Immunity to poliovirus after infection and vaccination

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Afweer tegen poliovirus na infectie en vaccinatie

(met een samenvatting in het Nederlands)

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Voor Tante Riet

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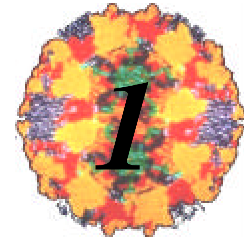

General Introduction

The early days of poliomyelitis: some historical notes

Sporadic cases of paralytic poliomyelitis have been reported for at least as long as recorded history [62]. Despite its long history, however, poliomyelitis has had its most notable effect on humanity within the past one hundred years. While the cumulative number of poliomyelitis patients world-wide had reached an estimated 10 million cases by the beginning of this century [79], no effective vaccine for this disease existed before the 1950s and ignorance about the route of transmission often hampered attempts to control its spread.

Poliomyelitis was, therefore, greatly feared, and the paralysis produced by this disease (especially in the young) was a familiar sight during previous decades [62]. As societies have improved their methods of sanitation (thereby eliminating a number of diseases in the process) the likelihood has increased that individuals will be exposed to poliovirus later rather than earlier in life, if at all. These patterns of exposure have resulted in a situation whereby this paralytic disease is no longer endemic in the western world, but occurs instead in sporadic epidemics [102].

Today, thanks to increased levels of hygiene and vaccination, poliomyelitis is rare in western countries. The cases that do occur are caused mainly by vaccine-associated disease within countries that use the live attenuated vaccine, or by wild-type virus infections within groups that refuse vaccination for religious reasons [5,61,74]. Poliomyelitis is also rapidly decreasing in most developing countries due to the World Health Organisation's vaccination campaigns [20].

Poliomyelitis presented a challenge to the scientific world for many years, as scientists and epidemiologists struggled to understand the cause of this disease. The first breakthrough occurred in 1909, when Dr. Karl Landsteiner discovered that poliomyelitis was caused by a viral infection of unknown origin [62]. Dr. John F. Enders, along with his colleagues Dr. Thomas Weller and Dr. Frederick Robbins, laid the foundation for the development of poliovirus vaccines in 1949 when they demonstrated the growth of poliovirus in cultures of non-neural cells [19]. Work by other researchers using in vitro viral culture subsequently followed from this important discovery, and Enders and his colleagues were rewarded for their work with the Nobel Prize in 1954.

A second important discovery occurred in 1949, when investigators were able to differentiate between the three different serotypes of poliovirus [6]. In 1952, Dr. Jonas Salk succeeded in developing a formalin-inactivated poliovirus vaccine (IPV) and in 1955 this inactivated vaccine was approved for the vaccination of children against poliomyelitis [82,83]. Vaccination campaigns in the USA and Europe soon followed, with great success. In 1960, the live attenuated vaccine strains developed by Dr. Albert Sabin were incorporated into a live attenuated oral vaccine (OPV) [81]. This OPV vaccine, because of its low cost, ease of use, safe administration and effectiveness against infection is now the vaccine of choice in the world-wide vaccination campaigns run by the World Health Organisation (WHO) [20,103,104].

The structure of poliovirus

Polioviruses belong to the genus enterovirus within the family *Picornaviridae*. With a diameter of 27-30 nm they are among the smallest viruses known, and contain a single-stranded RNA molecule of positive polarity linked to a small protein at the 5' region of the genome designated as the genomic virion protein (VPg). The entire

nucleotide sequence has been determined and the total genome consists of 7440, 7440 and 7435 nucleotides for serotypes 1, 2 and 3 respectively [102]. The viral capsid consists of 20 copies of each of the four structural virion proteins (VP1, VP2, VP3 and VP4) [40]. These viral capsid proteins protect the encapsidated nucleic acids from degradation and interact with a specific cellular receptor on susceptible host cells: the CD155 molecule [37].

Polioviruses can be classified into three distinct serotypes based on their reaction to reference panels of neutralising antisera [8]. Virus neutralising antibodies against one of the three serotypes do not protect against the other types, although some cross-reactivity has been described between the serotypes 1 and 2 [36,83]. The epitopes responsible for inducing poliovirus neutralising antibodies are located at the end of the loops on the three structural proteins: VP1, VP2, and VP3 [18]. Because VP4 is located entirely on the inside of the viral capsid, it plays no known role in the induction of poliovirus-neutralising antibodies. VP1 is the most exposed surface protein and plays a major role in the induction of neutralising antibodies for all three poliovirus serotypes [94]. Three antigenic sites (epitopes) involved in virus neutralisation have been identified on polioviruses based on studies with Sabin-derived mutant viruses resistant to neutralisation by monoclonal antibodies [40,63] [Figure 1].

Dr. Jonas Salk

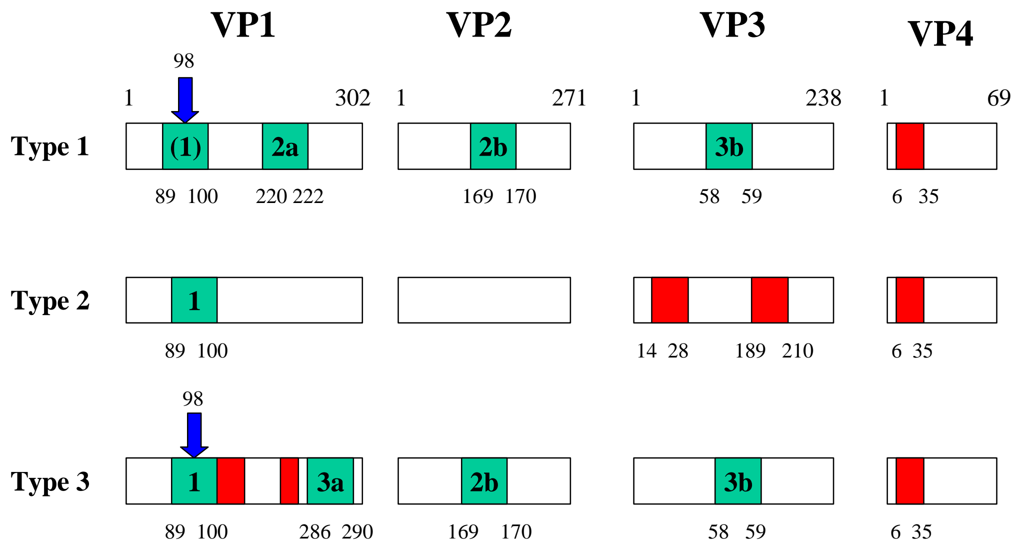

Figure 1. Identified B and T cell epitopes on the structural proteins of poliovirus

= trypsin cleaving site = B cell epitopes = T cell epitopes

Monoclonal antibodies induced in mice have determined that antigenic site 1, composed of amino acids 89 to 100 of VP1, is the major immunogenic site for serotype 2 and 3 polioviruses [63]. This site is usually immunorecessive in serotype 1 [75]. Site 2 is a complex site combining residues 220 to 222 from VP1 (site 2a) with residues including 169 and 170 and others on VP2 (site 2b) [63]. Both site 2a and 2b have been detected in serotype 1 poliovirus, while only site 2b has been detected in serotype 3 poliovirus. Site 3 is also a complex site and includes the residues 286 to 290 from VP1 (site 3a), and residues 58 and 59 from VP3 (site 3b). Both sites 3a and 3b have been detected in serotype 3 poliovirus, while as yet only site 3b has been detected in serotype 1 poliovirus [75]. The immunological relevance of these three antigenic sites in humans is not clear [Figure 1].

