Intracellular fate of *Mycobacterium avium* subspecies *paratuberculosis* in Monocytes From Normal and Infected, Interferon-Responsive Cows as Determined by a Radiometric Method

BenYang Zhao, Charles J. Czuprynski, and Michael T. Collins

ABSTRACT

The ability of *Mycobacterium avium* subsp. *paratuberculosis* to survive in bovine monocytes was studied using radiometric (BACTEC) culture, standard plate counting and microscopic counting of acid-fast stained monocyte monolayers. Results of microscopic counts sharply contrasted with results of viable counts determined both by plate counting and radiometric counting. We observed an early phase (the first 6 d after in vitro infection) of intracellular bacillary growth, followed by a later phase of mycobacteriostasis or killing (up to 12 d after in vitro infection) in monocytes from non-infected cows. The data suggest that multiplication and death of *M. avium* subsp. *paratuberculosis* occur simultaneously in bovine monocytes infected in vitro. Using the BACTEC method, we compared the ability of bovine monocytes from normal cows and cows infected with *M. avium* subsp. *paratuberculosis* and showing evidence of a strong Th1-like cellular immune response to ingest and inhibit the intracellular growth of *M. avium* subsp. *paratuberculosis*. There was a trend toward greater phagocytosis and faster killing of *Mycobacterium avium* subsp. *paratuberculosis* by monocytes from the infected, immune responder cows. However, the observed numbers of viable *M. avium* subsp. *paratuberculosis* at each time after monocyte infection were not significantly different between normal and infected cows.

RÉSUMÉ

La survie de *Mycobacterium avium* ssp. *paratuberculosis* dans les monocytes bovins a été étudiée à l'aide d'une technique de culture avec radiométrie (BACTEC), d'un énombrement sur gélose et du comptage en microscopie des bactéries sur monocouches de monocytes colorées. Les résultats du comptage en microscopie étaient très différents des résultats de comptes viables obtenus par comptage sur gélose et par radiométrie. Une phase initiale (durant les 6 premiers jours suivant l'infection in vitro) de croissance intracellulaire du micro-organisme était suivie par une phase de stase ou de destruction bactérienne (jusqu'à 12 j après l'infection in vitro) dans des monocytes provenant des vaches non-infectées. Les résultats suggèrent que la multiplication et la destruction de *M. avium* ssp. *paratuberculosis* se produisent simultanément dans les monocytes bovins infectés in vitro. À l'aide de la méthode BACTEC, des monocytes bovins provenant des vaches témoins ont été comparés à des monocytes provenant des vaches infectées par *M. avium* ssp. *paratuberculosis*. Les monocytes provenant des vaches infectées semblaient démontrer une meilleure capacité à phagocyter et une destruction plus rapide de *M. avium* ssp. *paratuberculosis*. Toutefois, il n'y avait pas de différence significative entre les animaux témoins et les animaux infectés dans le nombre observé de *M. avium* ssp. *paratuberculosis* viables aux différents temps après infection des monocytes.

(Traduit par le docteur Serge Messier)

INTRODUCTION

Paratuberculosis, or Johne's disease, is a chronic granulomatous enteritis of cattle and other ruminants. It is caused by gastrointestinal infection with *Mycobacterium* subsp. *paratuberculosis*, a member of the *Mycobacterium avium* complex (1,2). Animals are thought to be infected at an early age, usually 1–6 mo after birth (3). Clinical signs, which typically develop after a 2- to 5-year incubation period, are intractable diarrhea and emaciation which ultimately lead to severe malnutrition, dehydration, and death (3). Paratuberculosis is economically important, causing shortened herd-life and decreased milk production (4,5).

The interaction of mycobacteria with mononuclear phagocytes is important to understanding of the pathogenesis of mycobacterial infections. Research has demonstrated that *M. tuberculosis* and *M. avium* can inhibit phagolysosomal fusion (6), escape from fused phagolysosomes into non-fused vesicles or the cytoplasm (7), block vacuole acidification, (8,9) and resist oxidative metabolites (10). Interaction of *M. avium* subsp. *paratuberculosis* with bovine monocytes is not well understood, at least in part because of the lack of a precise method for quantifying numbers of viable intracellular *M. avium* subsp. *paratuberculosis*. The biological characteristics of *M. avium* subsp.

Department of Pathobiological Sciences, University of Wisconsin, Madison, WI 53706-1102 USA.

