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Resistance of high and low antibody responder lines of mice to the growth of avirulent (BCG) and virulent ($H_{37}Rv$) strains of mycobacteria

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SUMMARY

The resistance to *Mycobacterium bovis* (BCG) of lines of mice selected for high (*H*) or low (*L*) antibody responsiveness was estimated from the rate of BCG multiplication in the organs. During the first 2 weeks after i.v. infection with 5×10^6 CFU, BCG multiplied faster in the spleens of *H* than of *L* mice. Afterwards there was a more drastic reduction of viable BCG counts in *H* mice than in *L* mice so that the residual BCG counts were significantly lower in *H* mice than in *L* mice, not only in the spleen but also in the liver and lungs. On the 14th day of infection, the spleen and liver enlargement and the increase of phagocytic activity were similar in the two lines, suggesting an identical T lymphokine release. In contrast with BCG, during the first 2 weeks after infection with 7×10^5 CFU, *M. tuberculosis* ($H_{37}Rv$) multiplied in the spleens of *L* mice at a similar or a slightly faster rate than in the spleens of *H* mice. On the 4th week, the viable $H_{37}Rv$ counts were reduced in *H* mice whereas *L* mice did not survive the infection. In mice vaccinated with BCG 5 months before virulent challenge, the multiplication of $H_{37}Rv$ was inhibited in the *H* and *L* lines. The protective effect of BCG is therefore stronger in *L* mice taking into account their higher innate susceptibility to $H_{37}Rv$. This might be due to the higher level of living BCG found in *L* mice at the time of challenge.

Keywords mycobacteria infection resistance immunity genetics

INTRODUCTION

Increased attention has recently been given to the genetics of resistance to infections. The clear cut differences in resistance to intracellular pathogens, observed among inbred strains of mice are controlled by a single or a few genes (Plant & Glynn, 1976; Cheers *et al.*, 1980; Gros, Skamene & Forget, 1981; Skamene *et al.*, 1982; Plant *et al.*, 1982). The expression of these genes is probably not related with immunoresponsiveness. The defect in mice susceptible to *Salmonella typhimurium* or to BCG is at the level of macrophage control of early bacterial multiplication. Moreover acquired immunity can be obtained in these lines of mice (Nauciel, 1984; Orme & Collins, 1984). In fact in bred strains do not differ very much in their general capacities for immune responses, which are under polygenic regulation (Biozzi *et al.*, 1980). In outbred mice, the individual genotypes for polygenic characters, are normally distributed, therefore in inbred strains the more frequent median genotypes are preferentially fixed (Biozzi *et al.*, 1984).

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In contrast the lines of mice selected for high or low antibody production (*H* and *L* line, respectively) have extreme genotypes. They are therefore useful tools to evaluate the role of immunoresponsiveness in the resistance to infections. The resistance of *H* and *L* lines to various bacterial and parasitic infections has been studied in our laboratory and in several others (Biozzi *et al.*, 1978). As expected, H mice are more resistant than L mice against infections in which antibodies play a major protective role. This advantage is greatly increased after vaccination. However, the selective breeding modified macrophage functions in a direction opposite to antibody production: poor antibody responsiveness in *L* mice is due to the rapid catabolism of immunogens in the macrophages. Since *L* mice macrophages also have a stronger bacteriostatic activity, these mice are more resistant to infections with intracellular pathogens (Biozzi *et al.*, 1982; Plant & Glynn, 1982).

In this paper the resistance to BCG and to *Mycobacterium tuberculosis* infections was measured in *H* and *L* lines in terms of bacterial growth in various organs. It is generally admitted that cellular immunity is the main defence mechanism in mycobacterial infections (Rook & Stanford, 1979). A preliminary observation by Lagrange, Hurtrel & Thickstun (1979) indicated that *H* mice were more resistant to virulent *M. tuberculosis* than *L* mice and also more efficiently protected by living BCG vaccination.

More detailed data were needed for a complete understanding of *H* and *L* lines' resistance to mycobacteria. The experiments reported here include the long course counts of BCG colony forming units (CFU) in the organs, and the modifications in macrophage phagocytic activity. The growth rate of the virulent *M. tuberculosis* was also measured in normal and in BCG vaccinated *H* and *L* mice.

MATERIALS AND METHODS

Mice. Male and female mice from *H* and *L* lines of selection I (50–55th generation) were used when 2–5 month old. F_1 hybrids were produced by reciprocal crosses; since no difference was noticed, pooled results in $(H \times L)F_1$ and $(L \times H)F_1$ are given.

