Pediatric HIV-1-infection: perspectives on vaccination strategies and immune reconstitution during long-term antiretroviral therapy
Bekker, V.
Persistent humoral immune defect in HAART-treated HIV-1-infected children: Loss of specific antibodies against attenuated vaccine strains and natural viral infection

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Hans Zaaijer
Taco W. Kuijpers
Abstract

Context: In the pre-HAART era a loss of specific antibodies was seen.

Objective: To describe the loss of specific antibodies during treatment with HAART.

Methods: In a prospective single-center cohort study on 59 HIV-1-infected children, we investigated the long-term effect of HAART on the titers and course of specific antibodies against measles, mumps and rubella (MMR) vaccine strains compared to wild-type varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein Barr virus (EBV).

Results: During HAART, age-adjusted CD4+ T cells and B cells increased, whereas total immunoglobulin levels declined. Although pre-immunized before the start of HAART, only 24 (43%) had antibodies against all three MMR components. Antibodies against each MMR component were lost in 14 (40%), 11 (38%), and 5 (11%) children seropositive at baseline. We also observed loss of VZV-IgG in 7 of 34 (21%), CMV-IgG in 3 of 45 (7%), but none of 53 EBV-seropositive children, respectively. During HAART, primary vaccination in 3 patients and 15 revaccinations in those with negative serology demonstrated incomplete seroconversion.

Conclusions: Humoral reactivity in HIV-1-infected children remains abnormal during HAART. Despite immune reconstitution, antibodies against live-attenuated vaccine and wild-type natural virus strains disappear over time in up to 40% of HIV-1-infected children.
Introduction

HIV-1-infection causes a progressive immune deficiency due to the loss of CD4\(^+\) T cells. Consequently several abnormalities in the B-cell compartment occur. These include a progressive decline in total CD19\(^+\) B-cells, with polyclonal hyperimmunoglobulinemia [1,2], impaired reactivity to immunization [3] and loss of specific antibodies [4]. After successful treatment with HAART, CD4\(^+\) T cell count increases and a reduction of the hyperimmunoglobulinemia is seen [5,6]. During the first 12 weeks of HAART an increase in absolute B cell count is found in most patients [7].

The function of the B-cell compartment is to produce neutralizing antibodies and to maintain serologic memory after primary infection. After MMR vaccination lifelong immunity is maintained in healthy individuals. Before the era of HAART it was found that in HIV-1-infected children the initial response to vaccination is weaker and transient compared to healthy children [3,4]. However, the long-term effect of HAART on the B-cell count and function in children is unclear. Vaccination has lead to a decline in the incidence of measles, mumps and rubella cases in otherwise healthy children, even though outbreaks still occur. MMR coverage as well as seroprevalence in the Netherlands is high at 94% with further increase after routine booster immunization at 9 years of age [8,9].

In this study we investigated whether the B-cell memory was restored during the treatment with HAART. As a surrogate marker for B-cell memory we determined whether the loss of specific antibodies against the components of the MMR vaccination, would be influenced by the treatment with HAART. We compared the MMR serology with the humoral response against natural viral pathogens, i.e. VZV, CMV and EBV, and tested whether any loss of specific antibodies would continue despite treatment with HAART and consecutive immune reconstitution and, if so, is this only seen against live-attenuated viruses or against wild-type viruses as well.

Materials and Methods

Patients

The Pediatric Amsterdam Cohort on HIV-1 (PEACH) consists of children and young adolescents under the age of 18. Since 1997, patients receive HAART consisting of 2 nucleoside-analogue reverse-transcriptase inhibitors (NRTI) and at least 1 protease inhibitor (PI) or a non-NRTI. For the present study we selected all children who had started therapy between 1997 and 2005. The Medical Ethical Committee approved the study for serotyping and (re)vaccination. Caregivers gave written informed consent.

Blood samples

During the routine blood tests, antibody levels against MMR were annually checked and children were (re)immunized when indicated. The Dutch national vaccination program
(Rijksvaccinatieprogramma (RVP)) includes MMR vaccination at the age of 14 months and 9 years of age [9].

