INHIBITION OF TUBERCULIN SKIN HYPERSENSITIVITY IN GUINEA PIGS BY INJECTION OF TUBERCULIN AND INTACT TUBERCLE BACILLI DURING FETAL LIFE

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The term "actively acquired tolerance" was coined by Billingham, Brent, and Medawar in 1953, and defined by them as "an induced, specific, central failure of the mechanism of immunological response brought about by an exposure of animals to antigenic stimuli before maturation of the faculty of immunological response" (1).

The possibility of inducing specifically acquired immunological tolerance was first suggested by Burnet and Fenner in 1949 (2). To explain the absence of antibody responses to autologous antigenic material under normal conditions, they postulated a "self-marker" mechanism by which antibody-forming cells learn to recognize, and not to react immunologically towards, antigenic tissue components present at the time of maturation of the antibody-forming system. They thought it possible that foreign antigenic material might also be recognized as autologous if brought into contact with antibody-forming cells at a critical stage in their maturation. As evidence for the natural occurrence of such a circumstance, Burnet and Fenner cited the observations of Owen (3–5) and Traub (6–8). Owen found that twin cattle, which exchange blood in fetal life through anastamoses of placental vessels, are frequently red blood cell chimeras, each twin possessing blood cells of its own as well as of its sibling's genetic type. Traub made the observation that mice naturally infected *in utero* with the virus of lymphocytic choriomeningitis produced no complement-fixing antibodies in adult life although they remained carriers of the virus, whereas healthy animals infected experimentally in adulthood produced such antibodies.

The experimental induction of immunological tolerance was first demonstrated indirectly by the prolonged survival of tissue grafts exchanged between embryonic or newly hatched birds (9–12). These experiments were not, however, designed as studies

of immunological tolerance, and the findings were thought to be demonstrations of the antigenic adaptation of transplanted tissues (13). The first experimental attempts directed at producing immunological tolerance by artificial means were reported by Billingham, Brent, and Medawar who found that mice injected in fetal life with tissue suspensions of mice of another, highly inbred strain were able in adulthood to accept skin grafts from animals of the donor strain (1).

Specifically acquired immunological tolerance has since been demonstrated in a variety of animals, towards a number of unrelated antigens. Billingham, Brent, and Medawar have recently reviewed the literature on this topic (14) and only a few of the diverse immunological situations in which tolerance has been achieved need be mentioned here to indicate that this is an immunological phenomenon of wide application.

Tolerance to skin homografts has been demonstrated in mice, rats, rabbits, and birds, and to heterologous cells in birds (14). Red blood cell chimerism, and inhibition of the formation of isoagglutinins even after chimerism has disappeared, has been achieved in chickens by artificial twinning of the embryos (14, 15), and in rats by the intravenous injection of the fetuses with hematopoietic cells of different genetic type (16). Tumor homografts have been successfully transplanted to mice of normally refractory strains by injecting the animals in utero or shortly after birth with the malignant cells (14, 17); tolerance to tumor homografts has also been induced by pretreating mice with fetal injections of normal tissue of animals of the donor strain (18). The formation of antibodies to chicken red blood cells has been inhibited in turkeys by injecting them with chicken blood in ovo, and antibody formation to turkey red blood cells has been similarly inhibited in chickens (19).

That specific immunological tolerance can be induced by non-living as well as by "living" cellular antigens, is suggested by the observations of Hanan and Oyama (20), Dixon and Maurer (21), and Cinader and Dubert (22) that the antibody response of rabbits to foreign protein antigens—bovine or human serum albumin— could be markedly reduced by injecting the newborn animals with moderate quantities of the foreign protein.

Evidence has also accumulated that immunological tolerance may occur widely in nature. Anderson et al. (23) and Billingham, Lampkin, Medawar, and Williams (24) found that twin cattle (which have a common fetal circulation) were usually tolerant of each other's skin grafts, as well as being red blood cell chimeras. Red blood cell chimerism has also been discovered in man (25) and lambs (26). Further indication of the natural development of tolerance in man comes from the observations of Owen, Wood, Foord, Sturgeon, and Baldwin (27) that sensitization to Rh antigen is considerably less frequent in Rh-negative women whose mothers are Rh-positive than in those whose mothers also are Rh-negative, the suggested explanation being that the mother's Rh antigen induced tolerance during the fetal life of her daughter. It has also been suggested by Billingham et al. (14) that the establishment in man of melanomatous tumors of maternal origin (28) may have a similar explanation: tumor cells passing the placental barrier may induce tolerance to themselves in the fetus and initiate progressive development. Other evidence that tolerance to cells of maternal antigenic specificity may come about by placental passage is suggested by the observation that guinea pigs occasionally tolerate maternal, but seldom, if ever, paternal skin homografts (14).

Tolerance to microbial antigens has also been indicated. Traub's findings (6–8) that mice infected *in utero* with lymphocytic choriomeningitis fail to produce complement-fixing antibodies have already been mentioned. Kerr and Robertson reported that the antibody response to *Trichomonas foetus* is reduced considerably in calves injected intramuscularly with this organism shortly after birth (29). Buxton found that injection of killed *Salmonella pullorum* early in life reduced the resistance of chickens to a later oral challenge with the living bacilli (30). On the other hand, Fox and Laemmert (31) and Burnet, Stone, and Edney (32) observed no reduction in the antibody response to influenza virus and bacteriophage of chickens first injected with these antigens *in ovo*, and subsequently 4 to 9 weeks after birth.

The possibility of rendering animals natively incapable of specific antibody responses to microbial antigens suggests a new approach to the study of acquired immunity to infectious disease: The role played by a particular antibody response in the host-parasite relationship might be ascertainable by comparing the immunogenesis and pathogenesis of the disease in normal animals with that in animals rendered specifically tolerant to a given microbial antigen.

A study was initiated to determine whether the faculty of responding hypersensitively to tuberculin could be abolished in guinea pigs by making them immunologically tolerant to tuberculoprotein, and to then observe the development of immunity and disease in such animals after vaccination and experimental infection (33). The purpose of this communication is to describe the results obtained in exploratory attempts to prevent by fetal injection of Old Tuberculin (the concentrated, filtered, and heated culture fluid in which tubercle bacilli have been grown for several weeks (34)) and intact tubercle bacilli the development of tuberculin sensitivity after vaccination in adult life with living and non-living vaccines.