It is reported that trypsin, present in the intestinal fluids, can cleave both serotype 1 and serotype 3 polioviruses at antigenic site 1 at residue arginine-98 [26,45,80]. While the virus in both cases retains its infectivity, the antigenic properties of the poliovirus are drastically altered, and trypsin-cleaved viruses are not neutralised or immunoprecipitated by monoclonal antibodies to site 1 of non-treated virions [45].

Pathogenesis of poliovirus infection

Polioviruses have a restricted host range and humans are the only reservoir of naturally circulating poliovirus. Poliovirus can infect and cause flaccid paralysis in chimpanzees and cynomolgus monkeys, but the remaining (wild) populations of these animals are not large enough to sustain poliovirus circulation in the absence of human infections [17]. In monkeys, paralysis is initiated most readily by direct inoculation into the brain or spinal cord, and infection by the oral route is usually asymptomatic [7,62]. Poliovirus is, however, excreted in the throat and stool after oral infection [86].

The incubation period of poliovirus is usually between seven and 14 days (range two to 35 days) [62]. Poliovirus can be detected in the stool for five to six weeks following infection and is present in the pharynx for one to two weeks after infection [1,41,62]. Transmission occurs mainly via the faecal-oral route and the virus can spread to other people through contaminated water or food [1]. Survival of poliovirus in the environment is highly variable, but viral inactivation is usually complete within months [17] Following oral ingestion, poliovirus first multiplies in the pharynx and the small intestine. After initial and continuing replication, probably in lymphoid tissue of the pharynx and gut (Peyer's patches), the virus is able to spread to other lymph nodes until it is eventually detected in the bloodstream (viremia) [8,99]. Electron microscopy has demonstrated that poliovirus particles specifically adhere to and are endocytosed by intestinal M-cells [87]. These data suggest that M-cells are the site of poliovirus penetration of the intestinal epithelial barrier in humans.

Viremia can be detected as early as two to three days after infection. Once the virus has reached the bloodstream, the anterior horn cells of the spinal cord are at risk for infection unless sufficiently high levels of neutralising antibodies are present in the circulation [7]. Not much is known about the manner in which polioviruses are able to cross the blood brain barrier. Data from transgenic mouse experiments show that polioviruses permeate through the blood brain barrier at a high rate, independently of the poliovirus receptor [3]. It has also been proposed that polioviruses may cross the blood brain barrier into the CNS via infected monocytes [39,25]. Meningitis or paralysis can occur when neutralising antibodies are not able to block infection of the

central nervous system. Inflammation occurs secondary to the infection of the nerve cells; the infiltrations are mainly lymphocytes, with some polymorph nuclear cells and plasma cells [7]. In addition to pathological changes in the nervous system, hyperplasia and inflammatory lesions of lymph nodes, Peyer's patches and other lymph follicles in the intestinal tract are frequently observed [62]. Some degree of recovery of motor functions may occur over the subsequent six months, but paralysis still present at the end of this time remains permanent.

Most poliovirus infections (90-95%), however, do not result in clinical symptoms. The infections that do become clinical can be divided into minor illness (4-8%) and more severe illness (0.1-1%) such as paralysis or meningitis [53]. Minor illness (or abortive poliomyelitis) is characterised by fever, malaise, sore throat, headache and vomiting—all symptoms that can easily be mistaken as flu-like. Paralysis and meningitis are relatively infrequent complications of poliomyelitis. The ratio of sub-clinical to clinical infection in primary poliovirus infections does not in itself affect the spread of wild type poliovirus but is important for an accurate assessment of the extent to which the poliovirus has spread. This ratio varies according to serotype, and is highest for serotype 3 (estimated at between 4000:1 and 500:1 [13,60,83]). The lowest ratio is detected for serotype 1 (between 60:1 and 175:1 [60]) and intermediate values are found for serotype 2 (1000:1 or higher [92]).

A sudden increase in muscle atrophy has been observed in 22% to 87% of persons who have suffered from poliomyelitis, long after their apparent recovery [12,11,53]. Remaining motor neurons take over the function of the lost neurons during the recovery phases after acute poliomyelitis. Depending on the severity of the damage, these remaining motor neurons have to innervate more than the usual amounts of muscle fibres. This compensation mechanism can become exhausted during subsequent years, leading to new symptoms of muscle weakness now known as post-polio syndrome [11,12,53]. An alternative explanation suggested by some investigators is that the reported presence of poliovirus-specific RNA in former poliomyelitis patients indicates chronic viral infection [50,65].

Laboratory diagnosis of poliomyelitis

Cell culture isolation of poliovirus from the stool or pharynx early in the course of the disease is diagnostic for poliomyelitis [104]. As the disease progresses, the detection of the virus in the blood or cerebrospinal fluid (CSF) is also considered to be diagnostic [62]. The WHO recommends that at least two stool samples should be obtained from patients suspected of having poliomyelitis in order to increase the probability of poliovirus isolation. These samples should be taken 24 hours apart as early as possible in the course of the disease (ideally within the first 15 days after the onset of disease) [104,105].

Polioviruses grow rapidly in cell culture, and cell destruction (cytopathic effect or CPE) is usually complete within a few days [104]. The serotype of the isolate is identified by virus neutralisation tests [104]. Intratypic strain differentiation is necessary to determine whether the poliovirus isolate is wild or vaccine-related [93,96]. An important benefit of virus isolation is that the molecular analysis of the isolated viral genome can help to reveal the origin of the isolated poliovirus. The use of molecular epidemiological methods has enhanced the precision and reliability of poliovirus surveillance [47]. Poliovirus genomes evolve rapidly ($\sim 10^{-2}$ nt

substitutions/genome/year) during replication in humans because of the lack of viral RNA polymerase proof-reading during viral replication [47]. Studies based upon nucleotide sequence comparisons have revealed the existence of numerous genotypes endemic in different regions of the world, enabling the study of transmission routes [47].

