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Environmental Effects on Experimental Aerogenic Tuberculosis in BCG-Immunized and Non-Immunized Guinea Pigs.* (28429)

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Identification of the physiological and biochemical mechanisms through which nonspecific stress may influence immunological responses has not yet been satisfactorily accomplished. The influence of environmental factors on establishment of acute or chronic disease is uncertain because sufficient basic information relative to the host-parasite interaction is lacking.

Studies of antibody formation in rabbits immunized with soluble antigens and exposed to environmental extremes or placed in endocrine imbalance(1,2,3) and work which indicated that animals subjected to simulated altitudes are more resistant to infection by virus but less resistant to bacterial infections (4), led us to investigate further the role of environment in controlled infection. The use of a quantitative system for estimation of tuberculosis infection and disease in guinea pigs induced by the airborne route(5) would satisfy several criteria. The aerogenic route of infection would closely approximate the "natural" one, relatively small numbers of animals could be used for the statistical evaluation of the results, and information might be obtained concerning the amount of disease and environmental influence on the *in vivo* growth of the infecting organism. In contrast to many experiments in which reliance is placed on subjective histological studies and gross pathology for interpretation of the data, the present study utilizes objective methods for quantitation of the extent of the tuberculous process. Previous work by Middlebrook(5) and collaborators (6,7) has indicated these advantages and pointed out that the quantitative method was not only time-saving, but more important, circumvented the effects of allergy and disease itself on the immunity produced by the vaccine(7).

Methods. Animals. Male and female adult guinea pigs weighing 400-600 g were used. These animals were taken from our own random-bred colony of albino guinea pig (Trapani-Campbell strain). The diet of each group was the same. Control and high-altitude groups were kept on dried prairie grass in similar cages; the cold-exposed group was kept on wire screen with no bedding. Each group consisted of 30 guinea pigs, and 3 animals were housed in each cage.

A) The control group was maintained in our Denver Laboratories (altitude = 5,280 ft; temperature = 20°C).

B) The cold-exposed group was housed in a specially constructed cold-box capable of maintaining an environmental temperature

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of –4°C. The animals were placed in the cold chamber, which was maintained initially at 10°C. The temperature was lowered gradually to –4°C over the next 4 weeks, and the animals were maintained thereafter at that temperature (for an additional 4 weeks of adaptation and throughout the remainder of the experiment). Food and water were provided *ad libitum*; water was changed several times a day because of freezing.

C) The high-altitude group was housed in a heated, insulated building at the Summit Laboratory (altitude = 14,150 ft; temperature = 13-18°C) of the Inter-University High Altitude Laboratories, Mt. Evans, Colo. The animals were brought to high altitude from Denver for 30 days of adaptation before being subjected to further treatment.

Immunization. After the animals had been maintained in their particular environment for 30 days, half of each group was immunized by intradermal injection of BCG vaccine (Research Foundation and Univ. of Illinois, Chicago, Lot. No. 132R). The total dose injected was 1.0 mg BCG in 0.1 ml divided into 2 sites. Thirty-eight days after immunization all animals were skin-tested with 250 tuberculin units intracutaneously, and the reactions read 24 and 48 hours later.

Aerogenic infection. On the forty-second day after immunization, all animals were aerogenically infected with virulent tubercle bacilli (H37Rv) in a Model II Middlebrook airborne infection apparatus (Tri-R Instruments Co., Jamaica, N. Y.). A single cell suspension of a 10-day-old culture of H37Rv strain was prepared by first dispersing the organisms by means of a Teflon-to-glass grinder and then filtering through an "F" fritted glass filter (Corning No. 36060) under positive air pressure. The single cell suspension obtained was read in a Coleman Nephlo-Colorimeter Model 9, using Coleman certified Nephelos standards, and the turbidity adjusted to 100 Nephelos units per ml. After 3 weeks of incubation 0.1 ml of 10^{-5} dilutions of this suspension on 7H10 oleic acid albumin agar medium(6) yielded mean plate counts of 73, 57, 68 and 33 culturable units. The suspension was diluted 1:400 for use in the nebulizer of the airborne apparatus. The animals were exposed for 30 minutes to a cloud of fine droplet nuclei containing the bacilli. After a 30-minute decay period of the ambient cloud, the apparatus and the guinea pigs were surface-sterilized by exposure to ultra-violet light for 15 minutes.

