pigs in special ways render the animals highly refractory for many months when later attempts at sensitization are made with the same chemicals. The animals do not develop delayed-type hypersensitivity as determined by contact tests and do not form antibodies specific for the hapten fed, though they can be sensitized typically by other, unrelated, chemical allergens. The principal method used to induce immunologic unresponsiveness in the past and in the work reported here is by feeding allergens over the course of several weeks; more recently unresponsiveness has been shown to be inducible by injecting allergens parenterally in minute amounts (4, 5).

The inability of allergen-fed guinea pigs to produce specific antibody appears similar to the inability of mice to form antipneumococcal antibody following injection of pneumococcal polysaccharide. This condition in mice, termed "immunologic paralysis" by Felton (24), was shown to persist for at least 15 to 18 months after the paralyzing dose. In examinations on mice within the 1st month of treatment, Dixon *et al.* (22) found prompt elimination from the circulation of rabbit antipolysaccharide antibody. Support was thus provided for the hypothesis that antibody synthesis was not absent in unresponsive mice but that antibody, as formed, was removed from circulation on foci of retained polysaccharide (29, 40, 22). Despite a marked reduction of polysaccharide shown to be extractable from spleens at 3 to 8 weeks (40) or visible in tissues by fluorescent antibody at 6 months (29), antipolysaccharide antibody was never infused at a time when residual polysaccharide is diminished and the mice are still unresponsive.

The present investigation, besides providing information on the duration of

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the unresponsive period in hapten-fed guinea pigs, was directed to ascertaining whether hapten-specific antibody is cleared from the circulatory systems of hapten-fed and normal animals at different rates.

In addition, to determine whether lymphoid cells of hapten-fed guinea pigs are immunologically inhibited because of the presence of extracellular haptenic depots, the cells were removed to a hapten-free environment of normal animals where their abilities to confer delayed-type sensitivity and to produce circulating antibody were tested. Portions of this work were cited in previous preliminary reports (2, 16, 17).

Materials and Methods

Induction of Unresponsiveness.—Following two "training" feedings of corn oil, recrystallized picryl chloride dissolved in corn oil was fed to adult guinea pigs daily (0.3 ml of 1 per cent solution) for 5 days and withheld for 9 days. This pattern was continued until a total of 15 or 20 feedings (45 or 60 mg) were given; precautions were taken not to contaminate the external mouth parts. Thereafter, the animals were rested for periods of 30 to 298 days before use in experiments or exposure to one of two sensitization procedures.

Sensitization Techniques.—The "intracutaneous method" of sensitization (14) consisted of daily intradermal injections of picryl chloride (2.5 μg in 0.1 ml saline-ethanol solution) until each animal received 15.

The "combination method" (14) involved application of hapten by two procedures and induced more intense delayed-type hypersensitivity than did the "intracutaneous method." One ml of a water-in-oil emulsion containing 0.75 mg picrylated guinea pig erythrocyte stromata and 0.75 mg killed dried human tubercle bacilli with paraffin oil and aquaphor (Duke Laboratories, Inc., Stamford) as emulsifier was injected among 5 sites in the nuchal muscles. Thereafter, on days 14, 21, and 28, adjacent areas of skin were painted with 2 drops of 1 per cent solution of picryl chloride in olive oil or dibutyl phthalate.

The degree of hypersensitivity engendered was examined 1 to 3 weeks later by contact tests.

Contact Tests.—Several hours after closely clipping the hair from 3 or 4 dorsal sites, single drops (0.025 to 0.03 ml) of 3 or 4 concentrations of picryl chloride in olive oil (1, $\frac{1}{3}$, $\frac{1}{10}$, $\frac{1}{50}$, $\frac{1}{150}$, $\frac{1}{450}$ per cent) were applied to the skin and spread with a glass rod over a 25 mm circle. Sites were examined at 18 to 24 and 42 to 50 hours, but only the peak reactions are given in the tables.

Normal animals of corresponding weight and sex were included in each test to measure nonspecific irritations.

Preparation of Picryl Conjugates.—Casein and guinea pig serum proteins, picrylated as described before (14), were used as antigens to elicit passive cutaneous anaphylaxis and for coating tanned sheep erythrocytes in the Boyden hemagglutination test. When the proteins were coupled with picryl chloride offered in a ratio of 1 gm to 120 mg, the products (picrylated casein batch V and picrylated guinea pig serum batch XVI) contained 75 and 23 mg of picryl groups per gm of conjugate.