The determination of the level of poliovirus-neutralising antibodies in serum is considered to be the most specific assay for the estimation of protection against poliovirus-induced disease. It is assumed that an antibody titer of 8 or higher is sufficient for protection against the induction of paralysis, but it is not clear if persons with lower titers are also protected [104]. Serological tests may be helpful in supporting or ruling out a diagnosis of poliomyelitis if serum samples are obtained early in the course of disease. However, the measurement of neutralising antibodies to poliovirus for diagnostic purposes is not recommended by the WHO [104], due to the fact that a) results are often difficult to interpret because antibody titers are similar in vaccinated and infected persons, and b) neutralising antibodies appear early in the course of infection, and seroconversion will have already taken place in many cases at the time of the first clinical symptoms [62]. Determination of neutralising antibody titers can, however, be helpful in assessing the level of protection against poliovirus within a population.

Immunity to poliovirus after natural infection

Upon infection, poliovirus replicates in the epithelium of the pharyngeal and intestinal mucosa and initiates a process that eventually results in mucosal immunity to poliovirus [70]. Poliovirus-specific secretory antibodies are produced by plasma cells originating in the gut-associated lymphoid tissues, mainly from the Peyer's patches [99]. The predominant class of immunoglobulin in the secretions of the alimentary tract is secretory IgA, which engages in neutralising activity against poliovirus [3,46,66,68]. The association between the presence of poliovirus-specific secretory antibodies and protection against re-infection with poliovirus has been clearly demonstrated [46,66-70]. Mucosal immunity provides a local barrier to poliovirus infection, and therefore forms a first line of defense, preventing the pathogen from entering the host [62,70,71]. Local immunity, however, is not absolute and can be overcome by a sufficiently large dose of challenge virus [73]. The persistence of poliovirus-specific secretory IgA has not been studied extensively. However, poliovirus-specific secretory antibodies have been detected in nasopharyngeal secretions 10-15 years after natural infection with wild serotype 1 poliovirus [70].

Following natural exposure, poliovirus-specific IgM and IgG appear in the serum about 7-10 days after infection, and sufficiently high levels of these antibodies can block poliovirus entry into the central nervous system [7]. The IgM response precedes the IgG response, and peaks at about two weeks after the onset of disease, disappearing from the serum within 60 days [66]. IgG levels increase steadily until approximately eight weeks after infection. IgA antibodies appear in the serum two to six weeks after exposure and remain at low levels [66].

It is generally believed that once a person has been naturally exposed to wild type poliovirus they are protected for life from further disease induced by that specific serotype [77]. The acquired immunity against re-infection, however, incomplete, as was illustrated by Gelfand et al [29] in their investigation of households containing

poliomyelitis patients in 1953-1955 (the beginning of the vaccination era). They demonstrated that 20% of the persons with naturally acquired immunity were undergoing a poliovirus infection [29]. Verlinde and Wilterdink [98] discovered in 1959 that excretion of poliovirus following challenge occurred in 31% of naturally-immune children when OPV serotype 1 was used as a challenge virus, 37% when serotype 2 was used, and 53% when serotype 3 was used.

Cell-mediated immunity to polioviruses in humans has been incompletely investigated. Poliovirus-specific cell-mediated immunity as determined with lymphocyte proliferation assays is seen in the early stages of acute poliomyelitis, but disappears in most patients after three months [52]. Poliovirus-specific CD4-positive cells (T-helper cells) could be found in the peripheral blood of participants in studies where persons were immunised with live attenuated poliovirus vaccine [35,88]. Immune lymphocytes proliferate to polyacrylamide gel purified-capsid proteins VP1, VP2 and VP3 and in some individuals, to synthetic VP4, indicating the presence of T cell epitopes in each of these proteins [9,88] [Figure 1]. T cell epitopes adjacent to each of the B cell antigenic sites in VP1 of poliovirus serotype 3 were identified. T cell lines generated in response to poliovirus infection were cross-reactive between the three serotypes. The response to the region adjacent to B cell antigenic site 1 (residues 97 to 114) was found to be immunodominant [33].

T cell clones induced in mice were found to be either serotype-specific or cross-reactive between two or all three serotypes [55]. As in experiments conducted with humans, the T cell clones recognised determinants on the surface capsid proteins VP1, VP2, and VP3 and the internal capsid VP4 [Figure 1]. One serotype 3-specific T cell clone recognised an epitope within amino acids 257 and 264 of VP1. Three T cell epitopes corresponding to residues 14 to 28, 189 to 203, and 196 to 210 were identified on VP3 of type 2 poliovirus. Four T cell epitopes were mapped to an immunodominant region of VP4, encompassed within residues 6 and 35. The VP4 epitopes were conserved between serotypes. In contrast, T cell clones that recognised epitopes on VP1 or VP3 were largely serotype specific.

The exact importance of T cell-mediated immunity to poliovirus and its role in recovery, protection from re-infection and the destruction of nerve tissue is not known. Neutralising antibodies are thought to be important for clearing poliovirus infections because children with agammaglobulinemia get persistent infections [108]. However, it is not possible to cure these patients or clear the poliovirus from the CNS even with the infusion of high titered antibody into the cerebrospinal fluid [59,78]. In addition, poliovirus persistence has occurred in persons with pure T cell deficiencies and normal immunoglobulin levels and antibody responses [34]. It would appear, therefore, that T cells or other cellular immune mechanisms play at least a partial role in the clearance of poliovirus from the CNS.

Vaccination against poliomyelitis

It goes without saying that both the development and the successful use of poliovirus vaccines have exerted a major influence on the spread of poliovirus. The principal aim of vaccination was originally to protect the vaccinated individual from disease. Within the context of vaccination programmes for larger populations, vaccination is able to enhance herd immunity to such an extent that the chain of transmission can be inhibited or even interrupted within a given community or country [62]. Two different

vaccines are currently used in vaccination campaigns against poliomyelitis: the inactivated poliovirus vaccine (IPV) and the live attenuated poliovirus vaccine (OPV).

Dr Jonas Salk developed the first inactivated polio vaccine (IPV) using polioviruses grown on monkey kidney cells that were subsequently formalin-inactivated [82]. After extensive field testing, IPV was licensed in the United States in 1955, using the Mahoney (serotype 1), MEF-I (serotype 2) and Saukett (serotype 3) poliovirus strains [82]. Dutch polio vaccination with IPV started in 1957, and IPV has been produced at the RIVM in The Netherlands since 1959. Today, The Netherlands, Finland, Sweden, France, Iceland, Norway and parts of Canada use this inactivated polio vaccine in their national vaccination programs. The same strains are still used by all manufacturers of IPV today with the exception of Sweden, where the Brunenders strain is used for serotype 1 [83].

