CONCOMITANT ANAPHYLACTIC SENSITIZATION AND CONTACT UNRESPONSIVENESS FOLLOWING THE INFUSION OR FEEDING OF PICRYL CHLORIDE TO GUINEA PIGS*

By JEROME R. POMERANZ,‡ M.D., AND PHILIP S. NORMAN,§ M.D.

(From the Allergy and Infectious Disease Division, Department of Medicine, The Johns Hopkins University and Hospital, Baltimore)

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Hypersensitivity to simple chemicals in guinea pigs has been considered as a model for human drug hypersensitivity, because cutaneous application or injection of simple chemical haptens regularly sensitizes this species. Although drug hypersensitivity in human beings often involves sensitization to drugs administered orally or intravenously, sensitization of guinea pigs by feeding or infusion of simple chemicals has not been recognized. Instead, the oral or intravenous administration of chemical haptens to guinea pigs induces a state of immunologic tolerance or paralysis to subsequent attempts to sensitize actively by intracutaneous injections (1, 2).

The drug doses that humans ingest or are given intravenously are generally quite large when compared to the amounts of simple chemical haptens necessary to induce unresponsiveness in guinea pigs. This raises the possibility that the administration of large doses of hapten to guinea pigs by the intravenous or oral routes might initiate the process leading to immunologic paralysis and yet actively sensitize the animal at the same time.

The following experiments show that intravenous injection or feeding of comparatively large doses of picryl chloride to guinea pigs results in the usually observed rate of immunologic unresponsiveness to delayed contact sensitization. Concomitantly, anaphylactic sensitization detectable by picryl protein conjugates occurs in many of the animals irrespective of the presence or absence of contact unresponsiveness.

Materials and Methods

Animals.—Randomly bred, male Hartly strain albino guinea pigs obtained from Lightner Enterprises, Thompsontown, Pennsylvania were used in all studies.

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‡ Present Address: Department of Dermatology, Cleveland Metropolitan General Hospital, Cleveland.

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Antigen.—Picryl chloride (Eastman Organic Chemicals, Rochester, New York) was recrystallized three times from a hot mixture of two parts absolute alcohol and one part benzol. After drying for several days in a desiccator over phosphorus pentoxide, the crystals were ground to a fine powder (3).

Solvents and Techniques Used for Intravenous Injection.—Picryl chloride was dissolved in the following solvent mixtures: (*a*) dimethylsulfoxide-saline, 1:1; (*b*) ethanol-saline, 1:1; (*c*) ethanol-glycerine-saline, 2:1:1; (*d*) ethanol-glycofurol[1]-propyleneglycol-saline, 1:1:1:2; (*e*) glycofurol-saline, 1:1; and (*f*) ethanol-glycofurol-saline, 1:1:2. In all instances the picryl was dissolved in dimethylsulfoxide, ethanol, or glycofurol first and then glycerine, propyleneglycol, and/or saline was added.

The guinea pigs were immobilized and an incision made on the inner aspect of the thigh to expose the subcutaneous vein. A new 26 gauge needle was inserted into the vein for each infusion and 1.0 ml of solvent containing the desired dose of picryl chloride was slowly administered. If there was any evidence of infiltration or extravasation during the injection the animal was discarded. After the needle was removed, pressure was applied for 30 to 60 sec and a thin layer of antibiotic ointment spread over the wound. When the animal resumed its normal stance, the skin margins were approximated, and the wounds generally healed without difficulty.

Solvents and Techniques Used for Feeding.—Picryl chloride was dissolved in the following solvents: (*a*) dimethylsulfoxide; (*b*) olive oil; (*c*) ethanol; (*d*) ethanol-glycerine, 3:1; and (*e*) ethanol-propyleneglycol, 1:1. The animals were deprived of solid food for 15 to 18 hr prior to feeding. Then they were suspended from a metal wire by the incisor teeth and a polyethylene tube inserted through the esophagus into the stomach. Three quarters or 1.5 ml of solvent containing picryl chloride was slowly administered and then flushed with 0.25 ml of solvent alone. Solid food was reintroduced 3 hr after the feeding.