**Lymphocytes, T cell subsets, and T cell proliferation**

Numbers of B cells (CD19+) and T cells (CD3+) and subsets (CD3+CD4+, CD3+CD8+) were determined by standard FACScan procedures, as described before in detail [8].

Age correction for CD4+ and CD8+ T cells and CD19+ B cells was done by dividing the counts by the mean of an age matched healthy control group [8].

**Plasma HIV-1 RNA determination**

Plasma HIV-1 RNA concentration was determined either using Nuclisens HIV-1 RNA QT (Biomérieux, Boxtel, the Netherlands) or Versant HIV-1 RNA 3.0 (Bayer, Tarrytown, NY, USA). All tests were performed according to the instructions of the manufacturers. Due to a different lower limit of detection in the 2 assays, all pVL below 400 copies/mL were considered as undetectable.

**Measles, mumps, rubella, VZV, CMV and EBV serology**

Specific antibodies to measles and mumps were determined by enzyme immunoassay (EIA) (Virotech, Rüsselsheim, Germany). Serology of measles and mumps is expressed as Arbitrary Units (AU)/mL. An antibody amount of 9.0 AU/mL or more was regarded as positive. Specific antibodies to rubella were determined by Axsym (Abbott, Illinois, USA), expressed as International Units (IU)/mL. An antibody amount of 10.0 IU/mL was regarded as positive. Specific antibodies to VZV were determined by Vidas immunoassay (Biomerieux, Lyon, France). The test values of this assay were converted to IU/ml using the conversion factor as determined by van der Zwet et al. [10]. An antibody amount of 0.139 IU/mL or more was regarded as positive. CMV antibodies were defined by Axsym assays (Abbott park, Illinois, USA), expressed as AU/mL. An antibody amount of 15 AU/mL or more was regarded as positive. Specific IgG against the viral capsid antigen (VCA) and against nuclear antigen of EBV (EBNA) was determined qualitatively using respectively the ‘anti-EBV VCA IgG ELISA’ and the ‘anti-EBV EBNA IgG ELISA’ of Biotest (Dreieich, Germany). All tests were performed following the instructions of the manufacturers.

Seropositivity was defined by the presence of a positive specific IgG after the age of 18 months to exclude any confounding contribution of maternal antibodies in the very young. Serological tests within 3 months after the administration of blood products were excluded from the analyses.

**Statistical analyses**

Statistical analyses were performed using SPSS for Windows version 11.5 (SPSS, Chicago, USA). All p-values were two-tailed. P-values smaller than 0.05 were considered statistically significant. Continuous data were analyzed using a Mann-Whitney-U test. Categorical data were compared with a Fisher’s exact test. Correlation was tested using the Spearman’s correlation test. The mean age-adjusted CD4+ T cells, CD19+ B cells and total IgG were modeled using a mixed model incorporating repeated measurements. This model handles missing data adequately by estimating the outcome using a ‘first
order autoregressive’ structure. Differences in these estimates between different levels of the variable were tested for significance using t-statistics.

**Results**

Since 1997, 59 children started treatment with HAART at a median age of 4.3 years, 49% of the children were male, 24 presented with a CDC-C classification. Median follow-up since the start of HAART at the time of analysis was 205 weeks (Table 1).

**Baseline serology**

Prior to the start of HAART, only 24 (43%) children had positive antibody titers against all 3 components of the MMR vaccine. Whereas officially reported to be immunized — either according to the national vaccination program or upon entry in the health care system — 8 (13%) of the included children starting with antiretroviral medication had no detectable antibodies against any of the MMR components and 24 (41%) children had a discordant response against 1 or 2 of the components in the vaccine (Table 1). Of the various components, 35 (63%) children had specific antibodies against measles, 29 (52%) against mumps and 45 (80%) against rubella (Table 2).

