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Impaired Delayed Hypersensitivity in Germ-Free Guinea-Pigs

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Summary. The delayed-type hypersensitivity response of germ-free guinea-pigs was found to be defective. Whereas almost all conventionally reared guinea-pigs became hypersensitive to an allergenic hapten (picryl chloride), most germ-free guinea-pigs did not. When injected with a fully antigenic substance, bovine γ-globulin (BGG), none of the germ-free animals acquired BGG-specific delayed hypersensitivity. Further, none of the germ-free guinea-pigs developed spontaneous iso-hypersensitivity for a beta globulin as do conventional guinea-pigs. In addition, germ-free guinea-pigs given Freund's complete adjuvant did not develop the characteristic induration or erythema normally seen at injection sites and most animals died within 21 days.

Germ-free guinea-pigs given competent lymphoid cells from highly sensitized conventional guinea-pigs were unable to translate adoptive hypersensitivity into delayed dermal reactions.

A permeability factor in aged guinea-pig sera, injected into the skin of germ-free and conventional animals to determine whether the skin of germ-free guinea-pigs was able to support reactions, initiated immediate dermal reactions of equal intensity in both sets of animals.

INTRODUCTION

Although numerous reports have appeared concerning circulating antibody responses of germ-free animals, none has dealt with delayed-type hypersensitivity. Germ-free animals of a variety of species including pigs (Kim, Bradley and Watson, 1967, 1968), mice (Stecher and Thorbecke, 1967; Asofsky, Ikari and Hylton, 1968; Horowitz and Bauer, 1968; Nordin, 1968; Thonard, Dalbow and Crosby, 1968; Wostmann, 1968), rats (Wagner, 1968; Wostmann, 1968; Legler and Gustafson, 1969) and guinea-pigs (Rozmiarek, Chorpenning, Gisler and Frederick, 1969) have shown abilities to synthesize immunoglobulins equal to those of conventional animals. Indeed, in one instance (Rozmiarek *et al.*, 1969) germ-free guinea-pigs that were colostrum deprived and not exposed to living bacteria, produced greater amounts of antibody than did conventional guinea-pigs. In contradistinction one group of authors (Miyakawa, Kishimoto, Itaya, Yei and Kashio, 1958) found that tissue transplanted between germ-free guinea-pigs persisted until the 6th day whereas transplanted tissue in conventional guinea-pigs necrosed within 48 hours. They attributed the prolonged interval of survival to the poorly developed lymphoid apparatus of the germ-free guinea-pigs.

The present study is concerned with the delayed-type immune response of germ-free guinea-pigs. It evolved out of an inquiry into whether germ-free guinea-pigs would be able to develop delayed-type hypersensitivity spontaneously to an heritable iso-antigen as has been reported for conventional guinea-pigs (Battisto, 1968). When it was found that germ-free guinea-pigs did not, the question arose whether these animals would display normal delayed hypersensitivity responses after deliberate sensitization with an antigen. We have found that germ-free guinea-pigs responded poorly or not at all to sensitization attempts with an allergenic hapten and with a fully antigenic protein. Further, germ-free guinea-pigs given lymphoid cells from highly sensitive conventional guinea-pigs did not translate passively conferred delayed hypersensitivity into detectable dermal responses.

MATERIALS AND METHODS

Obtaining and maintaining germ-free guinea-pigs

Guinea-pigs of the Rockefeller University, Pirbright, Abyssinian and Hartley strains were used. They were derived germ-free by the methods described previously (Lev, 1963). Briefly, pregnant females judged close to term by palpation of the pubic symphysis were killed, opened surgically and their uteri removed whole. The latter were then passed through a germicidal trap into nylon isolators that were modifications of those previously described (Lev, 1962, 1964). Within the isolators the young were removed from the uteri, their cords clamped and cut; and they were stimulated to induce breathing. Throughout the experiments they were fed Diet L478 (General Biologicals, Chagrin Falls, Ohio) supplemented with vitamin C and thiamine. The diet was sterilized by steam autoclaving. Bacteriological tests for sterility were performed at bi-monthly intervals and prior to experimentation (Lev, 1963).

