MEASUREMENT OF RESISTANCE TO EXPERIMENTAL TUBERCULOSIS IN MICE

The Hyperacute Phase

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(Received for publication, August 23, 1962)

During studies of nutritional effects on tuberculous mortality in NCS mice a consistent deviation was found in the frequency distribution of death. This was due almost entirely to the fact that a large number of animals responded to the infection in a hyperacute manner. Since a knowledge of this response would appear to be important to the understanding of mechanisms of resistance to tuberculosis, a report of our findings is given below.

Materials and Methods

Animals.—The new NCS colony of mice maintained at The Rockefeller Institute has been described elsewhere (1–6). Female mice were used in the majority of experiments, although occasional groups of male mice were included. All animals were obtained at 4 to 6 weeks of age, and were distributed at random in groups of three to twenty animals. The size of cage was selected to insure that crowding did not occur. The litter of wood shavings was changed weekly.

Diets.—Animals were maintained on one of several nutritional regimens. Unless otherwise noted, they were given commercial D & G pellets (Dietrich and Gambrill, Frederick, Maryland). In other experiments animals were fed commercial Rockland mouse pellets (A. E. Staley Manufacturing Co., Decatur, Illinois) or semisynthetic rations containing 15 or 20 per cent vitamin-free casein, 4 per cent Wesson salt mixture, 10 per cent alphacel, 0.4 per cent vitamin fortification mixture, 0.1 per cent cystine, 5 per cent fat (peanut or corn oil), and starch or dextrin to 100 per cent. The several semisynthetic diets used differed slightly, as indicated, but these differences were without effect on the results. Once placed on a given diet, the animals were maintained on it until termination of the experiment. Diets were fed *ad libitum*. Fresh tap water was available at all times.

Vaccines.—Animals received by parenteral injection killed mycobacterial cells or a commercially available lipopolysaccharide endotoxin prepared from *Escherichia coli* (Difco Laboratories, Inc., Detroit, lot 439277). The H37Rv strain of *Mycobacterium tuberculosis* and the Vallée strain of *Mycobacterium bovis*, grown in the same manner as for the infecting challenge (see below), were killed by exposure to ethylene oxide (7). The Phipps strain of BCG was killed by exposure to ethylene oxide or to heat at 80°C. All injections were by the intraperitoneal route; 0.2 ml being injected in each case.

* This investigation was supported in part by a Public Health Service fellowship (EPD-14,668) from the National Institute of Allergy and Infectious Diseases, Public Health Service.

Infection of Animals.—The H37Rv strain of *M. tuberculosis* was obtained from the Standard Culture Depot of the National Tuberculosis Association (August, 1961) and maintained at 0–4°C without transfer. Prior to use for infection, an aliquot of this culture was transferred to 20 ml of Kirchner's medium (8) containing 0.5 per cent bovine serum albumin and 0.05 per cent tween 80. This was incubated at 37°C until an optical density (OD) of approximately 0.200 was obtained. A second transfer was made using 2.0 ml of this culture and 20 ml of the medium without addition of albumin. After exactly 5 days' incubation the culture was concentrated or diluted to give an optical density of 0.370 to 0.600. These cultures contained approximately 10^7 viable units per ml. The Vallée strain of *M. bovis* was the virulent culture maintained in this laboratory for many years. Stock cultures were preserved at 0–4°C. A transfer of this stock culture was made to tween-albumin medium and incubated for 7 days. Animals were infected by intravenous injection with cultures diluted to a final volume of 0.2 ml.

Preparation of Substances to be Injected into Mice.—Dilutions of endotoxin and vaccines were made with pyrogen-free diluents. Precautions were taken that cultures used for challenge were free of pyrogenic material. Reagent grade chemicals were used throughout and all glassware was scrupulously cleaned. Occasional assay for pyrogenic activity was made by parenteral injection of mice. Neither weight loss nor suppression of water intake was ever observed. This assay system has been determined to be sensitive to 0.1 to 0.3 μg purified endotoxin (4).

Validity of Results.—Large numbers of animals were used to determine the individual time of death from any cause related to the injection of virulent mycobacteria. For this reason it was neither possible to carry out autopsies or cultural tests nor desirable to confirm exactly the cause of death of any animal. It was important, however, to ascertain that death by extraneous means or by accidental secondary infection was extremely improbable. To this end, stool cultures were made from uninfected animals from time to time to insure the absence of *Salmonella* sp. and other potential enteric pathogens. No deaths occurred in uninfected animals kept under the same conditions as infected animals.