Experimental Methods

Animals.—Guinea pigs of the smooth haired, albino Dunkin-Hartley strain, bred at the Sir William Dunn School of Pathology at Oxford, were employed. Their diet consisted of standard guinea pig pellets (diet 18C, Christopher Hill Ltd., Poole, Dorset, England), hay, fresh greens (cabbage, kale, and alfalfa), and water, *ad lib.*

For mating, one adult male was placed in a pen with six to eight females for 5 days. Pregnancy was determined by abdominal palpation 3 weeks after mating. The females found to be pregnant at this time were segregated individually in metal nesting boxes, and the non-pregnant animals were remated.

In an initial experiment, guinea pigs were injected intraperitoneally with Old Tuberculin (O.T.) within 24 hours after birth. In subsequent experiments, the animals were injected in fetal life. Unlike the pregnant mouse, whose embryos can be brought into view by gentle manipulation and injected through the mother's body wall after a skin incision, the guinea pig must be subjected to laparotomy to visualize and inject its embryos. The following operative procedure was employed:—

The hair of the abdominal area was removed with an electric razor 1 hour before operation. Anesthesia was introduced by the intraperitoneal injection of 1.8 cc. of a 1:10 saline dilution of Nembutal (pentobarbital sodium). When the animals had become drowsy—8

to 10 minutes after injection—they were secured on an electrically warmed operating table. The abdominal skin was washed with a detergent disinfectant (dettol). Anesthesia was continued, and maintained throughout the operation, by dropping anesthetic ether on a soft cloth so wrapped around the head of the animal as to form a cone raised 1 inch over its nostrils. A midline incision through the skin and body wall, from 1 inch below the diaphragm to one inch below the umbilicus, was made after the breathing of the animals had become deep and regular, and the uterine horns exposed by gentle digital manipulation. The exposed viscera was covered with pieces of sterile gauze soaked in an isotonic saline solution heated to 37°C., and care was taken to reduce manipulation of the intestines and other organs to a minimum. There was usually only very little bleeding when the incision was directly along the midline; the occasional hemorrhage was light, and could be stopped readily by clamping the bleeding vessel with a hemostat.

The fetuses were injected through the uterine walls with 0.1 cc. quantities of aqueous suspensions of the antigen. Injection was with a No. 27 needle, the length of the needle varying with the proximity of the fetus to the uterine wall. It was found early in the course of these experiments that injection into the skull or pelvic or pectoral girdles of the fetus almost always resulted in its death and abortion. The injections were always aimed, therefore, at the peritoneal cavity of the fetus; in the few instances where the fetus could not be manipulated into a position permitting abdominal injection, injection was made into the muscles of the thighs or back.

It was always attempted to inject all fetuses in a pregnant animal. When more than three were present, however, or when the fetuses were very large, it was occasionally impossible to reach the ones lying most anteriorly. In such cases it was impossible to ascertain which animals of a litter had been injected *in utero*. (In experiments currently in progress, trypan blue is added to the inoculum, so that the injected animals can be identified in litters in which not all can be injected.) The results reported in this communication are therefore limited to experiments in which every fetus of a pregnant animal was injected.

After injecting the fetuses, the incision was closed with interrupted stitches. The stitches were removed 8 to 10 days after the operation. The operations were performed under the usual aseptic surgical techniques.

In order to obtain a population of animals injected at different stages of embryological development, the time after mating at which laparotomy was performed and the fetuses injected was varied widely. As the period of gestation of guinea pigs of this stock varied from 64 to 72 days, and the females were placed in contact with a male for 5 days, only an approximation of the stage of gestation was possible at the time of operation. The age of the fetus at the time of injection could therefore be expressed accurately only as days before birth.

Antigens Injected in Utero.—Fetal guinea pigs were injected with one of the following substances: O.T., intact tubercle bacilli of the BCG strain killed by heating or by exposure to phenol, or living BCG.

The O.T. was derived from cultures of human type virulent tubercle bacilli, and was obtained through the courtesy of Dr. A. B. Paterson of the Veterinary Laboratory, Ministry of Agriculture, Fisheries, and Food, Weybridge, England. It was stored at 4°C., and was diluted in 0.85 per cent saline diluent immediately before use.

The BCG cultures were of the Phipps strain (35), and were obtained through the kindness of Dr. Cynthia H. Pierce of The Rockefeller Institute for Medical Research. The bacilli were grown in Dubos medium (36) containing 1.0 per cent glycerol, but neither tween nor albumin. The medium was distributed in 350 cc. quantities in 3 liter Blake bottles, and inoculated after sterilization with 20 cc. aliquots of an actively growing culture in tween-containing Dubos medium. The inoculated bottles were incubated statically for 4 weeks, at which time a pellicle covering the surface of the medium had formed.

Killing with phenol was accomplished by adding liquid phenol (88 per cent) to the cultures to a final concentration of 2 per cent. The phenolized cultures were kept at room temperature for 12 to 16 hours, with frequent manual shaking. The cultures were then decanted through a muslin cloth filter, and the phenol removed from the bacillary mass by washing three times with 500 cc. amounts of distilled water and three times with similar quantities of acetone. The removal of phenol was evidenced by a negative Folin-Ciocalteu reaction (37) on the last washing. The bacillary mass was then transferred in acetone to a large crystallizing dish, and the solvent permitted to evaporate under mild negative pressure at room temperature. The dry bacillary mass was ground finely with an agate mortar and pestle, and stored over anhydrous calcium chloride at 4°C. Suspensions of the phenolized bacilli were made by grinding them in saline diluent in a teflon tissue grinder, similar to that used in the grinding of mouse tissues (38), immediately before injection.

To obtain heat-killed bacilli, cultures were grown in Dubos medium containing 1.0 per cent glycerol and 0.05 per cent tween 80. The medium was distributed in 50 cc. quantities in 250 cc. Erlenmeyer flasks, and inoculated with 1.0 cc. aliquots of an actively growing culture. The cultures were incubated for 7 days at 37°C. with frequent manual agitation, and immediately before use they were placed for 20 minutes in a water bath heated to 70°C.