Corresponding author: Dr. Michael T. Collins, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin USA 53706-1102; telephone: (608) 262-8457; fax: (608) 265-6463; e-mail: mcollin5@facstff.wisc.edu.

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paratuberculosis (eg, very slow growth and a tendency to clump) make it very difficult to quantify. These difficulties were overcome by using a radiometric technique for quantification of mycobacteria. This method, referred to as the BACTEC culture method, relies on a growth model that relates the amount of growth measured in a BACTEC 12B vial as cumulative growth index to the number of mycobacteria in the inoculum (11). Its advantages over traditional plate counts are that it is easier to perform, is faster and is not affected by the clumping of the mycobacterial cells (11,12). The BACTEC method has been used to study mycobacterial growth inside murine macrophages in vivo and in vitro and to quantify the rate of killing of *M. avium* subsp. *paratuberculosis* after exposure to heat (12).

In the present study, we employed the BACTEC counting method to study phagocytosis and intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine monocytes from both non-infected and *M. avium* subsp. *paratuberculosis*-infected cows showing evidence of a strong cellular immune response to the infection. The results obtained by the BACTEC method were compared with those determined by colony forming unit (CFU) counts and microscopic (visual) counting of acid-fast stained bacilli in monolayers. Our results indicate that after an initial period of *M. avium* subsp. *paratuberculosis* growth, normal bovine monocyte-derived macrophages became capable of killing or inhibiting the organism in vitro, and that monocytes from cows infected with *M. avium* subsp. *paratuberculosis* appeared to initiate the killing or inhibitory activity sooner than did monocytes from non-infected cows.

MATERIALS AND METHODS

MYCOBACTERIA

M. avium subsp. *paratuberculosis* strain BO45 was isolated from a cow with clinical Johne's disease that was treated at the Veterinary Medical Teaching Hospital at the University of Wisconsin-Madison (11). The bacteria were grown to a concentration of 10^8 colony forming units (CFU) per mL in 7H9 broth supplemented with 10% OADC (Difco Laboratories, Detroit, MI, USA), 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO, USA), and 2 μg/mL of mycobactin J (Allied Monitor Inc., Fayette, MO, USA) in tissue culture flasks. The bacteria were harvested, washed 3 times with phosphate-buffered saline (PBS) with 0.05% Tween 80, and dispersed to form a predominantly single-cell suspension with a motor-driven overhead stirrer and glass-Teflon homogenizer (Wheaton Instruments, Millville, NJ, USA). The bacteria were resuspended in 7H9 broth containing 0.05% Tween 80, 10% OADC and 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), and stored at −70°C.

ANIMALS

One- to three-year-old Holstein cows were used as blood donors. Non-infected cows were used in initial experiments. In subsequent experiments, cows experimentally infected with *M. avium* subsp. *paratuberculosis* were also used. At the time of testing, the infected cows were fecal culture-positive, and their peripheral blood leukocytes responded to stimulation with *M. avium* antigens (PPD) by release of interferon-gamma as determined using a commercial diagnostic kit (IDEXX Laboratories, Westbrook, ME, USA) (13).

CULTURE OF MONOCYTES

Blood was collected from the jugular vein by venipuncture, using sodium citrate (0.38% v/v, final concentration) as anticoagulant. Blood was centrifuged for 20 min at 400 × *g*, and the plasma removed. Blood cells were centrifuged at 600 × *g* for 20 min, the buffy coat was carefully removed, and the cells were resuspended in 30 mL Hanks Balanced Salt Solution (HBSS; Gibco). This was layered over 15 mL Ficoll-Hypaque (density 1.077, Accurate Chemical and Scientific Corporation, Westburg, NY, USA) and centrifuged at room temperature for 30 min at 600 × *g*. Mononuclear phagocytes were collected from the interface and washed 3 times in HBSS. These cells, which had a viability of 95% as determined by trypan blue exclusion, were adjusted to 3×10^6 cells/mL in HBSS with 0.5% FBS, and were distributed into the wells (1.0 mL per well) of a 24-well tissue culture plate (Costar, Cambridge, MA, USA). In some wells, 10-millimetre sterile glass cover slips were added for subsequent acid-fast staining of the infected monolayers. Plates were incubated at 39°C in 5% CO_2 for 2 h to allow the monocytes to adhere to the plate, then the wells were washed 3 times with warm HBSS to remove non-adherent cells. The adherent monocytes were cultured in RPMI-1640 with 4 mM glutamine (Gibco), 10% FBS, penicillin G at 100 U/mL and polymyxin B sulfate at 15 μg/ mL, at 39°C in 5% CO_2.