The original IPV, given intramuscularly, contained 20, 2, and 4 D antigen units of poliovirus serotype 1, 2 and 3 respectively. In 1978, the RIVM introduced a new culture technique using cells on microcarriers to produce a more potent IPV [95,97]. The IPV used today in The Netherlands contains 40, 4 and 7.5 D antigen units per dose. A total of six IPV vaccinations are given at 3, 4, 5 and 12 months and 4 and 9 years of age. The enhanced IPV (eIPV) used in other countries contains 40, 8 and 32 D-antigenic units per dose of serotype 1, 2, and 3 respectively [62].

The antigenic site 1 of serotype 3 poliovirus is immunodominant [63] and intramuscular vaccination with the complete inactivated virion will, in theory, mainly induce antibodies to this antigenic site. Upon mucosal infection with wild-type poliovirus, trypsin (which is present in the gastrointestinal tract) cleaves the antigenic site 1 of serotype 3 poliovirus, leaving neutralising antibodies to this site useless [26,45,80]. For this reason it has been suggested that IPV might be supplemented with trypsin-cleaved serotype 3 poliovirus antigen in order to achieve a vaccine with sufficiently broad immunogenicity [43].

The attenuated polioviruses used in the live vaccines are no longer neurovirulent and rarely cause poliomyelitis (vaccine-associated poliomyelitis) [102]. This vaccine is applied orally with the advantage that it replicates in the host, inducing local immunity in the gut and at other mucosal sites. Further, the Sabin vaccine may contribute to the immunisation of subsequent contacts because is spread faecally [62]. OPV-induced mucosal immunity is important because it is able to reduce the spread of wild type polioviruses upon its (re)introduction into a population, thereby assisting in the creation of sufficient herd immunity [62]. In 1991, the success of controlling poliomyelitis in the Americas led the EPI Global advisory group to recommend, on a global basis, the formulation of trivalent OPV with 10^6, 10^5 and $10^{5.8}$ TCID50 per dose of serotypes 1, 2 and 3, respectively [21]. The schedule recommended today is one dose at birth and 3 doses of OPV at 6, 10 and 14 weeks of age.

There are some recognised problems with the distribution of OPV. Breaks in the cold-chain in developing countries, for example, can lead to loss of vaccine effectiveness in tropical countries [16]. Further, malnutrition and infection with other enteroviruses are known to interfere with the effect of OPV vaccination [56]. The greatest disadvantage of OPV, however, is the risk of back-mutation to neurovirulent strains and the introduction of a wide variety of live mutant viruses into the population [20,90,108]. However, the risk of vaccine-associated paralytic poliomyelitis (VAPP) is low, with only one case reported for every 3.3 million doses of trivalent OPV that are distributed [20,90,108]. Serotype 3 is most commonly associated with paralysis in

vaccine recipients, while serotype 2 has primarily been associated with paralysis among contacts of OPV recipients [20]. The risk for VAPP is highest following the first dose of OPV (1:570,000) and among persons with primary B cell immunodeficiencies [90,108]. Despite the risk of (OPV) vaccine-associated paralysis this vaccine was chosen by the WHO because of its low cost, ease of use, safe administration and effectiveness against infection.

Induction of systemic immunity by vaccination with IPV and OPV

Both the (e)IPV and the OPV vaccines are capable of inducing high levels of circulating neutralising antibodies. However, vaccination with IPV has been described as inducing high percentages of seroconversions with only one or two doses [4,64,76]. Vaccination with eIPV induces more than 90% seroconversion (titer ≥8) against all three types of poliovirus after only one dose and 100% seroconversion after two doses [4]. In contrast, vaccination with OPV requires three or more doses to reach similar levels of neutralising antibodies in the serum.

The responses to trivalent OPV in tropical countries are generally lower than those observed in the western world [16,56]. Accumulated data from 15 studies that have examined the response to three doses of trivalent OPV in developing countries reveal a wide variation in the number of children seroconverting, with rates ranging from 36% to 99% for serotype 1, 71% to 100% for serotype 2, and 40% to 99% for serotype 3 [76]. Serological surveys carried out 15 years or more after the beginning of national OPV coverage indicate at least 95% neutralising antibody seroprevalence against all three serotypes of poliovirus in persons two years of age and older [32,58,84]. The average number of seropositives and/or seroconversion is lowest for serotype 3, followed by serotype 1 for both the OPV and IPV vaccine [62].

Induction of mucosal immunity by vaccination with IPV and OPV

Several studies have investigated mucosal immunity after vaccination with OPV and IPV [see Table 1]. Most of these studies are limited in scope because they focus mainly on serotype 1 poliovirus in young children shortly after immunisation [31,49]. In addition, they use different vaccine compositions (such as IPV, eIPV and different OPV vaccines) and many different vaccination schedules. These factors make a comparison of the various studies difficult. It should also be noted that some of these studies were carried out shortly after the start of the vaccination era [15,46,81,100], in areas where wild type poliovirus still circulated, or where OPV vaccination was widely used [49,73]. It is impossible to exclude the effect of additional infection with live poliovirus (vaccine or wild-type) under these circumstances.

The mucosal immune response to OPV closely parallels that of natural infection. After the administration of OPV, the virus is expected to multiply in the same alimentary tract sites and related lymphoid tissues and is shed in the stool for several weeks [24]. Secretory IgA is induced in the nasopharynx and intestine approximately one to three weeks after immunisation [66]. IgA can still be detected in the nasopharynx at 60 to 100 days after vaccination [66]. The secretory IgA response rapidly returns after revaccination, but usually for a shorter duration, although some studies describe the persistence of secretory antibody activity for as long as five to six years [71,89]. The poliovirus-specific IgA induced after OPV vaccination is also found to be protective against poliovirus infection as demonstrated by several

challenge experiments with OPV, and after natural exposure [31,38,72,73] [Table 1]. Protection from re-infection is incomplete and re-infection remains possible in about one third of recipients [73].

One early study demonstrated that IPV vaccination was unable to induce a secretory IgA response in the nasopharynx or in stool samples [46]. Other researchers later demonstrated some induction of poliovirus specific IgA in the nasopharynx as well as reduced viral shedding after vaccination with the new eIPV [15,22,28,38,81,89,109]. However, the induction of sIgA described in these studies was at lower levels (9%-88%) than was the case after vaccination with OPV (26%-100%) [22,109].

