Heterogeneity in human IFN-γ responses to clinical *Mycobacterium tuberculosis* strains*, **

Heterogeneidade de resposta por IFN-γ a cepas clínicas de *Mycobacterium tuberculosis* em humanos

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Abstract

Mycobacterium tuberculosis is one of the most successful human pathogens. Highly virulent strains, which are more easily transmitted than are less virulent strains, elicit variable immune responses. We evaluated the Th1 responses (IFN-γ production) in healthy volunteers after stimulation with various strains. Our results show that the individuals with negative tuberculin skin test (TST) results were not necessarily naive to all of the strains tested, whereas individuals with positive TST results did not respond to all of the strains tested. Drug-resistant strains induced a lower mean level of IFN-γ production than did drug-sensitive strains. One possible practical application of this finding would be for the prediction of responses to treatment, in which it might be advantageous to have knowledge of the estimated IFN-γ production elicited by a specific isolated strain.

Keywords: Tuberculosis; Polymorphism, restriction fragment length; *Mycobacterium tuberculosis*; Immunity, cellular; Interferon-gamma.

Resumo

Mycobacterium tuberculosis é um dos mais bem sucedidos patógenos do homem. As cepas virulentas são mais facilmente transmitidas, induzindo respostas imunes variáveis. Avaliamos a resposta celular tipo Th1, através da produção de IFN-γ, como resposta a cepas com padrões diversos em voluntários sadios. Nossos resultados mostraram que indivíduos com teste tuberculínico (TT) negativo já tiveram contato com algumas das cepas testadas, ao passo que indivíduos com TT positivo não responderam a todas as cepas testadas. Cepas resistentes induziram uma média menor de produção de IFN-γ que aquelas sensíveis. Uma possível aplicação prática disto seria que a produção de IFN-γ, em relação a uma cepa isolada específica, poderia auxiliar na previsão da resposta ao tratamento dos pacientes.

Descritores: Tuberculose; Polimorfismo de fragmento de restrição; *Mycobacterium tuberculosis*; Imunidade celular; Interferon gama.

According to the World Health Organization,[1] based on a hypersensitivity response to PPD, approximately one third of the world population is infected with *Mycobacterium tuberculosis*. Paradoxically, the human immune response is quite efficient in defeating the growth of this pathogen but is not efficacious in its eradication. The natural history of tuberculosis indicates that only 10% of infected individuals are at risk of developing the disease at some point in their life. However, the majority of infected individuals might be in jeopardy of reactivation of the disease when the immune system becomes suppressed or when there is reinfection with a new strain. Some studies have demonstrated a correlation between *M. tuberculosis* virulence and lack of Th1 response in murine models. Others have suggested that certain strains, typically those with special features, such as the Beijing strain, interact distinctly with the host,

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inducing different immune responses according to their transmission potential.[2,3] In the present study, we assessed the ability of clinical and nonclinical isolates of drug-resistant or drug-sensitive *M. tuberculosis* strains to induce IFN-γ-mediated protective immune responses in healthy volunteers. The study design was approved by the institutional research ethics committee.

The clinical strains were isolated from patients with pulmonary tuberculosis. The H37Rv strain of *M. tuberculosis* was obtained from the American Type Culture Collection (Manassas, VA, USA). The characteristics of the various strains are described in Table 1. The susceptibility test was performed by the Löwenstein-Jensen (LJ) proportion method,[4] and the insertion sequence *6110* (IS*6110*) pattern was obtained by RFLP.[5,6] We recruited 11 healthy volunteers, all of whom underwent a tuberculin skin test (TST). Of those 11 volunteers, 6 tested positive (TST+, induration ≥ 5 mm) and 5 tested negative (TST–). None of the volunteers had a history of tuberculosis. All of the volunteers gave written informed consent.