Guinea pig erythrocyte stromata were picrylated as described in reference 30; the batches used in the water-in-oil emulsion for sensitizing animals were prepared by coupling 50 mg of picryl chloride per gm of stromata.

Preparation of Antipicryl Antibody Used in Transfusions.—Two separate preparations of picryl-specific antibody produced in guinea pigs by the different sensitization methods were used for the transfusion studies.

Serum pool "324" was rendered from guinea pigs sensitized by the combination method. The individual sera taken from blood drawn on the 72nd day of immunization were pooled and sterilized by filtration through a Berkefeld "N" candle. When tested for picryl-specific antibody by passive cutaneous anaphylaxis (PCA method given below), 0.1 ml amounts elicited minimal reactions at saline dilutions of 1:600 to 1:800. A reaction consistently measuring 14 to 17 mm in diameter was attained by a dilution of 1:150 and this was termed 1 PCA unit. The serum pool thus contained 1500 PCA units/ml, an antibody concentration large enough to remain detectable despite the dilution that occurred *in vivo* following transfusion of small (0.57 to 1.0 ml) volumes.

Serum pool "1431-33" was produced from animals sensitized by the intracutaneous method; *i.e.*, 18 intradermal injections of picryl chloride (2.5 μg each) over 16 weeks. Sera taken on the 117th day also showed a 14 to 17 mm PCA reaction when diluted 1:150.

Passive Cutaneous Anaphylaxis.—Serum samples to be tested for antipicryl antibodies were inoculated (0.1 ml) into skin sites on the backs of normal albino guinea pigs (220 to 280 gm); one site always received 1 PCA unit of the serum sample used in transfusion. Samples for comparison were most often diluted in saline and tested in a given recipient at constant concentration to avoid the variable suppressive effect upon PCA reactions induced by different concentrations of serum proteins (36, 25, 7). The total number of sites and the serum dilutions tested on each recipient were regulated so that excess antibody would not result, upon test, in any trace of systemic shock. For closest comparison, samples were tested in symmetrical sites on the skin in one recipient and reversed in position on another recipient. Reactions were developed 17 hours later by intrajugular injection of antigen (5 mg picrylated casein V in 1 ml saline) combined with 0.5 per cent Evans blue dye (Warner-Chilcott Laboratories, Morris Plains, New Jersey *ca.* 0.025 ml per 10 gm body weight) and read after 30 minutes. Test site reactions were compared with the standard site so that antibody content could be graded in PCA units; the practice of diluting sera to extinction of reaction (35) was not used.

Hemagglutination Test.—Surfaces of washed sheep erythrocytes were altered with tannic acid by the procedure of Boyden (8) and coated with antigen using the reagent proportions of Stavitsky (41): 1 ml tanned erythrocytes, 1 ml antigen (1.5 mg picrylated protein), and 5 ml saline buffered at pH 6.4. Picrylated whole guinea pig serum (preparation XVI) having 23 mg picryl groupings per gm of conjugate served as antigen; other preparations of picrylated guinea pig serum and picrylated bovine gamma globulin were as satisfactory, but picrylated bovine albumin, chicken albumin, and casein were not. The density of the antigen-coated cell suspension was ascertained by lysing an aliquot in five volumes of distilled water and reading hemoglobin at 550 mμ with a Coleman junior spectrophotometer in a 10 mm round cuvette. The aim was to adjust suspensions to give an optical density reading of 0.485; actual values obtained were between 0.465 and 0.493. (Deviation in the final concentration of erythrocytes altered markedly the end point of the test.) An aliquot of antigen-coated cells (0.05 ml) was added to each tube of test serum diluted in 0.5 ml buffered saline containing heat-inactivated guinea pig serum (1:100) and sedimented patterns were read usually after 16 to 20 hours of refrigeration. With this test, as with PCA, precision was apt to be blunted when serum was tested in slight dilution only; therefore, with few exceptions, dilutions less than 1:10 were avoided.

Cell Transfers.—Lymph nodes of the cervical and axillary regions as well as spleens were removed from etherized animals exsanguinated by severing vessels at the base of the skull.

(*a*) *Lymph nodes.*—Nodes dissected free of superficial fat were washed, blotted, weighed, and under gelatin-Hanks' solution[1] were ruptured peripherally by means of teasing tools bearing pairs and triplets of sharp needles. The cells were passed through nested strainers

[1] Containing 0.125 per cent gelatin, Knox Gelatine Protein Products, Inc., Camden, New Jersey, similar to their type 1592.