Most studies show decreased pharyngeal shedding of poliovirus in IPV recipients compared to non-immunised children [15,28,38,81]. The effect on reduced pharyngeal shedding was highest in IPV vaccinated children with neutralisation titers of 8 or higher [44,57]. Only 38% of these children excreted poliovirus from the pharynx. In contrast, 75% of the group of children with titers below 8 shed poliovirus from the pharynx [44]. Further analysis of the prevalence of wild-type poliovirus in household contacts of patients revealed that poliovirus was less frequently isolated from the nasopharynx in IPV recipients than in non-vaccinated children. [57,101]. In contrast, these studies have not consistently demonstrated decreased faecal shedding of poliovirus. A number of these studies [15,49] demonstrated a decrease in the duration of excretion and the amount of poliovirus present in the stool in IPV recipients compared with non-immunised children. Other studies, however, report no difference [2,31,38,81].

Several researchers investigated poliovirus infection in IPV vaccinated household contacts of patients with paralytic poliomyelitis during the first decade after the start of IPV vaccination in the USA. All studies showed that there was no significant difference between IPV-vaccinated and non-vaccinated children within the households in the duration, amount and proportion of persons excreting wild type poliovirus [14,23,42,57,100,101].

Gelfand et al [29] studied naturally-occurring poliovirus infection in IPV-vaccinated families, and reported no significant difference in the pattern of transmission between IPV-vaccinated and non-immunised family members within infected households. However, among IPV-vaccinated persons with neutralising antibody titers of 128 or higher, the duration of faecal shedding was shorter (although the proportion shedding virus did not differ) than in persons with lower neutralising antibody titers in the serum [57]. Several studies report a correlation between serum NT titers and a reduced shedding of poliovirus from the stool and nasopharynx [15,44,51,57]. In most of these cases, however, the possibility that part of the NT titers may have been induced by additional infection with OPV or with wild-type poliovirus cannot be excluded. Unfortunately, the NT test does not discriminate between antibodies that are induced by inactivated versus live poliovirus.

In summary, studies that investigate mucosal immunity after IPV vaccination show partly conflicting results with respect to the induction of IgA and differences in the reduction of viral shedding after an OPV challenge [Table 1]. Nevertheless, it is clear that all studies comparing responses of IPV- and OPV-immunised children show a far greater decrease in the excretion of challenge virus among those immunised with OPV.

Table 1. Summary of studies investigating mucosal immunity after IPV or OPV vaccination.

Mucosal IgA induction :

		% with poliovirus-specific IgA				
		Stool		*Saliva*		*Vaccine/Country*
		IPV	OPV	IPV	OPV	
Keller [46]	1968	0	nd	0	nd	trivalent/Switzerland
Smith [89]	1986	nd	nd	9*	26	trivalent/USA/Sweden
Zhaori [109]	1988	nd	nd	27*	70	type 1/USA
Faden [22]	1990	nd	nd	41-88*	75-100	trivalent/USA

Viral shedding after OPV challenge :

		% shedding challenge virus				
		Stool		*Pharynx*		*Challenge Virus/ Country*
		IPV	OPV	IPV	OPV	
Ghendon [31]	1961	74	37	nd	nd	type 1/USA
Dick [15]	1961	63	nd	0	nd	type 1/UK
Henry [38]	1966	83	32	nd	nd	type 1/UK
Onorato [73]	1991	63*	25	1*	4	type 1/USA
Kok [49]	1992	7.1*	3.3	0*	0	type 1/Kenya

* = eIPV, nd = not determined

The problem remains that methodological differences between these studies often result in data that are difficult to compare [15,46,81,100]. It is important to bear in mind that additional mucosal priming can not be excluded in most of these cases. Whether or not IPV alone is able to exert an effect on mucosal immunity is still unclear from the results of these studies, and many different opinions currently surround this topic.

Prospects for eradication of poliovirus

In 1988, the World Health Assembly decided to strive for the eradication of poliomyelitis by the year 2000 [103]. The WHO estimates that current levels of

immunisation coverage prevent some 550,000 cases of paralytic poliomyelitis each year. The eradication of poliovirus will be considered complete when a) no more cases of poliomyelitis caused by wild-type poliovirus occur, and b) poliovirus is no longer circulating in humans (vaccinated or non-vaccinated) or in the environment [104].

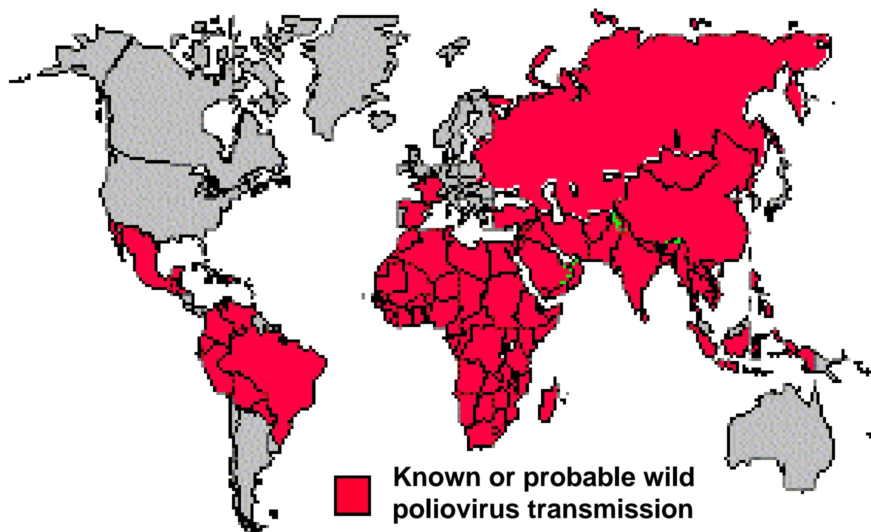

Figure 2. Wild Poliovirus in 1988

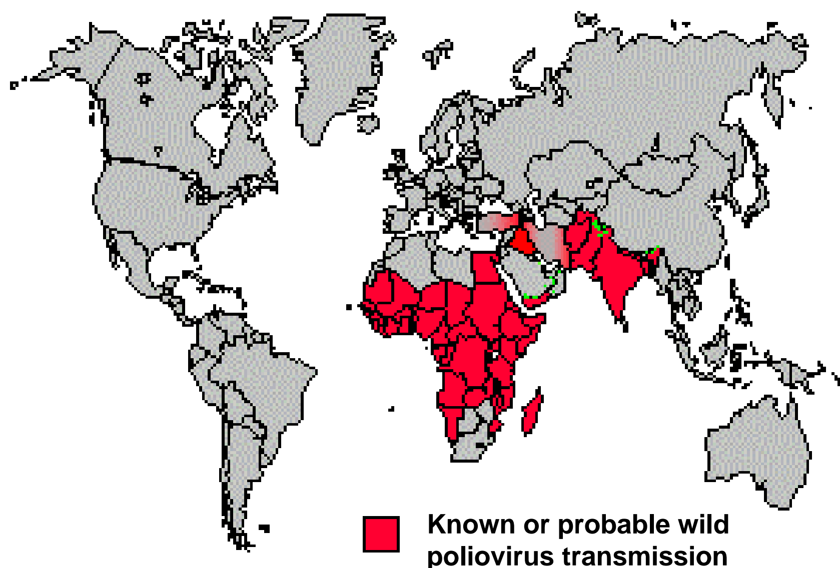

Figure 3. Wild Poliovirus in 1998

Methods of reaching this eradication goal include the implementation of a world-wide vaccine coverage of 80% or more of all new-born children, along with high quality clinical and environmental surveillance. Clinical surveillance recommended by the WHO includes the virological investigation of all patients with acute flaccid paralysis