Conventionalization

At appropriate times, germ-free animals were conventionalized by distributing cecal contents of a freshly killed conventional guinea-pig into their drinking water and food. Although some guinea-pigs survived this treatment, most died shortly afterwards.

Sensitization and testing procedures

For sensitizing guinea-pigs to picryl chloride, animals were injected intradermally with 0·1 ml of saline containing 2·5 μg of picryl chloride daily for 6–8 days. A week following the last injection the animals were tested for contact sensitivity to the hapten by applying picryl chloride in olive oil to separate skin sites (500, 167 and 50 μg on conventional animals and 500 μg on germ-free animals). Animals were grouped on the basis of intensity of response at each site: zero = no reaction to 500 μg hapten; low = very faint pink to 500 μg hapten; inter = very faint pink to 167 μg hapten; and high = very faint pink to 50 μg hapten.

Since germ-free guinea-pigs were tested with only the 500 μg dose, an estimate of the degree of sensitivity developed by five of twenty-nine animals, that proved to be reactors was necessary (cf. Battisto, 1968).

For sensitizing guinea-pigs to bovine γ-globulin (BGG), each animal was injected with 2·5 μg of BGG into each hind footpad. They were tested intradermally 12 days later for delayed cutaneous responses to 100 μg of BGG. Sites were examined for induration and erythema at 24 and 48 hours.

Reactions to permeability factor (PF)

The PF studied was that in serum rendered from clotted blood and aged for several days in the cold. It is detected in serum without dilution. A second PF in guinea-pig sera detectable upon dilution of serum in saline at 1 : 50 to 1 : 200 has not yet been examined in germ-free guinea-pigs. Both factors were originally described by Miles and Wilhelm (1955). Germ-free and conventional animals were injected intravenously with 1 ml of 0·5 per cent Evans Blue dye just prior to intradermally injecting 0·1 ml of saline as control and undiluted sera containing PF. Diameters of reactions and intensity of colour were read on the external skin after 15 minutes.

Passive transfers of delayed hypersensitivity

For each experimental trial, lymph nodes and spleens were taken from two to four conventional guinea-pigs. The latter had been sensitized to PPD by injection of Freund's complete adjuvant containing killed *Mycobacterium tuberculosis* H37Rv. The donor animals, when challenged with 10 μg of PPD, were capable of responding with reactions ranging from 13 to 20 mm in diameter of erythema and marked induration. After teasing and washing in Hanks's solution, the lymph node cells and spleen cells were equally divided among a conventional and two germ-free guinea-pigs. They were given intraperitoneally and the recipients were tested 2 days later by injecting 25 μg of PPD intradermally. Sites were read at 24 and 48 hours (cf. Battisto, 1968).

RESULTS

ISO-HYPERSENSITIVITY TO A SERUM FACTOR

It is now well established that a known proportion of guinea-pigs of certain strains become sensitive to a β-globulin serum component (SF) present in the sera of other guinea-pigs (Battisto, 1968). In order to determine what, within the conventional environment, contributed to the acquisition of the sensitivity it was decided to rear the animals in a germ-free state. Accordingly, guinea-pigs of the Rockefeller University and Abyssinian strains, which have been bred in our laboratories to the point where 20–50 per cent of the animals are known to develop SF iso-hypersensitivity, were delivered by Caesarian section into the sterile nylon isolators where they were reared. When 2 months of age or older the

TABLE 1

LACK OF SPONTANEOUS ISO-HYPERSENSITIVITY TO SERUM FACTOR (SF) IN GERM-FREE GUINEA-PIGS

Trial	Conventionalization	No. in group	Dermal response (mm) to:		
			SF+	SF-	Rab-serum
1 and 2	Pre-	8	0	0	
	Post-	6	0	0	
3	Pre-	8	0	0	0
	Post-	3a	18	5	
		b	4	2	
		c	2	5	