The results described are from experiments repeated a number of times and since the results were remarkably consistent and reproducible, a high degree of significance can be attached to them.

RESULTS

1. The Response of NCS Mice to Infection with M. tuberculosis. The Early-Acute Phase.—It will be useful to describe briefly the overt course of tuberculous infection in NCS mice as observed during a number of experiments.

Following infection with human-type bacilli no change was noted in the condition or behavior of any animal for the first 7 days. Animals gained weight normally and looked healthy and thrifty. No deaths were ever noted during this period. On the 8th to 10th day following infection one or two animals were found dead. Many other animals appeared ill. Contrary to the usual behavior of mice, the animals kept themselves isolated from their cage mates. Food and water intake appeared appreciably diminished. The following day three to five more animals had succumbed. No improvement was noted in the appearance of the surviving animals. It was common on the following day to find one or two dead animals. The remainder of the animals showed marked improvement and most of them appeared essentially normal. Only rarely did an occasional animal die following this time until the 16th to 20th day. During the following week or 10 days, death was a frequent occurrence until essentially all animals were dead. A small number of animals often survived beyond this period.

The response of mice to bovine-type bacilli differed from their response to the human-type

by the complete absence of illness and death in the early period. Animals appeared normal until the 16th to 20th day following infection. From then on some were found dead almost daily and within 10 days essentially all had died.

The deaths of animals in a typical experiment are recorded in Figs. 1 *a* and 1 *b* as a logarithmic probability plot. In this particular experiment female mice,

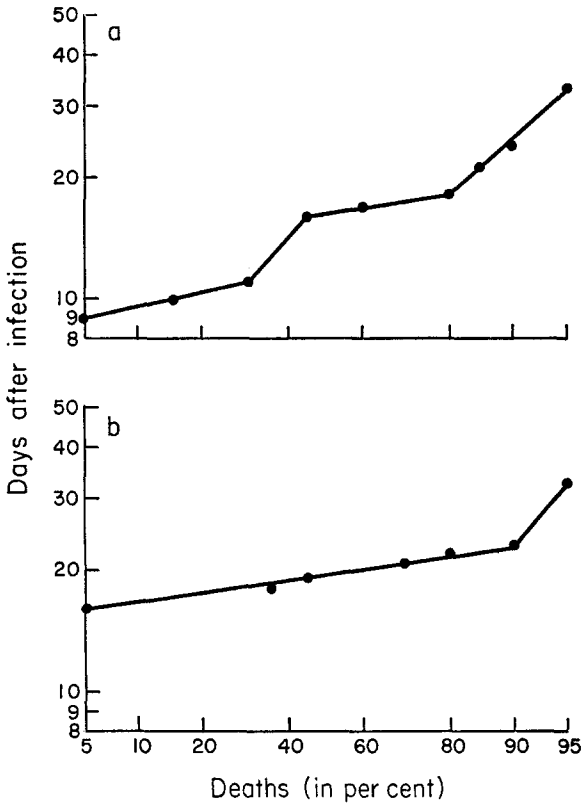

FIG. 1 *a*. Deaths of NCS mice following infection with human-type tubercle bacilli recorded as logarithmic probability.

FIG. 1 *b*. Deaths of NCS mice following infection with bovine-type tubercle bacilli recorded as logarithmic probability.

in groups of twenty, were 9 weeks old when infected with a suspension of human bacilli having an optical density of 0.500 or bovine bacilli having an OD of 0.080.

The deviation of the rate of death from normal in the case of human-type infection as compared with bovine-type infection is readily apparent. It is to be noted that deaths occurred in three easily distinguishable phases. The group of

mice dying during the 2nd week following challenge was entirely distinct from the group dying during the 3rd and 4th weeks. Both groups differed from the small group of animals dying irregularly following the 4th week of infection.

It is useful to accept the postulate (9) that mice dying during the first few weeks following heavy infection with the H37Rv strain succumb as a consequence of rapid multiplication of the tubercle bacilli in their lungs: reacting therefore in an "acute" manner. Using this terminology the first two phases of death may be considered as the early- or hyperacute phase and the late-acute phase. These terms will be used throughout this paper.