Picryl Conjugates.—The following conjugates were prepared by the method of Benacerraf and Levine (4): picrylated bovine albumin, 13 groups/mole, picrylated guinea pig serum, 93 mg/g conjugate.

Detection of Circulating Antibody.—Blood was obtained from the orbital sinus 13 to 14 days after infusion or feeding of picryl chloride and the sera were stored at —20°C for later testing. The presence of antipicryl antibody was determined as follows:

Passive Cutaneous Anaphylaxis (PCA).—0.1 ml of each sera (undiluted or diluted with saline) was injected intradermally into the back of a normal guinea pig weighing 270 to 320 g. 16 to 18 hr later the animal was given an intravenous injection of 1 ml 0.5% Evans blue (Matheson, Coleman, and Bell, Cincinnati, Ohio) containing 5 mg picryl bovine albumin. The reactions were read at 30 min. The antibody titer was determined by comparison with a positive control prepared by the injection of picrylated guinea pig serum in adjuvant.

Detection of Contact Reactivity.—Each guinea pig was tested by allowing 0.4 ml of 1, 0.3, and 0.1% concentrations of picryl chloride in acetone-olive oil (4:1) to spread from the tip of a pipette onto the shaved flank. This covered an area of about 7 $cm.^2$ They were read at 24 hr as follows: ±, faint pink spots; +, faint pink confluent macular erythema; ++, pink confluent macular erythema; +++, bright pink confluent erythema with a slightly thickened elevated edge; ++++, confluent bright pink erythema with distinct thickening, elevation, and necrosis.

Detection of Immunologic Unresponsiveness.—Active sensitization was attempted by the intracutaneous injection of 0.1 ml saline containing 5 μg of picryl chloride into each of six sites in the nuchal region, followed by a booster of 3 additional injections 7 days later. 2 wks

[1] Tetrahydrofurfurylalcohol, generously supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

after the initial series the animals were contact tested and bled to obtain sera for later determination of PCA antibody. This series of intracutaneous injections regularly induced contact reactivity, and often anaphylactic reactivity as well in the strain of guinea pigs used. Failure to develop contact reactivity was interpreted as indicating the presence of immunologic unresponsiveness.

Sensitizing Capacity of Plasma or Red Cells.—Plasma and red cells were obtained at intervals after an infusion or feeding of picryl chloride. The red cells were washed three times with saline prior to use. 1 ml of plasma or washed, packed red cells was emulsified with an equal amount of complete Freunds adjuvant $H_{37}Ra$, (Difco Laboratories, Inc., Detroit), and injected into two recipients; 0.1 ml into each foot-pad and 0.2 ml into three places in the neck. 14 days later the recipients were bled from the orbital sinus to obtain sera for later PCA antibody determination and then they were tested for contact sensitivity. When indicated, they were intravenously challenged with 0.5 ml of picryl bovine albumin 13 groups/mole to determine the presence of anaphylactic reactivity.

RESULTS

Anaphylactic Sensitization by Infusion or Feeding of Picryl Chloride

A major obstacle to giving picryl chloride in large amounts is its poor solubility in water and the necessity to use organic solvents, most of which are toxic. Dimethylsulfoxide (DMSO), an unusually nontoxic solvent, overcomes this problem. Guinea pigs infused or fed comparatively large doses of picryl chloride in DMSO, commonly develop anaphylactic reactivity (5). However, it was later noted that picryl chloride reacts with DMSO and is partially converted to picric acid, a less effective sensitizer of different specificity (6). The reaction of picryl chloride in DMSO tends to make dosage data only approximate, but does not alter the fact of sensitization. It was found that other solvents such as ethanol, glycerine, propyleneglycol, and glycofurol or combinations thereof did not react with picryl chloride and could be employed as relatively nontoxic solvents.

