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Cellular and humoral responses to influenza in Gabonese children living in rural and semi-urban areas


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Summary

With the current attention to the pandemic threat of avian influenza viruses, it is recognized that there is little information on influenza in Africa. In addition, the effect of influenza vaccination in African countries could be very different from the outcomes in regions with less exposure to micro-organisms and parasites. To monitor the presence of influenza viruses and investigate the immunological response to influenza vaccination, children in a semi-urban and a rural region of Gabon were studied. Influenza-specific antibody responses to the three strains represented in the vaccine were determined in the serum. Further, cytokine responses were measured after in vitro stimulation of whole blood with influenza antigens, before and after vaccination. Pre-vaccination antibody titres to H3N2 were high. Upon vaccination the titres to the three influenza strains increased significantly. Weaker anti-H1N1 and anti-B responses were found in rural versus semi-urban school children after vaccination. Influenza-specific cytokine responses were induced within a week, showing significantly lower IFN-γ and significantly higher IL-5 in the children from rural areas. Pre-vaccination antibody levels indicated influenza viruses circulate in Gabon. Altogether influenza vaccination induces weaker immune responses in a rural than in a semi-urban population of Gabonese schoolchildren.

Introduction

Little information is available on influenza in Africa. The influenza surveillance capacity is weak on this continent and only two countries, South Africa and Senegal, engage in active monitoring of the infection and disease [146]. Along with the global anxiety over the spread of H5N1 avian influenza, concerns have been raised on the lack of accurate data from the African continent [146-149]. From the few sporadic studies conducted in Africa, involving virological assessments, it appears that the problem of influenza virus is probably greatly underestimated. The virus was found to be present and circulate throughout the year or peak seasonally in the studied areas [147]. Even the reporting of influenza, based on clinical manifestations, is difficult in Africa, as symptoms are shared with many other infections that are prevalent in the region, making collection of data on morbidity and mortality particularly difficult. Such data are needed to trace the virus globally and also to identify people at the highest risk who would benefit from preventive vaccination.

Considering vaccination, even less is known about the effectiveness of influenza vaccines in Africa. With the current attention to the pandemic
threat of avian influenza viruses, its global spread and the preparation of preventive and curative vaccines, it is important to start asking the question of what the immunological consequences of influenza vaccination are in African populations. These vaccines are mostly developed and tested in Europe, in North America and in some parts of Asia. It is known that vaccines that have proven efficient in high income countries might perform less well in populations living in low income countries [150, 151]. In Africa, conditions in urban centers (those with high/middle wealth) might approximate those in western and industrialized countries, however, in rural areas lifestyle and exposures to infections are widely different. Many infectious diseases prevalent in Africa, particularly present in rural areas and chronic in nature, are known to be associated with profound alternations of the innate [152-154] and the adaptive [155-157] immune system, which may affect responses to third party antigens. For example, chronic helminth infections, highly prevalent in many rural areas in Africa, are known to be associated with skewing of immune responses towards Th2 [158]. Moreover, infections with some parasitic helminths and protozoa along with mycobacterial, malarial as well as viral hepatitis infections have been shown to induce regulatory immune responses that are characterized by production of high levels of suppressory cytokines such as TGF-β and IL-10 [6, 159-162]. These suppressory molecules might affect responses to vaccines as already documented for oral vaccines as well as tetanus and BCG vaccination [28, 32, 36].

To our knowledge influenza surveillance has never been conducted in Gabon and as symptoms of influenza infection resemble closely that of prevalent infections such as malaria, influenza is clinically not distinguishable. Therefore, antibody and cellular responses to influenza A and B strains were analysed in serum of schoolchildren residing in rural and semi-urban areas in Gabon. Subsequently the children received one dose of influenza vaccine and immune responses at various intervals after vaccination were monitored.

**Materials and methods**

**Study population**

The study was conducted in and around Lambaréné in Gabon, Africa. Children from two schools, one in a semi-urban area (Lambaréné) and another in a rural area (PK15), and their parents were informed of the study and written informed consent was received prior to inclusion. Exclusion criteria were: absence of informed consent, absence of stool, urine or blood samples for parasitological analysis or presence of any clinical symptoms. A total of 33 children from the semi-urban school and
22 children from the rural school agreed to participate in the study and met the inclusion criteria. The details of the children are shown in table 3.1. The children included in the study in the rural and semi-urban school, were similar with respect to age and sex ratio. The nutritional status was determined by measuring weight by age and sex and by comparing this with age and sex specific values provided by the Center for Disease Control and Prevention (CDC) [163]. For comparisons, we considered well-nourished children as those with a weight by age and sex >90% of the median corresponding weight by age and sex of CDC reference data.

