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The Serum Antibody Response to Bacteriophage ΦX174 in Germ-Free and Conventionally Reared Mice

II. KINETICS OF THE SERUM ANTIBODY RESPONSE FOLLOWING PRIMARY IMMUNIZATION

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Summary. The kinetics of the serum antibody response to various amounts of bacteriophage ΦX174 were compared in germ-free and conventionally reared mice using a 50 per cent neutralization procedure for the assay of neutralizing antibody. Ten- and 100-fold increases in the amount of ΦX174 used to immunize resulted in seven and ten-fold increases, respectively, in the amount of antibody produced in conventionally reared mice; however, the same amounts of antigen produced only 1·3- and 1·9-fold increases in germ-free mice.

Greater SD_{50} values, i.e. the reciprocal of the highest dilution of serum which neutralized 50 per cent of the added bacteriophage, were obtained in conventionally reared than germ-free mice during the later stages of the antibody response; no other significant differences were noted in the kinetics of the response produced in both groups of animals immunized with high doses of antigen. However, neutralizing antibody was produced at a more rapid rate and in larger amounts in germ-free than conventionally reared mice immunized with a low dose of antigen.

Regardless of the amount of antigen used to immunize, the time of onset of antibody formation was essentially the same (32–35 hours after injection) in both groups of mice. Conventionally reared mice eliminated antigen at half the rate observed in germ-free mice similarly immunized with high doses of ΦX174, but with low doses of antigen, no significant differences in elimination rate were noted.

INTRODUCTION

In earlier studies on the antibody response to bacteriophage ΦX174 by Uhr, Finkelstein and Bauman (1962), the question was raised as to whether the rapidity and magnitude of the response observed may have been influenced by prior sensitization of the animals used in their studies, either to bacteriophage ΦX174 or to cross-reacting antigenic materials encountered in the natural environment. In this context, studies on the secondary antibody response to bacteriophage ΦX174 indicate that in previously sensitized animals, antibody synthesis begins very early after re-immunization and proceeds at a very rapid rate (Uhr *et al.*, 1962); also, non-immunized animals frequently possess in

serum low levels of background neutralizing activity, presumably natural antibody, specific for coliphage T2 (Hajek, 1966; Hook, Toussaint, Simonton and Muschel, 1966), actinophage MSP-2 (Kim, Bradley and Watson, 1966) and bacteriophage ΦX174 (Hajek, 1966).

Although the basis for the presence of natural antibody is not known, Uhr *et al.* (1962) attempted to assess the role of prior sensitization to bacteriophage ΦX174 in their studies by several means: comparisons were made of the levels of background neutralizing antibody detected in the sera of conventionally reared adult and newborn animals; newborn animals, which were protected from prior exposure to the antigens of ΦX174, were immunized, and the kinetics of the antibody response compared to that obtained in conventional adult animals; and, the kinetics of the antibody response in minimally sensitized animals were assessed. On the basis of the results obtained in these studies, Uhr *et al.* (1962) concluded that the observed response produced in conventionally reared adult guinea-pigs was typical of a primary response.

An additional approach which might help to resolve this issue would be to compare the kinetics of the antibody response to ΦX174 obtained in germ-free and conventionally reared animals immunized with different amounts of antigen. In the case of germ-free animals, one would expect that the opportunities for prior exposure and sensitization to bacteriophage antigens would be, although not entirely lacking, greatly limited by virtue of the absence of an intestinal flora, particularly, members of the coliform group of bacteria which serve as host propagating organisms for bacteriophage ΦX174 and other coliphages found in nature. In this work, the antibody response to various doses of ΦX174 was compared in groups of similarly immunized germ-free and conventionally reared mice. Bacteriophage specific neutralizing antibody was assayed by a 50 per cent neutralization method and by an immune elimination technique. Of the parameters associated with antibody response, particular attention was directed to: the time of onset of antibody synthesis following immunization with different amounts of bacteriophage ΦX174; the rate of increase of neutralizing antibody; and the maximum amounts of antibody produced following immunization with different amounts of antigen.