The animals residing at high altitude were transported to Denver for aerogenic infection, and returned to the mountain environment within 6 hours. Cold-exposed animals were removed from their environment for approximately 3 hours during the infecting procedure.

Quantitation of culturable units of tubercle bacilli in the organs. Half of each group was sacrificed at 17 days, and the remaining half at 28 days after infection. The animals were killed by electrocution, and exsanguinated by cardiac puncture immediately after death. The spleen and the lower right lobe of the lungs were removed aseptically and used for quantitative estimation of culturable units of tubercle bacilli(8).

Post mortem examination. The number and size of macroscopically-visible surface tubercles on the lungs and the size and nature of the spleen and tracheo-bronchial lymph node were recorded. The number of surface tubercles on the right lower lobe of the lungs was estimated from the corresponding number of tubercles on the left lower lobe.

Histopathology. Sections were made of lung, liver, spleen and tracheo-bronchial lymph node of representative animals sacrificed 17 days after aerogenic infection using conventional histopathological techniques. Both Ziehl-Neelsen and haemotoxylin-eosin stains were employed.

Electrophoretic analysis. Pooled samples from each group of animals were prepared by using equivalent amounts of protein from each serum and dialyzed against 2 changes of barbital buffer (pH 8.6; μ=0.1). Moving boundary analyses were made in a Spinco Model H electrophoresis instrument (1.3°C; potential gradient=5.4 volts/cm). Electrophoretic patterns of descending boundaries were analyzed by conventional techniques(9, 10) to determine mobilities and relative percentages of each component.

Protein analysis. Appropriate dilutions of

TABLE I. Average Culturable Units of Tubercle Bacilli in Right Lower Lung and Whole Spleen of Non-Immunized and BCG-Immunized Guinea Pigs.

	Non-immunized				BCG-immunized†			
	Lung		Spleen		Lung		Spleen	
Environment	17 days	28 days	17 days	28 days	17 days	28 days	17 days	28 days
Control (5,280 ft; 20°C)	2.30*	22.03	.035	4.73	.11	.098	.00029	.00680
Cold (5,280 ft; −4°C)	5.38	35.67	1.250	34.60	.44	.725	.00088	.01100
High altitude (14,150 ft; 18°C)	7.28	16.91	.523	3.05	.06	.091	.00125	.00125

* $\times 10^5$.

† All animals in this group were tuberculin positive prior to infection.

serum samples were analyzed for nitrogen in quintuplicate by the micro-Nessler method (11). Protein values were calculated by multiplying nitrogen values by 6.25.

Results and discussion. The data recorded in Table I indicate the efficacy of BCG immunization in guinea pigs. Statistical analysis of the data indicates that, regardless of the environment imposed, a significant degree of protection is afforded the BCG-immunized animal infected with tubercle bacilli. It should be pointed out, however, that the response of the animal is altered by an environment considered to impose a stress, and that different environments may impose stresses of different, and perhaps unknown quality.

Though there are certain obvious similarities in responses of these environmentally-stressed animals, there are also important differences. These are particularly apparent when comparisons are made on the basis of time. The culturable units of tubercle bacilli in the lungs and spleen of both cold-exposed and high-altitude non-immunized animals are greater than those of controls at 17 days ($P<0.01$). However, at 28 days, even in non-immunized animals, the increase in number of bacteria in the tissue of high-altitude animals is not as great as in the tissues of either control or cold-exposed animals. Thus, it would appear that infectivity of tubercle bacilli is unaltered by host environment but pathogenicity, as measured by extension of infection to the viscera by 28 days(8) is greatest in cold-exposed animals.

