

2000

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Li, Qingsheng; Mansfield, Keith G.; Lackner, Andrew; and Haase, Ashley T., "Quantitative Image Analysis of Simian Immunodeficiency Virus Replication in Macrophages Coinfected with *Mycobacterium avium* Complex" (2000). *Qingsheng Li Publications*. 15.  
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## Quantitative Image Analysis of Simian Immunodeficiency Virus Replication in Macrophages Coinfected with *Mycobacterium avium* Complex

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### Abstract

*Mycobacterium avium* is the most frequent cause of disseminated bacterial infection in patients with human immunodeficiency virus type 1 infection and in rhesus macaques with simian immunodeficiency virus (SIV) infection. This animal model of AIDS was used to test the hypothesis that this frequent association is the result of reciprocal enhancement of replication of both microorganisms. The replication of *M. avium* and SIV was analyzed in lymphatic tissues obtained from rhesus macaques experimentally inoculated with SIVmac who developed or remained free of overt *M. avium* infection. In situ hybridization, quantitative image analysis, and staining of *M. avium* and of macrophages were used to assess the effects of coinfection on the replication of SIV and *M. avium* in vivo. There was no correlation between virus load and *M. avium* load in coinfecting lymph nodes, and, with one exception, there was no evidence that *M. avium* coinfection of macrophages increased SIV replication.

*Mycobacterium avium* is the third most common cause of opportunistic infection in patients with human immunodeficiency virus type 1 (HIV-1) infection and the most frequent cause of disseminated bacterial infection in rhesus macaques inoculated with simian immunodeficiency virus (SIV) [1–3]. Because the tissue distribution and histopathology of *M. avium* infections in macaques closely mimic those seen in the human population, we undertook a systematic examination of the relationship in vivo between viral and bacterial replication to try to resolve conflicting evidence that mycobacteria and lentiviruses affect each other's growth. In experiments in which virus and bacteria have been cultured [4] and in 1 report of *M. avium*/HIV-1 coinfection of macrophages in lymphatic tissues [5], coinfection has been reported to enhance the growth of both *M. avium* and HIV; to enhance only the growth of *M. avium* [6, 7]; to have no effect on either HIV or *M. avium* [8]; and to have no effect on the replication of SIV [9]. We hoped that by extensively surveying lymphatic tissues in this animal model, we could establish consistent and reproducible correlations in vivo between coinfection and enhanced growth.

