High susceptibility for enterovirus infection and virus excretion features in Tunisian patients with primary immunodeficiencies.

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To estimate the susceptibility to enterovirus infection and the frequency of long-term poliovirus excreters in Tunisian patients with primary immunodeficiencies (PIDs), enteroviruses were assessed in stool specimens of 82 patients with humoral, combined, and other PIDs. Isolated viruses were typed and intratyped by standard molecular techniques, and the whole VP1 region of poliovirus isolates was sequenced. Polioviruses were detected in 6 patients; all isolates were vaccine related. Five patients rapidly stopped excretion; one excreted a poliovirus type 1 isolate for several months, and the isolate accumulated up to 14 mutations in the VP1 region. Nonpolio enteroviruses were identified in 6 patients; 4 of them kept excreting the same strain for more than 6 months. The rate of enterovirus infection was 13.4% of the PID patients and 20.7% of those with an IgG defect; it greatly exceeded the rates generally found in Tunisian supposed-immunocompetent individuals (4.1% during the study period; P = 0.001 and P < 0.0001, respectively). Interestingly, patients with combined immunodeficiencies were at a higher risk for enterovirus infection than those with an exclusively B cell defect. A major histocompatibility complex (MHC) class II antigen expression defect was found in 54% of enterovirus-positive patients and in the unique long-term poliovirus excreter. The study results also suggest that substitutive immunoglobulin therapy may help clearance of a poliovirus infection and that most PID patients have the ability to stop poliovirus excretion within a limited period. However, the high susceptibility of these patients to enterovirus infection reinforces the need for enhanced surveillance of these patients until the use of oral poliovirus vaccine (OPV) is stopped.

The oral poliovirus vaccine (OPV), containing live attenuated strains from the three poliovirus serotypes, was developed in the 1950s by Albert Sabin, in parallel with development of the Salk inactivated polio vaccine. Both vaccines are very effective in preventing poliomyelitis through durable humoral immunity, but OPV has the advantages of ease of administration and spread to unvaccinated contacts of vaccine recipients, increasing the impact of vaccination beyond those immunized (21). However, the genetic stability of OPV strains has always been one of the main concerns. Since its development, it has been established that OPV can cause vaccine-associated paralytic poliomyelitis in vaccinees. In the current, late steps of the poliomyelitis eradication program, vaccine-derived polioviruses (VDPVs) are among the major concerns for the final success of the program (22, 29). VDPVs are viruses that have genetically evolved from the parental Sabin strains during replication in the human gut and person-to-person transmission. Considering the poliovirus variation rate of 1 to 2% per year (9), the extent of sequence divergence from the parental Sabin virus in the VP1 capsid gene is used to estimate the duration of replication (28). VDPVs are those strains showing a more than 1% sequence difference in the VP1 gene (30). This genetic evolution sometimes generates virus strains that are similar to wild viruses in terms of neurovirulence and/or transmissibility among humans. Sporadic cases and outbreaks of poliomyelitis due to VDPVs have been reported in different regions of the world: Poland in 1968, Egypt in 1988 to 1998, Hispaniola in 2000-2001, the Philippines in 2001, Madagascar in 2002 and 2005, China in 2004, Indonesia in 2005, Cambodia in 2005-2006, Nigeria in 2005 to 2010, Niger in 2006, Myanmar in 2006-2007, the Democratic Republic of Congo in 2008 to 2010, Guinea in 2009, and India and Ethiopia in 2009-2010 (23).

Based on programmatic criteria, VDPVs were classified into different categories; the most important are circulating VDPVs (cVDPVs), showing evidence of person-to-person transmission, and immunodeficient VDPVs (iVDPVs), isolated from patients with defects in antibody production. In fact, immunodeficient patients exposed to OPV may excrete poliovirus strains for many months or years (5, 13, 14, 20); the excreted viruses are frequently highly divergent from the parental OPV strain. Thus, these patients represent a potential reservoir for potentially neurovirulent polioviruses after eradication of wild polioviruses. Until recently, chronic iVDPV excreters were detected in many countries but mainly in developed ones. It is difficult to estimate the number of iVDPV excreters worldwide in the absence of systematic screening of immunodeficient patients for enterovirus excretion, especially in the developing world.

