The guinea pig has recently proven to be a useful and realistic animal model in which to test new drug regimens for their potential to treat disease caused by Mycobacterium tuberculosis. We previously showed that two regimens reduced the bacterial load in this model to non-culturable levels, but about a year thereafter a significant portion of these animals underwent spontaneous disease reactivation. In the present report we show the results of a small study in which we took healthy remaining animals and attempted to induce disease reactivation using cortisone. Ten guinea pigs that had remained completely healthy 11-months after drug therapy were treated in this manner, and a month later two of the animals, which had originally received a “fast-acting” regimen of rifampacin, pyrazinamide, and the experimental drug TMC207, showed severe reactivation, with large numbers of bacilli culturable from the lungs and other organs. Despite this, while acid fast bacilli could be detected, their staining was unusually weak. Thus, while new drug regimens are being developed for tuberculosis that seems to be fast acting, our data suggests this does not absolutely guarantee organ sterility. In addition, the poor acid fastness observed in reactivating animals might be explained in the context of new information regarding the physiology of these persisting organisms.

Introduction

The continuing tuberculosis epidemic, and with it the increasing number of strains that are multi-drug resistant, has emphasized the urgent need to continue to search for active new drugs [1-3]. After initial testing in vitro, most compounds are then evaluated for activity in the mouse model [4]. While this is cost-effective, there is a growing appreciation that the lack of overt necrosis in mouse strains infected with Mycobacterium tuberculosis is a limitation of the model, reflecting the fact that increasing evidence is showing that bacteria capable of lengthy persistence, which is the inherent basis of the very lengthy drug treatment needed in humans, are believed to be sequestered in residual necrosis present in primary lesions [5,6], a process lacking in mice. As a result, there has been a growing interest in using the guinea pig model to test drug regimens given that, like in humans, primary lesion necrosis is a central hallmark of the disease pathogenesis.

Using that model we previously showed that guinea pigs infected by low dose aerosol infection with the virulent Erdman K01 strain of Mycobacterium tuberculosis and then treated daily with a regimen of rifampicin, isoniazid, and pyrazinamide [RHZ] showed a characteristic biphasic reduction in the bacterial load in the lungs, in which there was rapid early clearance, followed by a very lengthy second phase in which persisting bacilli were slowly destroyed [7]. This result was then compared to a second study, in which animals were treated with a regimen containing rifampicin and pyrazinamide, plus the experimental compound TMC207 [RZ/207]. Animals given this regimen showed much faster kinetics of clearance, with bacteria no longer detectable after six weeks of treatment [8]. Despite this apparently increased efficacy, some necrotic lesions could still be found in the lungs of these animals, and approximately a year afterwards we noted spontaneous relapse in some of the treated animals at a frequency no different to that seen in animals given RHZ.

In the small study described here, we decided to examine a group of ten guinea pigs that had received one of these two regimens but had shown no signs whatsoever [distress, weight loss, etc] of disease relapse over a subsequent period of 340 days. We selected five from each of these and treated these animals with hydrocortisone to immunosuppress them. As we show here, no animals in the RHZ group had any evidence of relapse, whereas two out of five in the RZ/207 group relapsed. In these latter animals, there were large areas of pulmonary consolidation with granulomatous inflammation, and >10^6 bacilli [per lung lobe] could subsequently be cultured from lung tissues. These data imply that short-acting, apparently highly efficacious drug regimens do not absolutely guarantee complete lung sterilization, and that unfortunately very expensive and lengthy studies are probably needed to properly verify this. Finally, while large numbers of bacilli could be cultured from the lungs, acid fast bacteria in the lung lesions were sparse and were difficult to see; as such, this unexpected finding may help support the growing concept that bacteria that can survive and persist despite drug treatment undergo physiological adaptations allowing them to remain viable in residual necrosis, adaptations that may include modifications [even loss] of their cell wall structure, leading to relatively poor acid fast staining.