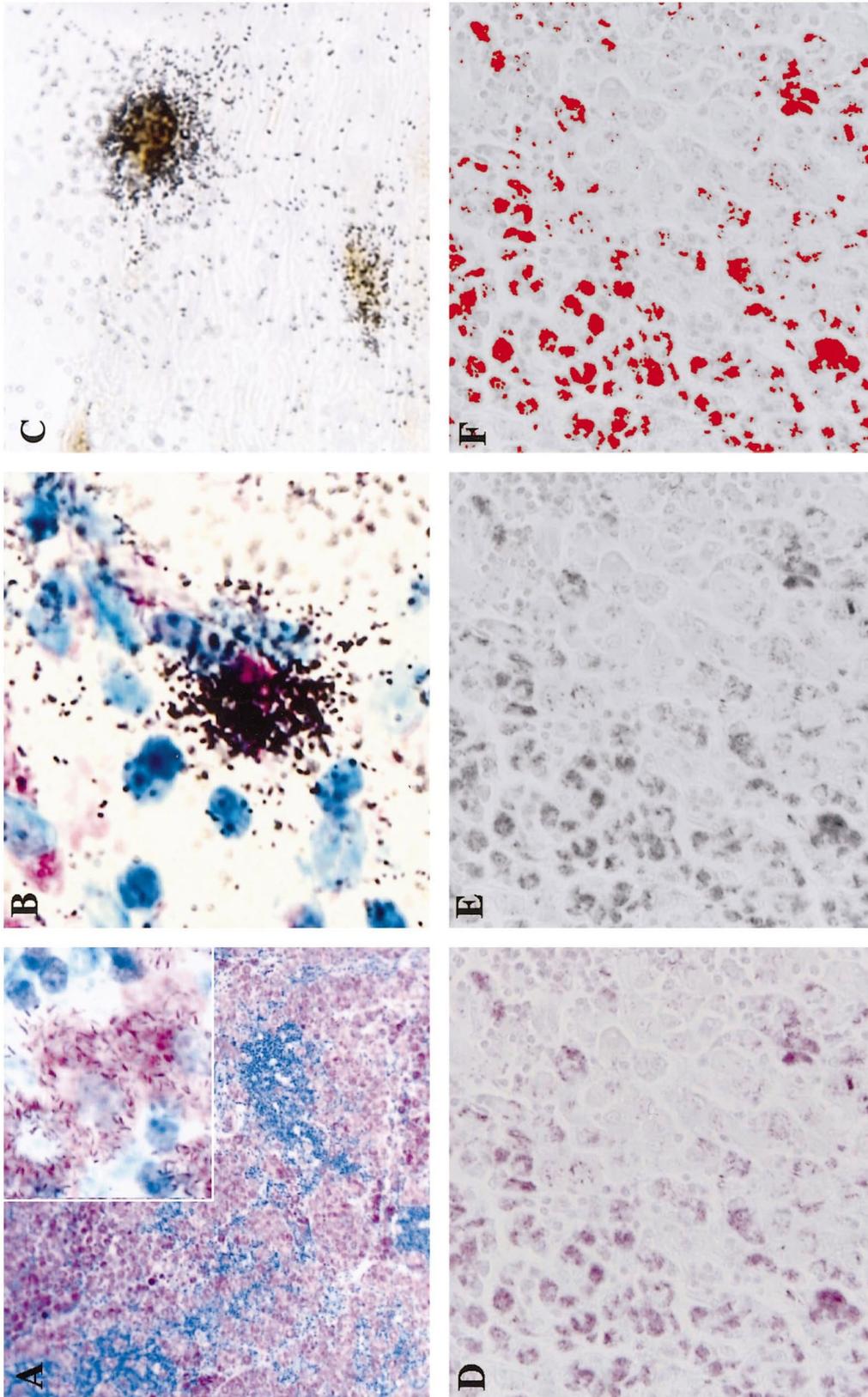
### Materials and Methods

**Animals, virus, and tissue specimens.** In this retrospective study, we examined mesenteric lymph nodes (LNs) of 11 macaques experimentally inoculated with SIV who received neither antiretroviral treatment nor antibacterial prophylactic therapy. All animals were infected and housed individually at the New England Regional Primate Research Center centralized biolevel III containment facility. Animals were inoculated

intravenously with 1 of the following 3 strains of SIV: SIVmac251, SIVmac239, or SIV-mac MER/YE. Although the in vitro growth characteristics of these strains differ, each of the strains causes essentially equivalent disease in vivo [3, 10, 11], with higher (SIVmac251) or lower (SIVmac239 and MER/YE) incidence with associated mycobacterial disease [3]. The mesenteric LNs were removed from the animals after spontaneous death or euthanasia and were snap-frozen and fixed in formalin.

**Isolation of *M. avium*.** Culture and isolation of mycobacteria from snap-frozen LN tissues were performed at the Massachusetts Department of Public Health Mycobacteriology Laboratory, as reported elsewhere [3]. Identification was confirmed by hybridization of an *M. avium*-specific DNA probe to ribosomal RNA.

**Quantitation of *M. avium* load in LNs.** *M. avium* in formalin-fixed and paraffin-embedded LN tissue sections was detected by use of a modified cold Kinyoun procedure [12]. Tissue sections 8 mm in diameter were attached to silanized glass slides, deparaffinized, placed in carbofuchsin for 5 min, decolorized with acid alcohol, and counterstained with methylene blue for 2 min. Acid fast, deeply red-stained mycobacteria were identified in sections, as illustrated in figure 1A. For quantitation of *M. avium* load by quantitative image analysis (QIA), the counterstain with methylene blue was omitted. MetaMorph software (Universal Imaging Corp., Westchester, PA) was used, as reported elsewhere [13], to set a threshold value for the staining intensity that discriminates the stained *M. avium* from the background (figure 1D–F). The computer program indicates the threshold and objects that will be measured by highlighting them in red. The area (in