BCG infection. The French 1173 P_2 BCG strain was chosen for vaccine preparation with regard to its high immunogenicity and because the maintenance of its phenotypic and genotypic characteristics are well known (Ladefoged, Bunch-Christensen & Guld, 1970; Mackaness, Auclair & Lagrange, 1973; Gheorghiu & Lagrange, 1983; Gheorghiu, Augier & Lagrange, 1983; Gheorghiu *et al.*, 1984). Fresh BCG vaccine was produced as described by Gheorghiu & Chambon (1976) by surface culture on Sauton medium, the bacillary mass was collected by filtration and homogenized with stainless steel balls. The vaccine was resuspended in Dubos medium containing 5% glycerol and 5% human albumin. It was stored at −70°C. The same batch was used throughout the experiments and 4×10^6 viable units (CFU) per mouse were given i.v. in a volume of 0·2 ml.

Growth of BCG in the organs. The number of CFU was measured in the inoculum (as described in Gheorghiu *et al.*, 1983) and in spleen, liver, lung, kidney and thymus. The organs, aseptically removed, were homogenized in Potter teflon glass tubes at 2,000 r/min for 1–2 min. Suitable dilutions from each individual mouse organ were plated onto Middlebrook 7 H 10 medium.

M. tuberculosis ($H_{37}Rv$) infection. Mice were infected i.v. with 7×10^5 viable units (CFU) from a 7 day culture in liquid tween albumin medium of the H_{37}Rv strain of *M. tuberculosis*, the virulence of which being maintained by regular passages in the mouse. The number of H_{37}Rv CFU in the spleens of mice was assessed by plating suitable dilutions of the organs onto Löwenstein–Jensen medium as described by Grumbach (1965).

Measurement of the phagocytic activity of reticuloendothelial macrophages of liver and spleen. The phagocytic index K was measured from the rate of blood clearance of 8 mg % g of colloidal carbon injected i.v. (Biozzi, Benacerraf & Halpern, 1953). Index K was calculated using the formula:

$$K = \frac{\log_{10} C_1 - \log_{10} C_2}{t_2 - t_1}$$

C_1 and C_2 being the blood concentrations of carbon at times t_1 and t_2 expressed in min.

The corrected index α is calculated as follows:

$$\alpha = \sqrt[3]{K} \times \frac{w}{wls}$$

in which w and wls are the body weight and the liver and spleen weight respectively. Index α expresses the macrophage activity for a constant liver and spleen weight.

Calculation of dominance effect. The dominance effect is expressed by the d/a ratio, d being the dominance deviation and a the additive effect:

$$d = \bar{x}\, F_1 - \frac{1}{2}(\bar{x}H + \bar{x}L)$$

$$a = \frac{1}{2}(\bar{x}H - \bar{x}L)$$

Calculation of protection index (PI).

$$PI = \frac{\text{number of } H_{37}Rv \text{ CFU in control mice}}{\text{number of } H_{37}Rv \text{ CFU in BCG vaccinated mice}}.$$

Statistical analysis. P values were calculated by the Student's *t*-test.

RESULTS

Kinetics of BCG-CFU in the organs of H *and* L *mice*

Groups of H and L mice (three males and three females per group) were sacrificed at different time intervals from 2 h to 180 days after i.v. BCG inoculation. The BCG-CFU were counted in spleen, liver, lungs, kidney and thymus. Since no evident sex related difference was noticed, the results are given as geometric means of individual counts per organ.

BCG-CFU in the spleen

The results concerning the spleen are shown in Fig. 1. There was a significant difference between *H* and *L* lines in the initial CFU counts (2 h after injection) which concerns the bacterial distribution after complete blood clearance. This difference is due to the larger spleen size constantly observed in *H* mice (See Table 1). In fact, the CFU counts per gram of spleen were similar in the two lines

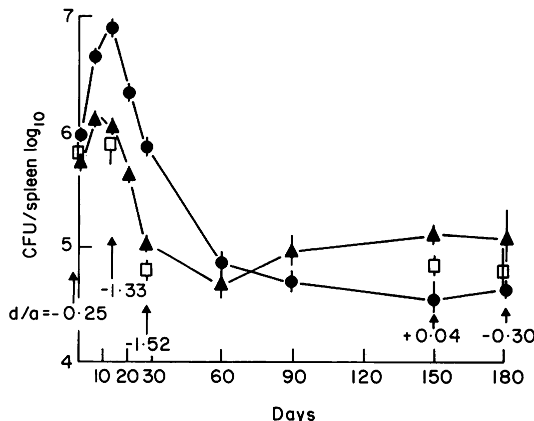

Fig. 1. Mean values ± s.e. of BCG-CFU recovered from the spleen after i.v. injection of 4×10^6 v.u. of living BCG in *H* (●), *L* (▲) and F_1 hybrids (□). H–L differences are significant: $P < 0.001$ on days 0, 7, 14 and 28 and $P < 0.01$ on day 150.