In Tunisia, the national immunization program has used OPV since 1979; the vaccine coverage rate is maintained over 90% since the early 1990s. The national surveillance of acute flaccid paralysis (AFP) cases started in 1991, and national immunization days were conducted from 1995 to 1997. The last paralytic cases due to wild polioviruses occurred in 1992, and the last wild isolate was detected in 1994. Ever since, all poliovirus isolates detected through
the national surveillance program have been vaccine related (2, 10).

The present work was performed in line with the recent recommendations of the World Health Organization (WHO) to conduct studies to better estimate the frequency of poliovirus excreters among immunodeficient patients, the dynamics of virus excretion, and the genetic evolution of excreted virus strains in different countries with different socioeconomic levels and different epidemiological and sanitary situations.

**MATERIALS AND METHODS**

**Specimen collection and protocol design.** A total of 82 patients with various primary immunodeficiencies (PIDs) were enrolled in the study. They originated with almost all the governorates of Tunisia. Their PID diagnosis was confirmed in the Laboratory of Immunology in the Pasteur Institute of Tunis, where advanced biological investigation of such patients is centralized. Samples were taken from 44 patients at the National Center for Bone Marrow Transplantation, where medical services are provided to patients with various PIDs. The samples were taken from hospitalized patients, or during regular follow-up medical visits for their immunodeficiency, from March 2006 to August 2010. Two initial stool samples were obtained from 21 patients; the remaining 23 patients had only one initial sample taken. Thirty-eight patients were sampled as part of a WHO collaborative between July 2009 and March 2010. The patients with previously known PID were visited at their home residences, and two initial samples were collected 24 h apart. The study protocol was approved by the Ethical Review Board of the Pasteur Institute of Tunis and the Ethical Review Board of WHO (Geneva, Switzerland). Written parental consent was obtained for all patients included in the study. Enterovirus-positive patients were followed up with serial subsequent stool samples, up to March 2011; 57 follow-up stool specimens were collected. Thus, a total of 198 stool samples were collected and investigated in the present work.

**Study population and type of PID.** The study population included 52 males and 30 females, ages 5 months to 33 years, diagnosed with 2 categories of PIDs: (i) humoral or combined immunodeficiencies (agammaglobulinemia, n = 11; common variable immunodeficiency disorders [CVID], n = 14; hyper-IgM syndrome, n = 8; MHC class II deficiency, n = 14) and (ii) other immunodeficiencies (X-linked chronic granulomatous disease [CGD], n = 8; disseminated Mycobacterium bovis BCG infection, n = 3; ataxia-telangiectasia [AT], n = 13; Buckley syndrome, n = 2; autoimmune lymphoproliferative syndrome [ALPS], n = 1; Chediak-Higashi syndrome, n = 1; familial hemophagocytic lymphohistiocytosis [FHL], n = 1). Fifty-eight patients had low IgG levels when their PID was diagnosed, and most of them were under substitutive intravenous immunoglobulin (IVIG) therapy when their initial stool sample was collected. Eight patients had bone marrow transplantation.

**Virus detection and serotype identification.** Virus detection was performed by specimen inoculation into cell culture, according to the WHO standard protocols for poliovirus surveillance (28). Stool extracts were inoculated into three cell lines: RD (human rhabdomyosarcoma cell line), L20B (a transgenic mouse cell line expressing the gene for human cellular receptor for poliovirus), and Hep-2C (a human larynx epidermoid carcinoma cell line, and inoculated cells were followed up for 10 days. Polioviruses were identified by the presence of a cytopathic effect (CPE) on L20B cell lines; they were then serotyped and intratyped by real-time PCR (16). Nonpolio enteroviruses were identified by the presence of a CPE on RD and/or Hep-2-C cells and typed by partial sequencing in the VP1 gene as described previously (25).

