EFFECT OF DIETARY FATTY ACIDS AND PROTEIN INTAKE ON EXPERIMENTAL TUBERCULOSIS¹

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Although there is much evidence which suggests that nutrition represents an important factor in tuberculosis, there has been little proof that any specific dietary factor exerts a singular effect on resistance to this disease. Studies have been made on the effect of high and low levels of protein intake on resistance to tuberculosis (Koerner *et al.*, 1949; Howie and Porter, 1950; Ratcliffe, 1951; Ratcliffe, 1952; Ratcliffe, 1953; Ratcliffe, 1954; Dubos, 1955). While it has been demonstrated that dietary protein does influence experimental tuberculosis, the above reports were not in agreement with respect to the specific effect of various levels on resistance. Likewise, there have been conflicting reports concerning the effects of dietary lipids on resistance to tuberculosis. Thus, the addition of egg yolk to the diet decreased the resistance of guinea pigs (Cirio and Rosso, 1947) whereas the inclusion of peanut oil in a low protein, high carbohydrate diet resulted in increased resistance to murine tuberculosis (Dubos, 1955).

Our previous work (Hedgecock, 1948) indicated that mice maintained on a synthetic ration containing coconut oil were more resistant to tuberculosis than animals which had received olive or linseed oil as a source of dietary fat.

In the present investigation, the fatty acids found in coconut oil have been explored in relation to appropriate levels of protein to determine their role in resistance to tuberculosis. This study on experimental tuberculosis has entailed the dietary administration of a group of fatty acid esters mixed in proportions which simulate coconut oil. The effect of varied levels of protein intake in the presence of these fatty acids has been determined. Evidence has been obtained which may clarify the previous diver-

gent results pertaining to the effect of diet on tuberculosis.

MATERIALS AND METHODS

Mice. Female, CF₁ mice (14 to 16 g) were housed in groups of 20 in metal cages. The cages and bedding, which consisted of sterilized sawdust, were changed weekly. The water was changed on alternate days, using sterilized bottles. The stock ration (Rockland Mouse Diet) was fed in pellet form whereas synthetic rations were administered in small feeding jars.

Diets. As a basal diet for incorporation of fatty acids and varied levels of protein, a synthetic ration (Hubble and Hedgecock, 1953) of the following composition was employed: vitamin-free casein, 20 per cent; Wesson salt mixture (Wesson, 1932), 4 per cent; Alphacel, 10 per cent; corn starch, 55.7 per cent; sucrose, 5 per cent; fat, 5 per cent; and supplement, 0.3 per cent. The following components of the supplement are expressed in terms of mg per 100 g of synthetic ration: pyridoxine hydrochloride, 1.0; nicotinic acid, 1.0; thiamin chloride, 0.6; riboflavin, 0.6; calcium pantothenate, 2.0; folic acid, 0.05; biotin, 0.05; *p*-aminobenzoic acid, 30; inositol, 50; choline chloride, 100; cystine, 100; α -tocopherol, 3.5; menadione, 2.5; vitamin A acetate, 300 μ g and calciferol, 5 μ g. Adjustments in the concentration of protein or fat in the ration were accomplished by the addition or omission of an equal weight of starch. An equivalent (percentage) adjustment in the concentration of cystine was made when the content of casein was altered. Rockland Mouse Diet was utilized as the control ration in all experiments. The rations were furnished *ad libitum* to the mice and equal weight gains were observed in groups of animals fed the above synthetic ration and the commercial diet.

Methyl laurate, methyl myristate and methyl palmitate (Matheson, Coleman and Bell Chemi-

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Figure 1. Relation in size of infective dose of *Mycobacterium tuberculosis* to alterations in rate of death of mice fed fatty acid mixture and varied levels of protein. Infective dose: A, 0.2-ml. culture; B, 0.05-ml. culture; C, 0.01-ml. culture. Curve 1, synthetic ration, 5% fatty acids, 10% protein; curve 2, synthetic ration, 5% fatty acids, 20% protein; curve 3, synthetic ration, 5% fatty acids, 30% protein; curve 4, synthetic ration, 5% fatty acids, 40% protein; curve 5, Rockland mouse diet.

icals Co.) were purified by fractional distillation under reduced pressure. Methyl stearate, methyl oleate (Fisher Scientific Co.) and methyl linoleate (Nutritional Biochemicals Co.) were used without further purification.

The mixture of fatty acid esters utilized in this investigation (formulated in proportions which simulate coconut oil) had the following composition expressed as per cent: methyl laurate, 50; methyl myristate, 25; methyl palmitate, 20; methyl stearate, 2; methyl oleate, 2; and methyl linoleate, 1.

Lard was of the commercial grade produced by Armour & Co.

Infection of animals. The H37Rv strain of *Mycobacterium tuberculosis* was cultured in Kirchner's medium containing "tween 80" and albumin (Fraction V) and maintained in this

medium at 5 C between experiments (McKee *et al.*, 1949). The inoculum which was used to infect mice consisted of 0.1 ml² of a suitable dilution of a 5-day culture of tubercle bacilli which had been cultivated in Kirchner's medium containing "tween 80" without albumin. Inoculation of the animals was made intravenously following prefeeding periods of 10 to 11 days.