MATERIALS AND METHODS

Bacteriophage and the assay for neutralizing antibody to bacteriophage

The procedures used for the preparation and assay of bacteriophage ΦX174 as well as for the assay of neutralizing antibody by the 50 per cent neutralization method have been described in detail in the previous article (Stashak, Baker and Roberson, 1970). The amount of neutralizing antibody present in the sera of immunized mice was expressed as the SD_{50} value, i.e. the reciprocal of the highest dilution of serum which neutralized 50 per cent of the bacteriophage added to the assay mixture.

Animals

The mice used and the conditions of maintenance have been described by Stashak *et al.* (1970).

Immunization

Recently assayed stock suspensions of purified bacteriophage were diluted appropriately in tryptone broth to the desired concentration to be used for immunization. Each

preparation was filtered through a Millipore HA Filter (0·45 μ porosity) to remove bacteria and dispensed aseptically into sterile, 2-ml glass ampoules. The ampoules containing bacteriophage, as well as all needles and syringes used for immunization, were introduced aseptically into the germ-free isolators. Unless otherwise stated, both conventionally reared and germ-free mice were immunized by a single intraperitoneal injection of 0·1 ml of a similarly prepared bacteriophage suspension. The amount of bacteriophage used for immunization was carefully standardized in terms of the number of plaque-forming units (PFU) present per unit volume.

Immune clearance studies

Three concentrations of bacteriophage, namely 2×10^7, 2×10^8 and 2×10^9 PFU were used in the antigen elimination or the immune clearance studies. The desired amount of bacteriophage in 0·1 ml tryptone broth was injected into the tail vein of each mouse with the aid of a tuberculin syringe equipped with a 27 gauge needle. Blood samples, taken from the retro-orbital sinus of individual mice by means of disposable 10-μl micropipettes, were collected immediately (zero time) and at designated time intervals after immunization. The entire contents of each 10-μl micropipette were expelled into an appropriate volume of tryptone broth from which further dilutions were made, depending on the dose of antigen used and the time of sampling, to give countable numbers of PFU for each sample by the soft agar layer assay method (Adams, 1959). On the basis of the volume of blood used and the number of dilutions made, the number of PFU/1·0 ml of blood was calculated at each time interval for each immunized animal. Mean values on the rate of decrease of viable circulating bacteriophage and on the time of onset of antibody synthesis were calculated from the data obtained with five individual, similarly treated mice.

Statistical methods

The data were analysed by the method described in the previous article (Stashak *et al.*, 1970).

RESULTS

COMPARISON OF THE SERUM ANTIBODY RESPONSE TO BACTERIOPHAGE ΦX174 IN MALE AND FEMALE GERM-FREE MICE

Since the germ-free mice used in these studies are generally supplied to us in lots of sixty weanling mice (ten to twelve litters) of similar age, approximately equally divided as to sex, it was necessary to determine whether the use of animals of both sexes would influence greatly the results obtained in studies on the kinetics of the antibody response. For both practical and economical reasons, it would be desirable to use all of the germ-free mice supplied in a given lot. Groups of five male and five female germ-free mice were immunized with 2×10^9 PFU of bacteriophage ΦX174, and at 4, 5, 7 and 10 days post immunization, the mice were bled and their sera assayed for the amount of neutralizing antibody present at each time interval by mice of each sex. The data presented in Table 1 indicate that the differences between male and female germ-free mice in respect to the SD_{50} values obtained, were rather small and represented differences of 23, 10, 16 and 16 per cent at 4, 5, 7 and 10 days post immunization, respectively. With the exception of the SD_{50} values obtained at 4 days post immunization, male germ-free mice appeared to produce slightly more neutralizing antibody than females at corresponding time intervals.

TABLE 1

COMPARISON OF THE NEUTRALIZING ANTIBODY LEVELS IN THE SERUM OF MALE AND FEMALE GERM-FREE MICE IMMUNIZED WITH 2×10^9 PFU OF BACTERIOPHAGE ΦX174

Sex	Day post injection			
	4	5	7	10
Male	43*	430	3450	4800
Female	56	380	2850	4000

* SD_{50} value obtained using pooled serum samples from five mice.