**Sequencing of the entire VP1 gene and sequence analysis.** The entire VP1 genomic region of the poliovirus isolates was amplified by PCR using previously described primers and protocol (27). PCR products were purified using the QIAquick gel extraction kit (Qiagen, GmbH, Germany) and sequenced in both directions using an automated DNA sequencer (3130; Applied Biosystems) and the ABI BigDye Terminator cycle sequencing kit, version 3.2 (Applied Biosystems). Sequence data were analyzed using the ClustalX software program for multiple alignments.

**Statistical analyses.** Analyses of statistical data were performed using the Epi Info7 software program. Pearson’s χ² test was used to compare percentages, and the test was considered significant for P values less than 0.05.

**RESULTS**

**Virus detection, typing, and intratypic differentiation.** For the total of 198 stool specimens collected from the 82 patients enrolled in the study, polioviruses were detected in samples from 6 patients (21 specimens) and nonpolio enteroviruses were detected in samples from 6 patients (31 specimens); one patient excreted polioviruses and nonpolio enteroviruses (11 enterovirus-positive patients in total). The 6 poliovirus-positive patients were previously vaccinated with OPV according to the national immunization schedule. All poliovirus isolates were identified as “Sabin-like” with the intratypic differentiation real-time PCR test.

**Enterovirus-positive patients.** Excreters of enteroviruses were among PID patients with humoral or combined immunodeficiencies known to be due to a defect of B cells or both T and B cells. Their distribution was as follows (Table 1): 1 out of 11 with agammaglobulinemia, 2 out of 14 with CVID, 2 out of 8 with hyper-IgM syndrome, and 6 out of 14 with combined immunodeficiency due to a defect of MHC class II molecule expression. Thus, enterovirus-positive patients with an MHC class II defect represented 54% of all positive patients.

Six patients with severe combined immunodeficiency (SCID) were enterovirus negative. No enterovirus-positive patient was identified among the other PIDs, including phagocytic defects (CGD and disseminated BCG infection) or other, more complex syndromes (AT, Buckley, ALPS, Chediak-Higashi, and familial hemophagocytic lymphohistiocytosis).

Table 2 shows the demographic and clinical data for the 11 positive patients, as well as the types of viruses they excreted and the dates for all the samples collected. Three patients died during the study course. All patients except one had a minimum of two successive negative samples before they died or sampling stopped. Only patient 8, who was excreting an echovirus type 30 isolate, could not be followed beyond day 299. On the 11 positive patients, 4 were infected with more than one enterovirus serotype during the study course. Concomitant infections with two different serotypes were detected in two patients: patient 1 at day 1 and patient 11 at day 1. Patients 7 and 6 were successively infected with 2 to 4 different enterovirus serotypes. Patient 9 excreted an echovirus type 11 isolate over 528 days, starting in August 2006, but had been previously identified as poliovirus type 2 positive in France a few months before his enrollment in the present study. Thus, a total of 17 different enterovirus infections were detected in 82 studied patients; this corresponds to a prevalence of 20.7%.

**Excretion features and genetic characteristics of poliovirus isolates.** Of the 6 polio-positive patients, 1 died rapidly after collection of the first sample; 4 patients stopped excreting polioviruses starting with the second or the third sample (Table 2). Prolonged virus shedding was found only in patient 6, who excreted a poliovirus type 1 isolate during a minimum of 134 days. All the poliovirus isolates (n = 15) from the six poliovirus-positive patients were tested for possible recombination by real-time PCR; all did amplify with the 3D genomic region using the homotypic Sabin-specific primers, ruling out the evidence of recombination.
with other vaccine-related or wild polio or nonpolio enteroviruses (Table 3). Genetic drift in the VP1 region was assessed by real-time PCR and sequencing; the numbers of mutations and mixed bases with comparison to the VP1 sequence of homotypic parental Sabin viruses are indicated in Table 3. The 15 isolates accumulated 0 to 14 mutations in the VP1 region. Seven isolates did not amplify with the VP1 real-time PCR primers; all had several mutations and mixed bases in the VP1 region, with several mutations inducing changes in the amino acid sequence (Table 3).