INFECTION OF MONOCYTES

To facilitate ingestion of bacilli, adherent monocytes were incubated with *M. avium* subsp. *paratuberculosis* at a 10:1 ratio of bacteria to monocytes in the presence of 10% fresh autologous serum for up to 120 min at 37°C in 5% CO_2. Subsequently, the monolayers were washed 3 times with warm HBSS to remove extracellular bacteria, and were cultured in complete RPMI 1640 for up to 15 d. The culture medium was replaced with fresh medium at 3, 6, 9, and 12 d post-infection (p.i.). Conditioned media were retained for bacterial quantification when the media was changed. The integrity of the monolayers was monitored by inverted light microscopy. Viability of monocytes was 85–90% at 12–15 d p.i. as determined by trypan blue exclusion. The number of adherent monocytes decreased approximately 20–30% during the 15-day incubation.

ASSESSMENT OF NUMBER OF INTRACELLULAR *M. AVIUM* SUBSP. *PARATUBERCULOSIS*

The number of intracellular *M. avium* subsp. *paratuberculosis* was determined by BACTEC, plate counting (CFU) and microscopic counting of acid-fast stained coverslips. For radiometric and CFU counting, duplicate monolayers were harvested immediately after infection, and at designated times, by lysing the monolayers with 0.25% sodium dodecyl sulphate (SDS). The conditioned media and monolayer lysates were stored at −70°C until all lysates were collected. The conditioned media and cell lysates for each time point were

then thawed, pooled, and centrifuged at 700 × *g* for 30 min.

For counting by the BACTEC method, cell pellets were resuspended and inoculated into BACTEC 12B vials supplemented with 2 μg/mL of mycobactin J and 1 mL of egg yolk. The growth index (a measurement of $^{14}CO_2$) was recorded every 24–48 h using the BACTEC 460 instrument until the cumulative growth indices reached 2000 units (usually in 20 d). The cumulative growth indices for each BACTEC vial was converted into an estimate of the number ($\log_{10}$) of viable *M. avium* subsp. *paratuberculosis* in the inoculum based on a growth model described previously (11):

$$\hat{Y}_{(x,t)} = \frac{Y_m}{1+B(C_0C_1^x)^t(D_0D_1^x)^{t^2}(E_0E_1^x)^{t^3}}$$

In effect, this technique uses a plot of cumulative growth index vs CFU of *M. avium* subsp. *paratuberculosis*. Solving for x permits a calculation of the number of *M. avium* subsp. *paratuberculosis* in an inoculum from a single growth measurement, as shown by the following equation:

$$x = \frac{\ln[Y_m/(Y-1)] - \ln(BC_0^tD_0^{t^2}E_0^{t^3})}{\ln(C_1^tD_1^{t^2}E_1^{t^3})}$$

In this equation, $Y_{(x,t)}$ is cumulative growth units as a function of inoculum size (x) and incubation time (t), with $t > 0$ and $x > 0$. Total growth is bounded by Y_m and below by $Y_m/(1+B)$, where $Y_m = 12\,950$. The letters B through E are regression coefficient constants determined to have the following values: $B = 10\,340$, $C_0 = 1.2217$, $C_1 = 0.84345$, $D_0 = 0.98959$, $D_1 = 1.004644$, $E_0 = 1.00008339$, and $E_1 = 0.99996559$. These coefficients were defined by regression analysis of plate count data on single-cell suspensions of *M. avium* subsp. *paratuberculosis*. Thus, in effect this technique uses a standard curve of cumulative growth index versus colony counts for single-cell suspensions of *M. avium* subsp. *paratuberculosis* inoculated into BACTEC vials.

For CFU determinations, cell pellets were resuspended with 2 mL PBS containing 0.05% Tween 80, and dispersed into predominantly single cell suspension with a motor-driven overhead stirrer and glass-Teflon homogenizer (Wheaton Instruments, Millville, NJ, USA). Ten-fold serial dilutions of the suspensions were plated (0.1 mL) on 7H9 agar plates supplemented with OADC and 2 μg/mL of mycobactin J. The plates were incubated for 30 d, and then the CFU were enumerated. We confirmed that the tissue culture medium used in this study neither supported nor inhibited the growth of *M. avium* subsp. *paratuberculosis* (14). We also verified that the concentration of SDS (0.25%) needed to lyse the monocytes did not adversely affect the viability of *M. avium* subsp. *paratuberculosis*. To do this, suspensions of the bacilli were treated with SDS for 40 min at 39°C, frozen at −70°C overnight, and then thawed the next day and inoculated into BACTEC vials. The results indicated that exposure to up to 1% SDS for 40 min did not affect the viability of *M. avium* subsp. *paratuberculosis*.