($9{\cdot}6 \times 10^3$). The CFU number increased faster in *H* than in *L* mice during the first 2 weeks. The multiplication coefficient between initial and peak values was 8·3 in *H* mice (between 0–14 days) and 2·45 in *L* mice (between 0–7 days). Thus, in the initial phase, the control of BCG multiplication was more efficient in *L* mice, which indicates an innate resistance higher in the *L* line than in the *H* line.

After 2 weeks there was a rapid drop in CFU counts in the two lines, corresponding to the phase of immune resistance, and later on the residual BCG counts remained significantly lower in *H* than in *L* mice. The faster initial growth of BCG in the *H* line had probably induced a stronger immunization which permitted the reduction of BCG-CFU and explains their lower residual level in the advanced phase of the infection.

The BCG-CFU counts in F_1 hybrids are indicated in Fig. 1 together with the values of the dominance effect measured by the d/a ratio. An overdominance of the low character was observed during the early period (negative d/a values) whereas in the advanced phase, F_1 values were intermediate between the parental ones.

BCG-CFU in other organs

The results obtained in liver, lung, kidney and thymus are shown in Fig. 2. No significant interline difference was observed in the initial distribution of BCG in these organs. The kinetics of BCG-CFU recovered from liver, lung and kidney were roughly parallel in the two lines: during the first 2 months a progressive reduction in the CFU numbers (about 1 log) occurred. For these three organs however, the number of CFU in the late phase of infection was constantly higher in *L* than in *H* mice. The observed differences are significant in lung and also in liver in spite of the large intraline variability.

A peculiar feature was observed in the thymus, namely a progressive increase in CFU numbers during the first month of infection. Afterwards the level of BCG counts remained stable. There was no marked interline difference. A high rate of BCG growth in the thymus, without any further decrease up to 6 months, has been observed in other strains of mice: specific pathogen free OF_1 (Gheorghiu *et al.*, 1984) and C57B16 and C3H (unpublished results). This less known pattern of BCG growth deserves further investigation since it could be important for resistance to *M. tuberculosis* and/or other aspects of cell-mediated immunity.

CFU counts in F_1 hybrids are only given at the times when the interline differences were significant. Otherwise they did not differ from the parental ones. In the 3rd and 5th month of the

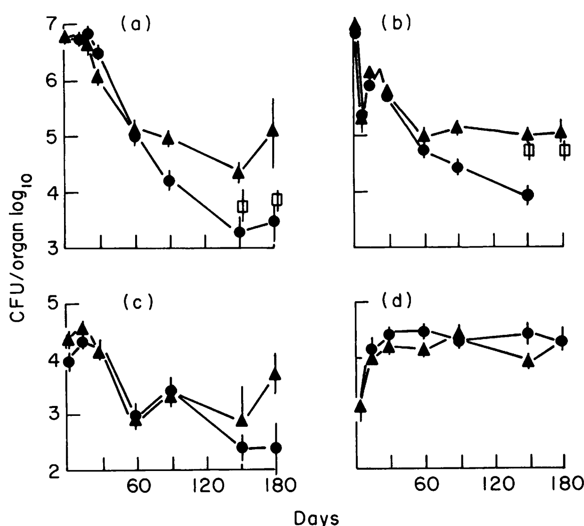

Fig. 2. Mean values ± s.e. of BCG-CFU recovered from (a) liver, (b) lung, (c) kidney and (d) thymus after i.v injection of 4×10^6 v.u. of living BCG in *H* (●), *L* (▲) and F_1 hybrids (□). H–L differences are significant $P<0{\cdot}001$ on day 30 in liver, on day 90 in lung; $P<0{\cdot}05$ on days 150–180 in liver, on day 150 in lung.

