Contact Sensitivity and Immunologic Tolerance in Germfree Guinea Pigs (36813)

MITCHELL H. FRIEDLAENDER, HAROLD BAER,[1] AND PHILIP R. B. MCMASTER

Laboratory of Bacterial Products, Division of Biologics Standards, and Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda, Maryland 20014

Recent investigation of the mechanisms of delayed contact sensitivity and immunologic tolerance have focused attention on the role of the lymphatic system in these processes (1–5). Intact lymphatic connections between the skin and regional lymph node have been shown to be necessary for the development of tolerance to simple chemicals placed on the skin but are not required for sensitization (5). The poorly developed lymphoid apparatus of the germfree animal (6, 7) led to the present investigation of cellular immunity in germfree guinea pigs. This study compares the delayed skin reactivity and immunologic tolerance of germfree and conventional guinea pigs to two simple chemicals: dinitrochlorbenzene (DNCB), a sensitizer when injected in complete Freund's adjuvant (CFA) or when applied to the skin of guinea pigs, and dinitrothiocyanatebenzene (DNTB), a sensitizer when injected in CFA, but a toleragen when applied to the skin (5). This latter chemical induces no tolerance when applied to alymphatic skin islands of guinea pigs (5).

Materials and Methods. Chemicals. DNCB was obtained from *Eastman Organic Chemicals, Rochester, New York,* and DNTB from *K and K Laboratories, Plainview, New York.* Purity was established as described earlier (8).

Guinea pigs. Male Hartley strain guinea pigs weighing 350 ± 50 g, and pregnant female Hartley guinea pigs, were obtained from the NIH Rodent and Rabbit Production Section.

Germfree guinea pigs were derived by cesarean section within an operating unit of the Reyniers germfree system and transferred to stainless steel or flexible film isolators for the duration of the experiment. Neonates were fed diet regimen L-445 (9), supplemented with vitamin C (10 mg/day) and thiamine (0.5 mg/day). At 10 days of age, they were weaned onto pelleted diet L-477 (General Biochemicals, Inc., Chagrin Falls, OH) with supplemental vitamins. All materials entering the isolators were either steam autoclaved or chemically sterilized with 2% peracetic acid. Bacteriologic cultures of stools were carried out at weekly intervals on tripticase soy agar or blood agar and in fluid thioglycollate broth and incubated at 25, 37, and 55°. To establish the growth promoting properties of these media, feces from conventionally reared guinea pigs was cultured periodically during the experiment.

Sensitization. At 6 wk of age, hair was removed from an area 30 mm in diameter, from the left flank with an electric clipper in the case of conventional guinea pigs, or by plucking in the case of germfree animals. Ten micromoles of DNCB dissolved in 0.1 ml of acetone were slowly applied with a pipette to the shaven skin of 11 germfree and 16 conventionally reared guinea pigs. Ten micromoles of DNTB were applied in the same manner to the skin of 7 germfree and 16 conventional guinea pigs. Skin tests were performed 21 days following skin application of DNCB and repeated at biweekly intervals (8). By the end of the experiment, 6 germfree animals that received DNCB were still alive as were 5 animals treated with DNTB. DNTB did not induce sensitization in conventional animals when applied to the skin, and therefore skin testing was performed after injec-

[1] Author to whom reprint requests should be sent.

tion of DNTB in complete Freund's adjuvant.

Skin testing. Five microliters of serial dilutions of a chemical in acetone were dropped on the skin, and the smallest dose (nmoles) which elicited an observable reaction 48 hr after application was recorded. For conventional or germfree animals receiving the same treatment, geometric means of the threshold reacting doses were calculated. For purposes of calculation, nonresponding animals were assigned a threshold value five times the highest skin test dose.