(80, 120 mesh[2]) and washed three times by centrifugation (800 to 1100 RPM) from 250, 100, and 50 volumes of gelatin-Hanks' solution. A sedimented volume was determined before the cells were taken up as a 10 per cent suspension in Hanks' solution containing 7 per cent normal guinea pig serum, passed through another 120 mesh strainer to eliminate cell aggregates, and injected immediately into recipient animals via the jugular vein.

(*b*) *Spleens.*—Spleens, dissected free of mesenteric attachments, were also washed, blotted, weighed, and in groups of five were scissored in a shallow dish under gelatin-Hanks' solution. The cell suspension, notably enriched in white blood cells because of exsanguination, was passed through three strainers (40, 80, 120 mesh) and processed further like the lymph node cells.

For judging viability and gross morphology, 0.05 ml of final cell suspension was mixed with 0.2 ml normal guinea pig serum and 0.05 ml of 2 per cent aqueous trypan blue. About 85 per cent of the lymph node cells and 75 per cent of the spleen cells were usually found unstained.

One day after receiving cells, the recipients were tested daily for delayed dermal hypersensitivity to picryl chloride (1 per cent in olive oil); on the 2nd day some were tested for sensitivity to tuberculin.

Tuberculin Testing.—Animals sensitized by the combination method received killed tubercle bacilli in the continuous oil phase of the injected water-in-oil emulsion and thus developed reactivity to tuberculin. Their dermal reactions to 0.5 μg of PPD-S or 1:2500 dilution of old tuberculin (in 0.1 ml) were read at 24 to 48 hours.

Following cellular transfer, recipient guinea pigs were tested with 50 μg of PPD-S in 0.1 ml.

The PPD-S used in these studies, lot 1112 made available by the kindness of Dr. Florence Seibert, was maintained in stock solution (5 mg/ml) from which fresh dilutions were prepared for each test.

RESULTS

Duration of Unresponsiveness.—That unresponsiveness to contact-type sensitization persists in allergen-fed guinea pigs for at least 9 months after the final feeding, may be seen substantiated by the data in Table I. Of the animals rested 48 through 94 days before sensitization was attempted (groups A to D) only 2 of 21 developed contact type reactions of very faint pink or greater to 1 per cent picryl chloride in contrast to intense reactions given by 19 of 22 non-fed, control animals. A waiting period of 276 to 298 days after the last feeding (groups E to G) allowed 6 of 19 or about a third of the total to develop sensitivity, a result still unlike that found among control animals, of which 11 out of 12 responded. When a second attempt to induce sensitivity was imposed upon some of the animals after 277 or 399 days (A, C), 2 of the 8 unresponsive at the first test became reactive. Whether their acquisition of reactivity was due to the second sensitization attempt or to the long elasped interval or to the two combined is unknown. Thus, only a slight decrease in the number of animals showing unresponsiveness was discernible at about 9 to 10 months despite vigorous and even repeated attempts to induce sensitivity.

Though the bulk of observations on duration of unresponsiveness was confined in this study to delayed dermal hypersensitivity, two tests were made for

[2] Lectro-mesh, nickel, 0.003 inch hole size as supplied by C. O. Jeliff Manufacturing Co., Southport, Connecticut.

circulating antibody production. Animals fed picryl chloride and later given several daily intradermal injections of the compound (2.5 μg in 0.1 ml) failed to show local reactions of the Arthus-type associated with circulating antibody, regardless of the interval between feedings and injections. In addition, intraperitoneal injections of picrylated guinea pig serum proteins totaling 25 mg and given in 5 divided doses at various times after the cessation of feeding did not easily provoke an anaphylactic state, although instances of anaphylaxis were

TABLE I

Persistence in Guinea Pigs of Unresponsiveness to Sensitization with Picryl Chloride (PCl) Induced by Feeding the Hapten

Guinea pig group	Time between last PCl feeding* and attempted sensitization‡	Contact-type sensitivity to PCl in animals§	
		Fed PCl	Not fed PCl
	days		
	First attempt		
A	48	0/4	5/5
B	73	1/8	6/8
C	85	1/5	5/5
D	94	0/4	3/4
E	276	3/6	5/6
F	277	0/4	6/6
G	298	3/9	
	Second attempt		
A	277	1/4	6/6
C	399	2/5	

* Animals of groups A, C, F, G received 15 feedings; those in B, D, E received 20.

‡ By the intracutaneous method.