Table 1. BCG-induced modification of the phagocytic activity of liver and spleen macrophages, in *H* and *L* lines

Line of mice	BCG infection (days before test)	Number of mice*	Phagocytic index K† (mean ± s.e.)	Organ weight mg/20g Liver	Spleen	w‡ / wls	Corrected index α
H	—	28	0·048±0·003	1,010±13	103±3	18±0·2	6·5±0·15
	7	10	0·116±0·011	1,076±23	170±8	16·1±0·4	7·8±0·2
	14	18	0·105±0·010	1,820±65	470±21	9·0±0·4	4·8±0·2
L	—	28	0·048±0·003	980±13	58±2	19·3±0·2	7·0±0·2
	7	10	0·119±0·023	1,096±36	95±7	16·8±0·6	8·2±0·4
	14	17	0·123±0·018	1,450±48	216±17	12·1±0·5	6·0±0·3

* Equal numbers of male and female mice.
† 8 mg % g colloidal carbon.
‡ Body weight/liver + spleen weight.

infection, the CFU numbers in F_1 hybrids, in lung and liver, were intermediate between the parental values. There was a small incomplete dominance of the high character for liver counts and of the low character for lung counts.

Modification of macrophage activity in BCG infected H *and* L mice

The augmentation of phagocytic activity during BCG infection is mediated by T cell released lymphokines. It has been shown, in conventional mice, that this effect is maximal on the 14th day after BCG inoculation (Biozzi *et al.*, 1954). The results in *H* and *L* mice are shown in Table 1.

The phagocytic index K was similar in *H* and *L* mice. The increase produced by BCG was also similar on day 7 and remained unchanged on day 14. Liver and spleen weights were significantly larger in *H* than in *L* control mice. This interline difference persisted in BCG infected mice: considering the maximum values (on day 14) the spleen weight was increased by 4·5 times in *H* and 3·7 times in *L* mice, as compared with control values. The fluctuations in the corrected index α, which are related to changes in spleen and liver weights, are also parallel in *H* and *L* lines. In both lines the α values are lower than those in controls on day 14, indicating a decrease in the specific phagocytic activity per unit of weight.

On the whole, the results in Table 1 indicate that a similar modification of the phagocytic activity of liver and spleen macrophages occurs in *H* and *L* mice, during the early course of BCG infection.

Resistance to M. tuberculosis *($H_{37}Rv$) infection in control and BCG vaccinated* H, L *and F_1 hybrid mice*

The multiplication rate of *M. tuberculosis* was determined by the counts of H_{37}Rv-CFU recovered from the spleen 14 and 28 days after i.v. inoculation of 7×10^5 H_{37}Rv-CFU. The innate resistance of *H*, *L* and F_1 hybrid mice was compared with the acquired resistance induced by living BCG administered 5 months before. This time interval was chosen in order to give the virulent challenge when a steady level of BCG-CFU is reached in the two lines and in F_1 hybrids. CFU counts in all organs were then higher in *L* than in *H* mice (See Fig. 1).

The results are shown in Fig. 3. The mean H_{37}Rv-CFU number recovered in the spleen of mice killed 24 h after inoculation (established in 10 mice of the different groups) was 5×10^5 ($5{\cdot}7 \pm 0{\cdot}4$ $\log_{10}$). The H_{37}Rv-CFU counts increased similarly in control *H*, *L* and F_1 mice during the first 2 weeks. Afterwards a striking difference between the three groups appeared. The CFU numbers decreased significantly in *H* and F_1 mice whereas in *L* mice, they probably continued to increase because the *L* mice died within 22 days. The 100% mortality in the *L* line significantly differs ($P < 0{\cdot}01$) from the low mortality rate observed in *H* and F_1 hybrids (about 20%).

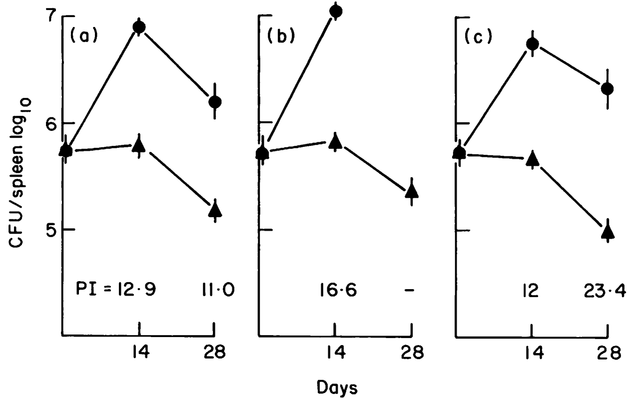

Fig. 3. CFU counts of *M. tuberculosis* H37 Rv in spleens of (a) *H*, (b) *L* and (c) F_1 hybrid mice, controls (●) or vaccinated by i.v. injection of living BCG 5 months previously (▲).