In the cohorts investigated in this study, 33% of the children in the semi-urban school were infected with *Schistosoma haematobium* whereas in the rural school 100% of children were infected. These results were obtained after 3 independent urine samples were tested. The prevalence after testing one urine sample was 19% and 78% in semi-urban and rural school, respectively. The prevalence of intestinal helminth infections in the semi-urban school and rural school was also significantly different for both *Ascaris lumbricoides* (15% and 55%, respectively, p= 0.002) and *Trichuris trichiura* (12% and 64%, respectively, p< 0.001). These values were obtained after two stool samples were examined using the Kato Katz method.

In total 12 out of 55 children were found to be positive for malaria during the study. 2 were infected with *P. malariae* and were treated with chloroquine 10 mg/kg/day for 2 days and 5 mg/kg/day the third day. The remaining 10 children were found to be positive for *P. falciparum* and were treated in case of clinical manifestations. Two children presented symptoms of malaria infection and were treated with sulfadoxin (25mg/kg, single dose) and pyrimethamin (1.25 mg/kg, single dose) (SP) as well as artemesunate 4mg/kg/day for 3 days. An additional 4 children whose positivity was reported during the study were treated with SP due to physical complaints.

The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné.

**Parasitological diagnostics**

Infection with *S. haematobium* was determined by passing 10 ml of urine through a filter with 10 μm pore size and staining the eggs with a ninhydrin solution. From children tested negative two additional urine samples were analysed, to increase the accuracy of the diagnosis. Infection with malaria parasites was determined by staining of thick blood smears with Giemsa, as described elsewhere [164]. The presence of the intestinal helminths *A. lumbricoides* and *T. trichiura*, was determined by analysing stool samples using the Kato Katz method on at least one and when possible two stool samples [165]. In total 35 out of 55 children were
infected with one or more species of helminths examined. Subsequent to blood withdrawal at day 28 after vaccination, all children were treated with a single dose of albendazole (400mg) and those with eggs of S. haematobium detected in the urine samples, were additionally treated with a single dose of praziquantel 40 mg/kg.

Table 3.1. Study population

<table>
<thead>
<tr>
<th></th>
<th>Semi-urban</th>
<th>Rural</th>
<th>p-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years; median age (range)</td>
<td>9 (7-12)</td>
<td>9 (7-11)</td>
<td>0.3</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>14/19</td>
<td>10/12</td>
<td>0.8</td>
</tr>
<tr>
<td>Nutritional status; well-nourished childrenb</td>
<td>17/29 (59 %)</td>
<td>5/20 (25 %)</td>
<td>0.02</td>
</tr>
<tr>
<td>S. haematobiumc</td>
<td>11/33 (33%)</td>
<td>22/22 (100%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A. lumbricoidesd</td>
<td>5/33 (15%)</td>
<td>12/22 (55%)</td>
<td>0.002</td>
</tr>
<tr>
<td>T. trichiurid</td>
<td>4/33 (12%)</td>
<td>14/22 (64%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Any helminth</td>
<td>13/33 (39%)</td>
<td>22/22 (100%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasmodium infectione</td>
<td>4/33 (12%)</td>
<td>8/22 (36%)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

aPearson Chi-Square test.
bWell nourished is defined as a weight by age and sex above 90% of CDC reference data.
cResults of testing 3 independent urine samples.
dResults of testing 2 independent stool samples.
ePlasmodium falciparum or Plasmodium malariae, at day 0, 2, 4, 7 and/or 14.

Vaccination and sample collection

On day 0, before vaccination, 3 ml of heparinated blood was used for immunological tests. Children were then vaccinated against influenza (Begrivac 2004/2005; Chiron Behring GmbH, Marburg, Germany) and tetanus (NIPHE, Bilthoven, The Netherlands). Subsequently, 3 ml of heparinated blood was used for immunological assays, drawn on day 2, 4, 7, 14 and 28 after vaccination. At all time points plasma was frozen and kept at -20°C, and all days except at day 28, whole blood stimulations were performed to collect supernatants for cytokine analysis.

Antibody measurement

For the detection of serum antibodies to influenza virus the haemagglutination inhibition (HI) assay was used. The HI assay was performed in duplicate according to standard methods [166, 167], using turkey erythrocytes and four haemagglutinating units of the vaccine strains, which were propagated in 11-day old embryonated chicken eggs. Ferret sera raised against the test antigens were used as positive controls.
Serum samples were treated with cholera filtrate to remove non-specific anti-haemagglutinins. To ensure comparability, all serum samples collected at different time points, were tested at the same time. For sera with titres below the detection level, the value of 5 was assigned. The virus neutralization assay was performed according to standard procedures as described previously [168].

