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Susceptibility to tuberculosis: Composition of tuberculous granulomas in Thorbecke and outbred New Zealand White rabbits

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Abstract

We sought to characterize the lung cellular immune responses to inhaled *Mycobacterium tuberculosis* (Mtb) of the susceptible inbred Thorbecke rabbit (the genomically sequenced strain, now unavailable) and compare it to outbred, Mtb resistant, New Zealand White rabbits. Using Mtb CDC1551, we confirmed that the inbred rabbits allowed establishment of infection with this low virulence strain, compared to poor establishment in outbred rabbits. With a more virulent strain, Mtb Erdman, that establishes infection well in both rabbit strains, we analyzed granulomas from rabbit lungs 5 weeks after aerosol infection. The lung granulomas of inbred rabbits had significantly higher frequencies of cells expressing MHC Class II and CD11b, and lower frequencies of CD8⁺ T cells than the outbred controls. Macrophage-sized cells expressing MHC Class II in inbred rabbit granulomas showed significantly decreased intensity of expression, suggesting impaired maturation. Although the inbred dermal tuberculin reactions were decreased, the *in vitro* IFN- γ mRNA responses of hilar node lymphocytes to tuberculin were higher than those of outbred rabbits. Further delineation of the outbred rabbit's resistant immune response to Mtb infection is warranted.

Keywords

TUBERCULOSIS; RABBIT; HOST SUSCEPTIBILITY; ANIMAL MODEL; DENDRITIC CELL

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Introduction

Globally, tuberculosis claims over 2 million lives per year. The global disease reservoir is also impressive with an estimated one-third of the world's population latently infected with *Mycobacterium tuberculosis* (Mtb). Among immunocompetent persons, 10% of these infected individuals will develop active, transmissible tuberculosis in a lifetime. Environmental factors (such as malnutrition, poor economic conditions and overcrowding), human immunodeficiency virus (HIV) infection and genetic variability in host defense play important roles in the development of active disease (Comstock, 1978; Schaible and Kaufmann, 2007).

In contradistinction to laboratory species such as mice or guinea pigs, outbred New Zealand White rabbits are relatively resistant to infection with Mtb like humans. Therefore, the rabbit is a very appropriate model to assess susceptibility to tuberculosis. Unlike many other commonly used animal models, the clinical pathologic course of Mtb infection in outbred rabbits mimics that of human tuberculosis well with the formation of pulmonary granulomas with caseous necrosis, and ultimately, containment of infection to a paucibacillary state (Dannenberg and Collins, 2001; Lurie, 1964).

Inbred animal models of tuberculosis with susceptible and resistant phenotypes have been used to elucidate host immune mechanisms that are important to the control of tuberculosis. Susceptible strains of mice, guinea pigs, and rabbits not only develop more severe disease after primary infection, but are also less likely to mount a protective immune response after immunization with a live vaccine (Lewis and Loomis, 1928; Lurie et al., 1952b; Medina and North, 1999).

Using rabbits, Lurie and colleagues compared and contrasted the response of resistant and susceptible inbred strains to aerosol infection with virulent tubercle bacilli (Lurie et al., 1952a). These studies were primarily accomplished through painstaking enumeration of bacillary burdens, as well as histopathological studies. They concluded that the resistance to disease was due to the capacity of macrophages to inhibit bacterial growth and directly related to the development of hypersensitivity reactions, which they defined in the publication as allergic sensitivity.

We have previously used an inbred New Zealand White rabbit developed by the late Dr. G. Jeanette Thorbecke in our Mtb studies (Dorman et al., 2004). In those studies, we showed that the Thorbecke inbred rabbits were more susceptible to aerosol infection with Mtb than its outbred counterparts. The Thorbecke rabbits also developed significantly larger and more numerous lung granulomas 5 weeks after infection with Mtb (the time of peak organism burden and granuloma size in outbred rabbits). Moreover, these inbred rabbits had impaired delayed-type skin hypersensitivity responses to tuberculin and impaired peritoneal macrophage TNF- α responses to *in vitro* stimulation with both lipopolysaccharide (LPS) and infection with Mtb.

