MECHANISMS OF PATHOGENESIS

The influence of influenza virus infections on the development of tuberculosis

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A R T I C L E   I N F O

Article history:
Received 12 July 2012
Received in revised form
20 December 2012
Accepted 4 February 2013

Keywords:
Tuberculosis
Influenza
Co-infection
Interferon
Reactivation
Susceptibility

S U M M A R Y

Recently, it was shown that interferon-γ mediated immune responses, which play a major role in the control of infection with Mycobacterium tuberculosis (Mtb), can be inhibited by type I interferons. Since type I interferons are abundantly induced during viral infections, we hypothesized that infections with influenza viruses might play a role in the development of active TB disease either directly after exposure to Mtb or through reactivation of latent Mtb infection. To explore this hypothesis we investigated in a retrospective study whether newly diagnosed adult tuberculosis patients from Indonesia had had recent influenza infection. Plasma samples from TB patients and controls were assayed for antibodies against two subtypes of at that time relevant, seasonal influenza A viruses. Overall, no correlation was observed with the presence of antibodies and manifest tuberculosis. Still, antibody titers against circulating A/H3N2 influenza virus were slightly enhanced in tuberculosis patients as compared to controls, and highest in cases of advanced tuberculosis. This suggests that tuberculosis patients were recently infected with influenza, before clinical manifestation of the disease. Alternatively, the production of antibodies and susceptibility to tuberculosis may be influenced by a common confounding factor, for example the ability of patients to induce interferon-α. We conclude that in an endemic country like Indonesia, an influenza virus infection is not a major determinant for developing clinically manifest tuberculosis.

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1. Introduction

Tuberculosis (TB) is a severe disease caused by Mycobacterium tuberculosis (Mtb). Up to 10% of the Mtb infected individuals develop active tuberculosis, while the majority of those infected develop a latent state of infection for years. During latency, Mtb may stay quiescent, but when the immune system fails to control the bacteria, reactivation may occur and precipitate development of active disease, in most cases pulmonary TB disease.

Several risk factors that influence the susceptibility to TB have been described, such as host genetic factors, malnutrition, smoking, diabetes and infection with HIV. Besides these factors also the Mtb strain virulence may influence the course of TB, since virulent Mtb can inhibit the host immune system in various ways. Recently, IFN-α has been described as a putative factor, which may be induced by highly virulent Mtb strains, and can inhibit an effective IFN-γ mediated immune response. Mouse studies revealed that during Mtb infection IFN-α is induced and that IFN-γ mediated immune responses can be impaired by IFN-α. In humans, a typical IFN-α/β transcript signature was found in the blood cells from TB patients. However, it is not clear whether Mtb infection in humans leads to the production of type I interferons or whether production of type I interferons leads to TB. It is possible that in TB patients type I interferons are more abundantly induced due to infections with viruses, such as pneumotropic influenza viruses.

After influenza virus infection a period of enhanced susceptibility to bacterial infections is commonly seen in humans. In mice, Toll-like receptor-induced responses of alveolar macrophages to bacterial ligands remain desensitized for months after an influenza virus infection. This explains why mice are highly susceptible to bacterial pneumonia for several weeks after influenza virus
infection.\textsuperscript{21} In humans, post-influenza bacterial pneumonia is a major cause of morbidity,\textsuperscript{19} with \textit{Streptococcus pneumonia} as the main pathogen associated with post-influenza pneumonia.\textsuperscript{22} TB is usually not diagnosed shortly after influenza infections and although some anecdotal reports suggest that the occurrence of TB was also high during influenza pandemics,\textsuperscript{23–25} a causal relationship between the epidemics of the two infectious diseases has not been investigated.

If indeed an influenza virus infection leads to the (re)-activation of a latent Mtb infection, this may remain unnoticed, because the period between (re)-activation of the bacteria and the first presentation of the clinical symptoms of TB is long. TB develops slowly, due to the slow metabolism and replication rate of Mtb. Thus it may be that within the latency period of TB, during the primary infection or after re-infection, a transient inhibitory effect on the antibacterial responses by an influenza virus infection influences the course of TB and leads to active disease, possibly in conjunction with other risk factors. In mice, co-infections of influenza viruses with Mtb enhanced the development of TB in the lungs.\textsuperscript{26} In humans, it is still unclear whether influenza virus infections can influence the course of TB.

We hypothesized that influenza virus infections may promote the development of active disease after exposure to Mtb, or might play a role in the reactivation of latent Mtb infection. To explore this hypothesis we investigated in a retrospective study whether patients with clinically-manifest TB had an influenza virus infection recently. Plasma samples collected from TB patients at time of diagnosis and from controls were screened for the presence of antibodies against influenza viruses in order to investigate a putative association between TB and influenza virus infections.