A considerable degree of immunity is conferred upon animals vaccinated with BCG. The cold-exposed group is not as well protected as the group at high altitude, either at 17 or 28 days. High altitude does not diminish the protective effect of BCG, since there is no significant difference when the high-altitude animals are compared with controls ($P>0.4$).

All cutures from tissues of non-immunized animals were positive for tubercle bacilli at 17 and 28 days. Negative spleen cultures from infected BCG-immunized animals were found in 4/7 and 1/7 controls at 17 and 28 days, respectively; in 2/4 cold-exposed animals at 17 days; and in 2/6 and 3/6 high-altitude exposed at 17 and 28 days, respectively. Lung tissue of all BCG-immunized animals gave positive cultures for mycobacteria. Livers of all BCG-immunized animals were negative on gross inspection, whereas, livers of non-immunized animals had from a few to multiple tuberculous foci. The size of the spleen at sacrifice was normal to slightly enlarged in both immunized and non-immunized animals at 17 days; 1.0 to 5.0 times normal size in non-immunized animals at 28 days; and 1.0 to 2.5 times normal size in BCG-immunized at 28 days.

Microscopic examination showed that the vascular structures of the tissues of all animals were within normal limits regardless of environment. Tubercles in the lungs of BCG-immunized guinea pigs were smaller than in the lungs of non-immunized animals and showed little or no necrosis. Seventeen days after aerogenic infection, liver and spleen from animals of all groups had no microscopically demonstrable tubercles or bacteria.

Alterations in the electrophoretic distribution of serum proteins were more pronounced in samples obtained 28 days after infection than at 17 days and were greater in non-im-

munized than in BCG-immunized animals. The major change was seen in α_1-globulin, which was increased in all animals at 28 days. A decrease in α_2-globulin seemed to be on the borderline of significance. Changes in the percentage distribution of other serum components were not significantly different from controls.

In considering the effect of environment, it seems unlikely that cold exposure *per se* exerts its primary effect at a cellular level, since no change in body temperature was noted. Thus the major pathway might be one involving several coinciding events which secondarily give rise to the index measured. High altitude, however, might be considered as a double-edged sword, especially in regard to pulmonary tuberculosis. The hypoxia of mountain altitudes may directly influence the growth of bacteria in the pulmonary spaces (12) and also may affect secondary pathways influencing resistance to disease and the immune response(1,3,4). Even though the decreased oxygen tension of mountain altitudes might provide an environment conducive to depression of the growth of mycobacteria, the additional respiratory embarrassment imposed by mountain altitudes in the tuberculous animal may create a respiratory cripple incapable of sufficient physiological adjustments to decreased levels of oxygen(13).

In all instances, the average increase in culturable units between 17 and 28 days post infection in the lungs and spleens of both non-immunized and BCG-immunized animals was less in animals at 14,150 feet than at 5,280 feet. These results seem consistent with the hypothesis(12) that a diminished O_2 tension diminishes the growth of tubercle bacilli.

It is well known that under environmental stress physiological changes occur in the thyroid and adrenal glands. The increased metabolic rate required to maintain body temperature of the cold-exposed animal in the presence of an increased heat loss has been well documented(14). The stress of high altitude appears to involve changes in adrenal cortical activity(15). The interrelationship of thyroid and adrenal cortical activity, especially under stress conditions, has not been adequately investigated and must be considered when hypothesizing about the activity of either gland.

Summary. BCG immunization confers immunity to guinea pigs under cold or high-altitude stress. Cold-exposed BCG-immunized animals had lower levels of immunity than controls, whereas those at high altitude were not significantly different from controls. Non-immunized animals at high altitude may be less susceptible to tuberculosis than those kept at –4°C. Moving boundary electrophoresis of serum samples showed an increase in the α_1-globulin of all animals 28 days post infection, and the α_1-globulin was greater in non-immunized than in immunized animals.

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