**Whole blood culture and cytokine measurement**

Cellular immunological analysis involved culturing of whole blood. 100 μl of blood was cultured with 100 μl of RPMI-1640 medium or influenza vaccine in medium (predetermined optimum of 1 μg HA/ml). Supernatant was collected after 72 hours of incubation and was kept at -20 °C until analysis.

Levels of IFN-γ, IL-5, IL-13, TNF-α and IL-10 were determined simultaneously in the supernatants by using the Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA), equipped with StarStation software (Applied Cytometry Systems, Dinnington, UK). Buffer reagent kits and Luminex cytokine kits (BioSource, Camarillo, CA, USA) were used and cytokines were measured according to the protocol, with slight modifications. Briefly, assays were performed in 96-well round bottom plates (Nunc) at room temperature. A mix of beads was incubated with a standard, samples, or blank in a final volume of 50 μl for 2 hours under continuous shaking. Plates were washed twice and incubated with a cocktail of biotinylated antibodies (25 μl/well) for 1 hour. After removal of excess biotinylated antibodies by washing twice, streptavidin-RPE was added and incubated for 30 minutes. Subsequently plates were washed a final time and analysed using the Luminex-100 cytometer. The lower detection limit of the assays was 3 pg/ml for IL-5, 5 pg/ml for IL-10 and IFN-γ and 10 pg/ml for IL-13 and TNF-α. Samples with concentrations below the detection limit were assigned the value of this threshold. To determine influenza specific cytokine production, background cytokine production (cultured with medium only) was subtracted from the values obtained after stimulation of the blood cells with influenza vaccine.

**Statistical analysis**

The distribution of age, sex, nutritional status and prevalence of parasitic infections between the study groups were tested using Pearson’s Chi square test. Cytokine and antibody levels were not normally distributed and the Mann-Whitney test was used to analyse these data. Logarithmic transformation of cytokine and antibody levels resulted in a normal distribution and these values were used for linear regression analysis. Results from statistical analyses, using SPSS or Graph Pad Prism, were considered significant when the p-value was below 0.05.
Results

Seroprevalence of antibodies to influenza viruses prior to vaccination

Results obtained with the haemagglutination inhibition (HI) assay showed that influenza virus specific antibodies were already present in the majority of sera before vaccination (figure 3.1), indicating that influenza A viruses of the H1N1 and H3N2 subtypes and influenza B virus have been circulating in Gabon prior to the study. Interestingly, a high seroprevalence was found of antibodies specific for the H3N2 strain A/Wyoming/3/2003 compared to that for the influenza H1N1 and B strains before vaccination. The presence of high titres of pre-existing antibody levels was confirmed using an alternative method for influenza serology. A virus neutralization assay was performed with the sera obtained from a subset of 19 children from both groups, showing similar titres; 1593 (range 160-20 480) and 1481 (range 80-10240) for the HI and neutralization assay, respectively. Thus the pre-vaccination A/Wyoming/3/2003 X147 (H3N2) specific antibody levels indicate that an outbreak of infection caused by a strain related to A/Wyoming/3/2003 must have occurred recently.

Figure 3.1. HI titres to the three influenza strains present in the vaccine before and 14 and 28 days after vaccination. Graphs a-c show the individual data; in d-f the kinetics of the responses are shown, geometric mean titres per group are given. a, d: A/New Caledonia/20/99, IVR-116(H1N1); b, e: B/Jiangsu/10/2003; c, f: A/Wyoming/3/2003 X147(H3N2). Closed triangles (and lines): semi-urban (sU) subjects; open circles (and dashed lines): rural (R) subjects, * p < 0.05 (Mann-Whitney test).
Efficacy of vaccination: antibody production

The antibody responses to the three different influenza virus strains present in the vaccine were determined not only before, but also 14 and 28 days after vaccination (figure 3.1). Upon vaccination, HI titres to the influenza A (H1N1) strain increased in both the rural and the semi-urban children, but reached significantly higher levels in the semi-urban than in the rural children on day 28 (figure 3.1a and d). Two groups of children could be identified, one group whose anti H1N1 titres remained at a level only slightly higher than the pre-vaccination values (low responders) and another group of children that showed a prominent increase in antibody responses (high responders) (figure 3.1a). The latter group was significantly larger in the semi-urban population (figure 3.2a), resulting in the overall higher titres in the semi-urban children.

