The Incidence and Genetic Variability of Small Round-Structured Viruses in Outbreaks of Gastroenteritis in The Netherlands

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Small round-structured viruses (SRSV) are a group of RNA viruses that can cause gastroenteritis in persons of all ages. To determine the incidence of SRSV-associated gastroenteritis in The Netherlands and to study the genetic variability of outbreak strains, all outbreaks that were reported to the epidemiologists of the regional health services in 1996 were investigated using a standardized protocol. In 60 (87%) of the 69 reported outbreaks, SRSV could be detected, showing the etiologic significance of SRSV in outbreaks of gastroenteritis in The Netherlands. Of these outbreaks, 84% occurred in semiclosed communities, such as nursing homes (59%) and hospital wards (25%). Sequence analysis of the outbreak strains revealed that the majority of the strains from January to November 1996 formed a tight cluster within genogroup II SRSV. In November 1996, a shift toward genogroup I SRSV occurred, suggesting a change to a new predominant strain.

Viral gastroenteritis contributes significantly to morbidity worldwide and to mortality in developing countries [1]. Advanced age has been described as a risk factor, and >50% of diarrheal deaths in the United States are in the elderly [2]. On the basis of epidemiologic criteria, it has been predicted that small round-structured viruses (SRSV) are the single most important viral cause of outbreaks of gastroenteritis [3]. However, outbreaks of gastroenteritis due to SRSV infection are diagnosed infrequently, mostly because of the lack of simple methods for their detection.

The SRSV are a group of single-stranded RNA viruses that recently have been classified as members of the family Caliciviridae [4]. Within the Caliciviridae, at least three genogroups have been recognized in humans: SRSV-type or genogroups I and II [5] and classical calicivirus-type or genogroup III [6]. Six antigenic groups have been described so far [7], but several more antigenically distinct strains or clusters of strains exist [8]. Symptomatic infections with classical caliciviruses or genogroup III human caliciviruses are mostly restricted to childhood, whereas the genogroup I and II or SRSV-type infections are an important cause of gastroenteritis in adults as well as in children [9].

The diagnosis of SRSV infections has recently been advanced tremendously by the development of very sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) assays [10, 11]. By sequencing the amplified products (polymerase gene), all known antigenic types of SRSV could be grouped into one of the two genogroups. The diversity within each genogroup is probably greater [8], and little is known about the epidemiologic and biologic implications of this genetic variability.

In the present study, the incidence of SRSV-associated outbreaks of gastroenteritis in The Netherlands was investigated by analyzing all outbreaks of gastroenteritis that were reported to the regional public health services (PHSs) in 1996. In addition, the genetic variation of 1996 outbreak strains was analyzed by sequence analysis and was compared with that of 1994 and 1995 outbreak strains [10].

Materials and Methods

Outbreaks. Outbreaks of gastroenteritis were investigated using a standardized protocol. In the protocol, instructions were given about collecting stool samples according to Centers for Disease Control and Prevention (CDC, Atlanta) guidelines [12]. Inclusion criteria were acute diarrhea in at least 2 persons, accompanied by two or more of the following symptoms: nausea, vomiting, fever, or blood in the feces. Of all PHSs, 27 participated in the study. They were located in different parts of the country, covering 52% of the Dutch population. The remaining 33 PHSs were interviewed for nonresponse by telephone questionnaire. In total, 704 stool specimens were obtained. In 22 outbreaks, stool samples from both cases (n = 176) and controls (n = 128) were collected for laboratory investigation.

Diagnostic evaluation of outbreaks. Routine bacterial culture of the stool samples was performed in the local public health laboratories according to standard procedures. Group A rotavirus and adenovirus were detected by EIA as described [10]. Stool samples from outbreaks in which no pathogen could be detected by any of the assays were also analyzed by electron microscopy [10].

Molecular detection of SRSV by RT-PCR and Southern hybridization. Viral RNA was extracted from stool specimens after it...
bound to silica particles [13]. To reduce the risk of contamination, specimens from each outbreak were analyzed on separate occasions, and 1 negative control sample was included for every 2 stool specimens. A stool sample positive for SRSV by electron microscopy was included as positive control. Extraction, preparation of master mixes, preparation of reactions, and analysis of PCR products were done in different rooms with designated sets of pipettes. SRSV RT-PCR was performed with primer pair JV12/JV13 as described [10] with some modifications. Briefly, after reverse transcription with the specific primer JV13, 5 μL was used in the PCR assay. For optimal amplification, the PCR was done at pH 9.2 rather than 8.3 as described previously [10]. PCR products were analyzed by agarose gel electrophoresis and confirmed by Southern hybridization [14] with a set of 5′-biotin–labeled probes [10].