Microscopic counting of the intracellular *M. avium* subsp. *paratuberculosis* was done as previously described (14), with some modification. Briefly, infected monolayers adhered to glass cover slips were removed from the wells, allowed to air dry, acid-fast stained, and examined by light microscopy (1000× magnification). Duplicate cover slips were used for each time point, and 100 monocytes in 4–5 fields per cover slip were counted. Monocytes were scored for the percentage of monocytes with acid-fast bacilli (AFB), and for the estimated number of intracellular AFB. The mean number of AFB per infected monocyte was calculated by dividing the total number of intracellular AFB by the total number of infected monocytes.

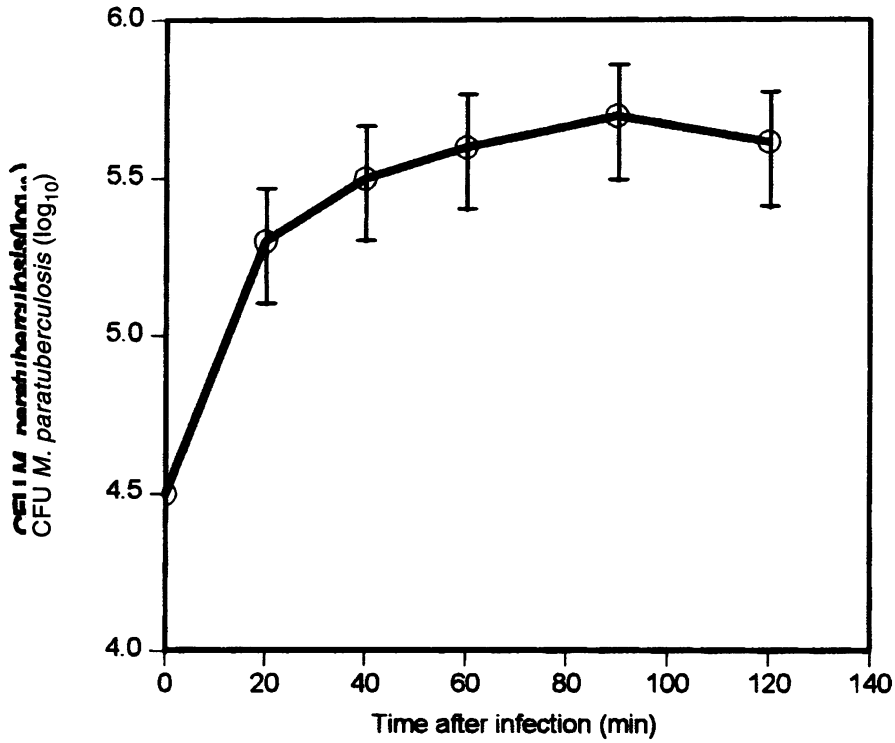

Figure 1. Time course of phagocytosis of *M. avium* subsp. *paratuberculosis* by bovine monocytes. Monocytes were infected and sampled as described in the materials and methods. Number of viable intracellular bacilli were determined by BACTEC. The results shown are the mean ± SD of 6 animals, each of which were sampled on 3 different days.

STATISTICAL ANALYSIS

All data were analyzed by a one-way analysis of variance (ANOVA), or repeated measures one-way ANOVA, using the InStat software package (GraphPad Software, San Diego, CA, USA). For all tests, $P < 0.05$ was considered to be significant.

RESULTS

UPTAKE OF *M. AVIUM* SUBSP. *PARATUBERCULOSIS*

The time course of phagocytosis was determined by incubating monocytes and *M. avium* subsp. *paratuberculosis* for up to 2 h in the presence of 10% autologous serum. Uptake

occurred rapidly, and peaked at 60 to 120 min post-infection (Fig. 1). To ensure maximal phagocytosis of bacilli, we used a 120-minute incubation period for phagocytosis in all subsequent experiments.

