Cellular and Humoral Responses to Influenza in Gabonese Children Living in Rural and Semi-Urban Areas


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Background. 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 effects of influenza vaccination in African countries could be very different from the effects in regions with less exposure to microorganisms and parasites.

Methods. To monitor the presence of influenza viruses and investigate the immunological responses to influenza vaccination, schoolchildren in semi-urban and rural regions of Gabon were studied. Influenza-specific antibody responses to the 3 strains represented in the vaccine were determined in the serum. Furthermore, cytokine responses were measured after in vitro stimulation of whole blood by influenza antigens, before and after vaccination.

Results. Prevaccination titers of antibody against H3N2 were high. At vaccination, the titers of antibody against the 3 influenza strains increased significantly. The anti-H1N1 and anti-B responses after vaccination were weaker in rural schoolchildren than in semi-urban schoolchildren. Influenza-specific cytokine responses were induced within a week, showing significantly lower interferon-γ and significantly higher interleukin-5 in the children from rural areas.

Conclusions. Prevaccination antibody levels indicated that influenza viruses circulate in Gabon. Altogether, influenza vaccination induces weaker immune responses in a rural population than in a semi-urban population of Gabonese schoolchildren.

Little information is available on influenza in Africa. Influenza-surveillance capacity is weak on this continent, and only 2 countries, South Africa and Senegal, engage in active monitoring of the infection and disease [1]. Along with the global anxiety over the spread of H5N1 avian influenza, concerns have been raised about the lack of accurate data from the African continent [1–4]. In the few sporadic studies conducted in Africa, involving virological assessments, it appears that the problem of influenza virus is probably greatly underestimated. In the areas studied, the virus was found to be present and to either circulate throughout the year or peak seasonally [2]. The reporting of influenza, based on clinical manifestations, is difficult in Africa, because 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, we find that 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 what the immunological consequences of influenza vaccination are in African popu-
lations. 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 performed well in populations living in high-income countries might perform less well in populations living in low-income countries [5, 6].

The conditions found in the urban centers of Africa (those with high- to middle-income populations) might approximate those found in the urban centers of western and industrialized countries. However, the conditions (i.e., lifestyle and exposure to infection) found in the rural areas of Africa vary widely. Many infectious diseases prevalent in Africa, particularly those present in rural areas and chronic in nature, are known to be associated with profound alternations of the innate [7–9] and the adaptive [10–12] immune system, which may affect responses to third-party antigens. For example, chronic helminth infections, which are highly prevalent in many rural areas of Africa, are known to be associated with the skewing of immune responses toward Th2 [13]. Moreover, infections with some parasitic helminths and protozoa, along with mycobacterial, malarial, and viral hepatitis infections, have been shown to induce regulatory immune responses that are characterized by production of high levels of suppressory cytokines such as transforming growth factor–β and interleukin (IL)–10 [14–18]. These suppressory molecules might affect responses to vaccines, as already has been documented for oral vaccines as well as for tetanus and bacille Calmette-Guérin (BCG) vaccination [19–21].

To our knowledge, influenza surveillance has never been conducted in Gabon, and, because the symptoms of influenza infection closely resemble those of prevalent infections such as malaria, influenza is clinically not distinguishable. Therefore, antibody and cellular responses to influenza A and B strains were analyzed in the sera of schoolchildren residing in rural and semi-urban areas of Gabon. Subsequently, the children received 1 dose each of influenza vaccine, and their 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. The children were from 2 schools, one in a semi-urban area (Lambaréné) and the other in a rural area (PK15). Their parents were informed of the study, and written informed consents were received prior to inclusion. The exclusion criteria were (1) the absence of informed consent; (2) the absence of stool, urine, or blood samples for parasitological analysis; and (3) the 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 1. The 2 cohorts of children who were included in the study were similar with respect to age and sex ratio. Nutritional status was determined by measuring age- and sex-adjusted weight, and by comparing this with age- and sex-specific values provided by the Center for Disease Control and Prevention (CDC) [22]. For comparisons, we considered well-nourished children as being those with an age- and sex-adjusted weight >90% of the median corresponding age- and sex-adjusted weight of CDC reference data.