§ Number of animals with minimum dermal reaction of very faint pink to 1 per cent PCl at 24 or 48 hours over the number subjected to sensitization and test. Most of the control animals shown (not fed PCl) exhibited high reactivity.

found among some of the long rested animals. At 149 days after the last feeding, 3 of 7 animals exhibited anaphylaxis (2 died) and the other 4 remained inert to the shocking dose (5 mg picrylated casein) while with a rest interval of 296 days, 2 of 5 animals succumbed to shock and 3 were negative. Thus, resistance to producing circulating antibody to hapten in hapten-fed guinea pigs appeared to be as long-lived as resistance to developing delayed-type hypersensitivity toward the hapten.

A Search among Unresponsive Guinea Pigs for Antibody Absorbing Hapten Depots.—

To determine whether depots of hapten exist in hapten-fed guinea pigs that might serve to absorb antibody from the circulation, the rate of clearance of passively administered,

hapten-specific antibody was compared in hapten-fed and control animals. Picryl chloride-fed animals rested 68 or 87 days and normal, weight-paired controls were given 0.57 to 1.0 ml of an antipicryl guinea pig serum pool known to contain 1500 PCA units per ml (Table II). In this way, 50 to 70 PCA units per ml of serum were established at the outset in animals

TABLE II

Persistence of Picryl-Specific Antibody Administered to Picryl Chloride-Fed and Normal Guinea Pigs, as Measured by Passive Cutaneous Anaphylaxis (PCA) and Hemagglutination (HA) Tests

Guinea pig			Time since last feeding	Anti-serum trans-fused*	PCA units per ml serum					Reciprocal of HA titer‡ (at hours)	Confirmation of unresponsiveness	
No.	Weight	Status			0 hrs	12 hrs.	17 hrs.	24 hrs.	216 hrs.		Time since last feeding	Final contact test§
	gm		*days*	*ml*						48 hrs.	*days*	
1	647	PCl-fed	68	1.0	70		40		5	20	93	U
2	636	Normal	—	1.0	70		40		4	20	—	R
3	756	PCl-fed	68	1.0	60		32		1	40	93	U
4	731	Normal	—	1.0	60		40		9	80	—	R
5	786	PCl-fed	68	1.0	55					20	93	U
6	794	Normal	—	1.0	55					20	—	U
7	854	PCl-fed	68	1.0	50		39		8	40	93	U
8	824	Normal	—	1.0	50		40		9	10	—	R
										24 hrs.		
9	712	PCl-fed	87	0.63	40	21		24		80	199	U
10	714	Normal	—	0.63	40	19		25		20	—	R
11	635	PCl-fed	87	0.57	40	21		18				
12	630	Normal	—	0.57	40	26		16				
										120 hrs.		
13	640	PCl-fed	87	0.57	40					20	199	R
14	638	Normal	—	0.57	40					20	—	U

* Guinea pig serum pool 324 containing 1500 PCA units per ml; 1 PCA unit, sufficient antibody to elicit 14 to 17 mm blue reaction at 30 minutes.

‡ Last tube to show a difference in sedimented pattern from that of controls when doubling dilutions of serum in saline (0.5 ml) were mixed with 0.05 ml tannic acid treated sheep erythrocytes coated with picrylated guinea pig serum (preparation XVI).

§ To one drop 1 per cent picryl chloride in olive oil applied dermally 2 weeks after attempted sensitization by intracutaneous method. Animals were judged unresponsive, U, when erythema read at 24 or 48 hours was less than very faint pink; responsive animals, R, showed erythema between faint pink and pink, usually the latter.

1 to 8 (636 to 854 gm) and 40 units in animals 9 to 1-4 (630 to 714 gm). Bleedings, taken from each animal at intervals between 12 and 216 hours, were tested by passive cutaneous anaphylaxis.

A comparison of the amount of antibody remaining in animals of pairs 1 and 2, 3 and 4, and 7 and 8 at 17 hours showed a slightly smaller quantity in

the picryl-fed individual of 1 pair (No. 3 of 3 and 4) but no significant differences were evident between individuals of the other 2 pairs. The results were essentially repeated with sera taken from the same animals at 216 hours, though in these samples fewer PCA units were detected. Bleedings at 12 and 24 hours from animals 9 and 10 and 11 and 12, given less antibody in an effort to increase the likelihood of detecting differences, also showed the hapten-fed individual of each pair to have as much antibody circulating as its non-fed partner.