The VP1 sequences of the poliovirus type 1 and poliovirus type 3 isolates from patient 1 were almost identical to those of homotypic Sabin viruses; the patient, age 18 months, received his 4th OPV dose a few days before sampling. The poliovirus type 3 isolate was not detected beyond the first sample. Poliovirus type 1 was detected 1 month later and then disappeared; the isolate accumulated 4 mutations during this period of time. Patient 2 also rapidly cleared a poliovirus type 3 isolate detected in his first sample. The 10-year-old patient received his last OPV dose 16 months before sampling, during a local vaccination campaign conducted in October 2008. Based on the number of mutations detected in the VP1 region and the admitted 1%-per-year rate of evolution of polioviruses, it is likely that the excreted virus is of more recent infection; the virus would have been acquired from the community. Two subsequent specimens from this patient were enterovirus negative; poliovirus type 2 was detected at day 249 and poliovirus type 3 at day 277. Family data revealed that his newborn brother was given the first and second doses of OPV 36 days and 3 days, respectively, before the patient again became polio positive. Poliovirus type 2 and poliovirus type 3 were detected only once and had few mutations over the VP1 region. Patient 6 was then reinfected with an echovirus type 30 isolate that he kept excreting up to publication time.

**DISCUSSION**

The first aim of this study was to search for chronic poliovirus excreters in patients with various PIDs from Tunisia, a country with a high rate of consanguineous marriages in its population resulting in a high prevalence of autosomal recessive forms of PIDs. There is no national registry, but in a first series recently published (4), 520 PIDs were identified in a period of 17 years, 40% of them with an IgG defect. Furthermore, most publications on long-term poliovirus excreters among immunodeficient patients are from developed countries; thus, it was interesting to estimate the frequency of such individuals in countries with different socioeconomic contexts and different health policies for poliomyelitis control. In Tunisia, OPV is given to more than 90% of neonates, and almost all patients with PIDs would have received this live attenuated vaccine or have been exposed to household contacts who received it. Individuals infected with wild polioviruses or receiving OPV usually excrete polioviruses for 2 to 6 weeks (1). It was suggested that excretion may last up to 137 days in apparently immunocompetent individuals (12). Among the 82 patients with various PIDs studied herein, Sabin-derived polioviruses were detected in 6 of them in the initial stool sample col-
TABLE 2 Demographic and clinical data for poliovirus- and nonpolio enterovirus-positive patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Isolated virus(es)</th>
<th>Ig deficiency</th>
<th>Age</th>
<th>Gender</th>
<th>Therapy</th>
<th>Duration of excretion (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PV</td>
<td>Hyper-IgM syndrome</td>
<td>18 mo</td>
<td>Female</td>
<td>Yes</td>
<td>90, Neg</td>
</tr>
<tr>
<td>2</td>
<td>PV</td>
<td>CVID</td>
<td>10 yrs</td>
<td>Male</td>
<td>Yes</td>
<td>148, Neg</td>
</tr>
<tr>
<td>3</td>
<td>PV</td>
<td>MHC class II</td>
<td>6 mo</td>
<td>Female</td>
<td>No</td>
<td>120, Neg</td>
</tr>
<tr>
<td>4</td>
<td>PV + EV</td>
<td>MHC class II</td>
<td>9 mo</td>
<td>Female</td>
<td>Yes</td>
<td>162, Neg</td>
</tr>
<tr>
<td>5</td>
<td>PV</td>
<td>MHC class II</td>
<td>26 yrs</td>
<td>Male</td>
<td>Yes</td>
<td>107, Neg</td>
</tr>
<tr>
<td>6</td>
<td>EV</td>
<td>MHC class II</td>
<td>7 yrs</td>
<td>Male</td>
<td>Yes</td>
<td>148, Neg</td>
</tr>
<tr>
<td>7</td>
<td>EV</td>
<td>MHC class II</td>
<td>3 yrs</td>
<td>Female</td>
<td>Yes</td>
<td>148, Neg</td>
</tr>
<tr>
<td>8</td>
<td>EV</td>
<td>CVID</td>
<td>24 yrs</td>
<td>Female</td>
<td>Yes</td>
<td>165, Neg</td>
</tr>
<tr>
<td>9</td>
<td>EV</td>
<td>MHC class II</td>
<td>15 mo</td>
<td>Female</td>
<td>Yes</td>
<td>165, Neg</td>
</tr>
<tr>
<td>10</td>
<td>EV</td>
<td>MHC class II</td>
<td>4 yrs</td>
<td>Female</td>
<td>Yes</td>
<td>165, Neg</td>
</tr>
<tr>
<td>11</td>
<td>EV</td>
<td>MHC class II</td>
<td>5.5 yrs</td>
<td>Male</td>
<td>Yes</td>
<td>165, Neg</td>
</tr>
</tbody>
</table>