Materials and Methods

Guinea pigs

Female outbred Hartley guinea pigs (approximately 500 g in

*Corresponding author: Ian M Orme, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado 80523, USA, Tel: +970-491-5777; Fax: +970-491-5125; E-mail: ian.orme@colostate.edu

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weight were purchased from the Charles River Laboratories (North Wilmington, MA, USA) and held under barrier conditions in a Biosafety Level III animal laboratory. The specific pathogen-free nature of the guinea pig colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University. Guinea pigs were infected using a Madison chamber aerosol generation device which delivered approximately 20 M. tuberculosis strain Erdman K01 bacilli into the lungs.

**Drug treatments**

Animals in this study were treated with rifampicin [R: 50 mg/kg of body weight], pyrazinamide [Z: 100 mg/kg body weight], and isoniazid [H: 30mg/kg body weight], or RZ plus TMC207 [207: 15mg/kg body weight]. The effects of these regimens on the clearance of the infection have been published previously [7]. As described previously, drugs were resuspended in 40% sucrose (wt/vol), 20% pumpkin (wt/vol) (Libby's 100% pure pumpkin) mixture supplemented with Vitamin C (50 mg/kg) and commercial Lactobacillus (BD lactinex) (all purchased from Walmart, Fort Collins CO). Guinea pigs were gently cradled and hand fed the respective drug regimen with a 1 ml syringe. A split-feeding protocol in which pyrazinamide and TMC207 was given in the morning and rifampacin in the afternoon was used to minimize drug toxicity and to avoid the known antagonism between rifampicin and TMC207.

**Confirmation of reactivation disease**

The bacterial load in the lungs of animals after immunosuppression was assayed by plating serial dilutions of homogenates of the lungs (right cranial lobe) on nutrient Middlebrook 7H11 agar plates (GIBCO BRL, Gaithersburg, MD). Colony-forming units were counted after 4 weeks incubation at 37°C. The bacterial load for each organ was calculated and converted to logarithms.

**Hydrocortisone-induced reactivation**

Guinea pigs were subcutaneously injected for 10 days with 100 mg/kg of hydrocortisone (Sigma-Aldrich, St. Louis, MO). Animals were closely monitored for acute effects using a modified Karnovsky scale [6].

**Histology**

Guinea pigs were subjected to full body necropsy in order to ensure detection of lesions. In addition to harvesting the left caudal lung lobe, spleen and mediastinal lymph nodes from each guinea pig (n=5), samples of brain, heart, liver, kidney and mesenteric lymph nodes were also collected. Tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Randomly selected tissue sections were embedded in paraffin and cut to 5 μm on a microtome. Tissue sections were mounted on glass slides, deparaffinized and stained with haematoxylin and eosin. To detect acid fast bacilli, slides were stained by the Ziehl-Neelsen method.

**Flow cytometry**

To look for possible biomarkers of reactivation flow cytometry was performed on leukocytes obtained from the blood. Briefly, before euthanasia, blood was collected in a heparinised syringe via a transthoracic cardiac puncture. Leukocytes were separated from red blood cells on a Ficoll (Sigma-Aldrich) gradient after centrifugation at 800 xg. Data acquisition and analysis was performed using a FACscalibur (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA). Analyses were performed with an acquisition of at least 100,000 total events. Single cell suspensions from the blood of individual guinea pigs were stained with Serotec antibodies to CD4, CD8, pan T cell, CD45, MIL4, B cell, macrophage and class II antibodies at 4°C for 30 minutes in the dark (23). In addition, membrane permeabilisation using Leucoperm (Serotec Inc, Raleigh, NC) was completed according to the instructions prior to staining with antibodies directed to macrophages and MHC Class II antibodies. Data acquisition and analysis were done using a FACscalibur (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA). Compensation of the spectral overlap for each fluorochrome was done using CD4 or MIL4 or CD3 antigens from cells gated in the FSC<sup>low</sup> versus SSC<sup>low</sup>, FSC<sup>mid-high</sup> versus SSC<sup>mid-high</sup>, SSC<sup>low</sup> versus MIL4<sup>+</sup>; SSC<sup>high</sup> versus MIL4<sup>+</sup> and SSC<sup>high</sup> versus MIL4<sup>−</sup> region respectively. Analyses were performed with an acquisition of at least 100,000 total events.