Among the cohorts investigated in this study, 33% of the children in the semi-urban school were infected with *Schistosoma haematobium*, whereas 100% of the children in the rural school were infected. These results were obtained after 3 independent urine samples were tested. The prevalence after testing 1 urine sample was 19% in the semi-urban school and 78% in the rural schools. The prevalence of intestinal helminth infections in the
semi-urban school and in the rural school was also significantly different for both *Ascaris lumbricoides* (15% and 55%, respectively; *P* = .002) and *Trichuris trichiura* (12% and 64%, respectively; *P* < .001). These values were obtained after 2 stool samples were examined using the Kato–Katz method.

Of the 55 children, 12 were found to be positive for malaria during the study. Of these 12, 2 were infected with *Plasmodium malariae* and were treated with chloroquine (10 mg/kg/day for 2 days and 5 mg/kg/day on the third day). The remaining 10 children were found to be positive for *P. falciparum* and were treated when clinical manifestations were present. Of these 10, 2 presented symptoms of malaria infection and were treated with sulfadoxin (25 mg/kg, single dose) and pyrimethamin (1.25 mg/kg, single dose), as well as with artesunate (4 mg/kg/day for 3 days). An additional 4 children whose positivity was reported during the study were treated with sulfadoxin and pyrimethamin because of 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 a 10-μm pore size and staining the eggs with a ninhydrin solution. From children testing negative, 2 additional urine samples were analyzed, to increase the accuracy of the diagnosis. Infection with malaria parasites was determined by the staining of thick blood smears with Giemsa, as described elsewhere [23]. The presence of the intestinal helminths, *A. lumbricoides* and *T. trichiura*, was determined by analyzing the stool samples, by the use of the Kato–Katz method on 1 stool sample at the very least but on 2 stool samples whenever possible [24]. Of the 55 children, 35 were infected with 1 or more species of the helminths examined. Subsequent to the collection of blood samples at day 28 after vaccination, all children were treated with a single dose of albendazole (400 mg), and those children whose urine samples had *S. haematobium* eggs detected in them were additionally treated with a single dose of praziquantel (40 mg/kg).

**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 (Begri vac 2004/2005; Chiron Behring GmbH) and tetanus (NIPHE). Subsequently, 3 mL of heparinated blood was drawn on days 2, 4, 7, 14, and 28 after vaccination, for use in immunological assays. At all time points, the plasma was frozen and kept at −20°C, and on all days except day 28 whole-blood stimulations were performed to collect supernatants for cytokine analysis.

**Antibody measurement.** For the detection of serum antibodies against influenza virus, the haemagglutination inhibition (HI) assay was used. The HI assay was performed in duplicate according to standard methods [25, 26], by the use of turkey erythrocytes and 4 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 nonspecific anti-haemagglutinins. To ensure comparability, all serum samples, collected at different time points, were tested at the same time. For sera with titers below the detection level, a value of 5 was assigned. The virus-neutralization assay was performed according to standard procedures, as described previously [27].

**Whole-blood culture and cytokine measurement.** Cellular immunological analysis involved the culturing of whole blood; 100 μL of blood was cultured with either 100 μL of RPMI 1640 medium or influenza vaccine in medium (predetermined optimum, 1 μg of HA/mL). Supernatant was collected after 72 h of incubation and was kept at −20°C until analysis.

Levels of interferon (IFN)–γ, IL-5, IL-13, tumor necrosis factor (TNF)–α, and IL-10 were determined simultaneously in the supernatants by using the Luminex-100 cytometer (Luminex), equipped with StarStation software (Applied Cytometry Systems). Buffer-reagent kits and Luminex cytokine kits (BioSource) were used, and cytokines were measured according to the protocol, with slight modifications. In brief, assays were performed in 96-well round-bottom plates (Nunc) at room temperature. A mix of beads was incubated with a standard or with samples, or left blank, in a final volume of 50 μL for 2 h under continuous shaking. Plates were washed twice and incubated with a cocktail of biotinylated antibodies (25 μL/well) for 1 h. After removal of excess biotinylated antibodies by 2 washes, streptavidin–RPE was added and was incubated for 30 min. Subsequently, the plates were washed a final time and were analyzed 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 relevant threshold value. 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 the influenza vaccine.