Blood samples were drawn, and PBMCs were prepared as described by Tavares et al.[7] In-vitro stimulation of 10^6 cells/well was accomplished via incubation, for three days, with a suspension containing 10^5 bacilli/well, and, as a positive control, 5 µg/mL of PHA (Sigma, St. Louis, MO, USA). Supernatants of bacteria-free cell cultures were collected and stored immediately at –70°C for IFN-γ quantification using ELISA (R&D Systems, Minneapolis, MN, USA). Levels of IFN-γ > 100 pg/mL were considered positive. The suspension of bacilli was prepared by cultivating the strains for three weeks at 37°C on LJ medium. The fresh growth was removed, placed into a tube containing 3-mm glass beads, and vortexed for 1 min. Following the addition of 5% PBS-Tween 80, mycobacterial growth was dispersed (by multiple aspirations with a 25-gauge needle), vortexed, and twice washed thoroughly in PBS by centrifugation. Remaining large particles were allowed to settle for 30 min, after which the supernatant cell count was adjusted to a McFarland standard of 1 and then to the desired number. Clump-free cell suspension was verified by smear microscopy for AFB. Results are expressed as mean ± SE. Data were analyzed by the nonparametric Student's t-test. The cut-off point for IFN-γ was 100 pg/mL, unless the background levels were higher in the unstimulated control cells, in which case a value at least twice that of the control was considered positive.

Table 2 shows the IFN-γ production after stimulation with the various strains. As expected, most of the TST+ volunteers produced IFN-γ in response to stimulation with the strains tested. Despite the fact that some of the strains tested have yet to be recognized, the intensity of the response, in most cases, correlated positively with the size of the induration on the TST. The multidrug-resistant *M. tuberculosis* strain 046, harboring ten IS*6110* copies and belonging to a cluster, was the strain most often evoking an immune response in the sample as a whole and even evoked such a response in 1 (20%) of the 5 TST– volunteers. This indicates that some of the healthy individuals, despite being TST–, were not naive to all *M. tuberculosis* strains, although it might indicate a cross-reaction with environmental mycobacteria. It is known that PPD and H37Rv antigens induce Th1 responses in TST+ individuals. One TST– volunteer had a cytokine response to the H37Rv strain and to one other strain. However, in the sample as a whole, elevated IFN-γ production correlated with the TST induration, except in the case of one TST+ individual (volunteer I), in whom neither H37Rv nor any of the other strains elicited IFN-γ production. The fact that some TST+ individuals from endemic areas did not respond to mycobacterial strains might be

Table 1 - Genotypic and phenotypic characteristics of the *Mycobacterium tuberculosis* strains used in the study.

Characteristic	Strain									
	H37Rv	046	081	213	238	282	065	215	270	274
RFLP-IS*6110* copy number		10	10	11	2	8	10	4	9	9
Cluster	N	Y	Y	Y	Y	Y	N	N	N	N
Drug resistance	S	MDR	H/E/Z	Z	H	MDR	S	S	S	S

N: no; Y: yes; S: sensitive; MDR: multidrug resistant: H: isoniazid; E: ethambutol; and Z: pyrazinamide.

Table 2 - Tuberculin skin test induration and IFN-γ production, by *Mycobacterium tuberculosis* strain, in the 11 volunteers evaluated.