BMT, bone marrow transplantation; D, day of specimen collection; EV, nonpolio enterovirus; PV, poliovirus; PV-1, poliovirus type 1; PV-2, poliovirus type 2; PV-3, poliovirus type 3; CVB5, coxsackievirus B5; EV30, echovirus type 30; EV21, echovirus type 21; Neg, negative.

All together, these data suggest that T cells play a major role in enterovirus excretion in this series (only 1 patient out of 11 had a nonpolio enterovirus). This has already been observed by other authors (8). In the literature, most of the reported long-term poliovirus excreters were found mainly among PID patients with hypogammaglobulinemia and particularly CVID (5, 8, 12, 13, 15, 18, 20). Interestingly, we found that patients with combined immunodeficiencies (T and B cell defects) are at a higher risk for enterovirus infection than those with exclusively a B cell defect. In addition, the only long-term poliovirus excreter patient had a combined immunodeficiency due to a defect of MHC class II antigen expression. This combined immunodeficiency is also diagnosed in a majority (6 out of 11) of the enterovirus-positive patients identified in this series, corresponding to 54% of cases. Three out of the six patients were poliovirus infected; this finding is particularly important since vaccine-associated paralytic poliovirus excretion (i.e., agammaglobulinemia) was rarely associated with enterovirus excretion in this series (only 1 patient out of 11 had a nonpolio enterovirus). This has already been observed by other authors (8).
TABLE 3 Intratypic differentiation of poliovirus isolates and VP1 sequence data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type and ITD</th>
<th>VP1 sequence</th>
<th>No. of mutations</th>
<th>Position(s) of mutation(s)</th>
<th>No. of mixed bases</th>
<th>Position(s) of mixed base(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-D1</td>
<td>Polio1-SL</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>1</td>
<td>2781</td>
</tr>
<tr>
<td>1-D1</td>
<td>Polio3-SL</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-D32</td>
<td>Polio1-SL</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>4</td>
<td>T2749G*, T2799C*, A3175G*, G3235T*</td>
</tr>
<tr>
<td>5-D1</td>
<td>Polio2-SL</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>3</td>
<td>T2511C, T2548C*, A2680G*</td>
</tr>
<tr>
<td>5-D53</td>
<td>Polio2-SL</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>4</td>
<td>T2511C, C2856T, T2862C, A3216C</td>
</tr>
<tr>
<td>2-D1</td>
<td>Polio3-SL</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>5</td>
<td>C2493T*, C2637T*, T2790C*, T2975A*, T3130C</td>
</tr>
<tr>
<td>4-D1</td>
<td>Polio2-SL</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>3</td>
<td>C2540T*, T2909C*, G3048A</td>
</tr>
<tr>
<td>4-D52</td>
<td>Polio2-SL</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>4</td>
<td>C2540T*, G2892A, T2909C*, G3048A</td>
</tr>
<tr>
<td>3-D1</td>
<td>Polio2-SL</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>5</td>
<td>T2523C, C2595T, G2694A, T2814A, T2909C*</td>
</tr>
<tr>
<td>6-D249</td>
<td>Polio2-SL</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>1</td>
<td>T2909C*</td>
</tr>
</tbody>
</table>

a RT-PCR, real-time PCR; ITD, intratypic differentiation.
b Mutations and mixed base positions were defined with reference to the Sabin 1, 2, and 3 sequences, GenBank accession no. AY184219, AY184220, and AY184221, respectively. Letter at left, Sabin original nucleotide; letter at right, nucleotide recorded with the isolated strain. *, mutation inducing changes to an amino acid residue within VP1.