The further studies reported herein describe the phenotypic composition of lung granulomas and draining hilar lymph nodes 5 weeks after infection. Our results emphasize the key role that types, numbers and level of activation of granuloma phagocytes have in modulating susceptibility to Mtb infection and long-term control. Further studies elucidating the kinetics of accumulation and activation of mononuclear phagocytes in outbred resistant rabbits may lead to the discovery of new correlates of protective immunity and enhance the use of the rabbit as an attractive model for the study of tuberculosis immunopathogenesis. Although the Thorbecke colony is no longer available, our previous report is the only other published data on immunologic responses on this rabbit strain. It is the Thorbecke rabbit genome that was chosen for partial sequencing (2 $\times$)

(<http://www.broad.mit.edu/ftp/pub/assemblies/mammals/rabbit/>) with planned full (6.5 $\times$)

coverage. Such data may allow for a continued expansion of immunologic reagents and make the rabbit a more compelling model of other human diseases.

Materials and Methods

Microorganisms

Cultures for aerosol infection were prepared by thawing frozen stock aliquots of the Mtb CDC1551 and Erdman strains (Manabe et al., 2003). Bacilli were grown to log phase in Middlebrook 7H9 medium containing oleic acid, albumin, dextrose, catalase supplement (OADC, Becton Dickinson, Sparks, MD) and 0.05% Tween 80. Clumps were allowed to settle for 2–3 hours and were then suspended in a 10% dilution of oleic-albumin complex (Becton Dickinson, Sparks, MD) in 0.9% NaCl.

Animals and infection

Pathogen-free New Zealand White (NZW) rabbits (female, 2.5–3 kg) were inbred for more than 20 generations by the late Dr. G. Jeanette Thorbecke of New York University (previously available from Covance Research Products, Inc., Denver, PA). Market NZW outbred rabbits were also obtained from Covance Research Products, Inc. The rabbits were maintained in standard cages under Biosafety Level 3 conditions and were fed commercial rabbit chow and water *ad libitum*. All animals were housed and maintained in accordance with protocols approved by the Institutional Animal Care and Use Committees of Johns Hopkins University, George Washington University, and U.S. Army Medical Research Institute of Infectious Diseases. Plethysmography and aerosol exposure were performed as previously described (Manabe et al., 2003). Four weeks after infection, 0.1 ml of Old Tuberculin (OT) (Synbiotics, San Diego, CA) was injected intradermally into each rabbit. Two days later, the resulting skin reactions were measured with calipers as previously described (Manabe et al., 2003). Five weeks after infection, the rabbits were euthanized with an intravenous pentobarbital preparation (Euthasol, NLS Animal Health, Baltimore, MD). The number of grossly visible pulmonary tubercles was counted and their diameters measured (Converse et al., 1998). For each rabbit, hilar lymph nodes and the right upper lung lobe were homogenized, and aliquots were plated on 7H10 agar supplemented with OADC. Colony forming units were quantitated at 6 weeks. Tissue was fixed in Streck (Streck Inc., Omaha, NE) and embedded in paraffin. Standard histologic sections were cut and then stained with either hematoxylin and eosin or carbol fuchsin and methylene blue (Dorman et al., 2004).

Flow cytometric analysis of single cell suspensions of lung granuloma and hilar lymph node cells

Single cell suspensions of individually isolated lung granulomas and draining lymph nodes were prepared as previously described (Kesavan et al., 2005). In brief, lung granulomas were macroscopically located and excised with scissors from the rest of the lung, weighed, treated with 50 µg/ml liberase RI (Roche, Alameda, CA), incubated for 1 hour at 37 °C and then sieved through sterile strainers. The suspensions were then centrifuged, washed, fixed with 4% paraformaldehyde for 15 min and incubated with mouse antibodies to rabbit CD4, CD8, CD11b, CD11c (Antigenix America, Huntington Station, NY) and MHC Class I, MHC Class II or IgM (Serotec, Raleigh, NC).