That all bacilli were killed by heat or phenol treatment, was shown by the absence of growth when they were inoculated into a variety of fresh media.

Tuberculin Testing.—Hypersensitivity to tuberculin was ascertained by injecting the animals intradermally (i.d.) with 0.2 cc. quantities of saline dilutions of Weybridge O.T. or purified protein derivative (P.P.D.) derived from human type tubercle bacilli. The site of injection was in the abdominal area, the hair being removed with an electric razor 1 hour before injection. The animals were observed at frequent intervals for 72 hours after injection, and the reactions recorded by measuring the largest and smallest diameters of edema or induration; these data are presented as the average diameter of edema or induration when the reaction reached its maximum ("maximum average diameter of edema or induration").

At each testing, every animal was also injected at an adjacent site with 0.2 cc. of saline; in no instance was the area of edema developing at this site larger than 4 mm. in diameter. A maximum average diameter of edema of 4 mm. or less was therefore considered to be a negative reaction; of 5 mm. or more, a positive one (34).

RESULTS

Injection of Newborn Guinea Pigs with Old Tuberculin.—

Twelve guinea pigs from six litters (A to F) were injected intraperitoneally within 24 hours after birth with 0.2 cc. quantities of undiluted O.T. or with O.T. diluted 1:1,000 and 1:10,000 in saline. Three control animals (litter G) were injected with saline, and two others (litter H) with a 5 per cent solution of bovine serum albumin, fraction V. (Armour & Co., Chicago) to determine whether neonatal exposure to an unrelated protein would affect later tuberculin reactivity. Three animals (litters I and J) were left uninjected.

Six weeks after birth, all animals were tuberculin-tested by the intradermal injection of 0.2 cc. of a 1:10 dilution of O.T. Two weeks later, they were vaccinated by the subcutaneous injection of 0.25 cc. of a living 7 day old Dubos tween medium culture of BCG Phipps. Tuberculin tests were repeated 4 and 6 weeks after vaccination. The results of the tuberculin tests are shown in Table I.

The results presented in Table I show that none of the animals were tuberculin-positive previous to vaccination with living BCG. Four weeks after vaccination, 4 of the 5 animals which had received undiluted Old Tuberculin

TABLE I

Skin Reactivity to O.T. (0.2 Cc. of a 1:10 Dilution) of Guinea Pigs Injected at Birth with O.T. and Vaccinated 8 Weeks Later with Living BCG

Litter origin	Dilution of O.T. injected at birth, in 0.1 cc.	No. of animals tested	Tuberculin tests*		
			6 wks. after birth	12 wks. after birth (4 wks. after vaccination with living BCG)	14 wks. after birth (6 wks. after vaccination with living BCG)
			Maximum average diameter of edema‡		
			mm.	*mm.*	*mm.*
A	Undiluted	2	0	0	10
			0	0	10
B	"	3	0	0	10
			1	4	6
			2	8	6
C	1:100	1	3	6	17
D	"	2	0	8	9
			1	8	10
E	"	2	0	6	10
			1	12	12
F	1:10,000	2	0	6	20
			2	7	20
Control: G	Saline	3	0	8	12
			0	14	24
			4	12	18
Control: H	5 per cent bovine albumin, fraction V	2	0	7	10
			1	12	14
Control: I	Not injected	1	0	13	20
Control: J	"	2	1	13	12
			2	6	12

* An area of reaction having a maximum average diameter of edema of 4 mm. or less is considered to be negative.

‡ The maximum reaction in all instances developed between 30 to 36 hours after injection.

at birth failed to react to tuberculin while all others were tuberculin-sensitive. Two weeks later, the 4 negative animals had become tuberculin-sensitive as well. With the exception of 1 animal in litter E, the guinea pigs which had received diluted Old Tuberculin at birth appeared to have a smaller reaction when

tested 4 weeks after BCG vaccination than did most of the control animals, but the retest 2 weeks later revealed no such difference. These findings indicate that neonatal injection of tuberculin may produce a transient degree of tuberculin tolerance in a few animals.

In view of the relative maturity of guinea pigs at birth, it was thought that immunological tolerance could be bestowed more readily by prenatal exposure to antigen. In subsequent experiments, therefore, the animals were injected in fetal life.

TABLE II

Fate of Pregnant Guinea Pigs Whose Fetuses Were Injected in Utero with O.T.

Quantity of O.T. injected into fetuses, in. 0.1 cc.	No. of pregnant animals operated on	Died of postoperative complications	Survived but aborted or gave birth to dead litter	Gave Birth To living young
Undiluted	12	2	5	5
Diluted 1:10	15	1	1	13
Diluted 1:100	6	1	1	4
Diluted 1:1,000	3	1	0	2
Diluted 1:10,000	6	0	2	4
	42 +4*	5	9	28
Total.............	46			

* Died during operation.

Injection of Fetal Guinea Pigs with Old Tuberculin.—

Forty-six female guinea pigs at different stages of pregnancy were subjected to laparotomy. The fetuses of 12 were injected with undiluted O.T.; those of 15 with O.T. diluted 1:10; of 6 with O.T. diluted 1:100; of 3 with O.T. diluted 1:1,000; and of 6 with O.T. diluted 1:10,000. Each fetus received 0.1 cc. of inoculum. Four of the pregnant animals died during operation, before their fetuses were injected.

The fate of the pregnant females during and after operation is recorded in Table II.

As shown in Table II, 28 (60.8 per cent) of the animals survived operation and gave birth to living young; 4 (8.7 per cent) died during operation; 5 (10.8 per cent) succumbed to postoperative complications, in all instances a generalized peritonitis and bronchopneumonia; and 9 (19.5 per cent) aborted after operation or gave birth to dead litters. The abortion rate among the 37 animals surviving operation was thus 24.3 per cent. Five of the 9 abortions occurred among the 10 animals whose fetuses received undiluted O.T. (a rate of 50 per cent), and the other 4 abortions among the remaining 27 animals (a rate of only 14.8 per cent). It thus appears that fetal injection of undiluted tuberculin causes a high incidence of abortion because of a toxic effect on embryo or

mother, the trauma of injection alone resulting in relatively few cases of abortion. The 28 females which survived operation and did not abort gave birth to 66 young, of which 5 were stillborn (7.5 per cent). This proportion of dead young among litters of living animals was of approximately the same order as that found in our guinea pig breeding colony. It thus appeared that when injection of tuberculin killed a fetus, the entire litter was usually aborted.