Within the semi-urban children there was a clear difference in the H1N1 antibody response of those infected with helminths (S. mansoni, A lumbricoides and/or T. trichiuris) and those without helminth infection. Thus, when only the group of semi-urban children without helminth infections was compared to the rural cohort, the difference in levels of H1N1-specific antibody titres became highly significant (p < 0.01), at both day 14 and day 28 after vaccination. Indeed, also in terms of percentage high responders, it was clear that helminth infected children in the semi-urban area behaved more like rural children (figure 3.2a). In addition, infections with plasmodia affected anti-H1N1 titres in rural children but not semi-urban children. As shown in figure 3.2b only few rural children with plasmodia were high responders when anti H1N1 antibody titres were considered.

The antibody titres specific for the influenza B-strain increased upon vaccination, reaching a peak 14 days after vaccination, and slightly decreasing thereafter (figure 3.1 b and e). Also for this strain, responses were significantly higher in the semi-urban children on day 28.

The levels of antibodies to the influenza A (H3N2) strain, which were very high before vaccination compared to pre-vaccination antibody levels specific for the H1N1 or the B strain of the vaccine, still increased slightly upon vaccination. At day 14, antibody levels were significantly higher in rural children compared to the semi-urban group, but this difference was not significant anymore at day 28 (figure 3.1c and f).

For antibodies to influenza B or H3N2 strains, neither helminths nor malaria infection influenced the responses significantly.
Figure 3.2 Influence of helminth (a) and malaria (b) infection on antibody responses to influenza A (H1N1). As shown in figure 3.1, antibody responses to the H1N1 strain were either low or high. In a, the percentage of high responders are shown for the total group of semia-urban (sU) children (both infected and uninfected; in grey), the semi-urban children without any helminth infection (sU-; white bars), the semi-urban children with a helminth (S. mansoni, A lumbricoides and/or T. trichiuris) infection (sU+; black bars) and the rural (R; grey) children (all infected). In b the percentage of high responders are shown for the total group of semi-urban (sU) and rural (R) children (both infected and uninfected; grey bars), the semi-urban and rural children without malaria infection (sU- and R-; white bars) and the semi-urban and rural children with malaria infection (sU+ and R+ black bars). * p < 0.05, ** p ≤ 0.005; Chi Square Test.

Efficacy of vaccination: cytokine responses
The cytokine response to influenza was determined at different time points to follow the kinetics of cellular immune response development upon vaccination (figure 3.3). After day 2 following vaccination, cytokine responses started to rise. The IL-10 response was early and a tight peak was seen in both rural and semi-urban school children on day 4 (figure 3.3a).
The magnitude of the influenza specific IL-10 response was significantly higher in the semi-urban compared with rural school children. The TNF-α, IFN-γ and IL-5 responses peaked at day 7 (figure 3.3b-d). The TNF-α levels were significantly higher in semi-urban schoolchildren; the peak at day 7 was almost absent in the rural group (figure 3.3b). Starting from day 7 after vaccination IFN-γ responses were significantly higher in semi-urban children (figure 3.3c). In contrast, rural children showed a more Th2 skewed response, as they produced less IFN-γ, and higher levels of IL-5 compared to the semi-urban group (figure 3.3d). Interestingly, higher IL-5 responses to influenza were seen in the helminth infected semi-urban children compared to the non-infected semi-urban children (insert in figure 3.3d), indicating that the Th2 skewing that is more prominent in the rural group may, at least partly, be the result of the immune modulating effect of helminths. Malaria infection did not affect cytokine responses to influenza antigens.
Discussion

The current study indicates that influenza A and B viruses circulate in Gabon and the presence of high levels of A/Wyoming/3/2003 X147 (H3N2) specific antibodies before vaccination, as determined by a HI and a neutralization assay, provide evidence for a recent outbreak of a H3N2 virus. Sporadic monitoring of influenza in other African countries, has revealed outbreaks of H3N2 virus in South Africa in 2003 [169], in Madagascar in 2002 [147] and in the Democratic Republic of Congo in 2002 [147]. In addition, influenza B viruses have been reported to be circulating in South-Africa, Madagascar and Kenya [170, 171].

In some of these studies, a mortality of at least 3 percent of cases was reported, due to influenza A/Panama/2007/97-like (H3N2) infection, indicating that influenza can have a high impact also in African countries [147]. No data are available on influenza mortality or morbidity in Gabon and given our findings, it would not be surprising if considerable mortality in the study area may be attributed to influenza epidemics. This is particularly important for the area we studied, in fact for most of Africa, where malaria is endemic and high fever is often treated with antimalarials. Thus, influenza infections in these areas can lead to considerable malaria over-medication on the one hand and over-estimation of malaria deaths, on the other.