2. Patients, materials and methods

2.1. Study subjects

Patients and controls (Table 1) were recruited between March 2001 and December 2004 from the TB clinic “Perkumpulan Pembaratansan Tuberkulosis Indonesia” in Jakarta.\textsuperscript{8,27,28} Patients newly diagnosed with active pulmonary TB, between the age of 15 and 70, were included. TB diagnosis was based on WHO definitions including the presence of clinical symptoms, a chest X-ray examination (CXR), microscopic detection of acid-fast bacilli in sputum and a positive culture of Mtb. Based on the CXR examinations TB patients were classified into two groups; patients with mild to moderate TB and patients with advanced TB. Patients seropositive for HIV were excluded. Community control subjects were recruited from neighboring houses and matched for age, sex and socioeconomic class. Controls with a history of TB or with positive TB infection in the CXR were excluded. The control subjects underwent the same examinations as the patients, but were not tested for HIV, since the prevalence of HIV in the Indonesian population was low, as evidenced by the low prevalence of HIV amongst the TB patients in this cohort (1.8\%).\textsuperscript{29} The influenza vaccination status of our study cohort is unknown. However, at the time of our study, vaccination against influenza viruses in Indonesia was only rarely applied and based on the low socio-economic status of our patients and matched controls these individuals are extremely unlikely to have received such vaccinations. For this study, the patients and controls were matched for the date of inclusion. Patients and matched controls were only included if the dates of inclusion were not more than 14 days apart. Written informed consent was obtained from all subjects. The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia.

### Table 1

Description of the study population.

<table>
<thead>
<tr>
<th>Description</th>
<th>TB patients (n = 111)</th>
<th>Controls (n = 111)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (median)</td>
<td>18–67\textsuperscript{11}</td>
<td>17–69\textsuperscript{13}</td>
<td>0.153</td>
</tr>
<tr>
<td>Gender; males</td>
<td>72 (65%)</td>
<td>60 (54%)</td>
<td>0.132</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>24 (22%)</td>
<td>8 (8%)</td>
<td>0.001\textsuperscript{1}</td>
</tr>
<tr>
<td>Individuals with BCG scar</td>
<td>39 (35%)</td>
<td>45 (41%)</td>
<td>0.489</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Student t-test.
\textsuperscript{1} \chi^2 test.

2.2. Detection of antibodies against influenza viruses

Heparinized plasma samples were obtained from the patients and controls and stored at –80 °C. Thawed plasma were analyzed for the presence of total IgG and IgM antibodies against two subtypes of influenza A virus, a H3N2 virus (A/Moscow/10/99, vaccine strain ResVir-17) and a H1N1 virus (A/New Caledonia/20/99, vaccine strain IVR-116), using the hemagglutinating inhibition (HI) test. These strains were chosen because of their high antigenic similarity to the specific H3N2 and H1N1 viruses circulating during the sample period. For use in the HI test, the influenza virus strains were propagated in 11-day old embryonated chicken eggs. The HI test was performed in duplicate according to standard methods\textsuperscript{26} with turkey erythrocytes and four hemagglutinating units of virus. Ferret sera raised against the test antigens were used as positive controls. All plasma samples of all study subjects were tested simultaneously, in duplicate, and were only regarded positive when both analyses gave positive results. The threshold of detection was an HI titer $>10$.

2.3. Statistical analysis

Data were analyzed using SPSS software. The Pearson $\chi^2$ test and the paired samples $t$-test were regarded significant when $p < 0.05$.

3. Results

3.1. No correlation between the incidence of tuberculosis and the seroprevalence of antibodies against influenza viruses

To study the effect of influenza virus infections on TB we examined the plasma samples of controls and newly identified TB patients for the presence or absence of antibodies against two subtypes of influenza A virus, H1N1 and H3N2 (Table 2). The threshold of detection was an HI titer $>10$. Both influenza strains circulated in the population during the time of plasma sampling: 46% of the TB patients and 41% of the controls had antibodies against H1N1, while 82% of the TB patients and 82% of the controls had antibodies against H3N2 (Table 2). No significant differences in the number of individuals with antibodies against influenza viruses were thus observed between the control group and the group of TB cases.

### Table 2

Seroprevalence of antibodies to A/H1N1 and A/H3N2 influenza viruses.