FATE OF INTRACELLULAR *M. AVIUM* SUBSP. *PARATUBERCULOSIS*

We next determined the ability of *M. avium* subsp. *paratuberculosis* to multiply in cultured bovine monocytes. In the initial set of experiments, only radiometric and microscopic quantification were used. The radiometric data indicated that the numbers of viable intracellular *M. avium* subsp. *paratuberculosis* increased significantly by 0.5 $\log_{10}$ ($P < 0.01$) from Day zero to 6 d p.i., and then started to decline through 12 d p.i. The numbers of viable intracellular *M. avium* subsp. *paratuberculosis* decreased significantly ($P < 0.05$) from 6 d to 12 d p.i., but the numbers remaining at 12 d p.i. were higher than the numbers of *M. avium* subsp. *paratuberculosis* initially phagocytosed (Fig. 2). Microscopic examination of cover slips, however, indicated that the numbers of AFB per infected monocyte steadily increased to 12 d p.i. (Fig. 2). The apparent disagreement between the results of BACTEC and microscopic counting could be due to a decreased metabolic rate of the intracellular bacilli, or the inability of microscopic counting to distinguish between live and dead bacilli. Therefore, in another set of experiments, we compared the intracellular numbers of *M. avium* subsp. *paratuberculosis* determined by BACTEC, CFU, and microscopic methods.

BACTEC results indicated that the numbers of viable intracellular *M. avium* subsp. *paratuberculosis* increased by 0.8 $\log_{10}$ ($P < 0.001$) from Day zero to 6 d p.i., and then decreased significantly ($P < 0.05$) by 0.46 $\log_{10}$ from 6 d to 12 d p.i. However, BACTEC data suggested that the numbers of viable intracellular *M. avium* subsp. *paratuberculosis* at 12 d p.i. remained higher than the initial numbers of *M. avium* subsp. *paratuberculosis* phagocytosed (Table I). Plate count data exhibited a similar pattern: the CFU of viable *M. avium* subsp. *paratuberculosis* increased approximately 2-fold ($P < 0.01$) from Day zero to 6 d p.i., and then declined significantly ($P < 0.01$) from 6 d to 12 d p.i. (Table I). These experiments demonstrated that there was general agreement between BACTEC and CFU counting methods for evaluation of

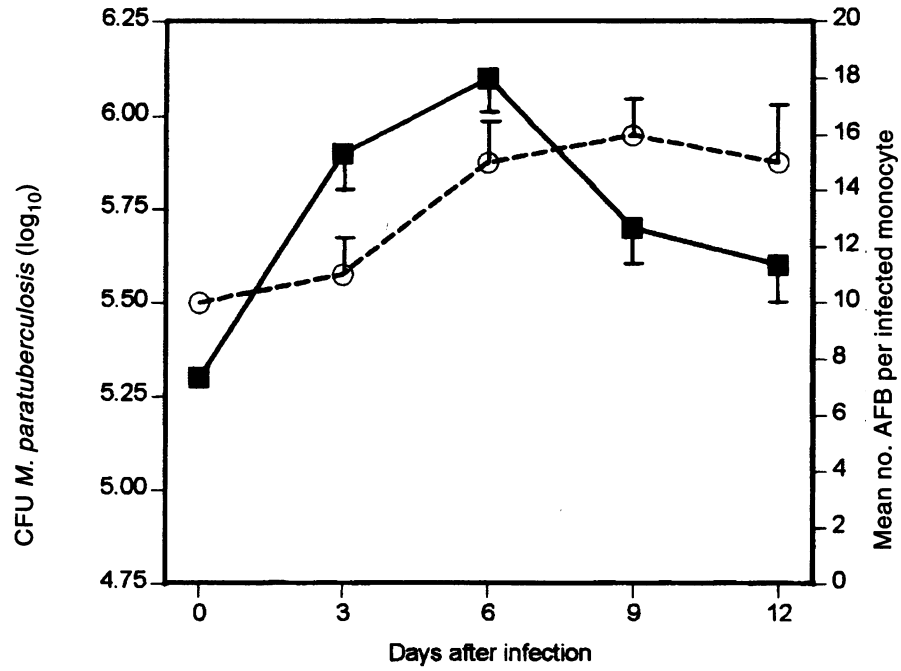

Figure 2. Comparison of BACTEC and microscopic counting of intracellular *M. avium* subsp. *paratuberculosis*. Monocytes were infected and sampled as described in the materials and methods. The $\log_{10}$ numbers of intracellular bacilli were determined by the BACTEC method (■). Mean numbers of AFB per infected monocyte were determined by microscopic counting of monocytes adherent to cover slips (○). Results shown are the mean ± SD of 3 animals sampled at each time point.