**Cycle sequencing and data analysis.** To determine the range of genetic diversity among the strains from the 1996 outbreaks, SRSV-positive RT-PCR products of strains from 53 outbreaks were sequenced using a dye terminator sequencing kit (Amersham, ‘s-Hertogenbosch, The Netherlands). Each RT-PCR product was sequenced in both orientations using the PCR primers. DNA sequences were edited and aligned as described [10].

**Statistical analysis.** Statistical analysis of data was done using Epi Info, version 6.02 (CDC).

**Results**

**Description of outbreaks.** In 1996, 69 outbreaks of gastroenteritis were reported to the epidemiologists from 27 of the 60 PHSs and were investigated using a standardized protocol. In each outbreak, at least 5 persons were affected. The outbreaks occurred in different parts of the country, and there were no obvious epidemiologic links between outbreaks. Most outbreaks (78%) occurred during the first 3 months of 1996. The majority of the outbreaks (84%) occurred in institutions, such as nursing homes for the elderly (59%) and hospitals (25%). All residents of nursing homes (40 of the 69 outbreaks) were ≥65 years old.

**Nonresponse investigation.** Epidemiologists from 33 PHSs were interviewed for nonresponse by telephone questionnaire. Twenty-two had not received any reports on outbreaks of gastroenteritis in 1996. The remaining 11 had, in total, 17 outbreaks reported. On the basis of epidemiologic criteria [12], in 12 of the 17 outbreaks, a viral etiology was suspected, but no virologic examination was done. *Salmonella enteritidis* was isolated from stool samples of 2 outbreaks. The cause of the remaining 3 outbreaks could not be identified, although food contamination by a bacterial toxin was suspected as the most likely cause.

**Diagnostic results of outbreaks.** A total of 704 stool samples was sent to the National Institute of Public Health and the Environment (Bilthoven, The Netherlands) for virologic investigation. A possible causative microorganism was detected in 94% of all outbreaks. By RT-PCR, SRSV were detected as the sole pathogen in stool samples from 60 (87%) of the 69 investigated outbreaks. In these outbreaks, 70% (range, 20%–100%) of the stool samples were positive by RT-PCR. In addition, individual stool samples from 3 outbreaks (2 day care centers, 1 boys camp) contained SRSV or rotavirus group A, indicating that both viruses did circulate independently in these settings. In 22 outbreaks, stool samples from both cases and controls were obtained. Control specimens were selected on the basis of absence of symptoms. SRSV were detected significantly more frequently in stool samples from cases (75%) than from controls (19%) (χ² = 91.6; P < .001, table 1).

Routine bacterial cultures were all negative, with the exception of 1 outbreak, in which *Clostridium perfringens* toxin was detected. In samples from this outbreak, no SRSV were found. All stool samples from the remaining 5 outbreaks were negative for SRSV by RT-PCR. Group A rotavirus was found in 9 of the 11 stool samples tested from 1 of these outbreaks, and no bacteria and viruses could be demonstrated in stool samples from 4 outbreaks (6%).

**Genetic variability.** To investigate the genetic variability of SRSV strains from outbreaks in 1996, we determined the nucleotide sequence of a 145-nucleotide stretch of the RNA polymerase region (pol). Strains from all outbreaks were sequenced (2 or 3 samples/outbreak). Strains from 53 of 55 consecutive outbreaks formed a tight cluster with the viruses circulating in The Netherlands in 1995 [10] and were closely related to the Bristol/Pilgrim cluster within genogroup II of SRSV [10]. The exceptions were a strain isolated from a day care center, clustering with Hawaii virus, and strains isolated from outbreak 96-61, which belong to SRSV genogroup I (figure 1A). The nucleotide sequences of the remaining 5 outbreaks also formed a tight cluster (Venlo-type, figure 1A) but clearly belonging to a totally different genotype within genogroup I viruses. These outbreaks all occurred in November and December 1996. Within outbreaks, identical sequences were found except in outbreak 96-61 (boys camp), in which 2 different sequences (genogroups I and II) were obtained from different stool samples. In addition, rotavirus group A was also detected in some stool samples. Between outbreaks of the NET96 (Netherlands 1996) cluster, 24 had identical nucleotide sequences, and small differences (maximum, 2.5%, figure 1B) were seen for 29 outbreaks.