Hilar lymph nodes were collected at the time of necropsy, mechanically dissociated and filtered through a 70µm strainer (Falcon, San Diego, CA) to generate single cell suspensions. Cells were stained in a 96-well round-bottomed microplate (Nalge Nunc Intl., Rochester, NY) with the same antibodies as above, for 30 min at 4 °C. All antibodies were titrated and working dilutions ranged from 1:50 to 1:200. For the Serotec antibodies, a secondary R: PE conjugated, donkey anti-mouse antibody (RDI Inc, Concord, MA) was used at 1:4,000. The data on

fluorescently labeled cells were acquired in a FACScalibur flow cytometer (Becton Dickinson, CA, USA). At least 50,000 events were counted.

Total RNA extraction and gene expression in rabbit lung by Real Time PCR

For total RNA purification, the lung tissues snap-frozen in Trizol were homogenized in liquid nitrogen. The total RNA was extracted with chloroform, precipitated with isopropanol and then treated with DNaseI. Synthesis of the first strand of cDNA was performed using 1 µg of total lung RNA using random hexamers (Bio-Rad, Hercules, CA) as primers. Expression of rabbit genes was assayed with SYBR Green I by real-time PCR using gene-specific primers: 18s rRNA (product size 174 bp) forward 5'-CGCGGTTCTATTTTGTTGGT, reverse 5' – CTTCAAACCTCCGACTTTTCG; TNF-α (product size 227 bp) forward 5' – CGCCATGAGCACTGAGAGTA, reverse 5' –GGCCACAGGGTTGACTAGAT; IFN-γ (product size 224 bp) forward 5'-AAAGACCATGAGGTCATCAAGAA, reverse 5'-AGCGTCTGACTCCTTTTTCG.

RT-PCR IFN-γ expression by hilar lymphocytes in response to tuberculin

Hilar node lymphocytes were counted and plated in 24 well plates at a concentration of 10^6 cells per ml/well. Twelve to 18 hours later, the cells were incubated in the presence of 2.5% Old Tuberculin (OT, Synbiotics) at 37 °C for 24 hours. The content of each well was centrifuged, the supernatant removed, and the pellet resuspended in Trizol. RNA extraction and RT-PCR were accomplished as above. For each of the 12 rabbits (6 inbred, 6 outbred), there were 3 control wells, and 3 wells containing OT. Each cDNA sample was set-up in triplicate for the IFN-γ RT-PCR. Therefore, 9 RT-PCR reactions per rabbit were performed. 18s rRNA expression was used to calculate the “fold-change” by the following equation: $\text{Fold-Change} = 2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = (C_T(\text{IFN}\gamma) - C_T(18s \text{ rRNA}))_{\text{stimulated}} - (C_T(\text{IFN}\gamma) - C_T(18s \text{ rRNA}))_{\text{unstimulated}}$.

Statistical Analysis

All comparisons of non-normally distributed continuous data were analyzed with the Mann Whitney U test using Statview 5.0.1 (SAS, Cary, NC) or GraphPad Prism software (San Diego, CA).

Results and Discussion

In this report we confirm that the inbred Thorbecke rabbit is more susceptible to aerosol infection with different strains of Mtb as we previously described (Dorman et al., 2004). We show that inbred rabbits develop a greater number of lung tubercles that are of larger diameter, and that the lung burden of bacilli is always higher than what is found in outbred rabbits aerosol-infected with the same dose. These outcomes occur irrespective of the strain employed. In particular, we have shown that Mtb CDC1551 is a low virulence strain for outbred rabbits (Manabe et al., 2003). Five weeks after aerosol infection (the time of peak bacillary burden and disease in the lung), most outbred rabbits have poor establishment of infection with healing granulomas and no caseous necrosis on lung tissues. To determine whether this low virulence bacterial strain would establish disease in the more susceptible inbred animals, 6 inbred and 8 outbred rabbits were aerosol-infected with an average inhaled dose of 1.5×10^4 CFU. At necropsy 5 weeks after infection, the inbred rabbits had significantly more tubercles (200 ± 23 vs. 48 ± 18 , $P=0.007$), which were larger (1.6 ± 0.2 mm vs. 0.8 ± 0.1 mm, $P=0.007$). Additionally, greater numbers of bacilli were present in homogenates of the right upper lung lobes in inbred rabbits (4.0 ± 0.1 logCFU vs. 2.8 ± 0.1 logCFU, $P=0.001$). As shown before, outbred rabbits had poor initial establishment of infection with the same strain; lung granulomas were smaller and abortive without caseous necrosis at the same time point (Figures 1A, B).

