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Immunological Unresponsiveness Produced in Adult Guinea Pigs by Parenteral Introduction of Minute Quantities of Hapten or Protein Antigen.* (27717)

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As has long been known (1-8), immunological unresponsiveness to haptenic chemicals may be established in adult guinea pigs by repeatedly feeding the chemicals. Animals fed a particular hapten no longer respond to injection of the hapten (*i.e.,* to the derivative antigens formed by conjugation *in vivo*) either with production of specific circulating antibody or delayed-type contact hypersensitivity. Early attempts to make animals unresponsive by dermal or intravenous hapten administration produced sporadic rather than reproducible results(1,7,9). Parenteral inoculation of adult animals with proteins and polysaccharides effects immunological tolerance but only when the antigens are injected in massive amounts(10,11).

The present authors have recently succeeded in inducing unresponsiveness to picryl chloride by introducing minute doses of the hapten into the mesenteric veins of adult guinea pigs(12). The following report shows the effectiveness of this method for making adult animals immunologically tolerant not only to a simple chemical hapten (picryl chloride) but also to a protein antigen (bovine gamma globulin).

Experimental. In a typical experiment demonstrating induction of unresponsiveness to a hapten, sexually mature guinea pigs were inoculated *via* mesenteric veins with 50γ of picryl chloride in 2 ml of 1% ethanol-0.85% NaCl solution on 2 occasions, one week apart; control animals were similarly injected with 2 ml of saline. To make the inoculations, an animal was positioned on its side upon a restraining board, the hair about its midsection clipped and, under ether anesthesia, a 2 cm ventrolateral incision made just posteriorly to the last rib. A loop of small intestine was exposed sufficiently to allow entry into one of the smaller tributaries of the mesenteric vein with a 30-gauge, 3/8-inch needle. Upon completing the injection, made slowly to prevent rupture of the tiny vessel, the vein was ligated above and below the puncture site; after ascertaining that no bleeding occurred, the intestine was returned to the peritoneal cavity, and the incision sutured. The wound was covered with a thin layer of bacitracin ointment; but left unbandaged. The second inoculation, 7 days later, was made from the opposite side.

After resting 25 days, the animals were examined for capacity to respond with delayed

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TABLE I. Specific Unresponsiveness of Guinea Pigs to Attempted Sensitization with Picryl Chloride Following Pretreatment with the Hapten.

Pretreatment with picryl chloride	Guinea pig No.	Delayed reactions following attempted sensitization with picryl chloride and BGG: Reaction to picryl chloride* 1%	1/3%	1/10%	Reaction to 5γ BGG*
2 x 50γ *via*	1	0	0	0	+±, 11 mm
mesenteric	2	0?	0	0	++, 12 "
vein (Day 0 and	3	0?	0	0	++, 12 "
7); animals	4	±	0?	0	++, 8 "
rested 25 days	5	+	±	±	++, 13 "
None. Saline	6	++	+	0	0 , 0 "
via mesenteric	7	+±±	+	0	+±, 11 "
vein (Day 0 and	8	+±±	+±	±	++, 11 "
7); animals	9	+++	++	±	+, 13 "
rested 25 days	10	+++	+++	+±	++, 4 "

* Intensity of erythema indicated as: 0 = negative; ± = negative at 24 hr, doubtful at 48 hr; + to +++ = very faint pink to pink at 24 hr. Diameters are listed only for the bovine gamma globulin reactions as each area is made uniform in the contact test with picryl chloride.

hypersensitivity. Test and control animals were inoculated intracutaneously with 2.5γ of picryl chloride in 0.1 ml of 1% ethanol-0.85% NaCl solution into each of 6 sites in the nuchal region; 8 days later, 2 additional intradermal injections of 2.5γ of picryl chloride were made in the same region. Estimation of the extent of sensitization induced was made a week later by applying 0.05 ml of each of 3 solutions of picryl chloride (1%, 1/3%, 1/10% in olive oil) to separate dorsal skin sites from which hair had been closely clipped. The response in each site was read at 24 and 48 hours. To control the specificity of refractoriness 2.5γ bovine gamma globulin (BGG, Armour Co. Lot No. S30005 in 0.05 ml saline) was injected into each hind foot pad of all animals. Eight days later each animal was tested intradermally with 5γ BGG in 0.1 ml saline and the sites read at 24 and 48 hours(13).