**Figure 1.** Mesenteric lymph nodes (LNs) of rhesus macaques infected with simian immunodeficiency virus (SIV) RNA. A, LN tissue from macaque no. 10. SIV RNA was detected with in situ hybridization (ISH; exposure time, 10 days), and *M. avium* was visualized (red-stained microorganisms in macrophages [15] in the inset) with Kinyoun cold staining. Bright-field, original magnification  $\times 100$  and high magnification  $\times 1200$  from the same section in the inset. B, Mesenteric LN of macaque no. 3. SIV RNA was detected with ISH (exposure time, 24 h), and *M. avium* was visualized with Kinyoun cold staining. In the developed radioautograph, the black silver grains overlying an SIV RNA-positive cell colocalize with the red-stained *M. avium* in the cell. Bright-field, original magnification  $\times 1200$ . C, Double-labeled ISH for SIV RNA and immunohistochemical staining for CD68 in the mesenteric LN of macaque no. 3. In the developed radioautograph, large numbers of black silver grains in viral RNA<sup>+</sup> cells colocalize to brown-stained macrophages. Bright-field, original magnification  $\times 240$ . D–F, Quantitative image analysis of *M. avium* in lymphatic tissues. *M. avium*-infected mesenteric LN of macaque no. 7 was visualized with Kinyoun cold staining without methylene blue counterstain, original magnification  $\times 400$ . D, Color image; E, black-and-white image; F, area of *M. avium* in the LN that will be measured highlighted by the red overlay by use of the MetaMorph threshold tool (Universal Imaging Corp., Westchester, Pennsylvania).

square pixels) of the highlighted objects is then measured. *M. avium* levels were expressed as a percentage of the area of the section, the latter being determined with the MetaMorph calibration tool (Universal Imaging).

**Quantitation of SIV RNA load in LNs.** The total number of copies of SIV RNA in infected cells per gram of LN tissue was determined by in situ hybridization (ISH) and QIA as described elsewhere [13, 14]. Briefly, cells in the section containing SIV RNA were identified after ISH by collections of silver grains over the cells significantly greater than those over the background. Infected cells in the section were counted, and the number of silver grains above background over the cells was determined by QIA and converted to copy number by a previously validated back calculation [13]. The weight of the sections was determined from the area multiplied by a predetermined density [13]. The average number of copies of SIV RNA per cell multiplied by the number of viral RNA positive cells in the sections per gram was taken as a measure of virus load. For ISH, 8- $\mu$ m tissue sections attached to silanized glass slides were deparaffinized and then sequentially immersed in 0.2 N HCl for 30 min, 0.15 M triethanolamine (pH 7.4) for 15 min, 0.005% digitonin for 5 min, and 5  $\mu$ g/mL proteinase K for 15 min. The sections were subsequently acetylated, and after dehydration, the sections were hybridized to <sup>35</sup>S-labeled SIV RNA-specific probes, with 10<sup>5</sup> cpm of antisense (or sense, as a control) per milliliter of hybridization mixture. SIV RNA antisense and sense probes were transcribed from the T7 or SP6 promoter of linearized clones of the 47-1130 sequence of SIVmac251 and 6868-8554 sequence of mac239 inserts in a pGEM-4z vector. After hybridization at 45°C for 24 h, the sections were washed, digested with ribonucleases A and T1, dehydrated in graded ethanols containing 0.3 M ammonium acetate, dipped in Kodak NBT-2 emulsion, and exposed at 4°C for variable times. From the specific activity and complexity of the probes, the equivalent of 1 full-length copy of SIV virion RNA would generate a signal of 0.6 silver grains per 24-h exposure time. For the longest exposure times in these studies (3 weeks), infected cells with as few as 2 copies of SIV RNA per cell would be detectable.