The sera from some of the animals were examined, as well, by the Boyden hemagglutination test in which tannic acid–treated sheep erythrocytes coated with picrylated guinea pig serum proteins served as antigen. Sera from 3 pairs of animals, 1 to 2 and 5 and 6 taken at 48 hours and 13 and 14 taken at 120 hours (Table II), showed identical agglutination titers of 1:20. Of the remaining animals bled at 24 and 48 hours, the hapten-fed individual of 1 pair (No. 3 of 3 and 4) had a smaller titer than its partner whereas No. 7, of 7 and 8, and 9, of 9 and 10, had larger titers than their partners.

Though the hapten-fed guinea pigs of Table II were not subjected to attempted sensitization prior to the infusion of antibody, other guinea pigs fed hapten along with them were, and they were shown to be unresponsive. Only after the rate of clearance of antibody had been determined (93 to 199 days from final feeding) was sensitization of the serum-infused animals undertaken by the intracutaneous method. Two weeks after the last picryl chloride injection, each animal was dermally tested with 1 per cent picryl chloride in olive oil. Five of 6 animals were unresponsive (final column of Table II); the one exception (No. 13) may have failed at the outset to convert to the unresponsive state or its status may have changed in the 112 days elapsed between the final feeding and the attempted sensitization. Notable is the fact that catabolism of the administered antibody led in these animals to no apparent loss of unresponsiveness. No reason is known, other than age why normal recipients 6 and 14 did not undergo allergic conversion.

Results of hemagglutination tests on consecutive bleedings from 2 pairs of hapten-fed and normal guinea pigs infused with hapten-specific antibody 68 and 33 days after the last hapten feeding are shown in Table III. Animals 1 and 2 (identical with those of Table II) received 1 ml of the serum pool 324 rendered from animals immunized by the combination method, whereas animals 15 and 16 received 2 ml antipicryl serum 1431–33 obtained from guinea pigs immunized by the intracutaneous method. A comparison of the post-transfusion titers of sera taken at 17 through 216 hours showed them to be essentially alike for animals of each pair and what differences were observed between guinea pigs 15 and 16 were considered to be within experimental error.

Another test for an extracellular hapten depot was made, though the data are not shown in the tables. From a pool of spleen cells, taken from immunized animals at a time when they were known to be producing antipicryl antibody, a

quota (0.37 ml packed volume) was injected into a picryl chloride fed and a normal guinea pig. The serum of both cell recipients, drawn on the 5th day, and examined for antibody by PCA, showed equal concentrations of specific antipicryl antibody present.

Attempt to Transfer Sensitivity with Cells of Unresponsive Guinea Pigs Subjected to Sensitization Procedures.—In normal animals, two types of immunologic response may be stimulated by injections of chemical allergen into the skin, namely, antibody production and delayed contact-dermatitis. The question naturally arose whether the immunologic apparatus of allergen-fed, unresponsive animals actually remains unstimulated when sensitization is at-

TABLE III

Detection, by Boyden Hemagglutination Test (HA), of Picryl-Specific Antibody Administered to Picryl Chloride-fed and Normal Guinea Pigs

Guinea pig			Time since last feeding	Antisera transfused*	Reciprocals of HA titers‡ for sera taken					
					Pretransfusion 24 hrs.	Post-transfusion				
No.	Weight	Status				17 hrs.	24 hrs.	48 hrs.	96 hrs.	216 hrs.
	gm		*days*	*ml*						
1§	647	PCl-fed	68	1.0	3	48		12	12	12
2§	636	Normal	—	1.0		48		12	12	12
15	510	PCl-fed	33	2.0	<20		320	320	160	
16	516	Normal	—	2.0	<20		640	640	80	

* Antipicryl serum pool 324 given animals 1 and 2 and pool 1431-33 given 1-5 and 1-6. See text for preparation of pools.

‡ Read as in Table II.

§ Data on these animals appear also in Table II.

tempted, or whether a mechanism exists for "masking" these two sorts of response. Although many observations were made in hapten-fed guinea pigs exposed to sensitization procedures which contravened the idea that excitation is elicited, tests upon the behavior of their spleen and lymph node cells in new hosts devoid of hapten were thought necessary.