In view of the slight differences in response noted, it seemed reasonable to conclude that germ-free mice of both sexes could be employed in studies on the kinetics of the antibody response to ΦX174; however, to minimize further the effects of the slight differences noted in response between sexes, serum samples from male or female immunized mice were collected on *alternate* days after immunization. By this procedure, data could be used to construct a curve describing the kinetics of the antibody response in germ-free mice which was in close agreement with the SD_{50} values obtained when mice of one sex were used. The variation encountered using germ-free mice of both sexes was certainly no greater than that obtained when conventionally reared mice of one sex (females) were used (see Figs. 1, 2 and 3).

SERUM ANTIBODY RESPONSE IN GERM-FREE AND CONVENTIONALLY REARED MICE IMMUNIZED WITH VARIOUS AMOUNTS OF BACTERIOPHAGE ΦX174

Data on the serum antibody response, as measured, by the 50 per cent neutralization method, in germ-free and conventionally reared mice immunized with various amounts of ΦX174 are presented in Figs. 1, 2 and 3. Because of the non-linearity of the response obtained, it is difficult to calculate the precise rate of antibody synthesis in each of the groups of immunized mice examined; consequently, comparisons made as to the differences between germ-free and conventionally reared mice in their response to different amounts of antigen are approximations based on a graphic interpretation of the data.

With the exception of the development in conventionally reared mice of greater amounts of serum neutralizing antibody during the later stages of the antibody response (12–14 days post injection), both germ-free and conventionally reared mice responded similarly when immunized with 2×10^9 PFU of ΦX174 (Fig. 1). Similarities in the kinetics of the serum antibody response between both groups of mice were even more apparent when 2×10^8 PFU of ΦX174 were used to immunize, although slightly higher levels of neutralizing antibody were detected in the serum of conventionally reared mice at 14 days post injection (Fig. 2); however, the most striking differences in the kinetics of serum antibody response were seen in mice immunized with 2×10^7 PFU of ΦX174 (Fig. 3). Here, the data suggest that the development of the antibody response was much more rapid and the levels of antibody obtained were much greater in germ-free rather than conventionally reared mice. With the exception of the response obtained in mice immunized with 2×10^7 PFU of ΦX174, the rate of antibody synthesis appears to be about the same for both groups of mice; however, on the basis of the amount of neutralizing antibody detected at 14 days post injection, the response in conventionally reared mice appears to be greater in magnitude than that obtained in germ-free mice. While immunization with 2×10^8 and

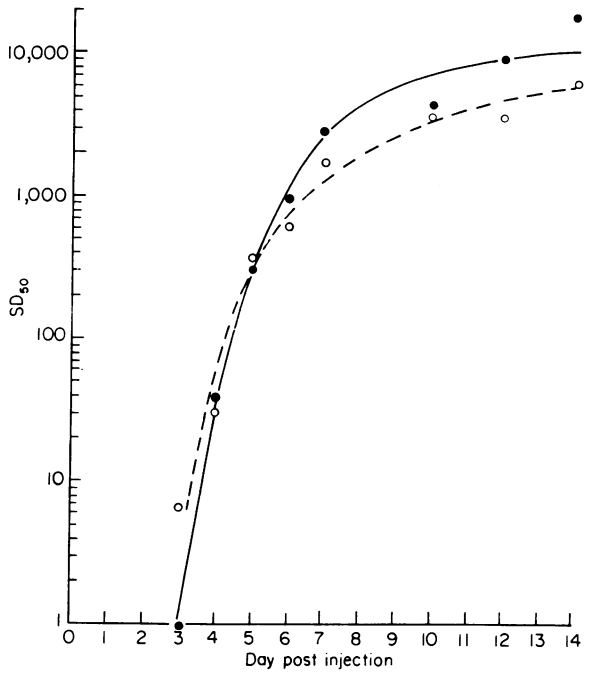

FIG. 1. Appearance of neutralizing antibody in the serum of germ-free (○) and conventionally reared (●) mice immunized with 2×10^9 PFU of bacteriophage ΦX174.

