Author's response to reviews

Title: Identification of a predominant isolate of Mycobacterium tuberculosis using molecular and clinical epidemiology tools and in vitro cytokine responses.

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Version: 3 Date: 29 Jan 2003

PDF covering letter
Reference no: TUBE/2001/000020

Re: In vitro cytokine responses to Mycobacterium tuberculosis Type 1 strain identified from Manitoba, Canada

Dr. Ellner:

Thank you for your comments. I have revised the manuscript according to reviewer’s comments. The corrections are listed below.

Answers to reviewer 1

1. The 0 hr referred to in the manuscript has been changed to 0.25 hr as suggested by the reviewer 1. This has been changed throughout the manuscript including figures.

2. The Type 1 strain of Mycobacterium tuberculosis from Manitoba, Canada is a different strain than M. tuberculosis Strain “O” from Tennesse-Kentucky, USA. The sentence has been rephrased to avoid this confusion (lines 155-161).

   The strain designation as “Type 1” is solely based on the Restriction Fragment Length Polymorphism patterns as described in lines 61-70. A sentence indicating this has been added in lines 70-71: “Numerical designations were arbitrarily assigned to strains according to their prevalence in the population
under study."

3. Authors agree that basal endotoxin contamination may alter the interpretation of our findings. To eliminate the interference of endotoxins in our experiments, we had used identical media lots for our experiments. Cytokine levels in *M. tuberculosis* infected cultures were compared against the levels in control cultures.

4. It was clearly evident that *M. tuberculosis* Type 1 strain was predominant in the tuberculosis positive patient population in Manitoba vs other strains, which is why it was the target of our study. Our objective was to look at the full profile of cytokines in cultures infected with this strain because this would provide additional information regarding its virulence.

Definition for ‘The First Nation People’ and ‘Reserves’ has been added (see page 15; line 256).

**Aboriginal/First Nations:** This term is usually used to describe the indigenous inhabitants of Canada and their descendants. It embraces those people registered as status Indians living on and off reserves as well as Metis and Inuit.

**Reserves:** A reserve is a tract of land, legal title to which is held by the federal Crown, which has been set apart by the Crown for the use and benefit of a specific First Nation.

5. IL-10 correction as suggested by reviewer in lines 244-247 has been made: “Following infection, IL-10 levels in Type 1 infected cells were briefly high and subsequently were same for H37Ra and H37Rv infected cells (Fig 4).” (Lines 231-232). The data presented in this paper is not contradictory with data in the literature.

The study conducted by Elliott A M *et al.*, 1999 (Ref 15) estimated the cytokine levels in Tuberculosis positive patients (22 HIV positive and 75 HIV negative). While Zhang M *et al.*, 1999 (Ref 39) studied IL-10 *in vitro* but the levels of IL-10 were estimated for Day 1, 7 and 14. Our findings at 0.25, 2, 4, 6 and 8 hrs have never been presented before in the literature.

6. Authors agree with the reviewers in regards to IL-10 being preformed. To eliminate this error, we compared cytokine (IL-10 and others) levels in *M. tuberculosis* (H37Ra, H37Rv and Type 1) infected cells vs control cultures.

7. The results of the experiment were listed as an average of three different experiments each conducted in duplicates. This is indicated in materials and methods (lines 99-100).

**Answers to reviewer 2**

- Assessment of mycobacterial growth:
  Smears of macrophage cells were stained using Kinyoun staining for acid-fast bacilli to observe
mycobacterial growth inside them. Our previous experiments did not show any difference between growth rates of H37Rv and the Type 1 strains in vitro (data not shown). The growth rates were also constant for these strains of M. tuberculosis on culture media (Middlebrook 7H10 plates, BACTEC liquid media and Lowenstein-Jensen egg based media).

• The epidemiological and clinical characterization of M. tuberculosis strains from Manitoba:

There is a clear predominance of this strain compared to other strains. The data presented for this paper focuses on the Type 1 strain only. Data from other strains would make this paper unwieldy as there are currently 194 M. tuberculosis strains from Manitoba, some of which are under investigation.

• IFN-γ data:

Macrophages represent the major habitat of Mycobacterium tuberculosis and they are first line of host defence in the adaptive immune response. This experimental model used macrophage cells in order to understand the initial stages of infection in vitro. We used THP-1 cells as well as other cell lines such as monocytic U937 cells to study the cytokines of interest in vitro such as IL-12, IL-1β, IFN-γ, TNF-α, IL-4 and IL-10 (data not shown). IFN-γ secretion from both the cells lines was quite similar (low). Furthermore, we are currently conducting studies in vivo using a mouse model that would better portray IFN-γ levels as well as levels of other cytokines in vivo.

• THP-1 cells were stimulated using M. tuberculosis strains H37Ra (ATCC 25177), H37Rv (ATCC 25618) and Manitoba Type 1 patient strain (lines 85-86). The control cultures were not exposed to M. tuberculosis.

• The ELISA protocol has been condensed (lines 93-103).

I am sending you four copies of the corrected manuscript (original and 3 copies) for final review. If you have any questions, please do not hesitate to contact me at phone (204) 789-6036 or fax (204) 789-2036 or e-mail me at meenu_sharma@hc-sc.gc.ca

Thanking you,

Sincerely,

Dr. Meenu Kaushal Sharma