**Table 1. Clinical characteristics of the patients at start HAART**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N=59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, median (IQR))</td>
<td>4.3 (1.4-8.8)</td>
</tr>
<tr>
<td>Male sex (n (%))</td>
<td>29 (49%)</td>
</tr>
<tr>
<td>CDC-classification (n)</td>
<td></td>
</tr>
<tr>
<td>Non-C</td>
<td>35</td>
</tr>
<tr>
<td>C</td>
<td>24</td>
</tr>
<tr>
<td>Total follow-up on HAART (median weeks (IQR))</td>
<td>205 (124-359)</td>
</tr>
<tr>
<td>Number of positive MMR components in previously vaccinated children (Total = 56) (n (%))</td>
<td></td>
</tr>
<tr>
<td>3 positive</td>
<td>24 (43%)</td>
</tr>
<tr>
<td>1 or 2 positive</td>
<td>24 (41%)</td>
</tr>
<tr>
<td>0 positive</td>
<td>8 (13%)</td>
</tr>
</tbody>
</table>

IQR: Inter quartile range ^ Clinical categories as defined by the US Centers for Disease Control and Prevention [29].

**Table 2. Immunity at baseline against life-attenuated measles, mumps, rubella and the natural VZV, CMV and EBV infection and loss of specific antibody titers during the treatment with HAART.**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Lost</th>
<th>Median time to loss in weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive, N</td>
<td>Negative, N</td>
<td>N (%)</td>
</tr>
<tr>
<td>measles</td>
<td>35</td>
<td>21</td>
<td>14 (40%)</td>
</tr>
<tr>
<td>mumps</td>
<td>29</td>
<td>27</td>
<td>11 (38%)</td>
</tr>
<tr>
<td>rubella</td>
<td>45</td>
<td>11</td>
<td>5 (11%)</td>
</tr>
<tr>
<td>VZV</td>
<td>34</td>
<td>25</td>
<td>7 (21%)</td>
</tr>
<tr>
<td>CMV</td>
<td>45</td>
<td>14</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>EBV (VCA)</td>
<td>53</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>
Virologic and immunologic response to antiretroviral therapy

Upon treatment with HAART, the HIV-1 replication is suppressed in most of the treated patients and immunologic recovery occurred in all. After 48, 96, 144 and 192 weeks, 40 of 56 (71%), 35 of 53 (66%), 26 of 45 (58%) and 18 of 35 (51%) children, respectively, had an undetectable plasma viral load of HIV-1 in on-treatment analyses. During 192 weeks of treatment with HAART total-IgG declined compared to baseline (P<0.001) (Figure 1A). The decline was most pronounced in the first 48 weeks, stabilizing thereafter. Total-IgM also declined during 192 weeks on HAART (p<0.001). Total-IgA showed a non-significant decline (p=0.067). On the other hand, age-adjusted CD4⁰ T cell and CD19⁺ B-cell numbers increased over 192 weeks on HAART (Figure 1B; both p<0.001), although the increase in B cells was more gradual. When defined as a normalization of the in vitro lymphoproliferative T cell response upon stimulation by the combination of CD3 and CD28 MoAbs [11], functional immune reconstitution was complete within 4-6 weeks after start of HAART, irrespective of the plasma viral load at start of treatment or during follow-up or age-adjusted CD4⁰ T cell count (data not shown).

Serology during treatment with antiviral therapy

Of the 35 children with specific antibodies against measles prior to the start of HAART, 14 (40%) lost their specific antibodies over time; mumps antibodies were lost by 11 (38%) of 29 and rubella antibodies by 5 (11%) of 45 the seropositive children (Table 2). The decline in total-IgG and the decline in specific antibodies against MMR were not correlated (r=0.9, p=0.7; r=0.3, p=0.13; r=0.2, p=0.24, respectively). The decline in total-IgG and the decline in specific antibodies therefore seemed unrelated to each other.