Of the 28 litters of living young, 11 were excluded from further study because the number of animals born in these exceeded the number of fetuses which could be injected *in utero*, and identification of the injected ones was not possible. The remaining 17 litters contained 28 living young. Six weeks after birth, these were tuberculin-tested by the intradermal injection of 0.2 cc. of a 1:10 dilution of P.P.D. One week later they were divided into two groups: One group of 10 animals (litters K to O) was vaccinated intraperitoneally with 200 γ phenol-killed, acetone-washed BCG suspended in 1.0 cc. saline; the other group of 18 animals (litters P to Z, and aa) was similarly vaccinated with 800 γ of phenolized BCG. Two control groups of 8 guinea pigs each of the same age received 200 and 800 γ of the phenolized bacilli, respectively, and a third control group of 12 uninjected guinea pigs was maintained in the same animal room to test for the possibility of "accidental" or "non-specific" sensitization to tuberculin.

Five weeks after vaccination, all animals were again tuberculin-tested with 0.2 cc. of a 1:10 dilution of P.P.D. The results of the tuberculin tests are shown in Tables III and IV.

It is seen from Table III that vaccination with 200 γ phenolized BCG sufficed to render all 8 control animals tuberculin-sensitive, 6 showing a reaction of 10 mm. or greater. None of the 12 unvaccinated controls became tuberculin-sensitive.

None of the 10 animals injected in fetal life with O. T. were tuberculin-sensitive when first tested 6 weeks after birth, and 7 were found to have remained tuberculin-negative after vaccination with the phenolized tubercle bacilli. Of the 3 animals which were sensitized by vaccination with phenolized BCG only one was highly sensitive (a reaction of 10 mm. or greater).

The results depicted in Table IV show that the larger dose of phenolized BCG rendered all 8 control animals highly sensitive to tuberculin. In contrast, 8 of the 18 animals injected in fetal life with O.T. failed to become sensitized after vaccination with phenolized BCG, 3 were sensitized to a moderate degree (5 to 9 mm.), and only 7 developed tuberculin reactions larger than 10 mm. Here again, none of the animals were found tuberculin-sensitive at the first postnatal test; tuberculin sensitivity displayed at the second postnatal test was specifically due, therefore, to vaccination with phenol-killed BCG, and the absence of sensitivity to a state of tolerance to tuberculin.

The numbers of animals injected with different quantities of O.T., at different periods of time before birth, were too small to permit an evaluation of the importance of dose of antigen or state of embryological development at the time of exposure in the induction of tolerance. The results indicate, however, that injection of tuberculin as late in gestation as 1 to 3 days before birth can

prevent sensitization to tuberculin following vaccination with killed tubercle bacilli in early maturity. The results also indicate that the higher concentrations of tuberculin were more effctive in inducing tolerance; thus, of the 7 animals

TABLE III

Skin Reactivity to P.P.D. (0.2 Cc. of a 1:10 Dilution) of Guinea Pigs Injected in Utero with O.T. and Vaccinated 7 Weeks after birth with 200 γ Phenol-Killed BCG

Litter origin	Dilution of O.T. injected *in utero*, in 0.1 cc.	Time of injection of fetuses (days before birth)	No. of animals tested	Tuberculin tests*	
				6 wks. after birth	12 wks. after birth (5 wks. after postnatal vaccination with phenol-killed BCG)
				Maximum average diameter of edema‡	
				mm.	*mm.*
K	Undiluted	3	2	1	0
				2	1
L	1:10	38	2	0	3
				3	12
M	"	38	2	0	0
				0	2
N	"	38	2	0	2
				1	4
O	1:10,000	41	2	0	8
				2	8
Controls: not injected *in utero* but vaccinated 7 wks. after birth with 200 γ phenol-killed BCG	—	—	8	—	6, 9, 10, 11, 11, 13, 14, 16
Controls: not injected *in utero* and not vaccinated	—	—	12	—	0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 2, 4

* An area of reaction having a maximum average diameter of edema of 4 mm. or less is considered to be negative.

‡ The maximum reaction in all instances developed between 30 and 36 hours after injection.

injected *in utero* with O.T. diluted 1:100 or more, only 2 were found to be tuberculin-tolerant, whereas 13 of the 21 receiving undiluted or 1:10 tuberculin became tolerant. (The lower incidence of tolerance in animals injected with undiluted tuberculin than in those given the 1:10 dilution may only be apparent; the rate of abortion of fetuses given the undiluted material was considerably higher, and the aborted animals probably included many of those

TABLE IV

Skin Reactivity to P.P.D. (0.2 Cc. of a 1:10 Dilution) of Guinea Pigs Injected in Utero with O.T. and Vaccinated 7 Weeks after Birth with 800 γ Phenol-Killed BCG

Litter origin	Dilution of O.T. injected *in utero*, in 0.1 cc.	Time of injection of fetuses (days before birth)	No. of animals tested	Tuberculin tests*	
				6 wks. after birth	12 wks. after birth (5 wks. after postnatal vaccination with phenol-killed BCG)
				Maximum average diameter of edema‡	
				mm.	*mm.*
P	Undiluted	29	1	0	16
Q	"	27	1	0	11
Y	"	1	1	0	0
Z	"	6	2	1	12
				1	14
T	1:10	32	2	1	15
				2	1
U	"	25	1	2	17
V	"	43	2	0	0
				0	16
W	"	43	1	4	2
X	"	29	2	0	0
				0	1
aa	1:100	29	2	0	3
				3	8
S	1:1,000	30	2	0	5
				2	7
R	1:10,000	32	1	0	0
Controls: not injected *in utero* but vaccinated 7 wks. after birth with 800 γ phenol-killed BCG	—	—	8	—	10, 11, 13, 14, 15, 17, 17, 19

* An area of reaction having a maximum average diameter of edema of 4 mm. or less is considered to be negative.