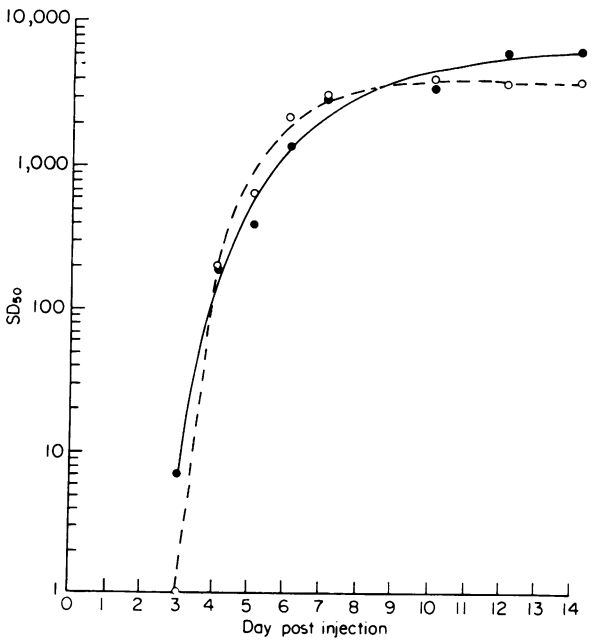

FIG. 2. Appearance of neutralizing antibody in the serum of germ-free (○) and conventionally reared (●) mice immunized with 2×10^8 PFU of bacteriophage ΦX174.

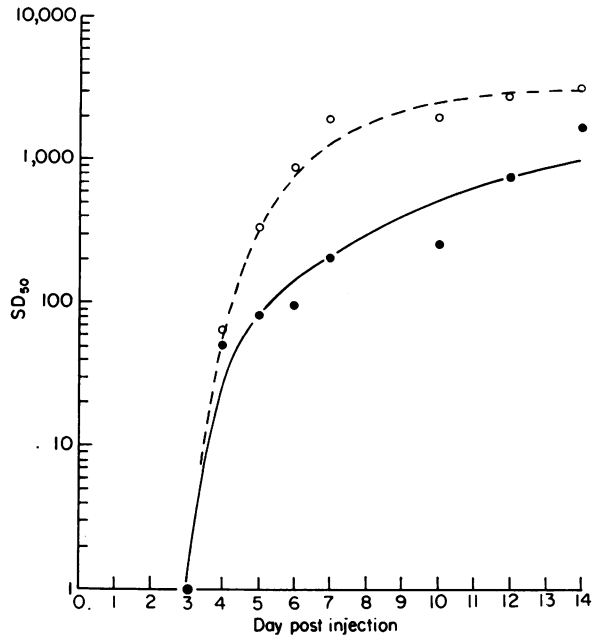

FIG. 3. Appearance of neutralizing antibody in the serum of germ-free (○) and conventionally reared (●) mice immunized with 2×10^7 PFU of bacteriophage ΦX174.

TABLE 2

MAXIMUM LEVELS OF NEUTRALIZING ANTIBODY OBTAINED IN THE SERUM OF GERM-FREE AND CONVENTIONALLY REARED MICE IMMUNIZED WITH VARIOUS AMOUNTS OF ΦX174

2×10^9 PFU		2×10^8 PFU		2×10^7 PFU	
Germ-free	Conventional	Germ-free	Conventional	Germ-free	Conventional
5,800*	10,000	4,000	6,600	3,100	1,000

* Values listed are the SD_{50} values obtained 14 days after immunization. Pooled serum samples obtained from five mice were used for each determination.

2×10^9 PFU resulted in seven- and ten-fold increases, respectively, in the SD_{50} values obtained relative to the response obtained with 2×10^7 PFU in conventionally reared mice, similar increases in dose gave only 1·3–1·9-fold increases, in the SD_{50}, respectively, in germ-free mice (Table 2).

ELIMINATION OF VIABLE BACTERIOPHAGE ΦX174 FROM THE CIRCULATION OF IMMUNIZED GERM-FREE AND CONVENTIONALLY REARED MICE

The presence of large numbers of viable bacteriophage in the circulation of immunized mice made it difficult to detect the presence of neutralizing antibody in serum prior to three days post injection by the 50 per cent neutralization method. Consequently, an immune elimination or clearance procedure was utilized to determine the time of onset of antibody formation and to detect the development of antibody during the first 48 hours after immunization.