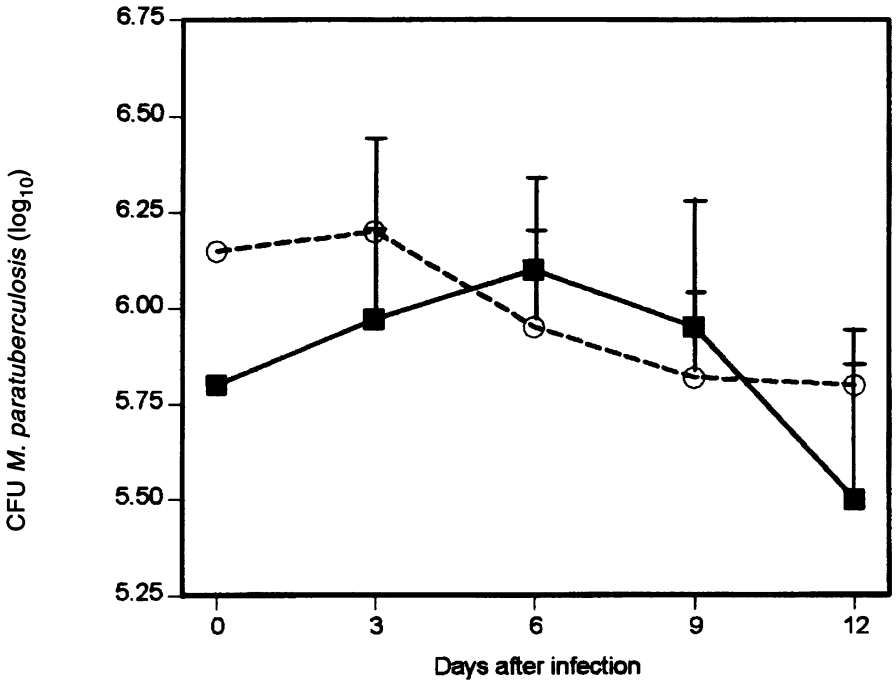

Figure 3. Comparison of the ability of monocytes from non-infected and infected, interferon-responsive cows to ingest and inhibit the intracellular growth of *M. avium* subsp. *paratuberculosis*. Monocytes were infected with *M. avium* subsp. *paratuberculosis* in vitro and sampled as described in the materials and methods. Results shown are the mean ± SD of 5 non-infected cows sampled on 2 separate days (■), and the mean ± SD of 2 infected cows sampled on 2 separate days (○).

TABLE I. Comparison of BACTEC, CFU and microscopic enumeration of intracellular *M. avium* subsp. *paratuberculosis* (mean numbers ± SD)

Days post-infection	BACTEC (log_{10})	CFU 10^4	AFB/Infected monocytes
0	5.3 ± 0.1	8.4 ± 2.1	10 ± 1
3	[a]5.9 ± 0.1**	10.4 ± 1.6	11 ± 2
6	[a]6.1 ± 0.2***	[a]15.3 ± 1.5**	15 ± 1
9	[b]5.7 ± 0.1*	[b]12.2 ± 2.4*	[a]16 ± 1*
12	[b]5.6 ± 0.2*	[b]5.5 ± 0.7**	15 ± 2

The results shown are the mean ± SD of 3 animals sampled on the same day
a*, $P < 0.05$, ** and ***, $P < 0.001$ compared with Day zero
b*, $P < 0.05$, ** and ***, $P < 0.001$ compared with Day 6 after infection

the intracellular fate of *M. avium* subsp. *paratuberculosis*. Microscopic examination of cover slips, however, indicated that the numbers of AFB per infected monocyte steadily increased up to 9 d after infection and then plateaued (Table I).