When 2 months of age germ-free guinea-pigs were injected intradermally with guinea-pig sera containing and lacking SF (0·1 ml of each). Sites were read at 24 and 48 hours. Injections were repeated with the same sera approximately 2 weeks after the animals were conventionalized by feeding cecal contents from conventional, freshly killed guinea-pigs. In trial 3, the animals were tested as well with 0·1 ml of whole rabbit serum before conventionalization.

germ-free guinea-pigs were injected intradermally with 0·1 ml of guinea-pig sera containing and lacking SF. In three trials utilizing sixteen guinea-pigs none demonstrated the delayed-type cutaneous reactions characteristically seen in conventional guinea-pigs (Table 1). Since virtually all conventional guinea-pigs are known to respond with delayed dermal reactions to cutaneously injected rabbit serum (0·1 ml, cf. Norton and Ziff, 1965; Battisto, 1968), eight of the animals were tested for this response; none reacted (Table 1).

To ascertain whether the germ-free animals would react following conventionalization, some were fed cecal contents of freshly killed conventional guinea-pigs. Our experience has been that most guinea-pigs die upon conventionalization. Two weeks later, the survivors of conventionalization were tested with the same guinea-pig sera used in the earlier tests. Only one guinea-pig in nine converted to delayed dermal reactivity for SF (Table 1).

ABILITY TO SUPPORT IMMEDIATE SKIN REACTIONS

In view of the fact that the reactions of germ-free guinea-pigs were so strikingly different in appearance from those of conventional animals, the question of dermal capability arose. To determine whether the skin of germ-free guinea-pigs would support an immediate-type response, they were tested with a permeability factor contained in aged guinea-pig serum (Miles and Wilhelm, 1955). Germ-free and conventional guinea-pigs were injected intravenously with Evans Blue dye just prior to intradermally injecting 0·1 ml of saline and of the undiluted guinea-pig sera containing PF. Observations made at 15 minutes revealed that although one germ-free animal failed to react, the remaining two produced cutaneous reactions equal to those of conventional guinea-pigs (Table 2).

TABLE 2

EQUAL IMMEDIATE CUTANEOUS REACTIONS TO PERMEABILITY FACTOR (PF) INDUCED IN CONVENTIONAL AND GERM-FREE GUINEA-PIGS

Guinea-pig recipients		Saline control	Dermal reactions (mm) to PF in guinea-pig sera	
			A	B
Germ-free	1	4, ±	9, +	8, +
	2	3, ++	11, ++	11, ++
	3	2, ±	2, ±	3, ±
Conventional	4	2, ±	9, ++	9, ++
	5	3, ±	7, +	7, +

Diameters of reactions measured at 15 minutes are given along with intensities of blueing: ±, +, ++, +++ equalling very light to deep blue.

DELAYED HYPERSENSITIVITY FOLLOWING ACTIVE SENSITIZATION

In order to find out whether the lack of delayed-type iso-hypersensitivity seen among germ-free guinea-pigs represents a general trait, the capability of these animals to develop usual degrees of delayed cutaneous reactivity upon purposeful sensitization was checked. Therefore attempts were made to sensitize germ-free guinea-pigs to an allergenic hapten picryl chloride. Following sensitization and cutaneous tests, grouping of the animals on the basis of the intensity of response to the hapten revealed that whereas conventionally reared guinea-pigs developed intermediate to high levels of sensitivity, germ-free animals did not

TABLE 3

ATTEMPT TO SENSITIZE GERM-FREE GUINEA-PIGS TO AN ALLERGENIC HAPTEN

Characterization of guinea-pigs	Degree of delayed contact dermal reaction to picryl chloride*				
	High	Intermediate	Low	Zero	Totals
Conventional	7	7	2	1	17
Germ-free	1	4	8	16	29

* After sensitization and testing, animals were grouped on the basis of intensity of response at each site (see 'Materials and Methods').

(Table 3). Only five of twenty-nine germ-free animals developed reactivity comparable to that of conventional animals and sixteen of twenty-nine failed to respond at all.