Picryl chloride-fed and normal guinea pigs that were to serve as cell donors were exposed to the combination sensitizing procedure for two experiments and to the intracutaneous sensitizing method in two others. The normal guinea pigs treated by the combination method became highly sensitive to contact with the chemical allergen; those selected as donors all responded well to $\frac{1}{10}$ per cent picryl chloride and half of these reacted well to $\frac{1}{150}$ per cent. By contrast, most allergen-fed guinea pigs treated in parallel developed a definite but moderate degree of sensitiveness only to the 1 per cent concentration. From

among them, donors with least sensitivity (responses of 0 to very faint pink) were selected so that any reaction evoked from recipients would reflect sensitivity masked in the donors; there was no reason to arrange the test to be a transfer of the positive rather low sensitivity that had been excited in some of the others. Both groups of animals became equally sensitive to tuberculin, for which no tolerance existed, due to the killed tubercle bacilli and paraffin

TABLE IV

Inability of Lymphoid Cells from Picryl-Tolerant Guinea Pigs to Transfer "Picryl" Dermal Sensitivity to Normal Guinea Pigs

Prior treatment of cell donors	Method of attempted sensitization	Cell origin	Sedimented cell volume‡	Donors per recipient	Contact dermal reactions to 1 per cent picryl chloride§				Tuberculin test‖ (50 μg PPD-S)
					T-1	T-2	T-3	T-4	
			ml						
None	Combination	Lymph nodes	0.10 to 0.20	1; 1.5	5/5	5/5	1/3		4/5
		Spleens	0.13 to 0.21	1; 1.5	4/5	5/5	2/3		
Picryl chloride fed 15 times	"	Lymph nodes	0.25; 0.57	3; 4	0/2	0/2	0/1	0/1	2/2
		Spleens	0.39; 0.57	3; 4	0/2	0/2	0/1	0/1	2/2
None	Intracutaneous	Lymph nodes	0.13 to 0.22	3; 4.5; 5	3/3	3/3	2/2	2/2	
		Spleens	0.10 to 0.57*	2.5; 3; 6	1/3	1/3	0/2	0/2	
Picryl chloride fed 15 or 20 times	"	Lymph nodes	0.30; 0.44	6.5; 7; 5	0/2	0/2	0/1	0/1	
		Spleens	1.00*	8	0/1	0/1	0/1	0/1	

‡ Cells were injected intravenously up to a cell volume of 0.4 ml; excess cells and splenic cells starred* were given intraperitoneally.

§ The first contact test (T-1) was made the day after transfer of cells. Recorded are the number of animals with reactions greater than any on toxicity control animals (*i.e.* very faint pink) over the total tested.

‖ Along with T-2, some recipients were tuberculin-tested; the number with 24 hour reactions of 8 mm in diameter or greater over the total tested are shown.

oil present in the injected material. Average reactions to 0.5 μg PPD-S were 14 to 17 mm of erythema and induration with 9 mm white margin around 5 mm of central livid area.

From normal guinea pigs subjected to the combination method of sensitization (Table IV), lymph node and spleen cells were transferred under minimal conditions so as to achieve a donor to recipient ratio of 1:1 or 1.5:1. This was accomplished in the first experiment by 0.1, 0.13, and 0.15 ml of packed lymph node cells and 0.13, 0.13, and 0.21 ml spleen cells from 3 individual guinea pigs into separate recipients. In the second experiment, pools of lymph node cells and of spleen cells from 3 individuals were used; 0.14, and 0.20 ml lymph node

cells, 0.14, and 0.20 ml spleen cells were given separate recipients. In contrast, pooled cells from unresponsive donors were always transferred in cell volumes equivalent to two or four times as many donors per recipient in order to favor detection of any reactivity. In two experiments, 0.25 and 0.57 ml lymph cells were given to recipients; 0.39 and 0.57 ml spleen cells to other recipients.

As may be seen in Table IV, the lymph node cells from sensitized guinea pig donors established picryl chloride contact dermatitis, detectable at tests 1 and 2, in all 5 normal recipients. The reactivity of some recipients diminished thereafter (T-3). Tuberculin sensitivity was detected, also, in 4 of the 5 despite use of small cell volumes. Splenic cells from the same donor animals transferred picryl chloride sensitivity to all 5 recipients (T-2), although reactivity in them did not arise altogether in parallel with that of the lymph node cell recipients. In sharp contrast lymph node and spleen cells of 3 and 4 allergen-fed, unresponsive, guinea pig donors failed to provoke any sure sign of contact sensitivity to picryl chloride in 4 recipients. Nevertheless, these lymph node and spleen cells were entirely competent to transfer hypersensitivity for tuberculin to each of the four, since the donors' unresponsiveness was directed only to picryl chloride. Indeed, the reactivity to tuberculin was greater in these recipients than in control recipients, undoubtedly due to the greater number of cells they had received.