Tolerance. Seven days following skin testing with DNCB, and 21 days following cutaneous application of DNTB, both germfree and conventional guinea pigs were injected with 1.0 ml of CFA (Arlacel:Drakeol 35:65) containing 1.0 mg of the chemical applied previously to the skin and 2.0 mg of *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI), distributed between the nuchal area, groins, axillae and footpads. Twenty-one days after these injections, skin testing was again carried out at biweekly intervals.

Controls. Groups containing eight conventional and five to seven germfree animals received 0.1 ml of acetone on the skin and 21 days later were injected with either 1.0 mg of DNCB or DNTB in CFA as described above. Skin tests were performed 21 days after the injections and repeated at biweekly intervals. By the end of the germfree experiment, 6 animals receiving DNCB in CFA were alive as were 5 animals treated with DNTB in CFA.

Serum protein electrophoresis. Serum protein electrophoresis of blood obtained from the ear veins of several germfree and conventional guinea pigs before and after injection of CFA was carried out on polyacetate strips (Gelman Instrument Co., Ann Arbor, MI). The strips were stained with Ponceau S and scanned with a Chromoscan densitometer (Joyce Loebel Co., Burlington, MA). Planimetry was used to determine the area of the pattern respresenting the gamma fraction, and this area was expressed as percentage of total serum protein.

Results. Sensitization. All conventional and germfree animals receiving 10 μmoles of DNCB in acetone on the skin developed delayed skin reactions (Fig. 1). All 16 conventional guinea pigs were sensitive by the first skin test, at which time 8 of 11 germfree guinea pigs responded. By the second skin test, all animals responded to skin testing and geometric means indicated that there was little difference in sensitivity between germfree and conventional animals.

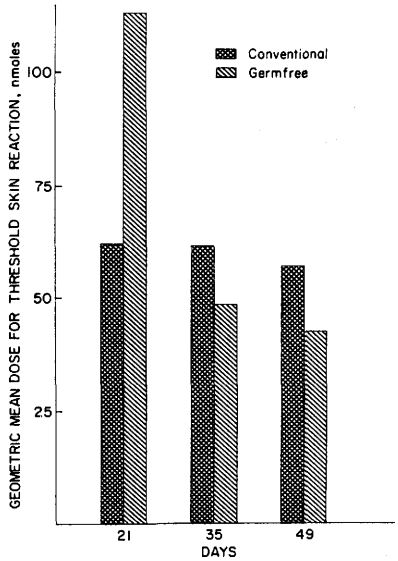

FIG. 1. Delayed skin sensitivity after application of 10 μmoles of DNCB to skin.

Controls. All germfree and conventional control animals became sensitive following the injection of 1.0 mg of DNCB or DNTB in CFA (Fig. 2). The degree of sensitivity

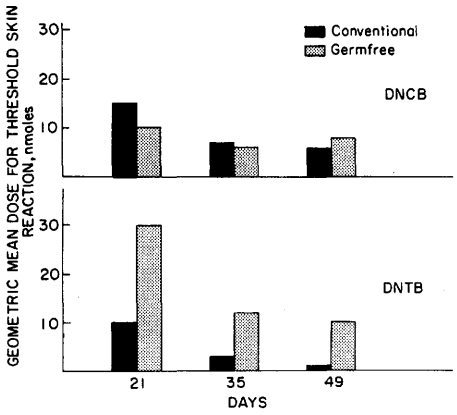

FIG. 2. Delayed skin reactivity of control guinea pigs injected with 1.0 mg of DNCB or DNTB in complete Freund's adjuvant.

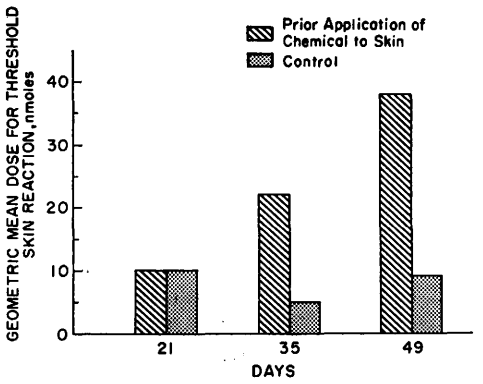

FIG. 3. Tolerance in germfree guinea pigs treated with DNCB.

tended to increase with successive skin testing. There was little difference in sensitivity between germfree and conventional animals injected with DNCB in CFA. Germfree animals injected with DNTB in CFA appeared slightly less sensitive than conventional animals receiving the same treatment.