**Triple-label ISH and Kinyoun and immunohistochemical staining to quantitate SIV RNA in coinfecting macrophages.** *M. avium* is known to infect macrophages [15]. To look for enhancement of SIV replication in macrophages coinfecting with *M. avium*, we combined ISH and the modified Kinyoun staining with immunohistochemical (IHC) staining of macrophages [16]. This allowed us to distinguish *M. avium*-positive macrophages with SIV RNA from *M. avium*-negative macrophages with SIV RNA. In portions of the LNs with prominent coinfection of macrophages, virtually all of the SIV RNA-positive cells were macrophages infected with *M. avium* identified simply by Kinyoun counterstaining of the developed radioautographs (figure 1B). In areas less heavily infected with *M. avium*, many more T cells than macrophages had SIV RNA. (In figure 1C there are 2 SIV RNA-positive cells that can be unequivocally identified as macrophages by IHC staining.) To compare the effects of coinfection in macrophages, we combined ISH with Kinyoun staining and IHC staining: 8- $\mu$ m sections were deparaffinized, pretreated with 1.2% hydrogen peroxide in methanol for 20 min, and microwaved in diethyl pyrocarbonate-treated 10 mM sodium citrate buffer (pH 6) at

800 W for 10 min. Sections were then cooled for 20 min, acetylated, and hybridized with antisense <sup>35</sup>S-labeled SIV RNA-specific probes at 45°C overnight. After posthybridization the sections were washed, blocked with 5% nonfat milk in PBS, and incubated overnight with anti-CD68 monoclonal antibody (KP1, 1:200 dilution; Dako, Carpinteria, CA) at 47°C. Antibody binding was visualized by use of the avidin-biotin-peroxidase method (ABC kit; Vector Labs, Burlingame, CA), with 3,3-diaminobenzidine used as chromogen. After washing in PBS containing 1 mM EDTA and dehydration in ethanols containing 0.3 M ammonium acetate, sections were dipped in emulsion and exposed at 47°C for a predetermined optimal exposure time. The slides were subsequently developed, Kinyoun stained, and mounted.

## Results

***M. avium* load and virus load are not correlated.** We screened 18 SIV-infected macaques with AIDS to identify 11 coinfecting animals, from whom we isolated *M. avium*. We could not directly evaluate enhancement of *M. avium* replication in vivo in macrophages coinfecting with SIV, because the signal from ISH in infected macrophages obscured the Kinyoun stain, but we could indirectly assess the effects of coinfection by determining whether higher virus loads were correlated with higher mycobacterial loads. We found, to the contrary, that the highest *M. avium* loads were present in animals 8 and 11, who had low virus loads (table 1). Moreover, *M. avium* loads were similar in animals 3 and 7, who differed in virus loads by 3 orders of magnitude. For the group of animals as a whole, there was no correlation between virus and *M. avium* load (Spearman rank order correlation,  $P = .60$ ).

***SIV replication in M. avium-coinfecting macrophages.*** To directly measure the effect of coinfection on SIV replication, we examined large numbers of triply labeled LN sections to find macrophages with SIV RNA that were or were not also infected with *M. avium*. Most of the SIV RNA-positive cells in regions of the LNs in which there was relatively little *M. avium* turned out to be CD3<sup>+</sup> T lymphocytes (data not

**Table 1.** *M. avium* and simian immunodeficiency virus (SIV) RNA levels in lymph nodes of macaques with AIDS.

Macaque no.	<i>M. avium</i> load (% thresholded area)	No. of copies of SIV RNA/g of tissue
1	0.7	1.6 × 10 <sup>7</sup>
2	21.2	4.5 × 10 <sup>6</sup>
3	10.9	4.6 × 10 <sup>9</sup>
4	7.6	6.1 × 10 <sup>6</sup>
5	0.6	6.5 × 10 <sup>7</sup>
6	6.3	2.1 × 10 <sup>6</sup>
7	10.5	3.5 × 10 <sup>6</sup>
8	27.8	NQ
9	1.5	NQ
10	13.4	NQ
11	46.6	NQ

NQ = not quantitated: SIV RNA levels could not be determined because there were few SIV RNA-positive cells in multiple sections even with 3-week exposures. In the other animals (1–7), SIV RNA levels were determined by multiplying the average number of copies in SIV RNA-positive cells by the frequency of SIV RNA-positive cells.

shown), but we were able to collect data on 61 unequivocally identified macrophages with SIV RNA that were not coinfecting, to compare with 61 coinfecting macrophages (table 2). The average SIV RNA copy number and range were not significantly different in the coinfecting macrophages. Data for animal number 3 are presented separately in table 2, because in screening sections from this animal it was obvious that the copy number was significantly higher. We found a >4-fold increase in SIV RNA in coinfecting cells in this animal.