Evaluation of results. The survival time of each animal was recorded in terms of days following injection of organisms. The mortality rate for the animals of each group was determined by plotting cumulative percentage dead on a probability scale against time on an arithmetic scale (Litchfield, 1949; Donovan *et al.*, 1949). When applicable, comparisons were made of the 50 per cent mortality time (T_{50}) of the mice in the various dietary groups. In certain groups of animals, sections of lung and spleen tissue were prepared and stained with hematoxylin-eosin and by the Ziehl-Neelsen method from which microscopic evaluation of the infectious process was made.

RESULTS

In the first phase of the investigation five rations were studied. Four of the rations contained the mixture of fatty acid esters at a concentration of 5 per cent and protein levels of 10, 20, 30 and 40 per cent. The rations were administered to groups of 20 animals with commercial chow as the control ration, and after 10 days each animal received an intravenous injection of 0.2 ml of a 5-day culture of *M. tuberculosis*. The deaths in each group of animals were recorded daily for a period of 27 days at which time the experiment was terminated. The rate of death of the animals in each group is shown in figure 1A. In each group straight lines were obtained which indicated that the death-time relationship followed a normal frequency distribution curve. The 50 per cent mortality time was 14.5 for the control group and 16.3, 19.0, 17.9 and 16.9 for the groups of animals which received synthetic rations containing 10, 20, 30 and 40 per cent protein, respectively. Although definite trends were indicated by these values, the differences were not statistically significant when 95 per cent confidence limits

² In the initial experiment 0.2 ml of the culture was used in order to obtain a concentrated inoculum.

were calculated. Since the infective dose of organisms had been relatively large and may have tended to overwhelm the resistance of the host, a similar experiment was performed (again using groups of 20 animals) in which the infective dose was reduced to 0.1 ml of a 5-day culture of *M. tuberculosis* diluted 1:1. The results are recorded in figure 1B. The T_{50} of the control group was 19.6 days. The rate of death of animals which received the control ration yielded a straight line while the rate of death of animals which received the synthetic rations changed during the course of the infection. An abrupt decrease in the rate of death occurred on the 15th to 16th day following infection and was maintained throughout the remainder of the experiment. At the termination of the experiment, 36 days after infection, there existed a mortality of 95 per cent in the control group in contrast to 61, 44, 56, and 69 per cent mortalities in the groups of animals which received the synthetic rations containing 10, 20, 30 and 40 per cent protein, respectively.

To allow freer expression of the factors involved in the development of resistance, a third experiment was performed in which the infective dose was reduced further (0.1 ml of a 5-day culture of *M. tuberculosis* diluted 1:10). The results of the experiment are recorded in figure 1C. The general pattern of an abrupt decrease in rate of death in the experimental groups was similar to that of the previous experiment; however, the major deviation did not occur until approximately the 30th day. A slight decrease also occurred in the rate of death of the control animals for which the T_{50} was 29.2 days. Ninety per cent of the control animals had succumbed when the experiment was

terminated 91 days following infection, whereas mortalities were 50, 26, 44 and 55 per cent in the experimental groups of animals which had been fed rations containing 10, 20, 30 and 40 per cent protein, respectively. Mice which had survived were sacrificed at the termination of the experiment. The lungs and spleens of these animals were sectioned and stained. The number of organisms in the tissues of the surviving mice as well as weight changes in the various groups are recorded in table 1. In the sections of lung tissue, the average counts of organisms per microscopic field correlated with the resistance of the groups as indicated by mortality. In the sections of spleen, average counts of organisms decreased as the content of protein in the diet was increased, and did not bear any apparent relation to the resistance of the various groups of animals. There was little difference in the average weight of the various groups of animals after they had been maintained on the rations for 10 days prior to infection. At the termination of the experiment it was found that the weight of the two surviving control animals had decreased approximately 17 per cent during the infection, while the weights of the surviving experimental animals had increased 15 to 20 per cent.

The data of the three previous experiments have been summarized in table 2. In a comparison of these data, a decrease in the infective dose of organisms resulted in an increased survival of animals in the experimental groups. When the infective dose did not exceed 0.05 ml of 5-day culture (T_{50} was 19 days or greater) all animals which had received synthetic rations (containing the fatty acid mixture) were more resistant to tuberculosis than control animals

TABLE 1

The effect of diet on weight and number of organisms in tissues of surviving mice following infection with Mycobacterium tuberculosis

Diet	Time of Infection		91 Days after Infection			
	Number animals	Weight animals	Number animals	Weight animals	Organisms per micro. field	
					Lung	Spleen
Synthetic, 10% protein	20	20.5	10	24.9	42	15
Synthetic, 20% protein	19	21.3	14	25.3	10	1.5
Synthetic, 30% protein	20	21.8	11	26.7	15	0.3
Synthetic, 40% protein	20	20.6	9	23.7	47	0
Rockland mouse diet	20	20.5	2	17	>1000	7

TABLE 2

Effect of diet on survival of mice infected with *Mycobacterium tuberculosis* at 3 different dose levels

Infective Dose (Ml of 5-Day Culture)	Per Cent Survival				
	Rockland mouse diet	Synthetic ration (per cent protein)			
		10	20	30	40
0.2	10	20	30	20	20
0.05	10	50*	60*	45*	50*
0.01	10	50*	74*	55*	45*

* Probability less than 0.02.

which had received commercial chow. In each experiment, animals which had received the synthetic ration containing 20 per cent protein were most resistant.