**Results**

**Consequences of cortisone-forced reactivation**

We previously reported similar rates of spontaneous reactivation in TB-infected guinea pigs receiving extensive treatment with a RHZ regimen or a shorter treatment consisting of 8 weeks of RZ/207 (in which no culturable bacteria were found by six weeks of therapy), with this reactivation beginning to be apparent about 330 days after drug treatment [8]. In a remaining group of guinea pigs however, no evidence of disease was seen and these animals remained completely healthy. We selected five animals from each of the two regimen groups based on high body weight and no evidence of any symptoms of infection, and immunosuppressed these animals with cortisone. This was given for ten days, then the animals were sacrificed a month later. Two animals in the RZ/207, but no animals in the RHZ group, underwent reactivation based on culture of bacilli from the lungs (Figure 1).

**Lung histopathology**

Full body necropsies were performed on each animal after cortisone immunosuppression. In the two reactivating animals, lesions were obvious in the lungs, draining lymph nodes, and the spleens. No lesions could be seen in these organs in the other animals, nor were any found in the brain, heart, liver, mesenteric lymph nodes, or the kidneys by gross examination, or by examining multiple random tissue sections.

![Figure 1: Bacterial colony counts in individual guinea pigs following forced reactivation. Bacteria could be cultured out of the primary target organs in two animals that had received RZ/207, whereas no animals reactivated after RHZ treatment.](image-url)
Histological examination harvested lungs was then performed. In non-reactivating RHZ animals we found sporadic lung fields containing small foci of mineralization, sometimes surrounded by minimal histiocytic inflammation, invariably fully calcified (Figure 2). In the two animals in the RZ/207 group that underwent reactivation, much of the pulmonary parenchyma was effaced by coalescing lesions were huge and consolidated much of the overall lung lobe tissues (Figure 3). These lesions were severe, multifocal and coalescing pulmonary granulomas that tended to track along airways. Inflammation was characterized by aggregates of epithelioid macrophages, and rare Langerhans giant cells. While there were occasional foci of mineralization, the larger granulomas had areas of central necrosis. Inflammation effaced much of the pulmonary parenchyma and within severely affected areas reduplication and proliferation of the airway epithelium was prominent.

Another prominent feature in the lungs of the reactivating animals was reduplication of airway epithelium, characterized by multiple cross sections of airways identified in areas of severe inflammation (Figure 4).

This finding was interpreted as a non-specific response to chronic lung disease resulting in parenchymal collapse with epithelial hyperplasia.

Surprisingly, despite the large numbers of cultivatable bacteria recovered from these animals, acid fast bacteria were often difficult to find throughout multiple lung sections, lung lesions and thus appeared to be on this basis at least very rare; small foci could be found here and there, but did not stain particularly brightly (Figure 5A). On the contrary, these bacilli were much easier to find within inflammatory cells and admixed with cellular debris within the upper airways (Figure 5B), which contained abundant mixed inflammation and cellular debris [bronchopneumonia].

**Cellular populations in the blood**

A further aspect of the reactivation process we investigated was the possible identification of biomarkers of this event. Because lungs cannot be taken from patients, we instead decided to look at leukocyte subsets in the bloodstream of these animals. CD4 and CD8 T cells in the lungs. Moreover, levels of CD4 and CD8 cells in the bloodstream of these animals were dramatically increased on both CD4 and CD8 cells in the blood of the two reactivating animals (Figure 6). No increases were seen in numbers of B cells, whereas, as anticipated, neutrophil levels were raised in the two reactivating animals (Figure 6).