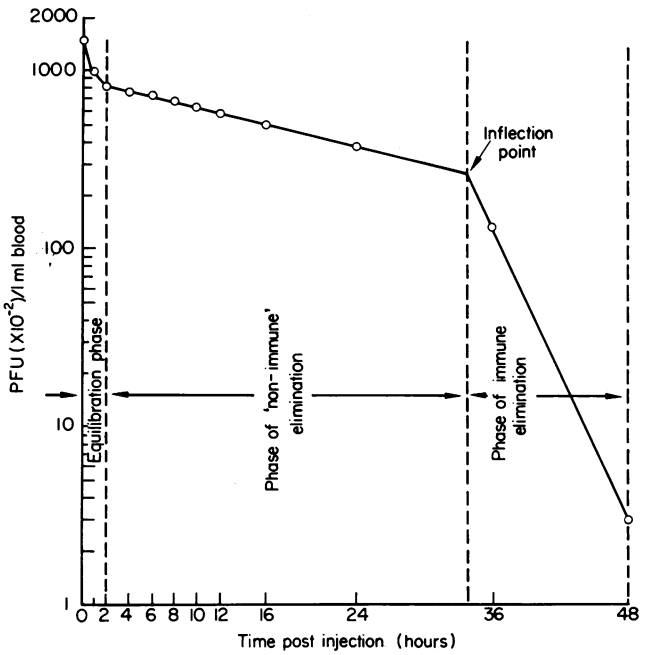

FIG. 4. Rate of decrease in the number of viable bacteriophage in the circulation of mice immunized with 2×10^7 PFU of ΦX174.

The data presented in Fig. 4 are typical of the results obtained using the elimination procedure with individual germ-free or conventional immunized mice employed in these studies. Following a very brief *phase of equilibration*, which was observed in only a few of the mice used in these studies, there occurs a phase of *'non-immune' elimination*. The latter is presumably the result of the uptake of bacteriophage by certain phagocytic cells of the reticulo-endothelial system and is thought not to be dependent upon the presence of specific antibody. Next, there occurs a phase of very rapid or accelerated neutralization and/or elimination of viable bacteriophage—the *phase of immune elimination*—which is antibody dependent (Uhr *et al.*, 1962). During this phase, one is witnessing the effects of both the neutralization of bacteriophage by newly formed antibody and the accelerated removal of bacteriophage–antibody complexes in which bacteriophage may or may not be neutralized. It is also quite possible that there is an accelerated removal of bacteriophage by newly formed antibody at the surface of antibody producing cells. Consequently, the time at which the phase of immune elimination begins (the inflection point) can be taken to represent the time at which the presence of specific antibody is first demonstrable.

The data of Table 3, in which are presented the rates of decrease in the number of circulating viable bacteriophage during the phase of immune elimination, indicate that there is no significant difference in the rate of disappearance of viable ΦX174 in germ-free and conventionally reared mice immunized with 2×10^7 PFU. These findings are in contrast to those obtained using the 50 per cent neutralization procedure in which germ-free mice immunized with 2×10^7 PFU of ΦX174 appeared to synthesize neutralizing antibody at a much more rapid rate than did similarly immunized conventionally reared mice (Fig. 3). However, significantly more rapid rates of elimination were obtained in

TABLE 3

RATE OF DECREASE IN VIABLE BACTERIOPHAGE IN THE CIRCULATION OF GERM-FREE AND CONVENTIONALLY REARED MICE IMMUNIZED INTRAVENOUSLY WITH VARIOUS AMOUNTS OF ΦX174

	2×10^9 PFU		2×10^8 PFU		2×10^7 PFU	
	Germ-free	Conventional	Germ-free	Conventional	Germ-free	Conventional
	2·98*	1·57	2·18	0·965	4·19	1·91
	3·51	1·40	2·69	0·913	3·21	3·04
	2·59	1·73	1·11	0·581	1·06	2·48
	2·78	1·47	2·92	1·34	3·96	2·88
	3·32	1·38	2·61	1·73	3·15	–
Mean	3·04	1·51	2·30	1·11	3·11	2·58
Test of significance (P)	<0.05		<0.05		>0.05	