‡ The maximum reaction in all instances developed between 30 and 36 hours after injection.

most successfully injected, and therefore most likely to have been tolerant.)

It also appeared that animals of the same litter varied in their ability to become tolerant to tuberculin. For example, one of the 2 siblings in litters T and V failed completely to respond to an intradermal injection of P.P.D. following vaccination 7 weeks after birth with 800 γ phenolized BCG, while the other developed an area of edema 15 mm. or more in diameter. Although variations in the exact site of deposit of the inoculum undoubtedly occurred in the process of fetal injection, it is unlikely that all such variations of response among litter mates can be accounted for by this experimental artifact. Variation in response of fetally injected litter mates has also been reported by Billingham,

TABLE V

Fate of Pregnant Guinea Pigs Whose Fetuses Were Injected in Utero with Phenolized Tubercle Bacilli

Quantity of killed bacilli injected into fetuses, in 0.1 cc. saline	No. of pregnant animals operated on	Died of postoperative complications	Survived but aborted or gave birth to dead litter	Gave birth to living young
mg.				
0.02	5	2	2	1
0.06	4	1	2	1
0.10	8	0	4	4
0.20	14	2	9	3
0.50	6	0	6	0
1.00	2	0	0	2
	39 +1*	5	23	11
Total.............	40			

* Died during operation.

Brent, and Medawar in several species of animals (14), and may be due to individual differences in immunological maturity at the time of injection.

Injection of Fetal Guinea Pigs with Phenol-Killed Tubercle Bacilli.—

Forty pregnant guinea pigs at different stages of pregnancy were subjected to laparotomy and their fetuses injected with quantities of phenol-killed bacilli of the BCG strain ranging from 0.02 to 1.0 mg. The bacilli were administered in 0.1 cc. saline. The fate of the animals during and after operation is shown in Table V.

As seen from Table V, the abortion rate of fetuses injected with intact phenolized bacilli was high. Of 34 animals surviving operation, 23 aborted (67.6 per cent), a considerably higher incidence of abortion than of animals whose fetuses were injected with Old Tuberculin (see Table II). Only one animal died during operation. The number of animals succumbing to postoperative com-

plications was of the same order as in the previous experiment (Table II). The higher abortion rate in this experiment does not, therefore, appear to be due to a cruder operative procedure, but rather to the greater toxicity of the inoculum.

The 11 surviving females which did not abort gave birth to 28 young, of

TABLE VI

Skin Reactivity to P.P.D. (0.2 Cc. of a 1:10 Dilution) of Guinea Pigs Injected in Utero with Phenol-Killed BCG and Vaccinated 9 Weeks after Birth with 200 γ Phenol-Killed BCG

Litter origin	Quantity of phenolized BCG injected in *in utero*, in 0.1 cc.	Time of injection of fetuses (days before birth)	No. of animals tested	Tuberculin tests*	
				8 wks. after birth	14 wks. after birth (5 wks. after postnatal vaccination with phenol-killed BCG)
				Maximum average diameter of edema‡	
	mg.			*mm.*	*mm.*
bb	0.02	23	3	0	7
				1	0
				2	12
cc	0.10	12	1	0	0
ff	"	30	2	0	3
				8	n.v.§
gg	"	32	2	1	4
				1	9
hh	0.20	31	2	3	2
				2	0
dd	1.00	13	1	0	5
ee	"	20	1	0	7

* An area of reaction having a maximum average diameter of edema of 4 mm. or less is considered to be negative.

‡ The maximum reaction in all instances developed 30 to 36 hours after injection.

§ n.v. = not vaccinated, because found tuberculin-sensitive at first postnatal test.

which 7 were stillborn (25.0 per cent). This proportion of dead young among litters containing living animals was greater than that observed in our normal animal colony, and suggests that at least in some instances death of one or more fetuses did not cause abortion of the entire litter.

Four of the 11 litters of living young were excluded from further study because the number of animals born exceeded the number injected *in utero*. The remaining 7 litters (bb to hh) comprised a total of 12 living young. These were tuberculin-tested 8 weeks after birth by the intradermal injection of 0.2 cc. of a 1:10 dilution of P.P.D. One week later, those animals found to be tuberculin-negative were vaccinated by an intraperitoneal injection of

200 γ phenolized BCG suspended in 0.5 cc. saline. They were again tuberculin-tested 5 weeks after vaccination. The results of the tuberculin tests are presented in Table VI.

It is seen from Table VI that one animal (from litter ff) was tuberculin-sensitive *before* postnatal vaccination with phenol-killed BCG. Of the 11 animals not sensitized by their fetal exposure to killed BCG, 6 failed to develop tuberculin sensitivity after postnatal vaccination, 4 became moderately tuberculin-sensitive, and 1 showed a high degree of sensitivity. These findings indicate that fetal injection with a sensitizing antigen may have one of three distinct effects: the animal may be sensitized, even when fetal exposure was early in development; it may not be sensitized, but remain fully capable of developing hypersensitivity after a further exposure to the antigen in adulthood; or it may not only fail to be sensitized, but also lose the ability, at least for some time, to respond hypersensitively to antigenic exposure in maturity.

That none of the animals receiving fetal injections of Old Tuberculin were rendered tuberculin-sensitive (Tables III and IV) is hardly surprising, because tuberculin alone does not induce typical, delayed type tuberculin hypersensitivity in normal adult animals.

The results presented in Table VI again indicate that embryos of the same mother may react differently to fetal antigenic exposure. Thus, fetal injection of 0.10 mg. phenolized BCG rendered 1 of 2 animals of litter ff tuberculin-sensitive, and exerted the opposite effect, tolerance, on its sibling. That such differences of immunological effect induced by identical fetal injection cannot be accounted for entirely by differences in the site of deposit of the inoculum is here indicated by the fact that both sensitization and tolerance can result only if the inoculum reaches sites of antibody formation.