(possible poliomyelitis cases) and their contacts [105]. Environmental surveillance includes the detection of poliovirus in the environment (for example, sewage water).

The last cases of wild-type induced poliomyelitis in the United States were reported in 1979. Since then, apart from rare cases of imported poliomyelitis, all reported cases of paralytic poliomyelitis in the United States have been vaccine-associated. The last case of poliomyelitis caused by wild-type poliovirus in the rest of the Americas was reported in August of 1991 in Peru. Despite improved surveillance, no other cases of poliomyelitis caused by poliovirus have been detected in this region, and in September 1994 the international Poliomyelitis Eradication Certification Committee certified the Americas to be free of wild type poliovirus [103].

Many other regions are now on their way to becoming certified [106,107]. The main problem areas for poliovirus circulation today are India and large parts of Africa—developing countries in which routine immunisation alone may not be sufficient for the interruption wild-type poliovirus transmission [106,107]. A set of national immunisation days, at which time two doses of OPV one month apart are given to all children under 5 years of age regardless of their immunisation status, may be more effective in these regions. The use of OPV during national immunisation days in these developing countries is either soon to be or has already been applied. This strategy has proved to be very successful in China and in the Pacific region. Figures 2 and 3 show the areas of known or probable wild poliovirus transmission in 1988 and 1998 respectively.

Outbreaks of poliomyelitis in The Netherlands and Finland

For the eradication program to succeed, transmission of wild-type poliovirus must cease completely [104]. Important information pertaining to the influence of IPV and OPV vaccination on the transmission of wild-type poliovirus can be obtained through analysis of recent poliomyelitis outbreaks. Outbreaks of poliomyelitis in countries using IPV or OPV have shown that epidemics can occur in areas that have been free of poliomyelitis for several years [43,48,68,74,85,91]. Despite the good clinical efficacy of both OPV and IPV and a high level of coverage, neither of these vaccines has been able to completely break the transmission of poliovirus. This can be concluded from the substantial proportion (between 21% and 39%) of fully vaccinated persons (both OPV and IPV) that appear to be involved in the chain of wild-type poliovirus transmission [43,48,68,74,85].

As previously noted, mucosal immunity is considered to be of particular importance for protection against (re)infection with poliovirus, thereby interrupting the chain of transmission of wild-type poliovirus [62,70,71]. IPV vaccination is thought to induce little or no mucosal immunity against poliovirus. For this reason, outbreaks in countries that use IPV exclusively in their vaccination programs are of special interest for the study of the transmission of poliovirus. Recent outbreaks in The Netherlands and in Finland are described below.

Between 1970 and 1980, the immunisation schedule in The Netherlands consisted of five doses of IPV during childhood. Coverage with three or more doses was higher than 95%. A type 1 epidemic with 110 notified cases (80 with paralysis) occurred in 1978 among non-immunised members of a religious group rejecting vaccination [85]. During this outbreak, 21% of fully immunised school children excreted wild type poliovirus, while 46% of non-immunised children excreted the virus [85].

Monovalent serotype 1 OPV vaccine was distributed among the risk groups to control the outbreak, which eventually spread to non-immunised overseas contacts among members of the religious group in Ontario (Canada) and the USA [27]. The Netherlands changed to a schedule of 6 IPV doses following the 1978 outbreak, attaining a level of coverage that exceeded 97%. The members of the religious group, however, continued to refuse vaccination. In 1992, after 14 years without endemic cases, a serotype 3 poliovirus outbreak occurred within this non-vaccinated group, with a total number of 71 patients [74]. This outbreak was investigated at schools, in the environment, at virus diagnostic laboratories and in the general population. No spread to other parts of the country was observed during the outbreak [10].

Six doses of IPV are used in the Finnish vaccination schedule and the coverage has been more than 90% for many years. An outbreak due to a serotype 3 poliovirus occurred in 1984 [43]. Nine cases of poliomyelitis were identified, two of which had received five doses of IPV in the past. Investigation of healthy contacts and other healthy persons showed the serotype 3 poliovirus to be widespread, and at least 100,000 persons were estimated to have been infected. A significant factor contributing to this outbreak was impaired herd immunity to the epidemic strain, which differed from the serotype 3 vaccine strain. Wild-type isolates had alanine 99 substituted by valine, and arginine 98 replaced by either serine or asparagine in the immunodominant region on VP1 [43]. In addition, the geometric mean titers of serum samples were lower against the epidemic strain when compared to the titers against the strains used to manufacture the inactivated vaccine, which also contributed to lower vaccine efficacy [54].

Outline of this thesis

The last outbreak in The Netherlands (in 1992/1993) raised a number of questions, such as whether the IPV vaccine provided sufficient protection against wild-type serotype 3 poliovirus, and whether the IPV-vaccinated population in The Netherlands was contributing to poliovirus circulation [30]. In this light, the induction of mucosal immunity by IPV vaccination in The Netherlands is of special interest, since mucosal immunity is considered to be of great importance for the interruption of transmission and a reduction in spread of poliovirus within the population. The experiments described in this thesis have been conducted to study the contribution of IPV vaccination to immunity from and protection against infection with poliovirus. We have developed new immunological tools for the rapid detection of poliovirus-specific antibodies and for the investigation of the induction of mucosal immunity after IPV vaccination.

An enzyme-linked immunosorbent assay-based Poliovirus-Binding Inhibition test to detect and quantify antibodies to polioviruses has been optimised and evaluated for use in population studies as an alternative to the virus neutralisation test in tissue culture (Chapter 2).

Chapter 3 presents research examining differences in response to the antigenic sites 1 and 3 of serotype 3 poliovirus between previously OPV- and IPV-vaccinated persons, to investigate possible gaps in the immune response to trypsin cleaved serotype 3 poliovirus induced by IPV vaccination.