To see if picryl-specific antibody was produced by the transferred cells, some recipients were bled (2 ml) on the 4th and 9th days after cell transfer and their sera tested by PCA. Though sera from recipients of either 0.57 ml nodal or splenic cells from unresponsive donors showed none, picryl-specific antibody was found in the 9 day sample from the recipient of only 0.2 ml nodal cells from the non-fed, sensitized donors.

In the remaining experiments of Table IV, sensitization of the animals was attempted by the intracutaneous method. The sensitivity developed in "non-fed" control animals was minimal (reactions to 1 or ⅓ picryl chloride solutions were seen), consequently, larger donor to recipient cell ratios were necessary to effect transfer of dermal reactivity. From cell pools of 5 animals, 0.18 ml lymph node cells and 0.1 ml spleen cells were given separate recipients; likewise, from a pool of 9 guinea pigs, 0.13 and 0.22 ml lymph node as well as 0.29 and 0.57 ml spleen cells were given separate individuals. Thus, for "control" lymph node cells, the donor-recipient ratios were 3:1, 4.5:1, and 5:1 and for "control" spleen cells they were 2.5:1, 3:1, and 6:1.

The picryl chloride–fed donors-to-be, with one exception, responded not at all or with mere trace reactions to 1 per cent picryl chloride. From one group of 7 selected as donors, 0.30 ml lymph node cells were given one recipient (the recipient of the spleen cells from this animal pool died) and from a second group of 8 animals, 0.44 ml lymph node cells were given one recipient and 1.0 ml spleen cells given another to effect donor to recipient ratios of 6.5:1 and 7.5:1 for the nodal and 8:1 for the spleen cells.

Again, lack of reactivity of the 2 recipients of lymph cells from allergen-fed donors contrasted markedly with the delayed contact reactions elicited in each of the 3 animals receiving nodal cells from "non-fed" donors. Though the splenic cells of the non-fed donors given in small amounts (0.1 and 0.29 ml) did not convert their respective recipients to reactivity, the largest quantity (0.57 ml) did. An even larger quantity of splenic cells from allergen-fed donors (1.00 ml) failed, however, to convert their recipient.

In none of the four experiments summarized in Table IV, therefore, was evidence found that white cells, from guinea pigs fed allergenic chemicals before receiving allergenic stimuli, induced onset of dermal hypersensitivity or circulating antibody when placed in new hosts. In addition, these cells were unable to confer unresponsiveness upon their new hosts; a series of intracutaneous injections of picryl chloride, started 10 days after transfer, converted each animal to sensitivity.

DISCUSSION

Guinea pigs fed simple chemical allergens have their future immunological response to these same chemical allergens markedly altered; they do not respond to intradermal injections of the particular allergen with contact-type hypersensitivity or with circulating antibody even though the sensitizing procedure causes normal animals to develop both. Allergen-fed animals rested prior to receiving injections of allergen remain unresponsive to developing contact hypersensitivity for periods in excess of 9 months; a few become slightly responsive between 9 and 11 months but none revert to the status of "new" animals. Indeed, second attempts to sensitize allergen-fed guinea pigs do not cause the majority to respond. The duration of such unresponsiveness was not ascertained with greater precision beyond one year primarily because of the large numbers of animals required and because older guinea pigs, in general, were found not to undergo sensitization with regularity.

Resistance to forming circulating antibody appears equally as durable as the inability to develop delayed sensitivity primarily when allergen-fed guinea pigs are injected intradermally with allergen or intraperitoneally with allergen conjugated to guinea pig serum proteins. When given allergen coupled to more highly antigenic moieties (*i.e.* bovine gamma globulin), unresponsive animals regain the ability to form allergen-specific circulating antibody (3, 17, 19), but not dermal (contact-type) hypersensitivity.

Of the picryl chloride fed, the little which succeeds in passing the gut wall would seem to be of chief importance for establishing unresponsiveness. The fate of such molecules, apart from the many hydrolyzed to picric acid, may well be to couple with cell membranes, fixed tissues, and soluble proteins or even to exist intracellularly, and in this way account for the long duration of unresponsiveness. As with pneumococcal polysaccharide for which there is no known *in vivo* enzymatic system for degradation, picryl radicals would seem to be stable for some time. In the first approach to understanding the mechanism of immunological unresponsiveness, a search for extracellular

depots of picryl radicals was made by infusing homologous antipicryl antibody to see whether it disappeared from the circulation of picryl chloride–fed animals faster than from the circulation of control animals. Despite use of antibody prepared by two different immunization techniques, no consistent difference in disappearance of antibody was found. In addition, lymphoid cells transferred from picryl-immunized animals produced antipicryl antibody equally in picryl chloride–fed and normal recipients. Transfer of minimally effective amounts of lymphoid cells from picryl chloride sensinized animals to normal and picryl chloride–fed recipients was already known to lead to identical degrees of contact-type hypersensitivity (13, 15). Thus, unresponsiveness appears not to be explained by absorption of antibody upon extracellular hapten or antigen depots (*cf.* references 29, 40, 22, 11) or by annihilation (stunting) of immunologically competent cells (*cf.* references 10, 31, 20, 37, 34, 26).