* Figures shown were obtained by calculating the slope of the elimination curve during the phase of immune elimination for individual mice. Values represent the $\log_{10}$ decrease in PFU/ml blood/hr.

germ-free than in conventionally reared mice immunized with 2×10^8 PFU or 2×10^9 PFU; here, the rate of elimination in conventionally reared mice was in general about one-half that observed in germ-free mice. Likewise, these findings are also in contrast to those obtained by the 50 per cent neutralization method (Figs. 1 and 2) and may serve to indicate that each procedure is assessing the result of one or a combination of several different processes, namely, neutralization in the case of the 50 per cent neutralization method and neutralization in addition to the selective elimination of bacteriophage in the immune elimination method.

The data of Table 4 fail to suggest substantial differences between germ-free and conventionally reared mice with respect to the time of onset of antibody synthesis. In both groups of mice, regardless of the amount of antigen used for immunization, antibody synthesis began on the average at about 32–35 hours after the administration of ΦX174. In separate studies, an attempt was made to demonstrate the presence of circulating bacteriophage–antibody complexes at 4, 8, 12 and 16 hours after immunization with 2×10^7, 2×10^8 and 2×10^9 PFU of ΦX174. A solution of dithiothreitol (DTT), a strong reducing agent capable of dissociating macroglobulin antibody, was added to appropriate dilutions of blood samples, obtained at the above time intervals, in a final concentration of 0·005 M. All solutions of DTT and dilutions of blood samples were made with 0·1 M Tris–0·1 M NaCl buffer, pH 7·4. After 10 minutes at room temperature, aliquots of the reaction mixture were assayed for the presence of additional PFU relative to blood samples

TABLE 4

TIME OF ONSET OF THE PHASE OF IMMUNE ELIMINATION FOLLOWING INTRAVENOUS IMMUNIZATION OF GERM-FREE AND CONVENTIONAL MICE WITH VARIOUS AMOUNTS OF ΦX174

	2×10^4 PFU		2×10^8 PFU		2×10^7 PFU	
	Germ-free	Conventional	Germ-free	Conventional	Germ-free	Conventional
	36*	36	36	35	36	34
	34	36	23	34	35	33
	36	36	31	33	35	36
	34	36	35	30	35	35
	36	36	36	34	35	–
Mean	35·2	36	32·2	33·2	35·2	34·5

* Values listed represent hours post immunization for individual mice.

not treated with DTT. Inasmuch as no additional PFU were detected following treatment with DTT, these findings suggest that few, if any, bacteriophage–antibody complexes were present in the blood of immunized mice prior to 32–35 hours following immunization with the above stated amounts of bacteriophage.

DISCUSSION

In a previous report (Stashak *et al.*, 1970), low but detectable levels of background neutralizing activity were noted in the sera of both germ-free and conventionally reared non-immunized mice assayed at 37° and at room temperature (25°). Although it was not possible to determine the precise amounts of antibody present, it did appear from the data obtained in assays conducted at 37° that the level of background neutralizing activity was higher in non-immunized conventionally reared than in germ-free, mice. If indeed natural antibody is responsible for the background neutralizing activity observed, and if it exerts an influence on the rate of antibody formation following immunization with bacteriophage (Kim, Bradley and Watson, 1966, 1967), then one should find differences between germ-free and conventionally reared mice with respect to the kinetics of the antibody response produced with low doses of antigen. The data obtained in the present work on the kinetics of the antibody response to various amounts of ΦX174 in germ-free and conventionally reared mice support such a concept. With high doses of antigen, there appeared to be no significant differences in the rate of antibody formation. Here, excess antigen might have discounted any possible influence attributable to the effects of background antibody. However, with a low dose of antigen (2×10^7 PFU), the antibody response in germ-free mice, perhaps due to their lower levels of background neutralizing antibody, was much more rapid and greater in magnitude, than that obtained in similarly treated conventionally reared mice.