Tolerance. Germfree guinea pigs treated with DNCB on their skin displayed no tolerance on the first skin test following the injection of DNCB in CFA (Fig. 3). Some differences in tolerance were observed with subsequent testing, including an apparent decrease in sensitivity of animals treated with DNCB on skin, however, considering the small number of animals in this group, it is not possible to say whether or not the sensitivity of these animals is different from that of the group receiving DNCB only in CFA. Germfree animals receiving DNTB on skin prior to being injected with DNTB in CFA exhibited marked tolerance on the first two skin tests (Fig. 4). While all five control animals reacted on the first skin test, four of seven animals receiving prior skin treatment with DNTB failed to react on the first skin test and three of six failed to react on the second skin test. Geometric means indicated that control animals were 8.3 times more sensitive on the first skin test and 10.9 times more sensitive on the second skin test than animals receiving DNTB on the skin. By the third skin test, all animals injected with DNTB in CFA were reactive and there was little difference in threshold skin responses between the two groups.

Conventional guinea pigs treated with DNCB and DNTB on their skin also developed tolerance (Fig. 5). Control animals injected with DNCB in CFA were 3.0 to 4.3 times more sensitive than those receiving prior skin treatment with DNCB. Control animals injected with DNTB in CFA were 12.1 to 43 times more sensitive than those animals treated with DNTB on skin prior to the injections.

Serum protein electrophoresis. Germfree guinea pigs had low levels of γ-globulin when compared with conventional animals (Table I). The mean value of γ-globulin in the conventional group was 3.4 times that of the germfree group. Following the injection of CFA, the γ-globulin fraction increased in both groups, however the mean value of γ-globulin for the conventional group was still 1.8 times greater than that of the germfree group.

Weekly cultures of the feces of germfree guinea pigs were negative on all media and at all temperatures. Feces from conventional animals produced marked bacterial growth in these same media.

Discussion. Germfree guinea pigs are capa-

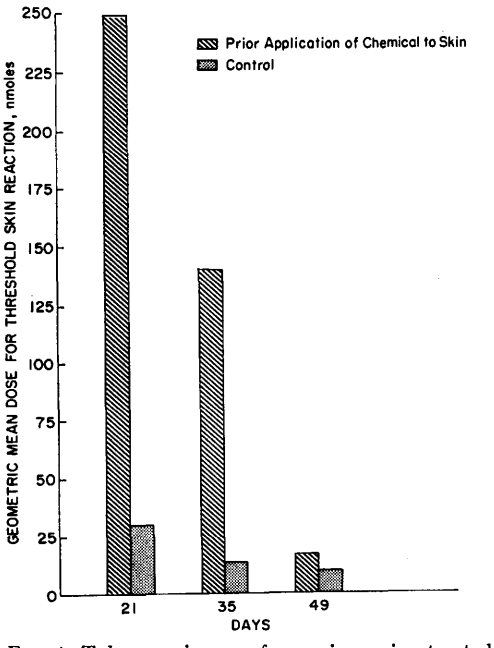

FIG. 4. Tolerance in germfree guinea pigs treated with DNTB.