The time of death of mice reacting in the hyperacute manner was remarkably

TABLE I

Time of Death of NCS Mice Following Infection with M. tuberculosis

Experiment	No. of mice	No. of deaths*											
		8	9	10	11	12	13	14	15	16–20	21–25	26–30	31–
1	20	1	1	5	—	1	—	—	—	1	4	3	4
2	19	—	—	5	1	—	—	—	—	4	2	1	6
3	20	—	—	—	5	—	1	—	—	2	9	2	1
4	20	—	—	5	1	—	—	—	—	10	2	0	2
5	19	—	—	3	4	—	—	—	—	2	1	2	7
Total	98	1	1	18	11	1	1	—	—	19	18	8	20

* Mice dead on given day or in period following intravenous injection of *M. tuberculosis*.

consistent. This is illustrated in Table I where the results of five similar experiments are given. Female mice were used and all animals were challenged with human bacilli.

It should be noted that the first phase of acute death always occurred between the 8th and the 13th day. The second phase of acute death never began before the 16th day.

In the experiments described a relatively large does of human bacilli had been injected. It was desirable to determine more closely the effect of the challenge dose on the early-acute phase of death. The results of one such experiment are given in Figs. 2 *a* and 2 *b*. In this experiment female animals, 8 weeks of age, were challenged with cultures of human bacilli having an OD of 0.960, 0.480, 0.240, and 0.120 respectively. Each group consisted of twenty mice.

As expected, occurrence of early-acute death was related to the challenge dose. The type of dose response obtained is of interest. With infections of a chronic type, such as tuberculosis, animals respond to a decrease in the infective dose with an increased survival time. In a general fashion this occurred here when the entire population was considered. However, when the animals

dying in the early-acute phase were considered apart from those dying in the late-acute phase, each group was noted to respond in a different manner. With a decreasing challenge dose a decreasing number of animals died of the early-acute disease, but those that died did so at essentially the same time irrespective of challenge dose. This finding reminds one of what is observed with certain

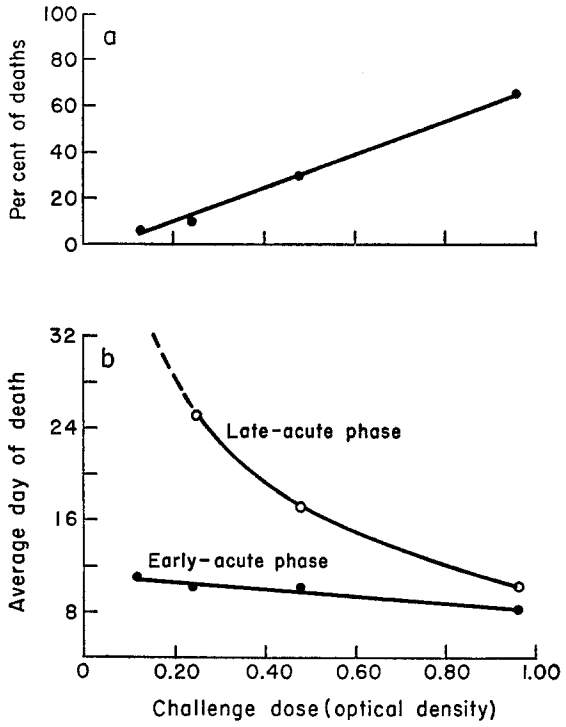

FIG. 2 *a*. Per cent of NCS mice dying during early-acute phase as a function of challenge dose (human tubercle bacilli).

FIG. 2 *b*. Average survival time of NCS mice dying during early-acute or late-acute phase as a function of challenge dose.

toxins and acute infections. On the other hand, animals which survived the initial phase of death responded to the decreasing dose by an increasing average survival time.

2. The Effect of Age, Diet, and Sex on the Early-Acute Response.—The results described in the preceding section indicated that NCS mice were prone to succumb to a hyperacute form of disease when injected with a heavy inoculum of human tubercle bacilli and that the time of death could be used to study the occurrence of this phase of disease. A number of experiments were designed to determine the optimal conditions for demonstrating this response.

Susceptibility of mice to tuberculous infection is known to change with age and nutritional history. The effect of these conditions on early-acute death was studied.

Female animals maintained on D & G pellets since weaning were changed to Rockland pellets, or to a synthetic casein diet, or continued on D & G pellets. At various ages they were challenged with human bacilli.

The results are recorded in Table II.

It may be observed that animals maintained on D & G pellets became increasingly resistant to early-acute death with age. A similar but more rapid

TABLE II

Effect of Diet on Per Cent of Animals Dying during Early-Acute Phase

Diet	Initial age of mice	Deaths* Time following initiation of diet, wks.				
		0	4	8	12	16
	wks.					
D & G pellets	5	50‡	45	40	25	25
Rockland pellets	5	50	30	20	0	—
Synthetic casein diet	5	50	80	55	50	—

* Per cent early-acute phase deaths following intravenous injection of *M. tuberculosis* after prefeeding diet for given number of weeks.