COMPARISON OF MONOCYTES FROM NON-INFECTED AND INFECTED COWS

Having established that the BACTEC method could be used to assess numbers of viable intracellular *M. avium* subsp. *paratuberculosis*, this method was used to compare the phagocytosis and intracellular fate of *M. avium* subsp. *paratuberculosis* in monocytes from non-infected and infected cows. Monocytes from infected cows phagocytosed slightly more bacilli (6.0 log_{10}) than did monocytes from non-infected cows (5.78 log_{10}) (Fig. 3). The numbers of *M. avium* subsp. *paratuberculosis* increased by 0.3 log_{10} ($P < 0.05$) in monocytes from non-infected cows by 6 d p.i. and then started to decrease through 12 d p.i. (Fig. 3). In contrast to these results, the numbers of *M. avium* subsp. *paratuberculosis* in monocytes from infected cows increased only 0.1 log_{10} within the first 3 d p.i., and then decreased through 12 d p.i. Although the decline in numbers of viable intracellular *M. avium* subsp. *paratuberculosis* in monocytes from infected cows began 3 days earlier than in monocytes from normal cows, the overall pattern of bacillary multiplication in monocytes from non-infected and infected cattle was similar (Fig. 3).

DISCUSSION

In this study, we demonstrated that there was general agreement between BACTEC and CFU counts of viable intracellular numbers of *M. avium* subsp. *paratuberculosis* (Table I). The advantages of the BACTEC method are that it is faster (20 d), easier (no dilution required), minimized the risk of contamination, and is not confounded by bacillary clumps (11). Data from Table I shows that the BACTEC counting technique was more sensitive to small changes in viable numbers of *M. avium* subsp. *paratuberculosis*, a feature essential when studying a bacterium with a generation time of roughly 2 d (11).

When we used the BACTEC method, we observed an early phase of intracellular growth of viable *M. avium* subsp. *paratuberculosis* in non-infected bovine monocytes, i.e., the first 6 d p.i. in vitro, followed by a later phase from 6 to 12 d p.i. of mycobacteriostasis or killing of intracellular bacilli. However, the estimated numbers of viable intracellular bacilli 12 d after monocyte infection remained higher than the initial numbers of *M. avium* subsp. *paratuberculosis* phagocytosed by monocytes (Fig. 2). This early phase of intracellular growth of *M. avium* subsp. *paratuberculosis* is consistent with previous reports. Zurbrick and Czuprynski (14) demonstrated by microscopic and plate count methods that *M. avium* subsp. *paratuberculosis* multiplied 200 to 250% in bovine monocytes over a 7-day incubation period. By electron microscopic examination, Bendixen et al (15) reported that heat-killed and live *M. avium* subsp. *paratuberculosis* appeared to remain intact in bovine monocyte-derived macrophages for up to 5 wk in vitro, although the viability of bacilli observed was not determined. The later phase of mycobacteriostasis or killing of intracellular bacilli observed in the present study has not been reported previously.

The biphasic survival curve for *M. avium* subsp. *paratuberculosis* we observed is the reverse of a reported biphasic growth curve of *M. avium* in human monocytes (16). The latter was characterized by bacteriostasis or killing during the first 4 d p.i., followed by a rapid intracellular replication (16). Differences between the 2 studies could reflect the different mycobacterial species used, or differences between bovine and human monocytes. The early phase of bacillary growth seen in our study, suggests that *M. avium* subsp. *paratuberculosis* may initially resist the antimycobacterial mechanisms of mononuclear phagocytes. Alternatively, perhaps, the antimycobacterial mechanisms of bovine monocytes are either not activated, or are suppressed, by intracellular *M. avium* subsp. *paratuberculosis*. There are several possible explanations for the later period of mycobacteriostasis or killing of bacilli. Monocytes may increase their antimycobacterial ability as they mature into macrophage-like cells. Alternatively, exposure of macrophages to autocrine or paracrine release of endogenous cytokines (eg, TNF-α or IL-1β) may lead to activation of microbicidal mechanisms (eg, ROI or RNI), or nutrient deprivation of *M. avium* subsp. *paratuberculosis* (eg, iron restriction). The seemingly disparate results obtained by radiometric and microscopic methods (Fig. 2) suggest that there may be multiplication of some intracellular *M. avium* subsp. *paratuberculosis* concomitant with the death or inhibition of other bacilli.

There was a trend for accelerated intracellular killing of *M. avium* subsp. *paratuberculosis* by monocytes from infected cows. However, the large variation among animals and replicate tests made the differences not statistically significant. Comparison of monocytes from non-infected and infected cows in ingestion and inhibition of intracellular growth of *M. avium* subsp. *paratuberculosis* (Fig. 3) has not been reported previously. If there is, in fact, no difference, it may be due to a lack of activation status of peripheral blood monocytes. This is consistent with a previous report by Adams et al (17), who observed no difference in TNF-α and IL-6 mRNA levels in whole blood from non-infected and *M. avium* subsp. *paratuberculosis*-infected

cows (17). Another possibility is that there is compartmentalization of macrophage activation in infected cows, in which case the activation status of peripheral blood monocytes would not reflect the activation status of intestinal macrophages.