Previous BCG vaccination completely inhibited the growth of virulent *M. tuberculosis* in the spleens of *H*, *L* and F_1 hybrids; the CFU levels did not increase between 0 and 14 days and decreased significantly on day 28. This result demonstrates that BCG had a long lasting protective effect on the subsequent virulent infection. This immune protection was stronger in *L* mice which have a higher innate susceptibility to the infection. This is suggested by the comparison of H37Rv CFU counts in the spleens of BCG vaccinated with those in control mice. On day 14 the PI was slightly higher in *L* than in *H* mice; this difference would probably have increased later on, due to a further rise of CFU in *L* mice spleens. Actually, on day 28 the PI is much higher in F_1 than in *H* mice. A very low mortality rate was observed in the three groups of BCG vaccinated mice (5%). Death occurred 6–9 days after infection and was not due to tuberculosis infection.

DISCUSSION

The course of BCG infection was different in *H* and *L* antibody responder lines of mice. The major interline difference when comparing the kinetics of BCG-CFU recovered from the organs of i.v. infected mice occurred in the spleen. In the early period (0–14 days) the counts were higher in *H* than in *L* mice. The difference was not increased when a smaller inoculum ($2{\cdot}5 \times 10^4$ v.u.) was used for infection (data not shown).

Differences in the early resistance to BCG multiplication were also observed among inbred strains of mice and were shown to result from the effect of a single gene (*Bcg* gene) located on chromosome 1 (Gros *et al.*, 1981). The *Bcg* gene was shown to affect macrophage function independently of T-mediated immunity (Pelletier *et al.*, 1982) and more precisely the bacteriostatic effect of the macrophage (Stach *et al.*, 1984). However, these two models are probably not comparable since it has been recently demonstrated that control by *Bcg* gene is restricted to BCG Montreal and is not found using BCG Pasteur (Orme & Collins, 1984).

Later on in the course of BCG infection, the decline of BCG-CFU in the spleen is more effective in *H* mice so that 5–6 months after infection, the level of residual BCG-CFU is significantly lower in *H* mice, not only in spleen, but also in liver, lungs and kidney. Such a converse interline difference clearly indicates that a different resistance mechanism intervenes in the advanced phase of the infection. In this respect it is worth noting that whereas in the early phase BCG-CFU counts in F_1 hybrids show an overdominance of the resistant character of the *L* line, later on they become intermediate between the parental values in all the organs investigated.

The decline of BCG-CFU in Bcg^s strains is attributed to the activation of macrophages by T cell released lymphokines (Pelletier *et al.*, 1982). The results on the BCG-induced stimulation of the

phagocytic activity of liver and spleen macrophages were similar in the *H* and *L* lines of mice. This finding is in agreement with the general view that the intensity of T-mediated immunity reactions has not been modified during the selective breeding for antibody production (Biozzi *et al.*, 1979). Nevertheless, it has been reported that delayed type hypersensitivity (DTH) reactions to PPD were stronger in *H* than in *L* mice (Lagrange *et al.*, 1979). This discrepancy could be explained since the measure of DTH reaction depends largely on the inflammatory cell infiltration and is not a direct estimate of effector T cells. Similarly, it has been shown that, in various inbred strains of mice there was no consistent variation in the release of different lymphokines, as well as in the expression of different cell-mediated immunity reactions, after injection of BCG cell walls (Neta & Salvin, 1981). The results reported here on the resistance of the *H* and *L* lines of mice to *M. tuberculosis* infection confirm the higher innate susceptibility of L mice reported by Lagrange *et al.* (1979).

The data in Fig. 3 demonstrate that *L* mice are unable to control the early multiplication of virulent *M. tuberculosis* whereas initial BCG growth is slower in *L* than in *H* mice. The resistance of *L* mice to bacterial growth was also observed during *S. typhimurium* infection. In these mice, the lack of innate resistance to *M. tuberculosis* might be due to the capacity of this virulent mycobacterial strain to multiply in the granulomatous tissue, outside the phagocytic cells. The resistance is completely restored in BCG vaccinated L mice.

BCG has a definite protective effect on infection in the two lines and in F_1 hybrids as well, even after a 5 month interval. By that time there is in fact a persistance of living BCG in all the organs investigated, the BCG-CFU counts being higher in *L* than in *H* mice and intermediate in F_1 hybrids. In the present results, the protection resulting from BCG vaccination was stronger in *L* than in *H* mice, whereas an inverse observation was reported by Lagrange *et al.* (1979). However, it should be remembered that in the experiment by Lagrange *et al.* (1979), the BCG vaccination was made by the s.c. route and the challenge was given on the 21st day when there were probably more BCG-CFU in the organs of *H* than of *L* mice. In a long lasting mycobacteria infection, different defence mechanisms intervene successively. It is therefore not surprising to find an alternating pattern of resistance in the H and L selected lines throughout the infection.

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