**Discussion**

The incidence of SRSV-associated outbreaks of gastroenteritis in The Netherlands has not been investigated previously.
Here we describe the results of a study in which we analyzed all outbreaks of gastroenteritis that were reported to the PHSs during a 1-year period. Twenty-seven of the 60 PHSs participated in this study, covering 52% of the total Dutch population. There was a clear selection bias in this study, since the 27 participating PHSs reported 69 outbreaks and the remaining 33 only 17; therefore, extrapolation of the data found in our study to all PHSs is not possible. Nonetheless, in 1996, SRSV were the single most common pathogen identified in outbreaks of gastroenteritis (87%).

The local epidemiologists at the nonresponding PHSs were interviewed by telephone questionnaire and reported 17 outbreaks, of which 2 could be attributed to *S. enteritidis*. On the basis of epidemiologic criteria, a viral etiology in 12 (71%) of the remaining outbreaks was very likely. Assuming that the figures in the telephone questionnaire are correct, there were 86 total outbreaks reported to all PHSs, and 70% of them were confirmed as SRSV outbreaks. This is a higher proportion than that described by Kaplan et al. [3], who estimated, on the basis of epidemiologic criteria, that 32%–42% of the total number of outbreaks may be viral, and that SRSV cause 67% of outbreaks in nursing homes.

Our study clearly indicates that SRSV outbreaks are quite common in The Netherlands. The total number of outbreaks of gastroenteritis is probably higher due to underreporting to the PHSs. This may also cause selection bias toward nursing homes and hospitals, where such outbreaks may be more alarming due to the presence of known risk groups. An important setting where underreporting is thought to occur is day care centers. Although, we analyzed 3 outbreaks from day care
centers, many PHS officers confirmed that only a fraction of the total number of outbreaks of gastroenteritis occurring in this setting is reported. Also, suspected foodborne outbreaks in The Netherlands are usually not reported to the PHS but to food inspection services.

The high number of PCR-positive samples and the findings that roughly half of the outbreak strains within the NET96 cluster had identical sequences raise concern about contamination of samples with PCR products. We feel, however, that this is not the case, since control samples (every third sample) were all negative, samples from outbreaks attributed to other pathogens were negative, there was a clear difference in positivity rates between cases and controls, and in stool samples from a sentinel physician-based survey that are analyzed in the same laboratory by the same person in parallel without breaks from a sentinel physician-based survey that are analyzed in the same laboratory by the same person in parallel without breaks from a sentinel physician-based survey that are analyzed in the same laboratory by the same person in parallel without breaks from a sentinel physician-based survey that are analyzed in the same laboratory by the same person in parallel without breaks from asentinelphysician-basedsurveythatareanalyzedinthe

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Our data support earlier findings in that the SRSV-associated outbreaks were clearly seasonal, as has been described previously for SRSV [15] and other enteric viruses. The majority of reported outbreaks occurred in homes for the elderly (59%) and hospitals (25%). Reportedly, wards with children were not affected during the hospital outbreaks, and in the day care center outbreaks that were studied, SRSV could not be considered the main cause of gastroenteritis. This finding was somewhat surprising. An explanation may be that the average age of the children in the affected groups was too low. SRSV infections do occur in older children, but infants are less commonly affected [9]. In a comparison of the attack rates for staff and residents, the number of affected residents (45%) was significantly higher than the number of affected staff (29%) (χ² = 64.1, P < .001; odds ratio = 2.1, 95% confidence interval = 1.7–2.5).

Apart from the high incidence of SRSV in outbreaks, a second striking finding was the limited genetic variability of outbreak strains, with a predominance of a single type of genogroup II strains in the first 10 months of the year, with a shift to a genogroup I strain in November. The 1996 predominant strains are highly similar to the Grimsby/Pilgrim-like SRSV-type strains that circulated in The Netherlands in 1995. This confirms and extends our previous studies, showing that the vast majority of outbreaks in institutions in 1994–1996 seem to be caused by a single predominant strain [10]. On the basis of this pattern, which we now have observed for 3 years, we predict that the genogroup I strain appearing in outbreaks in the early winter of 1996–1997 will be the new predominant strain circulating in The Netherlands in 1997. This Venlo-type SRSV is clearly distinct from all described genogroup I prototype strains. Although our generic primer pair (JV12/ JV13) is able to detect Venlo-like SRSV, the confirmation of these viruses can be missed by hybridization with the UK1–4 probes. Therefore, we included a Venlo-type–specific probe (JV5), which proved very useful in detecting this genogroup I type of SRSV (data not shown).

In conclusion, SRSV were the pathogen most frequently associated with outbreaks of gastroenteritis in The Netherlands in 1996. The epidemic spread of individual strains circulating in the population for a certain period, which we described previously [10], is confirmed in this study.