<table>
<thead>
<tr>
<th>Group</th>
<th>H1N1 positive</th>
<th>H3N2 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB patients (n = 111)</td>
<td>51 (46%)</td>
<td>91 (82%)</td>
</tr>
<tr>
<td>Controls (n = 111)</td>
<td>46 (41%)</td>
<td>91 (82%)</td>
</tr>
<tr>
<td>Total (n = 222)</td>
<td>97 (44%)</td>
<td>182 (82%)</td>
</tr>
</tbody>
</table>

\chi^2 test; p-value $p = 0.499$ $p = 1.000$.

The threshold of detection was an HI titer of $>10$. 
3.2. Titters of antibodies to influenza viruses correlated with the manifestation of tuberculosis

Because yearly influenza infection rates are estimated to be 5—10% \(^1\(^1\)\) the high percentage of individuals with H1N1 and H3N2 antibodies indicate that many of these individuals were already exposed to these viruses in the past. Therefore, the magnitude of the antibody responses might be a better indication of recent exposure as IgG antibody levels will be boosted upon reinfection and will only wane partially. Hence, we examined the titers of antibodies against H1N1 and H3N2 influenza viruses in the TB and control group. The results are displayed in Table 3 and Figure 1. The geometric means of the titers of antibodies against the H3N2 and the H1N1 influenza viruses were higher (respectively 1.7 and 1.4 times) for the total group of pulmonary TB patients, as compared to the titers of the control group. Statistical analysis using the paired sample t test showed that only for the titers of antibodies against H3N2 this difference was significant. We next determined whether there is a correlation between the severity of the disease and the antibody titers against H3N2. The cases of advanced pulmonary TB showed a higher geometric mean titer for H3N2 (1.8 times), while the group of cases with mild and moderate TB showed a smaller increase (1.4 times) in the geometric mean titer as compared to the matched controls. The difference in these titers was significant between the cases with advanced TB and their matched controls but not between the cases of mild to moderate TB and their matched controls. The geometric mean titer of antibodies against H3N2 influenza viruses for the extrapulmonary TB patients was even higher (2.0 times as compared to the matched controls). However, the latter patient group was small (n = 10) and the antibody titers of the cases with extrapulmonary TB were not significantly different from the titers of their matched controls.

3.3. The influence of diabetes mellitus on tuberculosis and the titers of antibodies to influenza viruses

In a previous study from our group, with the large cohort of patients and controls from Indonesia, Diabetes mellitus (DM) was identified as a risk factor for TB. \(^8\) Since having DM is correlated with the incidence of TB in this study (Table 1) we investigated whether having DM could be a factor of influence on influenza virus specific antibody levels. Hence, we compared the influenza virus specific antibody titers between the TB cases and their matched controls, while excluding individuals with DM from both groups.

The group of patients with pulmonary TB and without DM showed still significantly higher amounts of antibodies against H3N2 as compared to their matched controls, (paired sample t test, Table 3 and Figure 1F). The mean titer of antibodies against H3N2 of the TB cases with DM was on the other hand significantly lower (student t test, p = 0.049) as compared with the mean titer of the TB cases without DM (data not shown).

4. Discussion

The main finding of this report is that there is no correlation between the seroprevalence of antibodies against influenza A viruses and the development of clinically active TB in an Indonesian cohort. However, we observed an association between the level of antibody titers against influenza A/H3N2 virus and the stage of active TB lung disease. Compared to the control group the mean antibody titer of the group of TB patients was slightly enhanced, which may indicate that the TB patients were recently re-infected with an influenza virus strain, suggesting that an influenza virus infection precedes and influences the clinical manifestation of TB. However, such an epidemiological association may not be causal, and be confounded.

Previously, it was suggested that an association existed between the 20th century influenza pandemics and the incidence of TB. \(^23\)–\(^25\) The existence of this correlation can however be questioned because diagnoses in the early 20th century were based solely on clinical symptoms. In addition, an increased incidence of both diseases may also be explained by common susceptibility factors, such as malnutrition. Here, we tested the hypothesis that influenza virus infections enhance the susceptibility to develop active TB. Although we did not find an association between the number of people with antibodies against influenza viruses and the development of TB, the difference in influenza A/H3N2 virus specific antibody titers between TB cases and controls may suggest that more TB cases than controls were recently re-infected, or have been exposed more often, with these seasonal influenza viruses. A drawback of our study is the high (pre-existing) seroprevalence of the antibodies against H3N2. Detection of antibodies against specific epitopes of the most recent influenza subtypes was unfortunately not possible, due to the low antigenic drift between the latest seasonal influenza viruses.