Sensitization by Infusion of Picryl Chloride.—Significant numbers of guinea pigs receiving an intravenous injection of picryl chloride developed immediate reacting antibody by the 14th day as shown by positive passive cutaneous anaphylaxis (Table I). Sensitization required 13 to 14 days to appear as earlier bleedings did not show the presence of anaphylactic antibody. The titers were low, rarely more than 1:2, and usually 1:1 or less. The rate of sensitization was dose dependent, about 60% having been sensitized by a 3 mg dose. Larger doses could not be used because they were toxic; doses of 4 mg resulted in death in over a third of the animals and doses above 4 mg were uniformly fatal. The solvent used does not seem to play a specific role other than as a vehicle for delivering a relatively large amount of picryl.

A small proportion of the infused animals also developed contact reactivity. This may have been due to minimal extravasation through the vein into the subcutaneous tissue that was not noticed at the time of the injection. In contrast to the large dose needed to sensitize by the intravenous route, small

amounts of picryl will regularly induce contact reactivity and often antibody as well if injected subcutaneously in the femoral region (Table II).

Sensitization by Feeding Picryl Chloride.—Table III shows the results of

TABLE I

Intravenous Injection of Picryl Chloride

Dose	Solvent	Contact reactivity*	PCA*
mg			
0.5	Ethanol-saline, 9:1	0/6	1/6
	Ethanol-glycofurol-saline, 1:1:2	1/6 1+	0/6
1.5	Ethanol-saline, 1:1	2/6 2+, 2+	2/6
	Ethanol-glycofurol-saline, 1:1:2	2/5 1+, Trace	1/5
2.5	Ethanol-saline, 1:1	2/11 1+, Trace	4/11
3.0	Ethanol-glycerine-saline, 2:1:1	1/10 Trace	5/10
	Ethanol-glycofurol-propyleneglycol-saline, 1:1:1:2	1/6 Trace	4/6
	Ethanol-glycofurol-saline, 1:1:2	1/11 2+	7/11
	Glycofurol-saline, 1:1	0/4	2/4
4.0	Ethanol-glycofurol-saline, 1:1:2	0/4	3/4
Controls	Ethanol-saline, 1:1 Ethanol-glycofurol-saline, 1:1:2	0/6 0/15	0/6 0/15

* Numerator indicates number reactive; denominator indicates number tested.

feeding large doses of picryl chloride in various solvents to guinea pigs deprived of solid food for 15 to 18 hr previously. A significant number fed 20 or 30 mg developed anaphylactic reactivity. As in animals receiving intravenous injections of picryl chloride, the titers of PCA antibody were low. Again the response seems to be dose related in that feeding 10 mg did not result in antibody formation. Contact reactivity was virtually absent. In feeding experiments, it was

important to deny the animals solid food for 15 to 18 hr beforehand. Feeding large doses of picryl to unstarved animals never resulted in the production of anaphylactic type hypersensitivity. Presumably starvation enhances absorption and prevents the conjugation of picryl with stomach contents. Similarly,

TABLE II

Subcutaneous Injection of Picryl Chloride in Ethanol-Saline

Dose	Volume	Contact reactivity*	PCA*
mg	*ml*		
0.5	0.5	4/4 4+, 4+, 3+, 3+	4/4
0.05	0.05	3/4 3+, 3+, 1+	2/4

* Numerator indicates number reactive; denominator indicates number tested.

TABLE III

Feeding of Picryl Chloride

Dose	Solvent*	Contact reactivity‡	PCA‡
mg			
10	Ethanol-propyleneglycol, 1:1	0/10	0/10
20	Ethanol-propyleneglycol, 1:1	0/12	4/12
30	Ethanol-propyleneglycol, 1:1	0/16	8/16
30	Ethanol (absolute)	1/11 Trace	3/11
30	Olive oil	0/6	0/6
37.5	Ethanol-glycerine, 3:1	0/4	2/4
0.0	Ethanol (absolute)	0/5	0/5
0.0	Ethanol-propyleneglycol, 1:1	0/8	0/8

* Total volume of solvent was 1.75 ml except for absolute ethanol where it was 1 ml.

‡ Numerator indicates number reactive; denominator indicates number tested.

the use of a rapidly absorbed solvent was also necessary. Of those fed 30 mg of picryl in olive oil, which is slowly absorbed, none developed antibody.