**Statistical analysis.** Intergroup differences in age, sex, nutritional status, and prevalence of parasitic infections were tested using Pearson’s χ² test. Cytokine and antibody levels were not normally distributed, and the Mann–Whitney *U* test was used to analyze 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, by SPSS or Graph Pad Prism, were considered significant when the *P* value was < .05.

**RESULTS**

**Seroprevalence of antibodies to influenza viruses prior to vaccination.** Results obtained with the HI assay showed that influenza virus–specific antibodies were already present in the majority of sera before vaccination (figure 1), indicating that
influenza A viruses of the H1N1 and H3N2 subtypes and influenza B virus have been circulating in Gabon prior to the present study. Interestingly, before vaccination, the antibodies specific to the H3N2 strain A/Wyoming/3/2003 had a higher seroprevalence than did those specific to the influenza H1N1 and B strains. The presence of high titers of preexisting antibody 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 study groups, each showing similar titers: 1593 (range, 160–20,480) and 1481 (range, 80–10,240) for the HI and neutralization assays, respectively. Thus, the prevaccination 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.

Efficacy of vaccination: antibody production. The antibody responses to the 3 different influenza virus strains present in the vaccine were determined not only before but also 14 and 28 days after vaccination (figure 1). At vaccination, HI titers of antibody against the influenza A (H1N1) strain increased in both the rural and semi-urban schoolchildren, but, on day 28, they reached significantly higher levels in the semi-urban schoolchildren (figure 1A and 1D). Two groups of children could be identified, one whose anti-H1N1 titers remained at a level only slightly higher than the prevaccination values (low responders) and another that showed a prominent increase in antibody responses (high responders) (figure 1A). The latter group was significantly larger in the semi-urban population (figure 2A), resulting in the overall higher titers in the semi-urban children. Within the semi-urban schoolchildren, those with helminth infections (S. mansoni, A. lumbricoides, and/or T. trichiura) responded differently to the H1N1 antibody than did those without it. Thus, when only the group without helminth infections was compared to the rural cohort, the difference in H1N1-specific antibody titers became highly significant (P < .01), at both day 14 and day 28 after vaccination. Indeed, in terms of percentage of high responders as well, it was clear that helminth-infected children in the semi-urban area responded more like rural children (figure 2A). In addition, infections with plasmodia affected anti-H1N1 titers in rural children but not in semi-urban children. As shown in figure 2B, only a few rural children with plasmodia were high responders when anti-H1N1 antibody titers were considered.

The antibody titers specific to the influenza B strain increased at vaccination, reaching a peak at 14 days after vaccination and slightly decreasing thereafter (figure 1B and 1E). Also, for this strain, responses were significantly higher in the semi-urban children at day 28.

Compared with the prevaccination antibody levels specific to the H1N1 or B strain of the vaccine, the prevaccination levels of antibodies specific to the influenza A (H3N2) strain were very high, and they increased slightly at vaccination. At day 14, antibody levels were significantly higher in the rural schoolchildren than in the semi-urban children, but this difference was no longer significant at day 28 (figure 1C and 1F). For antibodies specific to influenza B or H3N2 strains, neither helminth nor malaria infection influenced the responses significantly.