Our data also show that both the establishment and the progression of the infection were more dramatic in the inbred Thorbecke rabbit. Inbred and outbred animals were infected with 2.5×10^4 CFU of Mtb H37Rv and monitored for up to 18 weeks post infection. At this time point, all the Thorbecke rabbits showed grossly visible granulomas, suggesting that not only the initial establishment, but also the ability of those animals to contain the infection and heal was also compromised (Supplemental Figure 1).

In this report, we also confirm that the Thorbecke rabbits have a diminished tuberculin skin test response compared to outbred rabbits (196 ± 52 vs. 750 ± 242 mm³, $P=0.028$), indicating that the delayed-type hypersensitivity (DTH) to Mtb antigens is impaired. This contrasts with the results obtained from inbred susceptible rabbits by Lurie *et al.* (Lurie, 1964). DTH responses to Mtb antigens were not diminished compared to resistant rabbits. This discrepancy suggests that significant differences in the immune response against Mtb may exist between the two strains of susceptible rabbits, and that they may not be directly comparable to previously described inbred rabbits.

To gain insight into the mechanism behind the enhanced susceptibility of the inbred rabbits, we characterized the phenotype of the different cell populations recruited to granulomatous lesions in the lung. To allow comparison, we used a highly virulent strain, Mtb Erdman for this experiment, which establishes disease well in both outbred and inbred rabbits (Manabe *et al.*, 2003). Following aerosol infection with 8.9×10^3 CFU of Mtb Erdman, both outbred and inbred rabbits developed caseous lesions 5 weeks after infection (Figures 1C and 1D, respectively). Again, necropsy revealed more tubercles in the inbred rabbits compared to outbred rabbits (220 ± 22 vs. 90 ± 23 , $P=0.016$), and more CFU in the homogenates of inbred rabbit right upper lung lobes (5.9 ± 0.2 logCFU vs. 4.0 ± 0.1 logCFU, $P=0.004$), confirming the increased susceptibility of the inbred rabbits.

The most novel data of our work was the study of the cellular composition of the lesions in both strains of rabbits. For this, granulomas were excised from the right lower lung lobe and single cell suspensions were analyzed by flow cytometry for the expression of the cell surface markers CD4, CD8, CD25, MHC Class I, MHC Class II, CD11b and CD11c. Granuloma-derived cells from the inbred Thorbecke rabbits gated on large, macrophage-sized cells (to avoid inclusion of lymphocytes) showed a significantly increased proportion ($P \leq 0.05$) of cells expressing MHC Class II and CD11b when compared to outbred rabbits (Figure 2A). The absolute numbers of MHC Class II- and CD11b-positive cells normalized for the weight of granulomas analyzed were also increased ($P=0.09$, $P=0.03$, respectively). Interestingly, inbred rabbits had a concomitantly lower number of CD11b+ and MHC Class II+ cells in the peripheral blood as early as 1 week after infection (Supplemental Figures 2 & 3). These results suggest that those cell types may have abandoned the circulation and been sequestered into the lung granulomas. Moreover, the analysis of the intensity of expression of CD11b, CD11c and MHC Class II (as determined by Geometric Mean Fluorescence) revealed that the relative expression of each of those markers was significantly reduced in the inbred rabbits ($P=0.001$, $P=0.0004$ and $P=0.0001$, respectively; Figure 3).