Within the group of TB patients the cases with extrapulmonary manifestation of the disease showed the highest mean antibody titers against A/H3N2 viruses. This association suggests that the clinical manifestation of extrapulmonary infections occurs in TB patients with stronger antibody responses after influenza virus infections. Dissemination of mycobacterial infections are more common in hosts with impaired immunity, for example in MSMD patients with impaired IFN-γ mediated immune responses \(^32\) or in HIV patients. \(^33\) In patients with extrapulmonary TB the plasma concentrations of IFN-γ are also reduced, as compared to patients with pulmonary TB. \(^34\) Therefore, we speculated that viruses other than HIV, like influenza viruses, affect the Th1 immunity against mycobacteria and in this way influence the course of TB. In mice it was indeed found that influenza virus infection aggravates the course of TB. \(^26\) It is unclear whether this is also the case in humans. Our cohort was too small to assess whether the risk of extrapulmonary manifestation is increased due to influenza virus infections.

The finding that TB patients had slightly higher plasma antibody levels to A/H3N2 influenza virus than the control group suggests a relation exists between influenza virus infections and the clinical manifestation of tuberculosis. However, the association may very well be confounded. One possible confounding factor is that a co-infection with an influenza virus in patients with TB may be more severe or lasts longer due to lung damage by Mtb, and as a result more antibodies against the virus are generated. Another possible confounding factor is that Mtb infection may result in the
polyclonal activation of B cells, as was observed in mice after infection with various microorganisms.\(^3\) Human memory B cells were also found, in response to a strong stimulus, to polyclonally produce small amounts of antibodies in the absence of specific antigens.\(^3\) Whether such non-specific antibody production also occurs during Mtb infection and results in a detectable rise in anti-influenza antibodies is yet unknown. Since plasma of TB patients before the manifestation of the disease is unavailable we were unable to determine whether the anti-influenza antibodies rise upon development of TB. The ability to produce high amounts of IFN-α may also be a confounding factor. Some individuals are relatively strong producers of type I interferons\(^3\) and because of this they may produce both higher amounts of antibodies against viral pathogens and may be more susceptible to developing TB.

In addition, TB may be aggravated if Mtb induced type I interferons are also more abundant in these individuals, as they may impair an effective IFN-γ mediated immune response.\(^3\) Another potential confounding factor may be diabetes mellitus (DM) since DM was previously found to be a risk factor for developing TB.\(^3\) We investigated whether DM cases had higher antibody titers against the viruses. Based on our results, we could exclude DM as a confounding factor.

Our findings are based on a retrospective study with TB cases in Jakarta (Indonesia) in the period from March 2001 till December 2004. This is the first study which investigates a potential role for viral infections in the development of TB by determining the presence of antibodies against influenza viruses in plasmas of TB patients and matched controls. In order to determine whether the...
observed association between TB and higher virus-specific anti-
body titers indeed indicates an increased risk for TB after influenza
virus infection and to exclude that confounding factors cause the
increase in the antibody response to influenza viruses, a prospec-
tive study is needed. Although such a study would be difficult to set
up, this should preferably investigate the influence of primary viral
infections on the manifestation of TB in childhood. In such a cohort
it would be possible to prove new, primary influenza virus infections.

A prospective study for adult TB is also possible, but many
children already have antibodies against previous seasonal vi-
ruses (that may cross-react with contemporary viruses) novel
infections will have to be identified by demonstrating antibody
titers rises against a new seasonal strain. The current study was
cross sectional and identified a high seroprevalence of antibodies
against two subtypes (44% and 82% for H1N1 and H3N2 respecti-
vely) indicating that a large proportion of study subjects were
infected during previous epidemics, as normally about 5–10% of the
population are affected during annual influenza epidemics. Studies
of larger cohorts and screening for strain-specific anti-

bodies may provide more insights into the effects of co-infections
with influenza viruses on the occurrence and severity of TB.

Based on our results we conclude that influenza virus infections are
not a major determinant in the development of clinically active
TB in adults, either through reactivation of latent disease or directly
after exposure to the bacillus, nor of more severe manifestation of
the disease. The identified correlation between the titers of anti-
bodies against influenza viruses and the manifestation of TB, is still
in line with the suggestion that type I interferons may play a role in
the immunopathogenesis of TB.

Ethical approval: The study was approved by the ethical
committee of the Medical Faculty, University of Indonesia.

Funding: This study was supported partly by a grant from the
Royal Netherlands Academy of Arts and Sciences (KNAW99MED01) and received supplementary support from
NWO-PRIOR. The research also received funding from the Euro-
pean Union’s Seventh Framework Programme (FP7/2007–2013)
for projects NEWTBVAC; IDEA; and ADITEC (No: 280873).

Competing interest: The authors have no conflict of interest.

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