To determine whether this inability to develop delayed dermal reactions extended to a complete antigen, germ-free and conventional guinea-pigs were immunized with bovine γ-globulin (BGG). In two trials, nine of thirteen conventional animals responded at 24 hours with reactions 7 mm or greater to 100 μg of BGG whereas none of thirteen germ-free animals did so (Table 4).

TABLE 4

ATTEMPT TO SENSITIZE GERM-FREE GUINEA-PIGS TO AN ANTIGEN, BOVINE γ-GLOBULIN

Trial	No. with delayed cutaneous response to 100 μg BGG:	
	Conventional	Germ-free
1	4/5	0/9
2	5/8	0/4
Totals	9/13	0/13

When attempts were made to sensitize germ-free guinea-pigs by using methods involving Freund's complete adjuvant, two observations were noted that are not tabulated. Injections of 0·1 ml of Freund's adjuvant into the paws of conventional guinea-pigs ordinarily causes the paws to become erythematous and indurated within 2 weeks. These signs were not observed in the germ-free guinea-pigs injected in parallel. In addition, the germ-free guinea-pigs injected with 2·0 ml of Freund's adjuvant and those given 0·4 ml of the adjuvant all died within a 40-day interval. Conventional guinea-pigs commonly tolerate these doses quite well.

PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY

In an attempt to determine whether germ-free guinea-pigs would demonstrate delayed dermal hypersensitivity that was passively transferred, lymph node and spleen cells from highly-PPD sensitive conventional guinea-pigs were equally divided among conventional and germ-free recipients. Care was taken to include among the germ-free recipients animals that had already shown dermal sensitivity for the allergenic hapten, picryl chloride. Tested 2 days after receiving the cells, conventional guinea-pigs revealed adoptive

immunity for PPD; dermal reactions measuring 14–15 mm in diameter were indurated and erythematous (Table 5). In contrast, none of the germ-free recipients developed delayed dermal responses. Thus the germ-free animals, in addition to being unable to acquire delayed sensitivity actively, were unable to support adoptive sensitivity conferred upon them passively.

TABLE 5

INABILITY TO TRANSFER PPD-DELAYED HYPERSENSITIVITY ADOPTIVELY TO GERM-FREE GUINEA-PIGS

Trial	Dermal response* to 25 μg PPD in:		
	Conventional recipients	Germ-free recipients	
		Picryl sensitive	Picryl non-sensitive
1	15 mm, + + +	0	0
2	14 mm, + +	Died	0

* Responses at 24 hours are given in mm of diameter; + + and + + + refer to extent of induration and erythema with + = faint pink, flat; and + + + = pink, markedly thickened.

DISCUSSION

The fact that germ-free guinea-pigs were unable to develop spontaneous iso-hypersensitivity for SF as do conventional guinea-pigs implicates some factor of the conventional environment as the initiator of the iso-hypersensitivity. Further support for this view derived from the fact that one of the guinea-pigs converted to reactivity for SF after conventionalization. The conversion to a positive reaction in this instance could not be attributed to active sensitization by SF itself since we had already established that repeated cutaneous injections of SF does not sensitize guinea-pigs to SF (Battisto, 1964). The positive conversion following conventionalization is more likely due to microbial influence on the animals. The factors involved are unknown and are to some degree the results of a specialized reaction in that accidental contamination of the germ-free guinea-pigs with organisms such as Gram-positive rods or cocci had no effect on the ability to produce the delayed response. Individual organisms of the normal flora of the guinea-pig have not yet been tested in this system.

In contrast to conventional guinea-pigs which respond with delayed dermal reactions to intracutaneously injected rabbit serum (Norton and Ziff, 1965), germ-free guinea-pigs did not. Thus, the spontaneously developed reactivity for rabbit serum must also be attributable to an environmental factor responsible for inducing xenohypersensitivity.