An IgA ELISA was developed and evaluated in order to answer the question of whether vaccination with IPV induced IgA. The seroprevalence of poliovirus-specific

IgA in the circulation of poliomyelitis patients and in the general IPV-vaccinated population of The Netherlands was investigated, as well as the IgA response to poliovirus in naturally-exposed persons after a single dose of IPV. The results are presented in Chapter 4.

The kinetics of the IgA response in serum and saliva after IPV booster vaccination in previously OPV- and IPV- vaccinated subjects was studied to test the hypothesis that IPV vaccination is able to induce mucosal poliovirus-specific IgA in persons who have been previously primed with live poliovirus at mucosal sites. ELISA and ELISPOT-assays were used for the detection of virus-specific IgA responses (Chapter 5).

New assays that enable the detection of poliovirus-specific antibodies or viral RNA have been developed during the last decade. The 1992/1993 poliovirus type 3 outbreak in The Netherlands has provided an opportunity to examine the potential of various methods for poliomyelitis diagnosis and their value in the eradication program. The results of using these new methods and their implications for the future diagnosis and clinical surveillance of poliomyelitis are described in Chapter 6.

The results described in Chapters 2 to 6 will be discussed in Chapter 7.

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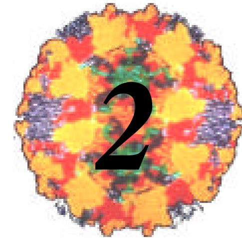

Evaluation of a Poliovirus-Binding Inhibition assay as an alternative for the virus neutralisation test

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Abstract

An enzyme-linked immunosorbent assay (ELISA)-based Poliovirus-Binding Inhibition test (PoBI) to detect and quantify antibodies to polioviruses was optimised and evaluated for use in population studies as an alternative to the virus neutralisation test (NT) in tissue culture. The sensitivity of the inhibition ELISA as compared with the NT in an inactivated poliovirus vaccine (IPV)-vaccinated population was 98.6%, 97.4% and 92.1% for serotypes 1, 2 and 3 respectively. The specificity of the PoBI test, as determined with sera from non-vaccinated persons, was also high for all three serotypes (99.0%, 95.8% and 100%). Antibodies to other enteroviruses did not cross-react in the serotype 1 or the serotype 3 PoBI, and only low levels of cross-reactivity were found for serotype 2. We found high correlations between the PoBI and NT titers for serotypes 1 and 2 in IPV-vaccinated blood donors (0.97 and 0.95), in oral poliovirus vaccine (OPV)-vaccinated blood donors (0.91 and 0.95) and in naturally immune persons (0.90 and 0.87). The correlation coefficient for serotype 3, however, was significantly lower in OPV-vaccinated blood donors (0.73) and in naturally immune persons (0.76) than in IPV-vaccinated persons (0.94; $p<0.01$). These results indicate that the antibody response to serotype 3 poliovirus in IPV-recipients is different from that in OPV-recipients and naturally infected persons. We conclude that the PoBI test is a suitable alternative for the NT to estimate the seroprevalence of neutralising antibodies to poliovirus, especially in large scale population studies.

Introduction

Poliovirus neutralising antibodies in serum are sufficient for protection against paralytic disease [2,4,12,16]. These neutralising antibodies, thought to be predominantly of the IgG isotype, prevent poliovirus from reaching the central nervous system [5,11]. The neutralisation test (NT) is used as the standard test for the measurement of immunity to the three serotypes of poliovirus after vaccination or natural exposure. Advantages of the NT are the high sensitivity, specificity and acceptance of this assay. The NT has been chosen by the WHO as the reference method for determining immunity against poliovirus [19]. However, the need to use cell culture and the long duration of the test (up to six days) make the NT expensive and less suitable for large scale screening of populations for protection against poliomyelitis. In addition, in view of the probable eradication of poliovirus in the near future, the use of live (wild-type) polioviruses in laboratory research and diagnostic assays (such as NT) will be discouraged or prohibited, and alternative methods for the immuno-surveillance of populations will be needed.

Recently Edevag et al. [1] described an inhibition-ELISA for the detection of neutralising antibodies using inactivated polioviruses as antigen. With this inhibition-ELISA (Poliovirus-Binding Inhibition assay; PoBI), a high correlation with the standard neutralisation test was found with a small set of sera in a pilot study. The specificity of the assay was not fully evaluated. Because the PoBI test is a promising alternative for the NT, we have optimised and evaluated the assay for use as a replacement of the NT in large scale population studies. In order to do so, we tested sets of sera from 1] persons vaccinated with inactivated poliovirus vaccine (IPV) or live attenuated oral poliovirus vaccine (OPV); 2] persons with documented infection with poliovirus; 3] a known seronegative population; 4] rabbits, each immunised with one of 41 different enteroviruses and 5] a cross-sectional epidemiological survey aimed at the evaluation of the national vaccination program in the Dutch population.

Materials and Methods

PoBI

The PoBI test was performed according to the method described by Edevag et al. [1] with modifications, and was conducted in two steps. For the first step (pre-incubation of serum and virus), microtiter cell culture plates (Greiner, Alphen aan den Rijn, The Netherlands) were blocked with 150 µl of 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 37°C. Two-fold dilutions of serum samples were made directly into the wells (75 µl/well, 1:2 to 1:4,096) in dilution buffer (PBS with 0.5% Tween 20, 0.5% BSA, 0.5 M NaCl). Monovalent, inactivated vaccine virus produced at the National Institute of Public Health and the Environment (RIVM) was used as antigen. The formalin-inactivated poliovirus was added to each well at concentrations of 20, 4 and 16 D-antigen units/ml for serotypes 1, 2 and 3, respectively, in a volume of 75 µl per well. The concentration of D-antigen was quantified with a direct ELISA [18]. Poliovirus serotype 1 was Mahoney, serotype 2 was MEF, and serotype 3 was a Saukett strain. Serum-virus mixtures were incubated for 2 h at 37°C.