## Discussion

The relationship between *M. avium* and HIV-1 has been most extensively examined in vitro, in cultured monocytes/macrophages or macrophage cell lines in which, under some conditions, growth of one or the other microorganism has been found to be increased or unaffected. Relating these results in vitro to the situation in vivo is difficult because of uncertainties whether the experimental conditions in vitro reliably reproduce the intracellular milieu in tissues or whether the cell lines precisely mimic macrophages in tissues. Cytokines such as tumor necrosis factor- $\alpha$ , interleukins, and interferons can have suppressive or enhancing effects, and the discrepancies in studies described by Ghassemi et al. [4], Meylan et al. [8], and others may be a consequence of the timing and extent of coinfection of the cultures that affected cytokine levels. The development of quantitative single-cell techniques [13] and access to tissues in the SIV model in rhesus macaques with AIDS coinfecting with *M. avium* [2, 3] provided an opportunity to directly assess the effects of coinfection in vivo. We found, in agreement with Meylan et al. [8], that SIV replication was usually not increased by coinfection with *M. avium*. We also did not find any correlation between viral replication and *M. avium* levels.

Although *M. avium* did not generally enhance SIV replication in vivo, it is clear that under some conditions coinfection is associated with increased replication of SIV (animal 3) and HIV-1 [5]. In the report by Orenstein et al. [5], where data are presented to directly evaluate the effects of coinfection, in 1 LN from a patient with mycobacterial infection the levels of HIV-1 replication were strikingly higher than the levels we have documented in productively infected mononuclear cells in lymphatic tissues in the presymptomatic stages of infection [13]. It is possible that highly productive infection of macrophages occurs under conditions associated with the most advanced stage of HIV infection. Disseminated *M. avium* infection generally occurs in HIV-1 infection at CD4<sup>+</sup> T-cell counts of  $\leq 100$  cells/mm<sup>2</sup>, with the highest incidence at counts of  $< 10$  cells/mm<sup>2</sup>, as was the case for the 1 patient described by Orenstein et al. [5]. *M. avium* infection in SIV-infected macaques occurs at higher CD4<sup>+</sup> T-cell counts, quite possibly reflecting a stage of infection, preceding the preterminal stage, in which the enhancing effects of opportunistic infection would have been evident [3]. Beyond these speculations, the real incidence and underlying mechanisms of reported enhancing effects of opportunistic infection, and explanations for the high level of SIV RNA in the coinfecting macrophages in 1 animal in this study, have yet to be determined.

**Table 2.** Simian immunodeficiency virus (SIV) RNA levels in macrophages coinfecting with *M. avium*.

Macaque no.	No. of copies of SIV RNA in macrophages per cell, mean $\pm$ SD	
	<i>M. avium</i> positive	<i>M. avium</i> negative
1, 2, 4-11	62 $\pm$ 23	65 $\pm$ 30
3	962 $\pm$ 160	221 $\pm$ 160

Triply labeled sections of lymph node (LN) were screened to identify the relatively infrequent macrophages (compared with T cells) free of *M. avium* of coinfecting macrophages. There were no obvious differences in 10/11 animals, and this was borne out by quantitative image analysis, by which the average and range of copy numbers in 61 macrophages coinfecting with *M. avium* were not significantly different ( $P = .52$ , Student's *t* test) from those in 61 macrophages infected with SIV but not *M. avium*. The quantitative comparisons are presented separately for animal 3, because SIV RNA levels were obviously higher in the coinfecting cells from animal 3. The copy number in coinfecting cells is significantly higher ( $P < .001$ , Student's *t* test).

**Acknowledgments** – We thank Louise Shand and Tim Leonard for their assistance in preparation of the manuscript. Financial support was provided by the National Institutes of Health grants RR00168 and AI 38565.

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