Injection of Fetal Guinea Pigs with Heat-Killed Tubercle Bacilli.—

Thirty-one pregnant guinea pigs at different stages of pregnancy were subjected to laparotomy, and their fetuses injected with 0.1 cc. quantities of undiluted cultures of BCG grown on Dubos-tween medium for 3, 13, and 20 days, and then killed by immersion for 20 minutes in a water bath heated to 70°C. The fate of these animals during and after operation is shown in Table VII.

As seen from Table VII, none of the 31 laparotomized guinea pigs succumbed either during or after operation. Nine animals (29.0 per cent) aborted. The remaining 22 gave birth to 47 young, of which 6 were still-born (12.7 per cent). It thus appears that suspensions of heat-killed bacilli cause fewer abortions than suspensions of phenol-killed ones, and that, as was seen with fetuses injected with Old Tuberculin, the death of one was usually accompanied by abortion of the entire litter.

Two of the 22 litters of living young were excluded from further study because the number of animals born exceeded the number injected *in utero*. Eight other litters were lost 3 weeks after birth when the animals were accidentally fed with fresh alfalfa from a crop con-

taminated with *Atropa belladonna*. The remaining 12 litters (ii to tt) contained a total of 20 living young. These were tuberculin-tested 8 weeks after birth by the intradermal injection of 0.2 cc. of a 1:10 dilution of P.P.D. Two weeks later, those animals found to be tuberculin-negative were vaccinated by the intraperitoneal injection of 200 γ phenol-killed BCG suspended in 0.5 cc. saline. They were again tuberculin-tested 5 weeks after vaccination. The results of the tuberculin tests are shown in Table VIII.

The results presented in Table VIII confirm the findings shown in Table VI that fetal injection with intact killed bacilli may result in tuberculin sensitization, induce tolerance, or leave the animal immunologically unchanged with respect to tuberculin. Seven of the 20 animals were found to have been sensi-

TABLE VII

Fate of Pregnant Guinea Pigs Whose Fetuses Were Injected in Utero with Heat-Killed Suspensions of BCG

Heat-killed bacillary suspension injected into fetuses, in 0.1 cc.	No. of pregnant animals operated on	Died of postoperative complications	Survived but aborted	Gave birth to living young
Undiluted 3-day-old culture	3	0	0	3
Undiluted 13-day-old culture	15	0	5	10
Undiluted 20-day-old culture	4	0	1	3
12-day-old culture diluted 1:4	9	0	3	6
Total.................	31	0	9	22

tized by their fetal exposure; 3 were made tolerant; and 10 were sensitized by postnatal vaccination. Here, again, animals from the same litter were found to be affected differently by fetal injection of antigen.

It is interesting to note that whereas 3 of 8 animals injected *in utero* with killed bacilli of a 20-day-old culture were made tolerant, none of the 15 animals receiving fetal injections of younger killed bacilli were made tolerant. It has been shown by others (14) that in order to induce tolerance, an antigen must be fully antigenic (*i.e.* capable of inducing the specific antibody response in a normal adult animal), and, when tolerance is to be induced to an antigenically complex material, such as skin homografts, the tolerance-inducing inoculum must contain every antigen present in the "challenge" inoculum. Heat-killed, 3- or 13-day-old cultures of BCG are capable of inducing tuberculin hypersensitivity in normal adult guinea pigs, but they may differ antigenically from the older, phenol-killed BCG cultures which were used to vaccinate the animals some weeks after birth. That the *method of killing* did not destroy the anti-

TABLE VIII

Skin Reactivity to P.P.D. (0.2 Cc. of a 1:10 Dilution) of Guinea Pigs Injected in Utero with Heat-Killed BCG and Vaccinated 10 Weeks after Birth with 200 γ Phenol-Killed BCG

				Tuberculin tests*	
Litter origin	Heated BCG suspension injected *in utero* in 0.1 cc.	Time of injection of fetuses (days before birth)	No. of animals tested	8 wks. after birth	15 wks. after birth (5 wks. after postnatal vaccination with phenol-killed BCG)
				Maximum average diameter of edema‡	
				mm.	*mm.*
ii	3-day-old culture	43	1	13	n.v.§
jj	" "	35	4	0	5
				0	8
				1	10
				8	n.v.
kk	13-day-old culture	5	1	5	n.v.
ll	" "	1	1	2	7
mm	" "	1	1	0	12
nn	" "	2	1	1	18
oo	" "	41	1	0	13
pp	" "	34	1	1	6
qq	" "	38	1	0	10
rr	20-day-old culture	28	3	0	2
				1	3
				6	n.v.
ss	" "	37	2	0	2
				8	n.v.
tt	" "	27	3	2	7
				10	n.v.
				11	n.v.

* An area of reaction having a maximum average diameter of edema of 4 mm. or less is considered to be negative.

‡ The maximum reaction in all instances developed between 30 and 36 hours after injection.

§ n.v. = not vaccinated, because found tuberculin-sensitive at first postnatal test.

genicity of the bacilli is evidenced by the ability of fetally injected *heat*-killed, 20-day-old BCG to prevent tuberculin sensitization after postnatal vaccination with 4-week-old, *phenol*-killed BCG. The apparent inability of younger heat-killed BCG to do likewise suggests, therefore, that tuberculin hypersensitivity may be induced by more than one antigen (tuberculoproteins of different antigenic specificity?), and that younger tubercle bacilli do not possess all these antigens.

Injection of Fetal Guinea Pigs with Living BCG.—

Thirty-four guinea pigs at different stages of pregnancy were subjected to laparotomy, and their fetuses injected with 0.1 cc. quantities of living BCG grown for 10 days in Dubos-tween medium. The fetuses of some animals received undiluted culture, and those of others cultures diluted 1:10, 1:50, and 1:100 in saline. The fate of the animals during and after operation is shown in Table IX.