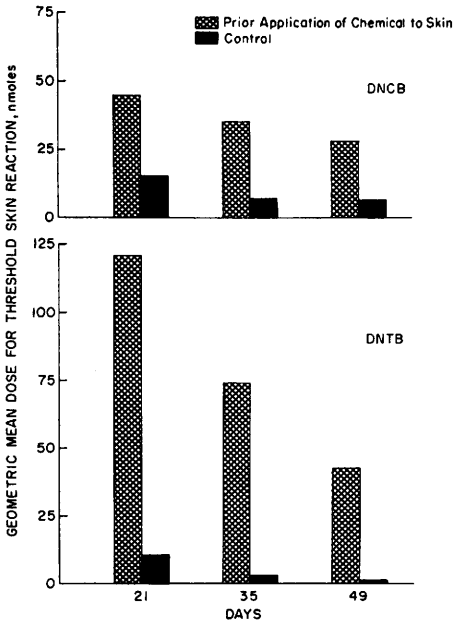

FIG. 5. Tolerance in conventional guinea pigs treated with DNCB or DNTB.

ble of developing contact sensitivity and immunologic tolerance as are conventionally reared guinea pigs. The level of sensitivity following application of DNCB to the skin and following the injection of DNCB and DNTB in CFA does not differ greatly from the sensitivity seen in conventional animals (Figs. 1 and 2). The observed differences in sensitivity between these two groups may be the result of a relatively small group of germfree animals, or the lesser reliability of a first skin test compared with subsequent skin tests. Application of DNTB to the skin prior to the injection of the homologous chemical in CFA induced tolerance in germfree guinea pigs. For germfree animals injected with DNCB in CFA, however, no clear cut differences in sensitivity developed (Fig. 3). Both germfree and conventional guinea pigs treated with DNTB exhibited striking tolerance during the first two skin tests. The germfree group had very little tolerance on the third skin test with DNTB, while the conventional group retained a high level of tolerance. Again, this may reflect the relatively small number of animals in the germfree group, but clearly the overall impression from the three skin tests is one of an ability of germfree guinea pigs to develop tolerance.

These observations are consistent with other studies on the immune responses of germfree animals which, in general, have been reported to be qualitatively the same as those of conventional animals (10–13). Some studies even suggest an enhanced cellular immune response in the germfree animal. Most germfree mice reject skin allografts following neonatal thymectomy while conventional mice retain their grafts twice as long before death from a wasting syndrome (11). Germfree rats have been shown to reject skin allografts more rapidly than their conventional counterparts, and lymphocytes from these germfree animals in the mixed lymphocyte reaction develop a burst of mitotic activity earlier than conventional animals (14).

Lev and Battisto (15) have reported an impaired ability of germfree guinea pigs to develop delayed-type hypersensitivity to picryl chloride, bovine gamma globulin or to an heritable isoantigen, as well as an impaired blastogenic response to PPD in animals sensitized to PPD (16). The differences between the results of these investigators and our

TABLE I. γ-Globulin Levels in Germfree (GF) and Conventionally Reared (C) Guinea Pigs Before and After Receiving Complete Freund's Adjuvant (CFA).

	Before CA		After CFA	
	GF	C	GF	C
	1.6[a]	6.3	4.6	7.8
	1.6	6.7	4.8	9.6
	1.7	7.4	4.9	11.4
	1.8	7.4	5.0	
	1.8	7.8	5.0	
	2.0		6.1	
	2.2			
	2.4			
	2.4			
	2.4			
	2.5			
	2.6			
	2.8			
Mean	2.1	7.1	5.1	9.3

[a] Percentage of total serum protein.

findings cannot be resolved at this time, but may reflect different immunization and skin testing schedules, different diets or rearing procedures, or possibly different strains of animals.

Miyakawa *et al.* (17) have reported histologic evidence of prolonged survival of skin allografts in germfree guinea pigs studied during a 6 day period after transplantation. Other studies (12, 18) suggest no difference between germfree and conventional animals in the rejection of tissue allografts or xenografts. Furthermore, germfree mice are known to survive longer if immunized with BCG prior to infection with *M. tuberculosis* (19), and this type of immunity is considered to be mainly cellular (20).