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References

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Table 2. Attack rates calculated from residents and staff in 12 SRSV-associated outbreaks of gastroenteritis.

<table>
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<th>Symptoms</th>
<th>No symptoms</th>
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<tr>
<td>Residents</td>
<td>603 (45%)</td>
<td>736 (55%)</td>
<td>1339 (100%)</td>
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<td>Staff</td>
<td>280 (29%)</td>
<td>699 (71%)</td>
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NOTE. Yates’s corrected χ² was used to compare no. of affected people between residents and staff (χ² = 64.1, P < .001; odds ratio = 2.1, 95% confidence interval = 1.7–2.5).
Lymph Node Expansion of CD4+ Lymphocytes during Antiretroviral Therapy

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The evolution of lymphocyte subsets was analyzed in sequential lymph nodes (LN) biopsies and compared with that in the blood of 25 human immunodeficiency virus type 1 (HIV-1)–infected patients receiving highly active antiretroviral therapy. An average of 3 biopsies were obtained from each patient, with a mean follow-up of 5.6 ± 0.6 months. A correlation was found between the CD4+/CD8 ratio in blood and in LN at baseline but not after ≥2 months of therapy. With therapy, there was a significant increase in CD2+ cells and a much higher CD4+ cell increase and CD8+ cell decrease in LNs compared with levels in blood. A subset of patients had increased expression of Ki-67 and a decreased expression of CD8/CD38 or CD3/HLA-DR. Expanded CD4+ cells in LNs were mainly CD45RO+, and changes were concomitant with a decrease in LN virus load. These data demonstrate that CD4 cell reconstitution in HIV-1 infection takes place primarily in secondary lymphoid organs and is not related to a simple redistribution of cells.

A major feature of human immunodeficiency virus type 1 (HIV-1) infection is the progressive decline in the peripheral blood CD4+ T lymphocyte count throughout the course of the disease. The use of potent inhibitors of HIV-1 protease causes, at least transiently, increases in circulating CD4+ T cells [1]. This could be the result of a high turnover of CD4+ T cells [2] or a redistribution from tissue compartments [3–7] in response to the reduced trapping of cells by a decreasing virus load. Others have argued that it may simply be a consequence of drug-induced stress [8]. To resolve this issue, which is important for establishing the pathophysiologic model of HIV-1 infection, we analyzed the lymphocyte subsets in blood, lymph nodes (LNs), and bone marrow obtained from 25 HIV-1–infected men receiving antiretroviral therapy.

Patients and Methods

Population. Twenty-five HIV-1–infected men agreed to have several LN biopsies before and after the introduction of a combination antiretroviral regimen including a protease inhibitor. Seven patients with no previous drug experience received a combination of zidovudine (200 mg 3 times daily), didanosine (200 mg twice daily), lamivudine (150 mg twice daily), and saquinavir (600 mg 3 times daily). Fifteen patients were receiving long-term zidovudine-didanosine or zidovudine-lamivudine therapy, and after enrollment, 3 of them received ritonavir (600 mg twice daily) and 12 also received indinavir (800 mg 3 times daily). Three patients were receiving long-term zidovudine-dideoxynosine therapy and were switched to stavudine (40 mg twice daily) plus lamivudine and indinavir. All 18 patients who were receiving therapy at study entry had plasma HIV-1 RNA levels >20,000 copies/mL when therapy was modified. Thus, the study subjects had different antiretroviral histories, but all had recently begun highly active antiretroviral therapy (HAART) that produced a dramatic decrease in plasma HIV-1 RNA levels.

LNs. LNs were biopsied surgically in the axillary region (13 cases) or in the inguinal region (12 cases). In 18 cases, subsequent biopsies were done in the same anatomic site, alternating on opposite sides. The LNs were minced with a scalpel, and the cells were teased out in RPMI 1640 (Eurobio, Les Ulis, France). LN mononuclear cells (LNMC) were then isolated by gradient centrifugation, using lymphocyte separation medium (Eurobio). Consequently, it is not possible to standardize the dilution of cells obtained, and only the relative number of lymphocyte subpopulations analyzed by flow cytometry have any significance.

Bone marrow cell suspensions. Bone marrow (2 mL) was aspirated from the posterior iliac crest into a syringe containing 3 mL of EDTA in RPMI as an anticoagulant. Routine smears were done to check the quality of the sample. Erythrocytes were lysed by incubating 1 mL of aspirate with 14 mL of lysing solution (1.68...