‡ Experimental groups consisted of eighteen to twenty mice. Most values refer to the average of results of two or more experiments.

loss of susceptibility occurred in animals changed to Rockland pellets. Animals fed the synthetic diet, on the other hand, showed no appreciable decline in susceptibility to early-acute death during the course of the experiment.

The manner of change in susceptibility is of interest. It is known that the effect of increasing age on resistance to tuberculosis is typically manifested in mouse survival experiments as an increase in average survival time. Contrary to this response, and to that of the mice surviving into the late-acute period, the survival time of animals dying during the early-acute phase was remarkably similar irrespective of age. This is illustrated in Fig. 3. The data given are for mice maintained on D & G pellets, but similar results were obtained with other nutritional regimens.

Again, as in the case of dose-response, it is noted that in older animals there is a decrease in the number of early-acute deaths. They occur, however, at the same time irrespective of age.

The effect of sex on early-acute death was determined. Virgin mice from the same litters were used. The experimental conditions were similar to those previously used. It is noted from the results given in Table III that although ani-

mals of both sexes died during the early-acute phase, female animals were more susceptible than were male. No difference related to sex was apparent in the average survival times.

3. Protection of Mice against Early-Acute Death.—Several experiments were carried out in an attempt to modify the occurrence of early-acute deaths in NCS mice.

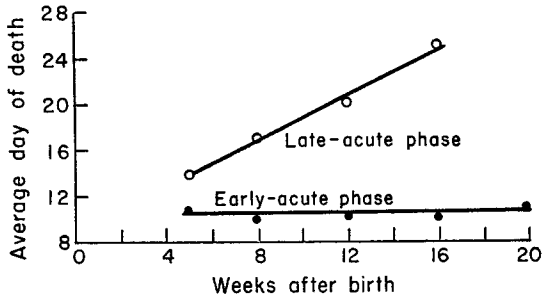

FIG. 3. Average survival time of NCS mice as a function of age. Early-acute or late-acute phase.

TABLE III

Effect of Sex on the Per Cent of Mice Dying during the Early-Acute Phase

Age of mice	Deaths*	
	Male	Female
wks.	*per cent*	*per cent*
5	30 (46)‡	50 (40)
8	24 (17)	42 (12)
15	27 (15)	35 (20)

* Per cent early-acute deaths following intravenous injection of *M. tuberculosis.*

‡ Figures in parentheses refer to total number of mice of given sex in experiment.

NCS mice were exposed to enteric organisms from "non-pathogen-free" mice. To this end, litters of 8-day-old animals were obtained. The ventral surfaces of the mothers were sprayed with a suspension of organisms prepared by homogenizing the intestines and intestinal contents of several CFW mice to which was added a dilute culture of *E. coli.* Stool cultures of weaned mice from these litters revealed a large, transient increase in the number of cultivable bacteria—chiefly *E. coli.* The bacterial content of stools returned to normal levels before the time of challenge infection. Certain of these animals were also given an intraperitoneal injection of 30 μg of endotoxin 3 weeks prior to challenge. The early-acute deaths in groups of female animals are given in Table IV. The results of two other experiments in which mice were parenterally injected with endotoxin prior to challenge are included.

The results suggest that brief exposure of animals to Gram-negative endo-

toxin or to a bacterial flora consisting predominantly of *E. coli* increased their resistance to early-acute death.

It is known that mice given a parenteral injection of mycobacterial products

TABLE IV

Effect of Enteric Flora or of Endotoxin on Early-Acute Phase Deaths

Experiment	Contamination with enteric organisms	Endotoxin*	No. of mice	Deaths‡
		μg		*per cent*
1	No	0	12	42
	"	30	10	10
	Yes	0	12	17
	"	30	11	9
2§	No	0	20	30
	"	30	12	0
3	"	0	19	37
	"	300	20	20

* The time between endotoxin injection and challenge infection was 3, 4, or 8 weeks respectively in the three experiments. Age of mice at challenge varied from 9 to 19 weeks.

‡ Per cent early-acute deaths following intravenous injection of *M. tuberculosis*.

§ Mice in this experiment were male and were fed synthetic diet.