On the other hand, the analysis of T cell populations in the granulomas revealed a statistically significantly lower frequency and absolute number (normalized for the total weight of granulomas analyzed) of CD8+ T cells (Figure 2A). No difference was found in the frequency of CD4+ T cells between the two groups. Taken together, the novel phenotypic analysis of the cells obtained from the lung granulomas of the two rabbit strains suggest that, despite the increased number of CD11b+ and MHC Class II-expressing cells in the lung granulomas of inbred animals, their phenotype and/or their level of activation were significantly different from outbred rabbits. Of these markers, the lower expression level of MHC Class II in inbred rabbit granuloma cells gated on macrophage size may suggest defective APC maturation, or

migration of more immature APCs. This observation supports the data published by Ordway *et al.* in tuberculous mice (Ordway *et al.*, 2006). These authors found that there was decreased expression of MHC Class II molecules on lung dendritic cells in unvaccinated controls compared to immune mice.

Hence, it is possible that differences in the maturation/marker expression of local APCs could be linked not only to poor bacterial killing, but also to the lack or delayed emergence of a proper adaptive immune response in inbred rabbits. Although we do not have an explanation for the increased number of APCs in the granulomas of inbred animals, it is possible that the increased bacterial burden may attract larger numbers of APCs to the granulomas that accumulate in the lung unable to control the infection.

Data obtained in rabbits and mice have suggested that susceptibility could also be associated with a delay in the trafficking of bacilli to the hilar lymph node, thereby delaying the priming of the entire T cell Th1 response (Chackerian *et al.*, 2002; Lurie *et al.*, 1952a). In our system, however, we could find no bacilli in the draining hilar lymph nodes of either group of rabbits at the time point tested. It is possible, however, that bacilli may have been present but they could not be cultured. Although no differences were found in the CD4+ T cell population, the lower numbers of CD8+ T cells in lungs of inbred animals points to the possibility of potential deficiencies in priming of specific components of the adaptive immune response.

We were unable to measure directly cytokine responses of lung granuloma cells to tuberculosis antigens. When we employed RT-PCR with RNA harvested from whole lungs, we could not find a statistically significant difference between the two groups of rabbits in TNF- α or IFN- γ transcripts (Supplemental Figure 4). It is possible, however, that the whole lung RNA analysis was biased by the higher number of bacilli and the tubercles that were larger and more numerous in inbred rabbit lungs. It is also possible that granuloma specific results (rather than whole lung RNA) may have been more informative.

We also studied the T cell responses using lymphocytes isolated from the draining hilar lymph nodes. We first determined the total numbers of lymphocytes in the hilar lymph nodes of rabbits infected with Mtb Erdman. The phenotypic analysis showed no significant differences in the numbers of CD4+, CD8+ or CD25+ T cells between the two rabbit strains (Figure 2B). In contrast, the number of B cells was higher in the resistant outbred rabbits ($P \leq 0.05$). Surprisingly, hilar node lymphocytes collected from infected inbred rabbits 5 weeks after aerosol infection showed significantly more IFN- γ mRNA expression than outbred rabbit lymphocytes at the same time point after restimulation *in vitro* with tuberculin, (Figure 4, $P=0.015$). It is important to note that no CFU were detected in the lymph node homogenates of either inbred or outbred rabbits. Moreover, the analysis of MHC Class II expression of cells gated on macrophage size recovered from these lymph nodes also revealed a lower mean fluorescence intensity per cell from inbred rabbits as shown in the lung granuloma cells (Supplemental Figure 5), suggesting that priming in the lymph node takes place by the same cell types present in the granuloma. Given the susceptibility of the inbred strain to infection with Mtb, it is a surprising finding that they also have higher levels of IFN- γ mRNA. It is possible that, despite the higher level of cytokine transcript, inbred rabbits fail to produce the cytokine; unfortunately this cannot be ruled out because to date, there are no reagents to detect lapine IFN- γ protein. As suggested by experiments conducted in murine models, another possible explanation is that fewer activated lymphocytes are recruited to the lungs of susceptible mice compared to resistant mice (Chackerian *et al.*, 2001; Lyadova *et al.*, 2000), implicating impaired lymphocyte recruitment, rather than impaired function. It is tempting to speculate that decreased specifically activated lymphocyte recruitment to the sites of infection due to impaired and/or delayed innate priming responses could explain larger lung granulomas with increased numbers of immature APCs (lower MHC Class II expression) and on-going

bacillary replication. In addition, diminished DTH skin responses to tuberculin antigens could correlate with impaired recruitment of specifically activated T cells to skin in inbred susceptible rabbits.