Immunologic Tolerance in Guinea Pigs Given one Infusion or Feeding of Picryl Chloride

In view of the well established phenomenon of immunologic unresponsiveness occurring in guinea pigs receiving multiple doses of hapten by infusion or feeding, an effort was made to determine the presence of tolerance in animals infused or fed one large dose of picryl chloride.

Tolerance in Picryl Chloride–Infused Guinea Pigs.—Following bleeding and contact testing at 2 wk, the animals that had received 0.5, 3.0, and 4.0 mg intravenously (solvent, ethanol-glycofurol-saline, 1:1:2) were given a series of intracutaneous injections in an attempt to induce delayed type contact reactivity (see Materials and Methods). Any animals that were shown to have

TABLE IV

*Contact Tolerance in Picryl Chloride–Infused Guinea Pigs**

Dose	Result of initial infusion	Result of attempted sensitization	
	PCA antibody	Contact reactivity to 1% PCl‡	PCA
mg			
0.5	0	+++	0
	0	+	Positive
	0	Trace	0
	0	0	0
	0	0	0
3	0	++	Positive
	Positive	+	0
	0	+	0
	Positive	0	Positive
	Positive	0	Positive
	Positive	0	0
	Positive	0	0
	0	0	0
4	Positive	+++	Positive
	Positive	+	Positive
	Positive	0	0
	0	0	0
Control	0	++++	Positive
	0	+++	Positive
	0	+++	Positive
	0	+++	Positive
	0	+++	0
	0	+	Positive
	0	+	0
	0	+	0
	0	+	0

* Tested 28 to 30 days following initial infusion and 14 to 15 days after first series of intracutaneous injections.

‡ Only animals that were contact negative as a result of the initial infusion were used in this experiment.

contact reactivity as a result of the initial infusion were excluded from this experiment. The results are shown in Table IV. In each dosage group a significant number of the animals demonstrated unresponsiveness. Among those that developed contact reactivity as a result of the trial sensitization, PCA antibody appeared in several that were not sensitized by the initial infusion. Six out of ten control animals developed picryl-specific antibody in addition to contact reactivity as a result of the intracutaneous sensitization.

Tolerance in Picryl Chloride-Fed Guinea Pigs.—Table V shows the results of attempted sensitization by intracutaneous injections in guinea pigs fed 10 or 30 mg of picryl chloride in ethanol-propyleneglycol, 1:1, or 30 mg in olive oil. In animals administered 30 mg picryl chloride in ethanol-propyleneglycol, unresponsiveness to contact sensitization was demonstrated irrespective of prior presence or absence of PCA antibody. Guinea pigs receiving picryl in olive oil were uniformly unresponsive.

Sensitizing Capacity of Plasma and Red Cells Obtained from Guinea Pigs Receiving an Infusion or Feeding of Picryl Chloride

Sensitization by the intravenous or oral routes presumably results from coupling with a tissue protein to form a complete antigen. Picryl chloride injected intravenously first comes in contact with components of blood and the vessel wall. Since it is not practical to study possible conjugation to the vessel wall by simple means it was decided to investigate the possibility of conjugation with blood components, specifically plasma and red cells. As a means of detecting conjugates formed in vivo, plasma or red cells from treated animals were injected with adjuvant into another animal. Microgram amounts of hapten protein complex can be detected by their ability to sensitize when used in this manner.

Sensitizing Capacity of Plasma or Red Cells following an Infusion of Picryl Chloride.—Plasma and red cells were obtained from guinea pigs that had received an intravenous injection of 2.5 mg of picryl chloride in ethanol-saline, 1:1, or 3 mg in ethanol-glycofurol-saline, 1:1:2, 6, 24, and 72 hr previously. The plasma or washed red cells from each donor were emulsified with adjuvant and injected into two recipients. Table VI shows the results in the recipients when tested at 14 days. If one of the recipients developed contact reactivity or PCA antibody, the donor was considered positive. It can be seen that red cells obtained up to 72 hr following an infusion will sensitize another animal when used with adjuvant. In contrast, plasma from such animals was not detectably antigenic.