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REFERENCES

1. **SAXEGAARD F, BAESS I.** Relationship between *Mycobacterium avium, Mycobacterium paratuberculosis* and "wood pigeon mycobacteria." Determinations by DNA-DNA hybridization. APMIS 1988; 96: 37–42.
2. **THOREL M-F, KRICHEVSKY M, LEVY-FREBAULT VV.** Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int J Syst Bacteriol 1990; 40: 254–260.
3. **CHIODINI RJ, VAN KRUININGEN HJ, MERKAL RS.** Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet 1984; 74: 217–262.
4. **COLLINS MT, SOCKETT DC, GOODGER WJ, CONRAD TA, THOMAS CB, CARR DJ.** Herd prevalence, geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. J Am Vet Med Assoc 1994; 204: 636–641.
5. **COLLINS MT, MORGAN IR.** Simulation model of paratuberculosis control in a dairy herd. Prev Vet Med 1992; 14: 21–32.
6. **CLEMENT F, HOROWITZ MA.** Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med 1995; 181: 257–270.
7. **MCDONOUGH KA, KRESS Y, BLOOM BR.** The interaction of *Mycobacterium tuberculosis* with macrophages: a study of phagolysosome fusion. Infect Agents Dis 1993; 2: 232–235.
8. **STURGILL-KOSZYCKI S, SCHLESINGER PH, CHARKRABORTY P, HADDIX PL, COLLINS A, FOK K, ALLEN RD, GLUCK SL, HEUSER J, RUSSELL DG.** Lack of acidification on mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase [published erratum appears in Science 1994; 263: 1359]. Science 1994; 263: 678–681.
9. **XU S, COOPER AM, STURGILL-KOSZYCKI S, VAN HEYNINGEN T, CHATTERJEE D, ORME IM, ALLEN P, RUSSELL DG.** Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium* infected macrophages. J Immunol 1994; 153: 2568–2578.
10. **CHAN J, FUJIWARA T, BRENNAN PJ, MCNEIL M, TURCO SJ, SIBILLE JC, SNAPPER M, AISEN P, BLOOM BR.** Microbial glycolipid: Possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci USA 1989; 86: 2453.
11. **LAMBRECHT RS, CARRIERE J, COLLINS MT.** A model for analyzing growth kinetics of a slowly growing *Mycobacterium* sp. Appl Env Microbiol 1988; 54: 910–916.
12. **REDDY VM, SRINIVASAN S, ANDERSEN B, GANGADHARAM PRJ.** Rapid assessment of mycobacterial growth inside macrophages and mice, using the radiometric (BACTEC) method. Tubercle 1994; 75: 127–131.
13. **ZHAO BY, COLLINS MT.** The temporal pattern of serum antibody production and interferon-gamma response by peripheral blood leukocytes to mycobacterial antigen stimulation in experimentally *M. paratuberculosis* infected cows suggests a Th1 to Th2-like shift in host immune response. (Abstract) Abstr Conf Res Workers Animal Dis 1995; #106.
14. **ZURBRICK BC, CZUPRYNSKI CJ.** Ingestion and intracellular growth of *Mycobacterium paratuberculosis* within bovine monocytes and monocyte-derived macrophages. Infect Immun 1987; 55: 1588–1593.
15. **BENDIXEN PH, BLOCH B, JORGENSEN JB.** Lack of intracellular degradation of *Mycobacterium paratuberculosis* by bovine macrophages infected in vitro and in vivo: light microscopic and electron microscopic observations. Am J Vet Res 1981; 42: 109–113.
16. **JOHNSON JL, ELLNER JJ, SHIRATSUCHI H.** Monocyte-*Mycobacterium avium* complex interactions: studies of potential virulence factors for humans. Immunol Serol 1994; 60: 263–279.
17. **ADAMS JL, COLLINS MT, CZUPRYNSKI CJ.** Polymerase chain reaction analysis of TNF-α and IL-6 mRNA levels in whole blood from cattle naturally or experimentally infected with *Mycobacterium paratuberculosis*. Can J Vet Res 1996; 60: 257–262.