Ten- and 100-fold increases in the amount of ΦX174 used to immunize conventionally reared mice, resulted in increases of seven- and ten-fold in the SD_{50} values, respectively, obtained at the peak of the antibody response. However, corresponding increases in dose resulted in only 1·3- and 1·9-fold increases in the SD_{50} values, respectively, in germ-free animals. Since previous investigations indicated that the antibody response to high and to low doses of ΦX174 is qualitatively similar (Stashak *et al.*, 1970), and that with high doses of bacteriophage ΦX174 there appears to be little difference in the rate of antibody synthesis in both groups of mice, it would appear, with the exception of possible differences in the rate of catabolism of antibody between germ-free and conventionally reared mice, that the differences noted with respect to the magnitude of the response produced with various amounts of bacteriophage might be accounted for on the basis of differences between germ-free and conventionally reared mice in the numbers of lymphoid cells capable of participating in the antibody response. In this context Olson and Wostmann (1966a, b) and Thorbecke (1959) have reported that germ-free animals possess fewer numbers of lymphoid cells and secondary lymphoid nodules than do their conventionally reared counterparts.

As assessed by the immune elimination procedure, no difference was noted between germ-free and conventionally reared mice, immunized with various amounts of ΦX174, with respect to the time of onset of antibody formation; the length of the inductive phase (32–35 hours) was very similar to that reported by Kim *et al.* (1967), who studied the development of neutralizing antibody and the rate of elimination of actinophage MSP-2

in germ-free, colostrum-deprived piglets. However in comparative studies on the immune response in germ-free and conventionally reared mice to horse ferritin and *Serratia marcescens* (Bauer, Paronetto, Burns and Einheber, 1966) and to Vi antigen (Baker and Landy, 1967), there appeared to be a considerable delay in the onset of antibody formation in germ-free mice. It has been reported that, although the macrophages of germ-free and conventionally reared mice phagocytize particulate antigens at about equal rates, the degradation or digestion of antigen proceeds much more slowly in the germ-free animal (Bauer, Horowitz, Watkins and Popper, 1964; Bauer *et al.*, 1966). While differences in the functional capacity of macrophages might result in substantial alterations in the antibody response when particulate antigens or antigens which are degraded with difficulty (Vi antigen) are employed, such alterations would not be evident upon immunization with an antigen such as ΦX174 which is easily degradable and highly immunogenic (Uhr and Möller, 1968).

With the lowest dose of antigen (2×10^7 PFU) used in the immune elimination studies, no significant differences were noted between germ-free and conventionally reared mice with respect to the rate at which viable ΦX174 was removed from the circulation. Indeed the rate of elimination of ΦX174 in germ-free mice was essentially the same with all three doses of antigen used in this study. However, with higher doses of antigen (2×10^8 PFU and 2×10^9 PFU), bacteriophage was removed from the circulation of conventionally reared mice at about half the rate ($P<0{\cdot}05$) observed with corresponding doses in germ-free mice. With higher doses of antigen, noticeable signs of endotoxaemia (diarrhoea, ruffled fur and general malaise) were noted in conventionally reared but not germ-free mice. Since the bacteriophage preparations used in these studies were isolated from culture lysates of the host bacterial strain of *E. coli*, the preparations may have contained small amounts of *E. coli* endotoxin which could not be removed by the bacteriophage purification procedure. Because of prior exposure and sensitization to the antigens of enteric micro-organisms, which normally are part of the intestinal flora, one would expect that the pharmacological effects of endotoxin, especially the endotoxin of *E. coli*, would be more pronounced in conventionally reared than in germ-free mice, thereby affecting adversely the functional activity of the reticulo-endothelial system with respect to the clearance and degradation of antigen by phagocytic cells. In this context Schaedler and Dubos (1961), Abernathy, Bradley and Spink (1958) and Wilson, Kolbye and Baker (1964) reported that the susceptibility of mice to the lethal and pharmacological effects of endotoxin is conditioned by prior exposure to microbial organisms possessing the antigen. In view of such considerations, it would appear that the germ-free animal is particularly well-suited for use in studies dealing with the kinetics of the antibody response to low doses of antigen.

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