TABLE IX

Fate of Pregnant Guinea Pigs Whose Fetuses Were Injected in Utero with Living BCG

Living BCG suspension injected into fetuses, in 0.1 cc.	No. of pregnant animals operated on	Died of postoperative complication	Survived but aborted	Gave birth to living young
Undiluted	20	1	10	9
Diluted 1:10	7	1	2	4
Diluted 1:50	4	0	1	3
Diluted 1:100	3	0	1	2
Total............	34	2	14	18

It is seen from Table IX that only 18 of the 32 animals surviving operation gave birth to living young, the other 14 (43.7 per cent) aborting. The 18 females gave birth to 45 young, of which 12 were stillborn (26.6 per cent). Four of the 33 living young died several weeks after birth; in two, numerous lesions swarming with acid-fast bacilli were found on the peritoneum, spleen, liver, and mesenteries.

The results presented in Table IX indicate that the number of abortions following fetal injection with living bacilli is greater than that induced by similar suspensions of heat-killed bacilli (see Table VII), but smaller than that induced by phenol-killed ones (see Table V). Death of a fetus injected with living BCG did not appear to cause abortion of the entire litter in all cases.

Four of the 18 litters of living young were excluded from further study because the number of animals born exceeded the number of fetuses that could be injected *in utero*. The remaining 14 litters (uu to zz, and Aa to Ah) contained a total of 24 living young. These were tuberculin-tested 8 weeks after birth by the intradermal injection of 0.2 cc. of a 1:10 dilution of P.P.D. One week later those animals found to be tuberculin-negative were vaccinated

TABLE X

Skin Reactivity to P.P.D. (0.2 Cc. of a 1:10 Dilution) of Guinea Pigs Injected in Utero with Living BCG and Vaccinated 9 Weeks after Birth with 200 γ Phenol-Killed BCG

Litter origin	Living BCG suspension injected *in utero*, in 0.1 cc.	Time of injection of fetuses (days before birth)	No. of animals tested	Tuberculin tests*	
				8 wks. after birth	15 wks. after birth (6 wks. after post-natal vaccination with phenol-killed BCG)
				Maximum average diameter edema‡	
				mm.	*mm.*
uu	Undiluted culture	14	3	6	n.v.§
				7	n.v.
				8	n.v.
vv	"	32	2	12	n.v.
				15	n.v.
ww	"	20	1	12	n.v.
xx	"	18	1	0	12
yy	"	21	1	0	11
zz	"	14	2	1	6
				15	n.v.
Aa	Culture diluted 1:10	34	3	0	10
				0	13
				15	n.v.
Ab	"	20	1	15	n.v.
Ac	"	29	1	16	n.v.
Ad	Culture diluted 1:50	22	2	6	n.v.
				10	n.v.
Ae	"	21	2	18	n.v.
				20	n.v.
Af	"	2	2	12	n.v.
				14	n.v.
Ag	Culture diluted 1:100	45	2	11	n.v.
				22	n.v.
Ah	"	46	1	11	n.v.

* An area of reaction having a maximum average diameter of edema of 4 mm. or less is considered to be negative.

‡ The maximum reaction in all instances developed between 30 and 36 hours after injection.

§ n.v. = not vaccinated, because found tuberculin-sensitive at first postnatal test.

by the intraperitoneal route with 200 γ phenol-killed BCG suspended in 1.0 cc. saline. They were again tuberculin-tested 6 weeks after vaccination. The results of the tuberculin tests are presented in Table X.

As seen from the results shown in Table X, 19 of the 24 animals were sensitized by the fetal inoculation with living BCG, and none of the remaining 5 were made tuberculin-tolerant.

To rule out the possibility that the procedure of fetal injection as such could affect future immunological response, a number of fetal guinea pigs were injected with a 1.0 per cent solution of bovine serum albumin, fraction V. None

TABLE XI

Skin Reactivity to P.P.D. (0.2 Cc. of a 1:10 Dilution) of Tuberculin-Tolerant and Non-Tolerant Animals 4 Weeks after Revaccination with Living BCG

Animals	No.	Maximum average diameter of edema of each animal*	Mean diameter of the tuberculin reaction
		mm.	*mm.*
Injected in fetal life; tolerant to tuberculin	24	14, 15, 15, 15, 16, 17, 17, 18, 18, 18, 19, 19, 20, 20, 22, 22, 23, 23, 23, 24, 24, 24, 24, 25	19.8
Injected in fetal life; not tolerant to tuberculin	33	13, 15, 15, 16, 16, 16, 16, 18, 18, 18, 18, 18, 18, 19, 19, 19, 19, 19, 20, 20, 20, 20, 20, 20, 20, 20, 21, 21, 21, 21, 21, 21, 22	18.7
Not injected in fetal life	20	13, 14, 14, 15, 16, 17, 17, 17, 18, 18, 18, 19, 19, 19, 19, 19, 20, 20, 20, 20	17.6

* The maximum reaction in all instances developed between 30 and 36 hours after injection.

of 17 animals so injected *in utero* were found to differ in their skin reactivity to P.P.D. following vaccination with phenol-killed BCG in adulthood from normal, fetally uninjected guinea pigs. The details of this experiment will be presented at a later date, in connection with a report on the induction of immunological tolerance with respect to the formation of circulating antibodies.

Revaccination of Tuberculin Tolerant and Non-Tolerant Animals with Living BCG.—

Of the 84 animals injected *in utero* with Old Tuberculin or killed or living BCG, 24 were found to be incapable of becoming tuberculin-sensitive following vaccination with phenol-killed BCG in early adulthood (Tables III, IV, VI, and VIII), and 33 were found to have retained the ability to become tuberculin-sensitive after adult vaccination (Tables III, IV, VI, VIII, and X). The 27 animals found to be sensitized by fetal injection of killed BCG (Tables VI, VIII, and X) were discarded. The 33 non-tolerant and the 24 tolerant animals were revaccinated by the subcutaneous injection of 0.5 cc. of a 7-day-old Dubos-tween cul-

ture of BCG 4 weeks after their last tuberculin test; they were thus 16 to 19 weeks old at the time of revaccination. A control group of 20 normal animals of the same age distribution was similarly vaccinated. Four weeks later, all animals were tuberculin-tested by the usual procedure. The results of the tuberculin tests are shown in Table XI.

As seen from Table XI, all animals were rendered tuberculin-sensitive to the same extent by vaccination with living BCG. It is not clear whether the tuberculin tolerance was overcome by vaccination with living BCG because this was a more "powerful" antigenic stimulus than killed BCG, or because the duration of the state of tolerance did not extend beyond the period of time during which the first postnatal vaccination with phenolized BCG, and the subsequent tuberculin test, took place.