In rabbits, the role and type of APCs recruited to the lung and the time course of that recruitment may play a crucial role. Due to the lack of reagents available for the study of the immune responses in the rabbit, however, we cannot determine whether the disparity in cell recruitment to both lung and skin was the result of differences in chemokine production or migratory capacity of T cells, macrophages and/or dendritic cells. Many questions remain about the interplay between specifically activated T cells and APCs in infected rabbit lung. Both the innate and acquired immune responses may be defective in these inbred rabbits. The lower expression levels of CD11b, CD11c, and MHC Class II surface molecules may represent less efficient or immature APCs in the lung granulomas of infected inbred animals despite increased numbers of cells expressing CD11b and MHC Class II, and strong, tuberculin-specific, local IFN- γ responses from hilar lymphocytes. Many of the granuloma-specific differences may be significantly influenced by differences in antigen/bacillary load, however. Further work on the early time course of bacillary and cell migration of resistant outbred rabbits is urgently needed to understand efficient and effective immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Lung granuloma from an outbred rabbit 5 weeks after aerosol infection with 4.18 logCFU of Mtb CDC1551 strain. A representative healing granuloma with central epithelioid macrophages and surrounding lymphocytes is shown. No evidence of caseous necrosis is seen (100×). **B.** Lung granuloma from an inbred rabbit 5 weeks after aerosol infection with the same dose of Mtb CDC1551 strain. The image shows a much larger and more extensive granuloma with surrounding macrophages and lymphocytes around an area of early central caseous necrosis (small arrow) with polymorphonuclear cells (100×). **C.** Lung granuloma from an outbred rabbit 5 weeks after aerosol infection with 3.90 logCFU of Mtb Erdman strain. **D.** Lung granuloma from an inbred rabbit 5 weeks after aerosol infection with 3.90 logCFU of Mtb Erdman strain. Central caseous necrosis is seen in both rabbit strains, although the lesion is much larger in the inbred animal with multiple well-formed pink areas of extensive caseous necrosis. Both lesions have epithelioid macrophages (arrows) and a mantle of lymphocytes (100×). Bars represent 100 μ

Figure 2.

A. Cell composition of pooled, representative, lung granulomas of inbred (grey bars) and outbred rabbits (white bars) 5 weeks after aerosol infection with 3.90 logCFU Mtb Erdman. The cell surface marker assayed is arrayed on the X-axis; the percentage of cells expressing each marker is shown on the Y-axis. **B.** Cell composition of a single hilar node from each of the same rabbits. The total number of cells per lymph node expressing each marker is shown on the Y-axis. Data represent average $\pm$ SD, n=6 rabbits. * denotes statistically significant, $P \leq 0.05$.

Figure 3. Relative geometric mean fluorescence of CD11b (A), CD11c (B) and MHC Class II (C) expression in lung granuloma cells from outbred rabbits (squares) and inbred rabbits (circles) 5 weeks after infection with Mtb Erdman.

Figure 4.

IFN- γ mRNA as measured by RT-PCR 5 weeks after aerosol infection with Mtb Erdman in 10^6 hilar lymph node lymphocytes stimulated for 24 hours with 2.5% Old Tuberculin (OT, Synbiotics). Each bar is the average of 6 animals; 3 RT-PCR reactions were measured for each of 3 wells of lymphocytes per rabbit. Data represent the comparison of RT-PCR values from stimulated and unstimulated lymphocytes normalized to 18s rRNA expression in each sample well as fold change as defined in the methods. The IFN- γ mRNA fold change in tuberculin-stimulated compared to unstimulated hilar lymphocytes of inbred Thorbecke rabbits (black bar) was significantly higher than that of outbred rabbits (white bar).