**Discussion**

Despite the small sample sizes used here, necessitated by the extreme expense of keeping animals under level-III conditions for such a long study, some important information was still obtained. Perhaps the most important was the finding that while the experimental regimen RZ/207 appears on face value to be highly efficacious [8] and certainly seems to support the goal of substantially reduced treatment duration, this is still no guarantee that the infection has been totally sterilized, even when the infected animal shows absolutely no signs of active disease. In contrast, in animals given the far more lengthy conventional RHZ therapy animals not showing evidence of spontaneous disease relapse over the next year did indeed appear to be fully sterilized. If this information can be extrapolated to therapy in humans, then it suggests an obvious trade-off between rapid therapy that may not fully ensure sterilization, versus very lengthy therapy that is far more costly but may result in full sterilization in individuals showing no signs of relapse.

To date, the efficacy of drug regimens has always relied on cultivation of the bacilli from infected organs, with the concept being that an animal can be regarded as “sterilized” if organs remain culture-
negative. Recently, however, we and others have begun to challenge this concept [4,6,9-11] by arguing that persisting bacilli may become physiologically altered, perhaps by biofilming, and as a result will remain as “unculturable” bacilli. In this regard, there is growing evidence in the relevant guinea pig model that bacteria that persist after chemotherapy do so by surviving in residual necrosis, even when such necrosis is minimal [5,8]. This in turn supports the contention that while the mouse model is very useful as an initial screening device [4], this model is limited by the fact that this species does not develop necrosis. Hence, in the context of studying drugs to remove persisting or latent bacteria, in which necrosis is the key event [12] the guinea pig model becomes the model of choice. We would propose based on our observations here that if a drug regimen is to be completely and thoroughly evaluated, then apparently cured guinea pigs [“culture negative”] should be allowed to spontaneously reactivate disease [which takes about a year] and those that show no evidence of this happening should then be immunosuppressed before a “sterilizing regimen” can finally be legitimately claimed.

In this regard, the experimental compound TMC207 is highly promising. It is highly effective, and has shown to be very active against multi-drug resistant isolates [13,14]. Given these data, it was hoped that the duration of therapy, a major drawback to current therapy given the cost, could be significantly reduced by adding this drug to existing regimens. As we recently showed [8] administration of TMC207 alone with rifampicin and pyrazinamide appeared to sterilize guinea pigs in just eight weeks of treatment (with no culturable bacilli found after six weeks). However, when followed for relapse, this event was seen at a similar rate in animals receiving RHZ or RZ/207. We should also point out that the usual protocol to follow spontaneous reactivation, in both mouse models and more recently in the guinea pig [15,16] is usually for 3-6 months. If we had followed this protocol we would not have observed reactivation that started after about 11- months in our study, nor is it likely we would have attempted to immunosuppress these animals. In other words, we could have easily come to the erroneous conclusion that animals given the RZ/207 regimen did not reactivate and thus appeared to be completely sterilized.

A further unexpected observation here was that despite our ability to cultivate very high numbers of bacilli from the lungs of the reactivating animals, we could barely see any at all when we first examined sections stained for acid fast bacilli. Although it can only be conjecture at this point, these observations allow us to speculate that in the animals undergoing forced reactivation disease, surviving viable bacilli were remaining in a structural state that rendered them poorly acid fast. Since the immune response of the host was intact to that point, we can speculate that immune pressure was a factor in preventing any regrowth of the infection, as of course would be anticipated. When released from this pressure the remaining bacteria expanded dramatically in numbers [we were able to measure >10^6 CFU a month later] and drove a florid granulomatous-like inflammatory response which created very large consolidating lesions in the lungs of the two reactivating animals.