Sensitizing Capacity of Plasma or Red Cells following Feeding of Picryl Chloride.—Similar experiments were carried out on plasma and red cells obtained at 2, 6, and 24 hr after a feeding of 30 mg picryl chloride in ethanol-propyleneglycol. Washed red cells from two of three donors bled at 2 hr were

TABLE V

*Contact Tolerance in Picryl Chloride-Fed Guinea Pigs**

Solvent	Dose	Result of initial feeding	Result of attempted sensitization	
		PCA antibody	Contact reactivity‡ to 1% PCl	PCA
	mg			
Ethanol-propyleneglycol, 1:1	10	0	+	0
		0	+	0
		0	+	0
		0	Trace	0
		0	0	0
		0	0	0
		0	0	0
	30	Positive	+	0
		0	+	0
		Positive	Trace	Positive
		Positive	0	Positive
		0	0	0
		Positive	0	0
		0	0	0
		Positive	0	0
		0	0	0
Olive oil	30	0	0	0
		0	0	0
		0	0	0
		0	0	0
		0	0	0
		0	0	0
Ethanol-propyleneglycol, 1:1	Controls	0	++++	Positive
		0	+++	0
		0	+++	0
		0	++	Positive
		0	++	0
		0	++	0
		0	++	0
		0	++	0

* Tested 28 to 30 days following initial feeding and 14 to 15 days after first series of intracutaneous injections.

‡ Only animals that were contact negative as a result of the initial feeding were used in this experiment.

able to anaphylactically sensitize the recipients (Table VII). Red cells obtained at later bleedings were not effective, nor was the plasma.

TABLE VI

*Sensitizing Capacity of Plasma or Red Cells Obtained at Intervals from Picryl Chloride-Infused Guinea Pigs**

Component	Solvent	Dose	Time after infusion of picryl chloride when blood was obtained from donor animals					
			6 hr		24 hr		72 hr	
			Contact reactivity‡	PCA‡	Contact reactivity‡	PCA‡	Contact reactivity‡	PCA‡
		mg						
Plasma	Ethanol-saline, 1:1	2.5	0/0	0/0	—	—	—	—
Red cells	Ethanol-saline, 1:1	2.5	0/3	2/3	0/3	3/3	—	—
Plasma	Ethanol-glycofurol-saline, 1:1:2	3.0	1/3 Trace	0/3	—	—	—	—
Red cells	Ethanol-glycofurol-saline, 1:1:2	3.0	2/3 +++, +	3/3	2/3 +, +	3/3	0/3	1/3

* Recipients tested at 14 days.

‡ Numerator indicates number reactive; denominator indicates number tested.

TABLE VII

*Sensitizing Capacity of Plasma or Red Cells Obtained at Intervals from Picryl Chloride-Fed Guinea Pigs**

Time after feeding of picryl chloride when blood was obtained from donor animals.

Component	2 hr		6 hr		24 hr	
	Contact reactivity‡	PCA‡	Contact reactivity‡	PCA‡	Contact reactivity‡	PCA‡
Plasma	0/3	0/3	0/3	0/3	—	—
Red cells	0/3	2/3	0/3	0/3	0/3	0/3

* All donor guinea pigs received one feeding of 30 mg picryl chloride in ethanol-propyleneglycol. Recipients tested at 14 days.

‡ Numerator indicates number reactive; denominator indicates number tested.

DISCUSSION

Sensitization of guinea pigs to unconjugated simple chemicals by the intracutaneous route usually results in both delayed contact hypersensitivity and the formation of antibodies detectable by protein conjugates of the chemical (3). In contrast, these experiments using the intravenous or oral routes have

more often produced anaphylactic hypersensitivity with relatively infrequent contact sensitivity. The absence of significant delayed contact reactivity in the experimental animals perhaps indicates that carefully infused or fed hapten does not come into contact with skin protein and form a conjugate capable of stimulating contact hypersensitivity. Instead, the process leading to specific unresponsiveness to contact sensitization is often stimulated.