After numerical and functional immune reconstitution as a consequence of HAART, the loss of specific antibodies was not anticipated. Several variables were tested for a correlation with the defective humoral B cell memory response; age-adjusted CD19⁺ B cell and CD4⁺ T helper cell counts, HIV load at start of HAART, age at start of HAART, mode of transmission, and gender. None of these variables correlated with the loss of specific antibodies against all 3 MMR components taken together. In contrast to the analysis for the MMR components combined, children who lost their measles antibodies were younger than children with sustained antibodies (median 2.5 vs. 6.2
years (p=0.04), when these components were analyzed separately. With the rather surprising exception of measles antibodies, almost all HIV-1-infected children who showed a loss of specific antibodies during follow-up against mumps and rubella already demonstrated lower antibody titers at baseline. Only in case of rubella this difference in antibody titers at baseline reached significance, comparing the HIV-1-infected children who lost these specific antibodies with those who sustained their specific antibody titer (median 18.1 vs. 73.9 IU/mL (p= 0.006)) (Figure 2A-C). These data suggest a weaker response upon primary vaccination before the start of HAART.

Fig. 2. Baseline serology in children who lose specific antibodies and children with sustained antibodies. In A. Measles antibodies at baseline (Arbitrary Units). In B. Mumps antibodies (Arbitrary Units). In C. Rubella antibodies (International Units). Rubella titers at baseline were lower in children who lost these specific antibodies than in children who had sustained antibodies (p= 0.005). In D. CMV antibodies (Arbitrary Units). CMV titers at baseline were lower in children who lost these specific antibodies than in children who had sustained antibodies (p<0.001). In E. VZV antibodies (International Units) at baseline.
Looking at vertical (n=52) and sexual (n=7) infection separately, one of the sexually infected adolescents was found to lose antibodies against a single component of the MMR vaccine during follow-up whereas 26 vertically infected children lost antibodies against at least one component (p=0.22).

Responses to vaccination during the treatment with HAART.
During this prospective study 3 additional HIV-1-infected children were vaccinated for the first time at the age of 14 months as part of their routine vaccination (RVP). HAART had been started prior to MMR vaccination and at vaccination immunity was already reconstituted in these 3 children. All responded well and showed complete seroconversion against all three MMR components. However, one child lost specific antibodies against mumps and another lost both mumps and measles antibodies during continuous treatment with HAART within the following 177 to 244 weeks after vaccination, respectively.

In contrast, 15 children received a second MMR immunization during the treatment with HAART because of the lack of specific antibodies against one or more components after their primary vaccination. Revaccination took place prior to the planned standard vaccination according to the RVP at 9 years of age. Characteristics of these children at the time of vaccination are shown in Table 3. Median age of the children was 7.3 years; median CD4+ T cell count prior to vaccination was 1050 cells/μL (interquartile range (IQR) 830-1190). For these, the median time between the start with treatment and the date of vaccination was 119 weeks. Of the 10 children negative for the measles component

<table>
<thead>
<tr>
<th>N</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median (IQR))</td>
<td>7.3 (4.2-9.0)</td>
</tr>
<tr>
<td>CD4+ T cell count (median cells/μL (IQR))</td>
<td>1050 (830-1190)</td>
</tr>
<tr>
<td>Weeks after start HAART (median (IQR))</td>
<td>119 (47-203)</td>
</tr>
<tr>
<td>Weeks between vaccination and serology testing (median (IQR))</td>
<td>48 (19-93)</td>
</tr>
</tbody>
</table>

IQR: Inter quartile range

Table 4. Effect of re-immunization against MMR during HAART.

<table>
<thead>
<tr>
<th></th>
<th>Serology at start HAART, after first vaccination</th>
<th>Re-immunization</th>
<th>Serology after re-immunization during HAART</th>
</tr>
</thead>
<tbody>
<tr>
<td>measles</td>
<td>Positive (n=5)</td>
<td></td>
<td>Positive (n=5)</td>
</tr>
<tr>
<td></td>
<td>Negative (n=10)</td>
<td></td>
<td>Negative (n=6)</td>
</tr>
<tr>
<td></td>
<td>Positive (n=6)</td>
<td></td>
<td>Positive (n=6)</td>
</tr>
<tr>
<td></td>
<td>Negative (n=9)</td>
<td></td>
<td>Positive (n=8)</td>
</tr>
<tr>
<td>mumps</td>
<td>Positive (n=10)</td>
<td></td>
<td>Positive (n=9)</td>
</tr>
<tr>
<td></td>
<td>Negative (n=5)</td>
<td></td>
<td>Negative (n=1)</td>
</tr>
<tr>
<td>rubella</td>
<td>Positive (n=10)</td>
<td></td>
<td>Positive (n=4)</td>
</tr>
<tr>
<td></td>
<td>Negative (n=5)</td>
<td></td>
<td>Negative (n=1)</td>
</tr>
</tbody>
</table>