The data (Table I) show that guinea pigs were made refractory toward developing delayed contact hypersensitivity to picryl chloride by 2 prior mesenteric vein inoculations of this hapten. Experimental animals No. 1-5 failed to react to 1/3-1% solutions of picryl chloride, whereas the sham-operated control animals No. 6-10 responded well to both the 1% and 1/3% solutions. The unresponsiveness was specific; test and control guinea pigs manifested equal sensitivity of delayed type to bovine gamma globulin.

To determine whether pretreatment with hapten given in different doses and by different parenteral routes would influence the extent of unresponsiveness, individual guinea pigs were given 2 doses, 7 days apart, of 50, 12.5 or 2.5γ of picryl chloride by mesenteric vein; others received 2 intraperitoneal or jugular vein injections of 50γ on the same schedule; an additional few were injected *via* mesenteric vein with 50γ on one occasion; still others were given a single inoculation of 50γ or 100γ into a jugular vein. After a 2-week rest period, these animals and sham-operated controls were tested for capacity to respond with, first, delayed hypersensitivity (intradermal injections of picryl chloride and contact tests were performed as for the experiment of Table I) and, second, immediate-type sensitivity (4 i.p. injections of 5 mg of picrylated pooled guinea pig serum proteins‡ were made over a two-week period, followed 18 days later by i.v. challenge with 1-15 mg of picrylated casein.)‡

As may be seen in Table II, the dose of hapten used to pretreat the animals influenced the acquisition of tolerance to contact hypersensitivity; 4 of 6 animals given 5γ of

‡ Picrylation of pooled guinea pig serum proteins and of casein was accomplished(14) by adding 120 mg picryl chloride per gram of protein; the resulting products contained approximately 25 mg and 75 mg of picryl groups per gram of conjugate.

TABLE II. Effect of Route and Dosage Schedule of Picryl Chloride Pretreatment of Guinea Pigs Upon Inhibiting the Development of Delayed- and Immediate-Type Hypersensitivity.

Group	Pretreatment with picryl chloride*		Contact skin reactions†	Anaphylactic reactions to subsequent i.v. dose of picrylated casein‡ 5-15 mg	1-2 mg
A	Mesenteric vein	2 × 2.5γ	4/6		
B	" "	2 × 12.5γ	7/15		
C	" "	2 × 50γ	4/17	+++, ++, +, ±, 0	
D	" "	1 × 50γ	3/4	D, +, +, 0	
E	Jugular vein	1 × 100γ	4/4		
F	" "	1 × 50γ	3/4	D, D	D
G	" "	2 × 50γ	4/7		
H	Peritoneal cavity	2 × 50γ	10/12	D, D, +, 0, 0	
I	Mesenteric vein	saline	28/29	D	D, D, +++, +

* Six animals of Group B received the second hapten injection after 14 rather than 7 days, but both sets of animals reacted alike.

† No. of animals showing + to +++ reactions to 1% picryl chloride over total No. of animals subjected to attempted sensitization and test. Tests and reading as in Table I.

‡ D = dead within 30 min. (usually within 5 min.); ++ to +++ = respiratory distress, incontinence, mild to severe convulsions, but with recovery; ± to + = mild to moderate respiratory distress and incontinence; 0 = no more than wiping or chewing. Shock dose given 18 days after attempted sensitization course of 4 i.p. injections of 5 mg picrylated guinea pig serum proteins.

hapten showed contact skin reactions (Group A), 7 of 15 pretreated with 25γ responded (B), but of those given 100γ of hapten only 4 of 17 reacted (C). In contrast, 28 of 29 controls developed delayed contact hypersensitivity (Group I). A single intravenous injection of picryl chloride did not produce tolerance to contact sensitivity, as reactions were seen in 3 of 4 animals pretreated with 50γ of hapten *via* the mesenteric vein (D), and in 7 of 8 given 50γ or 100γ *via* the jugular vein (E, F). Nor were intraperitoneal or jugular vein routes particularly effective even though two 50γ doses of hapten were used; 4 of 7 treated *via* jugular veins (G), and 10 of 12 pretreated intraperitoneally (H) reacted normally.