Considerable differences were observed in vaccine induced antibody responses between rural and semi-urban school children. The responses to H1N1 and influenza B strains were higher in the children from the semi-urban area. As the pre-vaccination titres did not differ between semi-urban and rural children, it is unlikely that differences in exposure to influenza could explain these results. Why children in rural areas would respond differently than those in semi-urban areas has yet to be fully investigated. However, some vaccines like BCG or tetanus, that were shown to be effective in non-tropical countries, were found to induce a weak response in tropical countries and this has been associated with the presence of helminth infections [35, 150]. Moreover, although to our knowledge the effect of helminth infections on the efficacy of influenza vaccination has not been investigated previously, studies on the efficacy of cholera vaccine [28], BCG [31] and TT vaccine [34, 36] suggested that immune skewing in response to vaccines is affected by pre-existing helminth infections. These studies were performed in different areas covering intestinal helminth [31], filaria or schistosome infections [34, 38]. One of the major differences between the Gabonese cohorts of rural and semi-urban schoolchildren investigated in this study is the extent of exposure to parasitic infections as shown in table 3.1. Helminth infections were shown to affect H1N1-
specific antibody titres in semi-urban children as shown in figure 3.2a. In addition it was found that malaria-infected children in the rural cohort were more often low responders for H1N1 than children without malaria infection. This effect was not found for the semi-urban group, raising the possibility that malaria infection in the helminth infected group (all children in the rural area were infected with helminths) has strong suppressory effect. Alternatively, malaria treatment could have affected the outcome, although several studies did not find a negative effect of malaria treatment on outcome of immunization [172-174]; only long term treatment with chloroquine has been associated with impairment of vaccination efficacy [175]. However, malaria infection or treatment did not seem to affect the antibody titres to the influenza A (H3N2) and the influenza B strain nor the cytokine responses in this study.

Another difference noted between semi-urban and rural children was the nutritional status as shown in table 3.1. However, the differences between antibody titres could not be explained by differences in nutritional status.

The anti-A/Wyoming/3/2003 X147 (H3N2) antibody levels, which were very high before vaccination, increased further after vaccination. Interestingly, the post-vaccination antibody titres were higher in the rural children, but on day 14 after vaccination only. Thus, post vaccination antibody titres to a virus strain that had circulated recently, were not different or even higher in rural children. This is different to what is observed with post vaccination titres to strains that had not caused a recent epidemic. The mechanism behind this is not clear but may be due to differential requirement of central or effector memory T-cells to become activated [176].

In terms of cytokine responses, the semi-urban subjects show a stronger influenza-specific Th1 response as determined by increased IFN-γ and reduced IL-5 levels compared to children from a rural area. It is possible that next to the effect of helminth infections on antibody responses in rural areas, the characteristic Th2-like immune responses evoked by helminths, as well as immunological hyporesponsiveness, affect the efficacy of vaccines [35, 150], which are expected to induce a Th1 response, by skewing cytokine responses towards Th2.

Interestingly, there was a positive correlation (Pearson correlation coefficient = 0.17, p = 0.034) between levels of IFN-γ production and nutritional status (weight by age) in the urban children, but not in the rural cohort (data not shown). This correlation was not influenced by helminth infection. The influence of nutritional status on cytokine production has not been studied extensively but in a mouse model the cytokine production in response to helminth antigens was determined, finding decreased IFN-γ levels in response to zinc deficiency [177].
TNF-α responses to influenza vaccination were significantly lower in rural children. An increase in production of IL-10 and/or regulatory T cell responses could explain downregulation of Th1 as well as pro-inflammatory TNF-α responses. However, we observed an increased rather than a decreased IL-10 response to influenza vaccination in the semi-urban group. Although it is known that high levels of IL-10 are often associated with helminth infections [36, 53], little is known of the IL-10 response upon influenza vaccination. More studies on the source of IL-10 early after vaccination are needed to fully understand the detailed cellular responses.

Taken together, we report here that influenza vaccination seems to be more effective in a semi-urban than in a rural population of Gabonese schoolchildren for influenza virus strains that did not cause a recent outbreak. With respect to cytokine production upon in vitro stimulation of whole blood with the vaccine, lower IFN-γ responses are elicited in a rural population. Moreover, a better diagnosis of influenza and its dis-tangling from co-endemic diseases such as malaria can have important implications for over/mis-medication. Finally, epidemiological data on influenza as well as immune responses to vaccination will be critical for managing epidemic influenza properly in Africa.

Acknowledgements
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