Finally, the negative fecal cultures of the germfree guinea pigs confirmed that the animals remained uncontaminated throughout the experiments. This was further supported by the low levels of γ-globulin (21, 22) compared with conventional animals both before and after injections with CFA.

The present study demonstrates that germfree guinea pigs have no impairment of cellular immunity as it involves sensitization and immunologic tolerance to simple chemicals. Although lymphatic pathways between the skin and regional lymph nodes have been shown to be involved in these two competing processes (4, 5), the poorly developed lymphoid system of germfree animals does not prevent them from responding in a normal fashion to antigenic stimuli. This lack of lymphatic development does not seem to represent any immunologic defect, but rather a lack of antigenic simulation. When an antigenic challenge does occur, germfree animals are capable of responding with intact cellular immunity.

Summary. The poorly developed lymphoid system of germfree guinea pigs did not prevent the development of contact sensitivity and immunologic tolerance to two simple chemicals, dinitrochlorbenzene and dinitrothiocyanatebenzene. Germfree animals responded in a manner similar to conventionally reared animals when these chemicals were applied to the skin or injected in complete Freund's adjuvant.

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1. Macher, E., and Chase, M. W., J. Exp. Med. **129**, 81 (1969).

2. Barker, C. F., *in* "Advances in Biology of the Skin" (W. Montagna and R. E. Billingham, eds.), Vol 11, p. 199. Appleton-Century-Crofts, New York (1969).

3. Tilney, N. L., and Gowans, J. L., J. Exp. Med. **133**, 951 (1971).

4. Friedlaender, M. H., and Baer, H., Science **176**, 312 (1972).

5. Friedlaender, M. H., and Baer, H., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **31**, 774 (1972).

6. Gordon, H. A., Bull. N.Y. Acad. Med. **31**, 239 (1955).

7. Olson, G. B., and Wostmann, B. S., J. Immunol. **97**, 267 (1966).

8. Godfrey, H. P., and Baer, H., J. Immunol. **106**, 431 (1971).

9. Phillips, B. P., Wolfe, P. A., and Gordon, H. A., Ann. N.Y. Acad. Sci. **78**, 183 (1959).

10. Olson, G. B., and Wostman, B. S., J. Immunol. **97**, 275 (1966).

11. Miller, J. F. A. P., Dukor, P., Grant, G., Sinclair, N. R. St. C., and Sacquet, E., Clin. Exp. Immunol. **2**, 531 (1967).

12. Bealmear, P. M., and Wilson, R., Cancer Res. **27**, 358 (1967).

13. Nordin, A. A., Proc. Soc. Exp. Biol. Med. **129**, 57 (1968).

14. McDonald, J. C., Zimmerman, G., Bollinger, R., and Pierce, W. A., Proc. Soc. Exp. Biol. Med. **136**, 987 (1971).

15. Lev, M., and Battisto, J. R., Immunology **19**, 47 (1970).

16. Gris, Y., Lev, M., and Battisto, J. R., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **31**, 796 (1972).

17. Miyakawa, M., Kishimoto, H., Itaya, J., Uei, Y., and Kashio, T., Acta Pathol. Jap. **8**, 177 (1958).

18. Smith, C. S., Pilgrim, H. I., and Steinmuller, D., Transplantation **13**, 38 (1972).

19. Hobby, G. L., Lenert, T. F., Maier-Engallena, J., Wakely, C., *in* "Advances in Germfree Research and Gnotobiology" (M. Miyakawa and T. D. Luckey, eds.), p. 269. Chem. Rubber Pub. Co., Cleveland, Ohio (1968).

20. Collins, F. M., RES, J. Reticuloendothel. Soc. **10**, 58 (1971).

21. Newton, W. L., and DeWitt, W. B., J. Nutr. **75**, 145 (1961).

22. Sell, S., J. Immunol. **93**, 122 (1964).

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