DISCUSSION

A limited degree of immunological tolerance to tuberculin could be induced in guinea pigs by injecting them during fetal life with Old Tuberculin. The state of tolerance was manifested by the failure of a proportion of animals so injected to develop skin hypersensitivity to tuberculoprotein (P.P.D.) following vaccination with 200 and 800 γ phenol-killed tubercle bacilli of the BCG strain several weeks after birth; such quantities of phenolized bacilli were sufficient to render normal guinea pigs tuberculin-sensitive.

Fetal injections of intact bacilli of the BCG strain, killed by heating or by exposure to phenol, also induced tuberculin tolerance, but in a smaller proportion of the animals. A number of animals injected *in utero* with intact killed bacilli were found to be tuberculin-sensitive when first tested several weeks after birth, prior to any post-natal vaccination.

Fetal injection of living BCG induced tolerance in none of the animals, and, indeed, rendered most of them tuberculin-sensitive, even when the injection was given as early as 46 days before birth. Such sensitization could not have been due to a transfer of antibody across the placenta. Tuberculin sensitivity is not passively acquired by a fetus from a hypersensitive mother (39). This was confirmed in an experiment in which 20 normal pregnant guinea pigs were injected subcutaneously with living BCG and tuberculin-tested at the same time as their young, 7 weeks after parturition. None of the 52 young, but all 20 mothers were found to be tuberculin-sensitive. It is also unlikely that the fetuses were sensitized by placental transmission of antigen, or by exposure to antigen at birth. While injection of fetuses with living BCG resulted in tuberculin sensitization of the mothers, probably because of the escape of part of the living inoculum in the process of injection through the uterine walls, none of the mothers whose fetuses received killed BCG were sensitized. The sensitization of animals by fetal injection with killed BCG could not have resulted, therefore, from a contamination with antigen from the mother.

A possible explanation for the sensitization of embryos injected early in their

development with intact tubercle bacilli might be that intact bacilli cannot induce tuberculin sensitivity until their structural integrity is interrrupted, and their proteinaceous antigens "exposed." In this event, intact bacilli injected in fetal life may remain immunologically inert for some time, until an immunologically significant amount of antigen is liberated, and by then the modality of immunological response may have changed from possible tolerance to sensitization. The greater frequency of sensitization of animals injected *in utero* with living rather than with killed BCG may reflect the greater structural integrity of the former. Another possible explanation might be that intact tubercle bacilli hasten the development of immunological maturity, and in order to induce tolerance must be injected still earlier in embryological development. Whatever the explanation, it is apparent that the induction of immunological tolerance to bacterial antigens requires more than the mere presence of the bacteria during fetal existence.

The development of immunological tolerance to tuberculin resembles the induction of tolerance to tissue homo- and heterografts, and to other antigens (14), in several respects. Individuals from the same litter were found to be capable of different modalities of immunological response to fetal injection of antigen, some becoming tolerant, others immune, and still others remaining unchanged with respect to further exposure to the antigen in adult life. That this diversity of response is due at least in part to individual variations in the time of immunological maturation is suggested by the observation that some animals of litters injected with antigen as late as several days before, to several hours after, birth were made tolerant, while others failed to become tolerant even when injected more than a month before birth. The degree of tolerance was limited, as seen by the greater number of animals found to be tuberculin-negative following postnatal vaccination with 200 γ rather than with 800 γ phenol-killed BCG, the larger quantity being a more powerful antigenic stimulus, and by the abolition of tolerance following further vaccination with living BCG. Experiments are in progress to determine whether this loss of tolerance is due to time or to the greater antigenic potency of living BCG.

In the experiments reported so far, tuberculin "tolerance" was tested for only by the absence of skin hypersensitivity to tuberculoprotein. It has not yet been determined whether failure to develop skin hypersensitivity to soluble tuberculoprotein is also accompanied by the absence of a hypersensitive response to the intradermal injection of intact tubercle bacilli, and by the absence of cellular hypersensitivity as revealed by tissue culture studies or passive transfer experiments, and whether the immunological unresponsiveness is truly a state of "central" tolerance, as would be indicated by the abolition of tolerance following transplantation of immune lymph nodes. Such studies are currently under way, as is a serological investigation of skin test tolerant and non-tolerant animals.

Experiments are also in progress designed to extend the degree and duration of tuberculin tolerance, by earlier and multiple injections of O.T. and unheated, purified protein derivatives in fetal life, and by the incorporation of soluble antigens and bacillary fragments in adjuvant substances.

SUMMARY

Female guinea pigs were subjected to laparotomy at different stages of pregnancy, and their fetuses injected through the uterine walls with one of the following preparations: Old Tuberculin, tubercle bacilli of the BCG strain killed by heat or exposure to phenol, and living BCG.

A large number of the animals injected *in utero* with Old Tuberculin failed to develop skin hypersensitivity to P.P.D. following vaccination with 200 or 800 γ phenol-killed tubercle bacilli (BCG) in early adulthood. Normal control animals were sensitized by vaccination with such quantities of phenolized BCG. The failure of animals which had been injected with Old Tuberculin in fetal life to respond hypersensitively to P.P.D. after adult vaccination with tubercle bacilli is ascribed to their acquisition of a state of immunological tolerance to tuberculoprotein (tuberculin tolerance).

Fetal injection with killed BCG conferred a state of tolerance on a few of the animals, and rendered others tuberculin-sensitive. Fetal injection with living BCG sensitized most of the animals to tuberculin, even when fetal exposure was as early as 46 days before birth, and induced tolerance in none.

Fetuses of the same litter, injected simultaneously with identical inocula, often responded differently, some becoming tolerant to tuberculin, others developing hypersensitivity, and still others remaining immunologically unaffected, becoming neither sensitive nor tolerant.

The state of tuberculin tolerance induced in these experiments was limited. When tolerant animals were revaccinated with living BCG several weeks after vaccination with phenol-killed bacilli, they developed as high a degree of tuberculin skin sensitivity as the originally non-tolerant animals.

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