Pretreatment of animals with one or 2 doses of picryl chloride into mesenteric veins suppressed acquisition of anaphylactic as well as delayed contact sensitivity (Table II) for, when challenged intravenously with 5-15 mg of picrylated casein, only 1 of 9 animals died and 2 others reacted severely but recovered. By contrast, 5 of 8 animals pretreated with hapten intraperitoneally or *via* jugular vein, and 3 of 5 sham-operated controls died in shock; the latter had been challenged with smaller, 1-5 mg doses of picrylated casein.

To see whether immunological tolerance in-

TABLE III. Suppression of Immunological Responses of Guinea Pigs to Bovine Gamma Globulin by Prior Injections of BGG.

Pretreatment with BGG *via* mesenteric veins	Delayed skin reactions to BGG* 10γ	100γ	Subsequent reactions to 5 mg BGG i.p. Death in 1-15 hrs	Antibody in survivors† 1000-5000	10,000-100,000
2 × 500γ Day 0, 7	1/5	–	2/5	2	1
2 × 50γ " 0, 2	1/6	1/6	–	–	–
2 × 50γ " 0, 7	1/11	3/6	0/5	5	0
2 × 50γ " 0, 14	0/6	1/6	–	–	–
2 × 5γ " 0, 7	1/5	–	2/5	0	3
	4/33	5/18	4/15	–	–
None	20/24	7/7	13/17	2	2

* No. of animals showing erythema greater than 6 mm at 24 hr over total No. of animals subjected to attempted sensitization and skin test.

† Reciprocals of titers *vs* BGG-coated tanned erythrocytes.

duced by divided intramesenteric vein doses of hapten might be applicable beyond chemical haptens that spontaneously couple to body proteins, attempts were next made to induce unresponsiveness using a complete antigen, bovine gamma globulin. BGG that had been centrifuged for 15 minutes at 30,000 *g* to remove undissolved particles(15) was injected in 3 different doses (5, 50 or 500γ) into the mesenteric veins of individual guinea pigs on 2 occasions from 2 to 14 days apart. Twenty-six days after the first injection, 2.5γ of uncentrifuged BGG was inoculated into each hind foot pad to induce delayed hypersensitivity(13). Skin tests made 11 days later with 10γ of BGG, and 7 days thereafter with 100γ, were read at 24 and 48 hours. Subsequent to skin testing, some animals were injected intraperitoneally with 5 mg of BGG in 1 ml saline to assess their ability to form circulating antibody; serums taken 14 days later were assayed for antibody content by Boyden hemagglutination tests(16) using BGG-coated sheep erythrocytes.

The data show clearly that delayed hypersensitivity to BGG was regularly inhibited by pretreatment of adult animals with the antigen *via* mesenteric veins in doses ranging from 10γ-1000γ (Table III). Only 4 of 33 experimental animals manifested delayed skin reactions to a test dose (10γ) of the antigen to which 20 of 24 sham-operated controls responded. The subsequent skin test with 100γ of BGG detected reactivity in 7 of 7 controls and only 5 of 18 experimental animals.

Unexpectedly, some of the animals that were given 5 mg of BGG intraperitoneally for the purpose of stimulating circulating antibody formation showed immediate signs of distress, *i.e.*, coughing, leaping, incontinence. Death occurred in 1-15 hours in 13 of 17 controls, but only in 4 of 15 experimental animals. Hemagglutination tests on the sera of surviving animals revealed circulating antibody to BGG in all; however, sera from 7 of 8 animals pretreated with 100 or 1000γ BGG showed low titers (1:1000-1:5000) compared with titers of 1:10,000-1:100,000 found for 2 of 4 surviving control animals. Even though some of the pretreated animals died and the remainder showed circulating antibody, a decided quantitative difference existed in the immunological responses of pretreated and control animals.