Figure 1. Haemagglutination inhibition (HI) titers of antibody against the 3 influenza strains present in the vaccine before and 14 and 28 days after vaccination. Graphs A–C show the individual data; graphs D–F show the kinetics of the responses, with the geometric mean titers per group. (A and D) A/New Caledonia/20/99, IVR-116 (H1N1); (B and E) B/Jiangsu/10/2003; (C and F) A/Wyoming/3/2003 X147(H3N2). The filled triangles and solid lines represent semi-urban (sU) subjects; the open circles and dashed lines represent rural (R) subjects. *P < .05 (Mann-Whitney U 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 following vaccination (figure 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 schoolchildren at day 4 (figure 3A). The magnitude of the influenza-specific IL-10 response was significantly higher in the semi-urban schoolchildren. The TNF-α, IFN-γ, and IL-5 responses peaked at day 7 (figure 3B). The TNF-α levels were also significantly higher in the semi-urban schoolchildren; the peak at day 7 was almost absent for the rural schoolchildren (figure 3B). Starting at day 7 after vaccination, IFN-γ responses were significantly higher in semi-urban children (figure 3C). In contrast, the rural schoolchildren showed a more Th2-skewed response, because they produced less IFN-γ and higher levels of IL-5 than did the semi-urban schoolchildren (figure 3D). Interestingly, IL-5 responses to influenza were higher in the helminth-infected semi-urban schoolchildren than in the non-infected semi-urban children (insert in figure 3D), indicating that the Th2 skewing that is more prominent in the rural schoolchildren may, at least partly, be the result of the immune-modulating effect of helminths. Malaria infection did not affect cytokine responses to influenza antigens.

Figure 2. Influence of helminth (A) and malaria (B) infection on antibody responses to influenza A (H1N1). As shown in figure 1, antibody responses to the H1N1 strain were either low or high. A, Percentage of high responders shown for the total group of semi-urban (SU) children (both infected and uninfected; gray bars), for the semi-urban children without any helminth infection (SU−; white bars), for the semi-urban children with a helminth (schistosome, ascaris, and/or trichuris) infection (SU+; black bars), and for the rural (R; gray bars) children (all infected). B, Percentage of high responders for the total group of semi-urban (SU) and rural (R) children (both infected and uninfected; gray bars) and the semi-urban and rural children without malaria infection (SU− and R−; white bars), and for the semi-urban and rural children with malaria infection (SU+ and R+; black bars). *P < .05 (χ² test); **P < .005 (χ² test).
Figure 3. Kinetics of influenza-specific cytokine production determined by ex vivo stimulation of whole blood before and after influenza vaccination. The mean values and the 95% confidence interval of interleukin (IL)-10 (A), tumor necrosis factor (TNF)–α (B), interferon (IFN)–γ (C), and IL-5 (D) are shown. The filled triangles and solid lines represent the semi-urban (sU) cohort; the open circles and dashed lines represent the rural (R) cohort. *P < 0.05 (Mann-Whitney U test); **P < 0.005 (Mann-Whitney U test). The inset in graph D shows the IL-5 responses of the semi-urban children with helminth infection (sU+) and of the semi-urban children without helminth (S. mansoni, A lumbricoides, and/or T. trichiura) infection (sU−).

DISCUSSION

The present study indicates that influenza A and B viruses circulate in Gabon and that the presence of high levels of A/Wyoming/3/2003 X147 (H3N2)–specific antibodies before vaccination, as determined by an HI assay and a neutralization assay, provides us with 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 [28], in Madagascar in 2002 [2], and in the Democratic Republic of Congo in 2002 [2]. In addition, influenza B viruses have been reported to be circulating in South Africa, Madagascar, and Kenya [29, 30].

In some of these studies, a mortality of at least 3% of cases was reported, because of influenza A/Panama/2007/97-like (H3N2) infection, indicating that influenza can have a high impact in African countries as well [2]. 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 where high fever is often treated with antimalarials. Thus, influenza infections in these areas can lead to considerable malaria overmedication, on the one hand, and to an overestimation of malaria deaths, on the other.