TABLE V

Effect of Vaccination with Mycobacteria on Percentage of Mice Dying during Early-Acute Phase

Vaccine*	Deaths‡	
	Control mice	Vaccinated mice
	per cent	*per cent*
Living BCG	40	10
Heat-killed BCG	35	0
Ethylene oxide–killed BCG	40	10
Ethylene oxide–killed Vallée	35	6
Ethylene oxide–killed H37Rv	30	0

* See Materials and Methods.

‡ Per cent early-acute deaths following intravenous injection of *M. tuberculosis*.

may develop acquired resistance to subsequent injection of living virulent tubercle bacilli. It was of interest to ascertain whether such products would afford protection against the early-acute phase of death. The results described in Table V are taken from a number of experiments. Female mice were used and were maintained on D & G pellets. The age of the mice at challenge time

ranged from 8 to 16 weeks; the vaccination period from 4 to 8 weeks. Each group contained from seventeen to twenty animals that were challenged with human tubercle bacilli.

All the vaccine preparations used gave significant protection against the early-acute form of death. As judged by usual survival data evaluation, the protection afforded by each of the vaccines would indicate a significant degree of acquired resistance to tuberculosis. However, the findings suggest that this resistance might be the expression of tolerance to the mechanisms resulting in early-acute death, as well as of a more specific immunity to other aspects of the disease. It is not improbable that the protection afforded by some vaccination procedures is partly due to the non-specific elimination of early-acute reactions.

DISCUSSION

This paper describes and briefly characterizes a hyperacute reaction occurring in NCS mice infected with human-type tubercle bacilli. This reaction frequently results in death.

There is some reason to believe that early-acute death may be a manifestation of a toxic-type reaction induced by substances produced by or present in the tubercle bacillus. The evidence for this is as follows. It has been demonstrated that injection of mice with killed BCG cells causes depression of water intake and weight loss similar to that caused by purified endotoxin from Gram-negative bacilli (4). The activity of such cells suggests the occurrence in mycobacteria of substances with endotoxin-like properties (10). Furthermore, both the dose-response and the age-response of mice to the early-acute reaction were similar to those of animals to certain toxins. Lastly, significant protection against early-acute death was afforded by acquisition of tolerance to endotoxin.

It should be noted that recognition of this phenomenon was due primarily to the proper, albeit fortuitous, selection of experimental conditions since, under these conditions, the reaction gave rise to the consistent and reproducible occurrence of early-acute death. Among these conditions may be listed the following: (*a*) the strain of mouse used, (*b*) the sex of the mouse, (*c*) the diet on which the animals had been maintained, (*d*) the type and size of challenge infection, (*e*) the age of the animals at challenge, and (*f*) the previous exposure of animals to enteric bacteria and other substances.

The experiments described herein were concerned solely with NCS mice. The choice of strain of mice, therefore, requires further discussion. CFW Swiss mice are related to NCS mice and animals from this strain were found to be equally susceptible to early-acute death. CF_1 (non-Swiss) albino mice, on the other hand, were much more resistant to the cause of this early-acute death. However, some early-acute effects probably did occur in these animals for many became exceedingly ill approximately 8 to 10 days following infection, but most of them recovered from this illness. These experiments suggest that early-acute reac-

tions are not unique in NCS mice although it may be more difficult to detect them in other mouse strains because death rarely occurs during this early phase.

The relevancy of early-acute death to the mechanism of resistance to experimental tuberculous infection seems of interest here. Much previous work has drawn attention to the importance of non-specific resistance mechanisms in determining the course of infectious diseases. Little is known, however, concerning the manner in which such mechanisms act. Some of the hypotheses advanced to explain the observed phenomena, such as alteration of phagocytic activity and protection against common antigens or related toxins, would appear to have little validity in tuberculous processes. Tubercle bacilli proliferate readily in phagocytic cells, and are not known to produce exotoxin. This report, however, provides evidence for a toxic-like manifestation of tuberculous disease in mice. It seems therefore possible that non-specific resistance to mycobacterial disease may depend in part on an enhancement of the host's ability to resist such toxic manifestation.

SUMMARY

NCS mice were found to be susceptible to a hyperacute form of disease induced by injection of relatively large doses of *Mycobacterium tuberculosis*. Susceptibility to this type of disease was conditioned by the sex, age, and dietary history of the animals. Unlike the more usual response of mice to mycobacterial disease, these conditions affected primarily the relative occurrence of early-acute deaths rather than average survival time.

Resistance to this form of disease was increased by previous exposure of the animals to Gram-negative bacteria, to their endotoxin, or to various preparations of killed mycobacterial cells.

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