In guinea pigs in the process of receiving chemotherapy, we have previously shown apparently intact very brightly staining AFB-positive bacteria can be still found in primary lesions, both in central necrosis and in cellular debris surrounding it [6]. These “necrosis-associated extracellular” bacteria [NECs] appear singly, and in small clumps or clusters [11] and almost certainly represent the “persistors” that remain after chemotherapy and which we currently believe [12] form the foci of any potential reactivation disease later. A very recent study [9] confirms this further, and verifies that after chemotherapy with the experimental drug TMC207 residual bacilli are confined to areas of residual necrosis, as we previously noted [8]. This “location” concept [12] which is far simpler than the more accepted “drug tolerance” viewpoint [17] holds that by persisting in residual primary lesion necrosis, these bacilli...
can escape the attentions of both the immune response, and that of a sufficient concentration gradient of drugs needed to kill them.

The NECs we have observed may represent some sort of biofilm, although probably not in the most conventional sense of how the term is used. As has been seen for multiple bacterial species, bacteria in biofilms have apparent drug resistance, in contrast to planktonic bacteria. In this regard, it has been firmly established in vitro that *M. tuberculosis* can indeed form biofilm-like structures [10]. Biochemical analysis of these structures has revealed that they appear to release she-like structures and analysis of these has shown these to comprise of mycolic acids [10]. Historically, the actual existence of non-acid fast, viable bacilli has to date been a point of conjecture. However, it was recently shown [18] that targeted deletion of kasB, one of two *M. tuberculosis* genes encoding distinct beta-ketoacyl-acyl carrier protein synthases involved in mycolic acid synthesis, resulted in the loss of acid-fast staining, an event attributed to synthesis of mycolates that had shorter chain lengths. These mutants were incapable of causing disease in immunocompetent mice, but persisted indefinitely. It is very reasonable therefore to speculate that bacilli persisting in our model after chemotherapy may have similar properties and that such observed changes may be the basis for their poor in vivo acid fast staining.

We can now hypothesize further that in the year or so after chemotherapy these remaining bacteria undergo further physiological and structural changes ensuring their survival. Such changes would have been missed in earlier studies because most were done in mice, which do not develop necrosis to any degree, and because no previous studies to our knowledge have been taken out to the extreme length of time we performed here. Because the availability of oxygen will obviously be very low, this represents true survival of the bacterium in the absence of any [energy requiring] attempt to regrow. If we assume that very weak acid fast-positivity represents changes in cell wall permeability to the stain, this in turn then suggests structural changes underlying this in these few long-term survivors. Moreover, our observations support the further hypothesis that once the bacilli are placed in a favorable environment which is nutrient rich, they can quickly recover and start cellular replication, thus explaining our ability to then detect CFU. This apparently rapid ability to regrow tends to argue against the concept that these bacilli are in a true state of "latency". To extend this line of argument further, one can propose that it is bacteria in this state of long term persistence that deserve further study, rather than bacteria simply exposed in vitro to extremely low [and hence probably irrelevant] levels of oxygen tension and nutrient starvation [12].

Two other sets of observations deserve comment. The first is that it proved far easier to detect acid fast bacteria in cellular debris in the large airways in the lungs. This suggests that these bacilli had restored the integrity of their cell walls, possibly triggered by the normoxic conditions; moreover this observation is also important from an animal husbandry point of view, given the general feeling that this animal species does not "shed". The second set of observations regard our analysis of leukocyte populations in the blood of the reactivating animals, as determined by flow cytometry. In these animals, we detected activated T-cell subsets in higher numbers, as well as an approximately 50-100-fold increase in cells expressing CT-4. This marker appears to be associated with cell influx into the lungs [possibly a selectin] and our detection here of CT-4+ cells in the blood is further evidence that this might be a useful biomarker of disease reactivation [8]. We should also note that an interesting aspect of the host response in naïve animals to *M. tuberculosis* aerosol infection is the surprising lack of influx of activated Class-IIα macrophages [19] an observation we make again here in terms of such cells in the blood. This could obviously reflect the effect of the cortisone treatment, but one would expect some degree of recovery of the immune system a month later.

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