both clearance and susceptibility to enterovirus infection. These observations are in accordance with data available from murine models and human studies, where T effector cells seem to play a major role in poliovirus clearance (19). Although it was well documented that the clearance of poliovirus infection involves antibodies (particularly mucosal IgA responses from gut-homing B cells), transfer experiments in poliovirus receptor transgenic mice have demonstrated an important role for virus-specific T cells (6). Furthermore, it has been shown in humans that booster immunization with an inactivated vaccine can expand gut-homing memory CD4+ T cell responses in mucosally vaccinated primed humans (17). Thus, there is strong evidence that a combination of various effector and helper functions is required for an efficient antienterovirus immune response.

Other PIDs have been associated with excretion of polioviruses and/or nonpolio enteroviruses, including SCID (3, 11, 15). The six SCID patients studied herein were enterovirus negative, but most of them died at early age and could not be followed for their virus excretion.

In addition to humoral and combined immunodeficiencies, it was also interesting to search for excreters among patients with other categories of PIDs. Accordingly, we have included in our study population 13 patients with ataxia-telangiectasia associated with complete IgA deficiency, but we did not find any to be enterovirus positive. These results join those of the series of Halsey et al. (12), in which 40 people with selective IgA deficiency were revealed to be enterovirus negative, suggesting that an associated T cell defect is probably required to increase susceptibility. We have also looked for enterovirus infection in immunodeficiencies with phagocytic defects or other well-defined PIDs, and no enteroviruses were isolated.

The mechanisms by which some patients will suddenly interrupt their enterovirus excretion, as well as those that will favor enterovirus persistence in the gut of some other patients, remain largely obscure. In the present study, most of our patients with humoral or combined immunodeficiencies were receiving substitutive immunoglobulin therapy at sampling time. Based on data in the literature, the possible role of this treatment in facilitating the clearance of poliovirus infection remains unclear because some prolonged poliovirus infections may have responded to treatment (7, 26) while others have not (5, 18, 20) or have resolved spontaneously (15). Most of the poliovirus excreters detected in this series (5 out of 6) had a limited period of excretion, while most of those re-excreting a nonpolio enterovirus (4 out of 6) had prolonged excretion despite the fact that they were receiving substitutive immunoglobulin therapy. Immunoglobulin concentrates may contain large amounts of antibodies against polioviruses but not as much of antibodies specific to the multiple other enterovirus serotypes; this may explain the fact that they may help clearance of a poliovirus infection but not clearance of infection by all other enterovirus serotypes. The role of other cofactors cannot be excluded. Whether or not the patient is under immunoglobulin therapy at sampling time. Based on data in the literature, the possible role of this treatment in facilitating the clearance of poliovirus infection remains unclear because some prolonged poliovirus infections may have responded to treatment (7, 26) while others have not (5, 18, 20) or have resolved spontaneously (15). Most of the poliovirus excreters detected in this series (5 out of 6) had a limited period of excretion, while most of those re-excreting a nonpolio enterovirus (4 out of 6) had prolonged excretion despite the fact that they were receiving substitutive immunoglobulin therapy. Immunoglobulin concentrates may contain large amounts of antibodies against polioviruses but not as much of antibodies specific to the multiple other enterovirus serotypes; this may explain the fact that they may help clearance of a poliovirus infection but not clearance of infection by all other enterovirus serotypes. The role of other cofactors cannot be excluded. Whether or not the patient is under immunoglobulin therapy, the question that remains to be answered is about the sudden interruption of virus excretion, sometimes after a short or longer period of virus shedding.

In conclusion, the present work joins several other studies showing that most of the patients with various PIDs have the ability to eliminate polioviruses within a maximum of a few months after infection. Factors contributing to chronic poliovirus infections in some patients are yet to be clarified; this will be of great interest for the endgame strategies of polio eradication.
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