In vaccine-induced antibody responses, considerable differences between rural and semi-urban schoolchildren were observed. The responses to H1N1 and influenza B strains were higher in the schoolchildren from the semi-urban area. Because the prevaccination titers did not differ between semi-urban and rural schoolchildren, it is unlikely that differences in exposure to influenza could explain these results. Why the schoolchildren in rural areas would respond differently from those in semi-urban areas has yet to be fully investigated. However, some vaccines, such as those for BCG or tetanus, which were shown to be effective in nontropical countries, were found to induce a weak response in tropical countries, and this has been associated with the presence of helminth infections [5, 31]. 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 the cholera vaccine [19], the BCG vaccine [32], and tetanus toxoid vaccine [20, 33] suggested to us that immune skewing in response to vaccines is affected by pre-existing helminth infections. These studies were performed in different areas and considered intestinal helminth [19, 32], falciparum, or schistosome infections [33, 34]. One of the major differences between the Gabonese rural cohort and the Gabonese semi-urban cohort investigated in the present study is the extent of exposure to parasitic infections, as shown in table 1. Helminth infections were shown to affect H1N1-specific antibody titers in semi-urban schoolchildren, as shown in figure 2A. In addition, it was found that malaria-infected schoolchildren in the rural cohort were more often low responders to H1N1 than were schoolchildren without malaria infection. This effect was not found for the semi-urban cohort, raising the possibility that malaria infection of the helminth-infected group (all schoolchildren in the rural area were infected with helminths) has a strong suppressory effect. Alternatively, malaria treatment could have affected the outcome, although several studies did not find that malaria treatment has a negative effect on outcome of immunization [35–37]; only long-term treatment with chloroquine has been associated with impairment of vaccination efficacy [38]. However, malaria infection or treatment did not seem to affect the titers of antibody against the influenza A (H3N2) or the influenza B strain, nor did it affect the cytokine responses in the present study.

Another difference noted between semi-urban and rural schoolchildren was nutritional status, as shown in table 1. However, the differences between antibody titers 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 postvaccination antibody titers were higher in the rural schoolchildren, but at only day 14 after vaccination. Thus, postvaccination titers of antibody against a virus strain that had circulated recently were no different from, or even higher than, those found in urban schoolchildren. This is different from what is observed with postvaccination titers of antibody against strains that have not caused a recent epidemic.
The mechanism behind this is not clear, but it may be based on the differential requirement for activation of central or effector memory T cells [39].

In terms of cytokine responses, the semi-urban schoolchildren show a stronger influenza-specific Th1 response than do the rural schoolchildren, as determined by their increased IFN-γ levels and their reduced IL-5 levels. It is possible that the effects that helminth infections have on the antibody responses in rural areas and on the characteristic Th2-like immune responses (as well as on immunological hyporesponsiveness) will affect the efficacy of vaccines [5, 31], which are expected to induce a Th1 response, by skewing cytokine responses toward Th2.

Interestingly, there was a positive correlation (Pearson correlation coefficient, 0.17; \( P = .034 \)) between levels of IFN-γ production and nutritional status (weight by age) in the urban schoolchildren but not in the rural schoolchildren (data not shown). This correlation was not influenced by helminth infection. The influence that nutritional status has on cytokine production has not been studied extensively, but, in a mouse model, the cytokine production in response to helminth antigens was determined by Shi et al., who found decreased IFN-γ levels in response to zinc deficiency [40].

TNF-α responses to influenza vaccination were significantly lower in rural schoolchildren. An increase in production of IL-10 and/or regulatory T cell responses could explain the down-regulation of Th1 as well as the proinflammatory TNF-α responses. However, we observed an increased rather than decreased IL-10 response to influenza vaccination in the semi-urban schoolchildren. Although it is known that high levels of IL-10 are often associated with helminth infection [20, 41], little is known about the IL-10 response at influenza vaccination. To fully understand the detailed cellular responses, more studies on the source of IL-10 soon after vaccination are needed.

In conclusion, we report here that vaccination against the influenza virus strains that have not caused a recent outbreak seems to be more effective in a semi-urban population than in a rural population of Gabonese schoolchildren. With respect to cytokine production at in vitro stimulation of whole blood by the vaccine, lower IFN-γ responses are elicited in a rural population. Moreover, a better diagnosis of influenza, as well as disentangling it from coendemic diseases such as malaria, can have important implications for over/mismedication. Finally, epidemiological data on influenza, as well as on immune responses to vaccination, will be critical for proper management of influenza epidemics in Africa.

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