Volunteer	Induration[a]	IFN-γ production (pg/mL)										
		by strain										
	mm	Control	H37Rv	046	081	213	238	282	065	215	270	274
A	0	28.76	42.69	37.71	6.72	10.77	0	8.45	33.60	30.63	3.61	0
B	0	3.89	0	0	0	17.5	0	0	0	0	0	0
C	0	149.21	43.63	88.95	97.51	65.14	95.84	111.13	88.75	137.55	139.65	110.46
D	0	0	0	6.72	14.17	8.81	0	8.07	0	7.31	0	1.72
E	0	120.24	240.43[b]	262.28[b]	135.24	0	7.92	111.64	121.75	192.80	128.74	37.89
F	5	100.12	121.75[b]	111.64[b]	101.55	41.23	11.24	91.46	108.28	81.39	51.25	44.56
G	10	97.13	124.25[b]	240.65[b]	151.28[b]	187.89[b]	141.22[b]	251.55[b]	144.64[b]	258.28[b]	244.82[b]	268.38[b]
H	13	225.66	902.40[b]	3955.24[b]	142.41	137.74	79.67	208.11	588.74[b]	2058.26[b]	1236.82[b]	194.13
I	13	36.11	76.28	48.72	30.55	8.79	8.64	51.31	33.75	75.32	95.92	50.38
J	15	130.76	1715.08[b]	2109.65[b]	2193.27[b]	208.35	157.10	740.21[b]	499.14[b]	2666.80[b]	2172.70[b]	263.99[b]
K	20	89.56	708.17[b]	745.17[b]	732.76[b]	209.78[b]	161.75[b]	109.08[b]	78.04	209.78[b]	1156.99[b]	572.12[b]

[a]Positive tuberculin skin test response defined as an induration ≥ 5 mm. [b]Positive result defined as IFN-γ > 100 pg/mL, unless background levels were higher in the unstimulated control cells, in which case it was defined as a value at least twice that of the control.

relevant in vaccine testing, as well as in the use of diagnostic methods based on the IFN-γ response assay, which have been commercially used to identify individuals with latent tuberculosis infection. Because certain clinical isolates, including H37Rv, produce a variety of responses in humans, as well as because virtually all new tuberculosis vaccines (most of which contain the H37Rv strain) are tested in animals, it remains unknown whether new vaccines (targeting other strains) will provide the same level of protection as that observed for those targeting this standard strain. Recently, clinical *M. tuberculosis* strains have been reported to produce a variable disease response in animal models.[8] Further studies are needed in order to elucidate the influence that the estimated sensitivity of the IFN-γ response assay has on INF-γ levels in individuals with latent tuberculosis infection.

The isoniazid-resistant strain 238, harboring a cluster with two copies of IS*6110*, induced IFN-γ production in only 2 of the volunteers (volunteers G and K), who presented with low positivity for IFN-γ and TST indurations of 10 and 20 mm, respectively. Among the TST+ volunteers, the mean level of IFN-γ production was lower for the drug-resistant strains than for the drug-susceptible strains (445.6 ± 155.6 pg/mL vs. 548.10 ± 153.9 pg/mL). The same was observed among the TST– volunteers (43.78 ± 12.73 pg/mL vs. 51.72 ± 14.25 pg/mL), although the difference was not significant. According to previous reports, some prevalent endemic strains, as well as those with low IS*6110* copy numbers, might have higher virulence and greater transmission potential, being associated with a lack of a protective immune response, as well as with the suppression of IL-1β, IFN-γ, and TNF-α production.[9-11] In the present study, clustered strains less often evoked an immune response from TST+ volunteers. However, all of the clustered strains isolated were drug-resistant strains. Decreased IFN-γ production has previously been described in patients infected with multidrug-resistant strains.[12,13] Our study produced similar results, which corroborates reports that drug-resistant strains downregulate the Th1 response, and that the degree of downregulation is strain-dependent.

In summary, we observed heterogeneous cytokine immune responses for a panel of clinical *M. tuberculosis* strains. This suggests a strain-to-strain difference in virulence factors. In general, an effective immune response is more likely to depend on the host than on the infecting strain. However, we observed some particular responses related to strain characteristics such as drug resistance. This suggests that an estimate of the IFN-γ production elicited by a specific isolated strain might help predict the response to treatment. In our study, some strains were shown to induce or mimic severe disease by downregulating the IFN-γ response, which can have implications for the efficacy of T cell-based diagnostic tests. In addition, our results suggest that new vaccines should be evaluated

as to whether they elicit immune responses to other *M. tuberculosis* strains, since we have demonstrated that the characteristic clinical strains evoke heterogeneous host responses.

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