The doses employed were huge when compared with those which sensitize by the cutaneous routes. As the dose increased, anaphylactic sensitization appeared more regularly. Feeding was a considerably less efficient method of producing anaphylactic sensitization, possibly because the hapten travels a more circuitous route before conjugation with a suitable carrier protein occurs. However, use of a rapidly absorbed solvent, partial starvation of the recipients beforehand, and a tenfold increase in dose resulted in a rate of anaphylactic sensitization by the oral route comparable to that seen with intravenous injection. Possibly starvation restricts loss through conjugation or alteration by gastric contents, and so enhances absorption of the hapten. When a slowly absorbed solvent was substituted none of the recipients formed circulating antibody. This failure to achieve sensitization may be due to the inability of picryl to reach appropriate combining sites in sufficient amounts.

As picryl chloride is a reactive chemical that combines readily with protein, presumably the infusion or feeding of large doses results in the formation of a hapten somatic protein complex capable of stimulating antibody formation. Infused picryl would be expected to combine with protein in the circulation and possibly the vessel walls. Similarly, fed picryl might also be expected to conjugate with protein in the circulation, if a sufficient quantity was rapidly absorbed so that some hapten entered the circulation while still unreacted. That red cells from picryl-infused or fed guinea pigs sensitized virgin animals when used with adjuvant demonstrates that the erythrocyte is a significant site of hapten attachment for picryl administered by those routes. Serum proteins were not similarly effective. The role of the white cells, vessel walls, and gastric mucosa will require further investigation.

The presence of picryl specific circulating antibody was often accompanied by unresponsiveness to contact sensitization to picryl by intracutaneous injection. Concomitant immediate type hypersensitivity and tolerance to contact sensitization would appear to represent divergent responses to a single antigenic stimulus. Battisto and Chase have suggested that delayed and immediate hypersensitivity are independent responses, although at times directed towards the same antigenic stimulus (7). Similarly, unresponsiveness has immediate and delayed components which appear to be independent of each other in that they can be overcome separately by appropriate sensitizing stimuli. An immunologically unresponsive guinea pig may be stimulated with heterologous protein hapten conjugate to synthesize specific circulating antibody without affecting contact tolerance. More intense stimulation with picrylated erythro-

cyte stromata combined with cutaneous application is required to modify contact tolerance, and then only a low level of sensitization is achieved.

In our experiments, increasing the intravenous or oral dose did not alter the rate of unresponsiveness to subsequent contact sensitization. Contact tolerance appeared with greater regularity when a single large dose of picryl was given in a slowly absorbed solvent to an animal deprived of solid food prior to feeding. Although the mechanism for the more uniform appearance of contact unresponsiveness is not apparent, this may be an efficient "one shot" method of producing unresponsiveness to contact sensitization by feeding the hapten. Further experiments are indicated to determine the minimum conditions necessary for the production of tolerance.

That hapten-protein complexes on cells induce immunologic unresponsiveness is suggested by the related observations of Battisto and Bloom, who reported that guinea pig erythrocyte stromata and spleen cells picrylated in vitro and infused without adjuvant have the capacity to induce immunologic unresponsiveness to contact sensitization (8). Furthermore, in one group receiving picrylated spleen cells, detection of circulating antibody by PCA was noted prior to the demonstration of contact unresponsiveness.

While it seems clear that an appreciable amount of infused or ingested picryl is linked to the red cell, the nature of the attachment remains to be shown. Infused or fed picryl may conjugate with a protein constituent of the red cell such as lipoprotein in the membrane or hemoglobin. Alternatively, the picryl may remain unconjugated, dissolved in the lipid phase of the red cell membrane (9). In any event, the removal of these "damaged" erythrocytes by the reticuloendothelial system could provide a stimulus for both antibody production and contact tolerance by effects upon different clonal populations of cells.

SUMMARY

Guinea pigs receiving one large dose of picryl chloride by the intravenous or oral routes commonly develop circulating antibody demonstrable by passive cutaneous anaphylaxis or by active anaphylaxis. They often concomitantly become unresponsive to the induction of delayed contact hypersensitivity by intracutaneous injections. Erythrocytes obtained from guinea pigs after infusion or feeding of picryl chloride may be used to sensitize other animals when injected with adjuvant.

It is concluded that guinea pigs may be anaphylactically sensitized to simple chemicals by the intravenous and oral routes if a sufficient dose is administered.

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