To demonstrate that the mixture of fatty acid esters was the basic factor responsible for the resistance observed in all animals fed the synthetic ration, a series of rations was prepared containing varied amounts of protein (10, 20, 30, 40 per cent), in which lard at a concentration of 5 per cent was substituted for the mixture of fatty acid esters. A similar series of rations was also prepared in which methyl linoleate at a concentration of 0.2 per cent was substituted for the mixture of fatty acid esters. The rations were administered to groups of mice for 10 days, at which time each animal was infected with 0.05 ml of a culture of *M. tuberculosis*. The results of the administration of these rations to the infected mice are presented in table 3. The T_{50} of the control group (19.3 days) was not different from those of the experimental groups. The rate of death in all groups of animals corresponded to a normal frequency distribution curve. The abrupt decrease in rate of death did not occur at 15 days or more following infection as had been charac-

teristic of animals fed the mixture of fatty acid esters.

DISCUSSION

The demonstration that the dietary administration of the particular group of fatty acids (found in coconut oil) enhanced resistance to tuberculosis when the infective dose was not excessive is in agreement with previous findings (Hedgecock, 1948). That the group of fatty acid esters was basically responsible for the development of resistance during infection with *M. tuberculosis* is evidenced by the lack of development of resistance in mice when 5 per cent lard or 0.2 per cent methyl linoleate was substituted for the fatty acid mixture.

The results of these experiments may clarify the divergent reports in the literature relating to the effect of dietary protein on susceptibility to tuberculosis. For example, when lard or methyl linoleate served as the sole source of lipid, variation in the protein-content of the diet from 10 to 40 per cent did not alter resistance of the animals to tuberculosis. However, when the fatty acid mixture was incorporated into the diet, resistance was greatest in groups of animals which had received rations containing 20 per cent protein. An increase or decrease from a normal level of protein in the diet (20 per cent) resulted in a decrease in resistance to tuberculosis. It thus appears that a normal level of protein is actually the optimal amount for enhancing the effect of dietary fatty acids on tuberculosis. Consequently, the divergent reports dealing with the effect of dietary protein on experimental tuberculosis might well be reexamined with respect to lipids in the diet.

Resistance to tuberculosis has been explained on the basis of two hypotheses: (1) activity of classical cellular and humoral factors involved

TABLE 3

Effect of 5% lard or 0.2% methyl linoleate as sole source of dietary fat on rate of death and terminal mortality of mice infected with *Mycobacterium tuberculosis*

	Rockland Mouse Diet	Synthetic Ration (Per Cent Protein)							
		10		20		30		40	
		Lard	Me. li.*	Lard	Me. li.*	Lard	Me. li.*	Lard	Me. li.*
Number animals	35	35	32	34	37	31	17	33	36
T_{50} (days)	19.3	19.3	20.5	20.4	20.2	20.2	21.6	20.5	21
Terminal mortality	94	100	94	94	84	87	77	85	86

* Me. li. = methyl linoleate.

in immunological mechanisms of host (Bloch and Segal, 1955), (2) alteration of biochemical environment in tissues of host such that conditions are unsuitable for proliferation of *M. tuberculosis* (Dubos, 1955). The results of the present investigation appear compatible with the theory that increased resistance to tuberculosis results from changes in the immunological state because the animals became refractory only after the organisms (*M. tuberculosis*) had been in the tissues for two weeks or longer. However, this theory may require the postulation of a (hitherto unknown) activation and stimulation of cellular and humoral immune mechanisms by the fatty acid mixture in the presence of *M. tuberculosis*, suggesting essentially an *in vivo* incorporation of antigenic material similar to Freund's adjuvant. The possibility that enhanced resistance may have resulted from slowly-developing alterations in the metabolism of infected tissues is not excluded by these data. In addition to alterations in resistance of the host, direct inhibitory action on *M. tuberculosis* by the fatty acids must be considered. Further investigation is required to elucidate the mechanisms involved in the action of dietary fatty acids on resistance to tuberculosis.

SUMMARY

The resistance of CF₁ mice to tuberculous infection was increased by dietary administration of a group of fatty acid esters (mixed in proportions simulating coconut oil). Enhanced resistance of mice was manifested as an abrupt decrease in rate of death which occurred two weeks or more following injection of *Mycobacterium tuberculosis*; this increase in resistance did not occur when lard or methyl linoleate replaced the group of fatty acid esters in the diet.

With the fatty acid mixture incorporated into the diet, resistance to tuberculosis was greatest in groups of animals which had received rations containing 20 per cent protein.

The resistance of mice was not altered by variation in the protein content of the ration from 10 to 40 per cent when lard or methyl linoleate was utilized as the sole source of dietary lipid.

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