Discussion. Guinea pigs pretreated with gamma amounts of hapten or protein *via* mesenteric veins have profoundly reduced abilities to respond immunologically to the specific substances used in pretreatment; they no longer can be provoked into delayed cutaneous hypersensitivity or stimulated to produce more than small quantities of circulating antibody. Although induction of delayed hypersensitivity appears more completely inhibited than induction of circulating antibody production, the apparent difference may simply reflect the use of more sensitive methods presently available for detecting serum antibody.

A precise comparison of incidence and degree of tolerance induced in guinea pigs pretreated with picryl chloride *via* mesenteric vein and oral routes cannot be made as no information exists concerning the amount of picryl chloride that enters tissues when the hapten is fed nor of the maximum duration of the altered response induced when the hapten is injected into mesenteric veins. Nevertheless, the tolerance obtained by using the mesenteric vein technic would appear to compare favorably with that produced by the feeding method (cf. 1,7). The mesenteric vein technic offers special advantages over the feeding method. It is simple, less time-consuming, permits precise control over the amount of hapten entering the tissues and allows tolerance studies upon haptens and hapten-protein conjugates that do not pass the gastrointestinal wall.

Though a small proportion of hapten administered orally has been stated to localize to guinea pig gastrointestinal wall through spontaneous conjugation(6,7), the relationship of intestinal wall-hapten conjugate to tolerance induction has never been clear. The successful production of unresponsiveness by injecting hapten into mesenteric veins indicates that conjugates in the gastrointestinal tract are not prerequisite for tolerance induction. Whether localization in the liver occurs and is of significance in effecting tolerance is unknown.

That gamma amounts of protein produce unresponsiveness in adult guinea pigs contrasts sharply with the massive doses of proteins needed for tolerance induction in adult rabbits(10). Should the mesenteric vein route for administration of minute amounts of antigens be successful in species other than the guinea pig and, perhaps, in inducing tolerance of homografts (experiments in progress), the necessary foundation will be provided to structure a single mechanism for the phenomenon of tolerance.

Summary. Delayed hypersensitivity and circulating antibody production to a hapten (picryl chloride) and to a protein antigen (bovine gamma globulin) were regularly suppressed in mature guinea pigs injected twice into mesenteric veins prior to sensitization, with hapten or protein in gamma amounts.

ADDENDUM: Following preparation of this manuscript, a report by D. W. Dresser has appeared (*Immunol.*, 1962, v5, 378) on induction of unresponsiveness in adult mice by parenteral administration of minute doses of a protein antigen.

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Measurement of Total Body Iron[59] in Animals Using Whole-Body Liquid Scintillation Detectors. (27718)

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The construction of large-volume, 4-pi liquid scintillation detectors permits direct measurement of total-body gamma radiation in humans and animals(1-3). The purpose of this paper is to describe the reliability of this method in determining the retention and rate of excretion of Fe^{59} in rats. The biologic problems encountered with iron are described.

Materials and methods. Adult male Canadian Hooded rats weighing 235-411 g were used in the study. Rats were raised in a pathogen-free environment and housed in wire cages. Their diet contained 15 mg of iron per 100 g of dry weight and average daily ingestion was estimated to be 1.5-2.5 mg of iron. Some rats were iron depleted by bleeding 4 ml weekly for 4 weeks prior to study and fed a milk-powder iron-deficient diet. Other rats were iron-loaded with 4 intramuscular injections of 25 mg of elemental iron as dextran-iron at weekly intervals.

The test dose of iron consisted of 0.1 or 0.25 μc of Fe^{59} as citrate in 0.25 ml of water with various carrier doses of elemental iron as ferrous sulfate. Oral test doses were administered through an olive-tipped 17-gauge endo-