A likely explanation for the inability of germ-free guinea-pigs to develop spontaneously sensitivities for either iso- or xeno-antigens may be that it is an extension of a general inability to develop delayed hypersensitivity. The germ-free guinea-pigs were unable actively to demonstrate delayed-type cutaneous reactions to picryl chloride, bovine γ-globulin or the organisms in Freund's complete adjuvant. Although lack of induration of the paws of germ-free animals injected with adjuvant may indicate that systemic delayed hypersensitivity is affected as well, no attempt has yet been made to ascertain whether germ-free guinea-pigs show systemic delayed hypersensitivity.

If the germ-free guinea-pigs were able to show systemic sensitivity then it would follow that they actively acquire delayed hypersensitivity but somehow lack the ability to convert the reactivity into dermal responses. This is seen in immature conventional guinea-pigs (Salvin, Gregg and Smith, 1962). Thus, it is possible that the immunological apparatus involved in delayed response of germ-free guinea-pigs parallels that of baby conventional guinea-pigs. However, it is worthwhile noting that an occasional germ-free animal did show a significant delayed response. Miyakawa *et al.* (1958) have noted a prolongation of survival of skin grafts in germ-free guinea-pigs which they attributed to lowered levels of γ-globulins. In view of the observations presented here their observations may be another manifestation of an inability to develop delayed hypersensitivity.

The skin of germ-free guinea-pigs appears to be competent for immediate type reactions. An experiment utilizing permeability factor of aged guinea-pig sera (Miles and Wilhelm, 1955), demonstrated that germ-free and conventional guinea-pigs responded to PF with immediate dermal reactions of comparable intensities.

What appears to be a second immunological defect of germ-free guinea-pigs became apparent when attempts were made to transfer hypersensitivities to them passively. Given competent lymphoid cells from conventional guinea-pigs highly sensitive to PPD, germ-free animals were unable to translate adoptive hypersensitivity into delayed dermal reactions. One of these germ-free recipient animals had been shown previously to be sensitive to picryl chloride. This would indicate that its immunological system and skin were competent to support an actively acquired delayed contact reaction. However, the animal's participation in converting passive immunity into a cutaneous response was defective. In this way the second deficiency was noted in the immunological system of germ-free guinea-pigs. What contributions conventional recipients of adoptive immunity make in the outward manifestation of delayed hypersensitivity have yet to be clearly enumerated.

It has been noted by Parkes (1959) that lack of pyridoxine in mice inhibited the normal homograft rejection. Whether the lack of delayed-type hypersensitivity in germ-free guinea-pigs can be attributed to a similar dietary deficiency is unknown.

The deaths of germ-free guinea-pigs given Freund's adjuvant are, as yet, unexplained. Although loss of weight and development of granulomas have been noted among conventional guinea-pigs given Freund's adjuvant (Chase, 1958), it is well known that death from this substance rarely ensues. The arthritis found in conventional rats following sensitization with Freund's adjuvant (Stoerk, Bielanski and Budzilovitch, 1954; Flax and Waksman, 1963; Pearson and Wood, 1964; Formanek, Rosak and Steffen, 1964) is found to the same degree in germ-free rats (Pearson, Wood, McDaniel and Doft, 1963). Since conventional guinea-pigs have not been noted to develop this form of arthritis, the deaths seen in the germ-free guinea-pigs may be due to an hitherto unsuspected effect of the adjuvant.

Notes added in proof

In a recent experiment, five germ-free guinea-pigs were tested for contact hypersensitivity to picryl chloride and only two responded. When the same animals were immunized intramuscularly with BGG (1 mg per animal on two occasions 3 weeks apart and serum taken 4 days after the second injection), four made antibody measurable by passive cutaneous anaphylaxis. Thus, germ-free guinea-pigs synthesize humoral antibody irrespective of their impaired capacity to show delayed hypersensitivity.

During presentation of a portion of these data (M. Lev, *Fed. Proc.*, **29**, 701 Abs, 1970), an abstract was brought to our attention (E. M. Lerner II, *Fed. Proc.*, **23**, 286, 1964) that contains results essentially in accord with our own.

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