Table 3. Characteristics of children revaccinated against MMR during HAART.
Longitudinal analyses of serology against CMV, EBV and VZV.

We compared the loss of specific antibodies against the live-attenuated vaccine strains with the humoral response against natural viral pathogens, i.e. VZV, CMV and EBV. Specific IgG antibodies against VZV were detected at start with HAART in 34 children (Table 2). During the treatment with HAART 7 children (21%) no longer showed detectable levels of VZV antibodies upon follow-up. CMV antibodies were detected in 45 children at baseline, which were no longer detectable in 3 (7%) children. In contrast, none of the 53 children with EBV VCA antibodies at baseline lost these antibodies during treatment with HAART. CMV antibody titers were significantly lower at baseline in HIV-infected children who lost these specific antibodies compared to the children who sufficiently sustained the specific antibody level (median 62.2 vs. 250 AU/mL (p<0.001)) (Figure 2 D and E), but did not differ in age. In contrast, children who lost their VZV antibodies were relatively younger than children with sustained antibodies (median 4.1 vs. 5.3 years (p= 0.04)).

Discussion

In the present study we evaluated the serologic responses against the live attenuated viral strains of the MMR vaccine as well as naturally occurring common viral infections in HIV-1 infected children (i.e. VZV, CMV, and EBV). We demonstrate that only 43% of the HIV-infected children had specific antibodies detectable against all three MMR components at baseline. During treatment with HAART, about 40% of the children lost specific MMR-antibodies...
present at baseline despite immune reconstitution. Although less frequently, specific antibodies against naturally occurring VZV and CMV were also lost in 21% and 7% of the children, respectively.

The discordant MMR responses prior to antiretroviral treatment are in line with the findings in the pre-HAART era [3]. However, the low rate of seropositivity and the loss of specific antibodies is in clear contrast with our previous cross-sectional study in over 200 healthy children of mixed racial background, in which we found that more than 90% of the children had specific antibodies against all three MMR components above the age of 3 years, further increasing to 100% after routine re-immunization at 9 years of age [8]. In a longitudinal study in more than 350 healthy children, specific antibodies against measles and rubella, when regularly measured over a period of 12 and 15 years, respectively, remained positive in 99-100% of the children [12,13]. Mumps antibodies were positive in 86% of the same after 9 years of follow-up [14]. These longitudinal data in pediatric controls support the relevance of the increased loss of specific antibodies in our cohort of HAART-treated children, irrespective of the immune reconstitution and normalized age-adjusted CD4+ T cell counts upon HAART. Age was not found to correlate with the loss of specific antibodies in our cohort. Therefore a relation between time since vaccination and start of HAART is not likely.

While being on HAART, HIV-1-infected children also showed vaccine failure upon boosting. Revaccination resulted in seroconversion in only 60-85% of the children. Also in the pre-HAART era it was found that HIV-1-infected children demonstrated both primary vaccine failure and loss of antibodies after an initial response [4]. In a recent study of 18 children treated with HAART without evidence of measles antibodies at baseline after previous immunization, the seroconversion rate was 83% after re-immunization [15]. Eleven children had antibody levels tested after 1 year of follow-up of whom only 8 (73%) showed sustained antibody levels. We further extend these data, showing that this loss of antibodies is not unique to measles [15-17], and is observed for wild-type viruses as well. In total, 27 children (46%) lost antibodies against at least one of the measles, mumps, rubella, VZV or CMV strains. Seventeen children lost antibodies against one, 5 against 2, 4 against 3 and 1 child lost specific antibodies against 4 of the above-mentioned viruses.