Hirata, Garvey, and Campbell (28), in a report on chickens made incapable of antibody production by injection of labeled bovine serum albumin at hatching, found little of the antigen retained (0.3 per cent of 20 to 180 mg). The authors state, "The smallness of the amount of antigen retention suggests that the suppression of antibody response is not due to 'absorbing out' of the antibody produced." Indeed, immunological paralysis in mice has long been known to be produced by minute doses of polysaccharide (24, see also reference 38) and more recently the same has been shown true of an allergen (4) and a protein antigen (23, 5).

Another approach to studying the mechanism of immunological unresponsiveness was to test the functional ability of lymphoid cells of allergen-fed and allergen-injected animals in normal guinea pigs. Some impetus for this test came from observing minimal, transient delayed reactions in hapten-fed animals given repeated intradermal injections of allergen; removal of their cells to a new hapten-free environment might allow maturation of any initiated immunological process. That such cells did not confer delayed dermal sensitivity or circulating antibody specific for picryl chloride upon their hosts, yet did transfer tuberculin hypersensitivity, was interpreted as an indication the cells had remained unstimulated in the presence of the chemical allergen. In repetitions of this type of experiment, Brooke and Karnovsky (9) found that lymphoid cells of mice with immunological paralysis toward pneumococcal polysaccharide did not protect normal mice from subsequent challenge with homologous-type pneumococci and Smith (39) found that lymphoid cells from protein-tolerant animals did not give rise to antibody formation in x-irradiated recipients.

Whether lymphoid cells from unresponsive animals are able to transfer unresponsiveness to normal recipients was not settled by these experiments. Though such recipients given picryl chloride injections became sensitive in a regular manner, the 10 day interval between cell transfer and initiation of stimulation with hapten may have been excessive. In addition, donor and recipient animals were not histocompatible. Though inbred mice were used by others (32, 1), claims for transfer of unresponsiveness *via* spleen cells appear premature. Experimental design in each instance leaves doubt about whether tolerance was induced *de novo* (*cf.* reference 32) and also about whether a mechanism for unresponsiveness was truly donated to immunologically inert cells of x-rayed recipients (1).

That animals may be rendered immunologically unresponsive to hapten (42, 12), to bacterial polysaccharides (24), to proteins (27), and to homografts (6) would seem

to point to a common underlying principle which may well be a successful penetration of antigen into sites of both mature and immature cells potentially capable of antibody production before the stimulus to make antibody is effectively received (17). Smith (39) too, has suggested that antigen encountered at a critical phase in a cell's development would allow access of antigen to later inaccessible sites where it unites with "pre-antibody" nucleic acid, to block subsequent steps in antibody synthesis.

Others have suggested that tolerance is simply insufficient stimulation of competent cells either through rapid degradation (leakage) of antigen (see Medawar, reference 33; Denhardt, reference 21) or because antigens lack "adjuvanticity" (see Dresser, reference 23; Claman, reference 18); or because haptens are carried on "self"-proteins (19).

The multiplicity of explanations for unresponsiveness attest to the fact that its underlying mechanism remains unknown.

SUMMARY

Guinea pigs fed picryl chloride to induce specific immunologic unresponsiveness cleared small amounts of venously infused antipicryl antibody at a rate equal to that of normal guinea pigs.

Catabolism of passively administered picryl-specific antibody did not alter the unresponsive state of picryl chloride–fed guinea pigs or the responsive state of normal guinea pigs.

Lymphoid cells of picryl chloride immunized guinea pigs produced equal amounts of picryl-specific antibody in picryl chloride–fed and normal animals.

Allergen-fed guinea pigs remained unresponsive to attempted sensitization with the allergen in excess of 10 months after the final feeding, though some became feebly sensitive between 9 and 11 months. Second attempts to make unresponsive animals hypersensitive were unsuccessful.

White blood cells of guinea pigs unresponsive to picryl chloride were unable to transfer delayed-type hypersensitivity for picryl chloride to normal recipients yet readily transferred tuberculin hypersensitivity.

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