Loss of specific antibodies determines an increased risk of serious infection. In areas with a high HIV-1 prevalence, HIV-1-infected children have high rates of mortality attributable to measles. Although the risk of waning specific antibody levels in communities with a high coverage rate of vaccination may be limited due to herd immunity, outbreaks still occur in the developed world [18]. Waning immunity can also be a potential problem for HIV-1-infected pregnant women who come in contact with rubella, VZV or CMV. Furthermore, in a study cohort of 1832 HIV-1-infected women an increased risk of shingles was found, irrespective of HAART [19]. After primary infection CMV and EBV persist for life and go into a stage of latency in epithelial tissue and immune cells from where they may reactivate unnoticed [20]. Whereas humoral immunity against EBV was not affected, CMV-specific antibodies were unexpectedly lost in 3 children.
Since the decline in total-IgG during HAART and the decline in MMR-specific antibodies are not correlated, these phenomena are unrelated and the consequence of different mechanisms. Hyper-IgG before start of HAART could be produced by low-avidity polyclonal B-cell reactivity, while specific memory B-cells are exhausted due to inappropriate stimulation as well as the loss of antigen-specific CD4+ T helper cells [21,22]. During HAART, an increase in total peripheral blood B cell counts was observed without any change in the relative memory B cell (IgD- CD27+) fraction [data not shown]. However, B cell memory may not recover functionally, as indicated by our specific antibody data and supported by reports on defective B cell function in vitro [5,23,24]. Since plasma cells originate from antigen-triggered B-cells, a shorter life span of plasma cells in HIV-infected children may result in the decline in specific antibody [25], which is a completely uninvestigated issue. Similar findings on vaccination responses and the loss of specific antibodies have been reported after chemotherapy and bone marrow transplantation [26,27]. The nature of the immune reconstitution is of course different in these settings.

Two important issues warrant further study. First, VZV-specific cell-mediated immunity has been shown to be unresponsive to HAART [28]. Thus, waning immunity against VZV may be both humoral and cellular in nature. Although beyond the scope of this study, proliferation tests against MMR and herpes viral antigens over time should give more insight in the biology of the loss of specific antibodies. Secondly, the order of HIV infection and prior vaccination status may be important for the induction of long-lasting B cell memory. Studies in adults should be performed to find evidence for the impact of “time of infection”.

A shortcoming of this study is the lack of a control group of healthy children followed prospectively. Such a control group was considered unethical for the longitudinal nature and multiple venapunctures in healthy children required for direct comparisons. Although MMR serology data in longitudinal cohorts of healthy children were reported previously [8], showing that 86-100% of the children maintained specific antibodies over a period of 9-12 years when measured every 1-3 years, this was at the time of vaccine introduction and the role of boosting by (sub clinical) exposure to the wild-type virus disease is difficult to assess. Using the same assays, our own cross-sectional study in healthy children (admitted to the hospital for unrelated minor trauma or elective surgery) indicated good levels of seroprotection [8]. MMR vaccination is part of the standard vaccination program in the Netherlands covering more than 95% of all children since its national introduction in 1987. Only rarely, local outbreaks confined to small regions occur in the Netherlands, which are neither considered to have any impact on the general population nor on the serology against all three MMR components.

The loss of specific antibodies as observed in more than 40% of HIV-infected children does not seem to occur to the same extent in healthy children. It still remains unclear whether the loss of specific antibodies poses a real threat to HAART-treated children. Regular testing for the loss of specific antibodies in HIV-infected children seems
mandatory. Repeated immunization may further support antigen-specific CD4\(^+\) T cell help in maintaining memory B and plasma cell function, irrespective of HAART.

**Acknowledgement**

We would like to thank E. le Poole and A. van der Plas for their support and care for the children and drs. R.A.W. van Lier and H. Schuitemaker for critically reading and commenting the manuscript.

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