For the second step, Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with the IgG fraction of bovine antipoliovirus hyperimmune serum in dilutions of 1:500 for serotypes 1 and 2 and 1:250 for serotype 3 in 0.04 M carbonate-bicarbonate buffer pH 9.6 (overnight at 4°C). Plates were blocked with 100 µl of 0.5% BSA in PBS for 1 h at 37°C. After blocking, 100 µl of the pre-incubated serum-virus mixture was transferred to the ELISA plate and incubated for 2 h at 37°C. For detection of bound antigen, serotype-specific monoclonal antibodies—type 1 (14D2E9, 1:3,000), type 2 (6-15C6, 1:10,000), and type 3 (2-13D9, 1:10,000)—in dilution buffer were added for 1 h at 37°C [13]. The monoclonals used for serotype 1 were directed against antigenic site 2a, while the monoclonals used for serotype 2 and 3 were directed against antigenic site 1 of the corresponding poliovirus capsid. Goat anti-mouse IgG alkaline phosphatase conjugate (Sigma, Zwijndrecht, The Netherlands) was subsequently added in a dilution of 1:500 and incubated for 1 h at 37°C. The substrate *p*-nitrophenylphosphate (Sigma) at a concentration of 1 mg/ml in 0.1 M glycine buffer (pH 10.4) was incubated at room temperature for 30 min. Plates were read at 405 nm by use of a Microwell System 510 spectrophotometer (Organon Teknica, Eindhoven, The Netherlands).

For the evaluation of the PoBI test, serum samples were considered positive if a reduction in extinction of ≥50% was reached. The reciprocal of the first serum dilution that was positive in the inhibition test was taken as the titer of the test sample. A standard in-house reference serum with known titers was included in each assay. Optimal dilutions of the coat, detector monoclonal antibodies, and conjugate were established by checkerboard titrations.

Neutralisation test

Poliovirus neutralising antibody titers of sera were determined in the standard NT as recommended by the World Health Organisation [19] by using the Mahoney (serotype 1), MEF (serotype 2), and Saukettt (serotype 3) virus strains as challenge viruses. In brief, serial two-fold dilutions of sera to be tested and 100 50% cell culture infective doses of virus were incubated in 96-microwell plates at 37°C for 3 h. After the

incubation, 1.75 x 10^4 Hep-2C cells were added per well. The plates were read after six days of incubation at 37°C. The titers are expressed as the reciprocal of the highest dilution showing complete neutralisation of the cytopathic effect of 100 50% cell culture infective doses. Samples were considered positive if NT titers were ≥8 ($\log_2$ titer, 3).

Serum samples

For evaluation of the PoBI test, the following groups of sera were examined: 1] Sera were obtained from IPV-vaccinated blood donors in The Netherlands (n=26) and from OPV-vaccinated blood donors in Belgium (n=42). 2] Sera were obtained from a group of non-vaccinated elderly persons (age 52 to 85 years) in The Netherlands that had been given a single dose of IPV (n=47). This group was presumably naturally exposed at a young age, when poliovirus was endemic in The Netherlands [8]. Sera were collected at the time of IPV vaccination, and at one and four weeks thereafter. 3] Negative-control sera were obtained from non-vaccinated children (n=96) from a population that refuses vaccination for religious reasons. None of these serum samples had detectable neutralising antibodies (titer, <2) against poliovirus. In addition, all sera were also negative for antibodies to other components of the vaccine cocktail (diphtheria and tetanus toxoid) that is used in the routine immunisation of children in The Netherlands. 4] Serum samples were obtained from rabbits hyperimmunised with either poliovirus serotype 1 (Brunhilde; NT titer: type 1, 20,480; type 2, 10; type 3, <10), serotype 2 (MEF-1; NT titer: type 1, 10; type 2, 81,920; type 3, <10), or serotype 3 (Saukett; NT titer: type 1, <10; type 2, <10; type 3, 40,960) or with other enteroviruses (coxsackievirus B serotypes 1 to 6, echovirus serotypes 1 to 9, 11 to 27, and 29 to 33, and enterovirus serotypes 68 to 71). 5] A total of 785 serum samples were obtained from a cross-sectional epidemiological survey in the province of Utrecht, The Netherlands, aimed at the evaluation of the national vaccine program.

Statistical methods

Regression analysis was used to determine the coefficients of correlation between results obtained by the PoBI test and neutralisation titers. *P* values of <0.01 were considered significant.

Results

Optimisation and properties of the PoBI test

We compared different incubation times in order to be able to reduce the duration of the PoBI test. Pre-incubation of the serum-virus mixture could be reduced to 2 h at 37°C (instead of an overnight incubation at 37°C) without loss of sensitivity or changes in PoBI titers. Incubation times of the detecting monoclonal antibody and conjugate were reduced to 1 h at 37°C without influencing the outcome of the assay.

The PoBI configuration (serum-virus pre-incubation) was compared to the direct binding of the antigen to the IgG in the ELISA plates. The PoBI titers were found to be 4- to 16-fold higher when the pre-incubation step was performed, and the correlation with NT was higher (result not shown).

Specificity of the PoBI test

When the previously described protocol [1] was used, 5.2%, 9.4% and 6.3% of the negative control samples tested positive in the PoBI test for serotypes 1, 2 and 3, respectively. The specificity of the assay was increased with the addition of 0.5 M NaCl in the ELISA dilution buffer to reduce aspecific binding, and this modification was used throughout the rest of the study. Using this assay format, the specificity was improved to 99.0%, 95.8% and 100% for serotypes 1, 2 and 3, respectively. Different blocking agents (BSA, fetal calf serum and milk powder) had no influence on false-positive results with sera from known seronegative donors. False-positive signals were strongly reduced or disappeared after $(NH_4)_2SO_4$ precipitation of the sample, indicating that the signal was not caused by cross-reacting IgG in the serum sample (data not shown).

Sensitivity of the PoBI test

The sensitivity of the PoBI test was determined with sera from IPV- and OPV-vaccinated blood donors and from naturally immune older persons (before and after IPV vaccination). Overall, the sensitivity was 99.5%, 100% and 96.2% for the serotype 1, 2 and 3 assays, respectively. The sensitivity of the PoBI test is dependent on the NT titer and was lowest for sera with low levels (titer, ≤16) of neutralising antibodies (95%, 100% and 75% for serotypes 1, 2 and 3 respectively). The sensitivity increased to 100% at neutralisation titers of 16 and 64 for serotypes 1 and 3 respectively (data not shown).

Cross-reactivity of the PoBI test.

Hyperimmune rabbit sera were used to check for possible cross-reactivity between antibodies to poliovirus and other enteroviruses in the PoBI test (Table 1). Sera to other enteroviruses (coxsackievirus B serotype 1 to 6; echovirus serotypes 1 to 9, 11 to 27, and 29 to 33; and enterovirus serotypes 68 to 71) did not react in the PoBI test for serotypes 1 and 3. In the serotype 2 test, low levels of cross-reactivity were observed with 13 of the 41 antisera that were tested. In addition, moderate levels of cross-reactivity were detectable between the polioviruses in the PoBI test and the NT (Table 1).

Correlation between the PoBI titer and the NT

We found a high correlation between the NT and the PoBI test similar to that described by Edevag et al. [1] with serum samples from IPV-vaccinated persons (0.97, 0.95 and 0.94 for serotypes 1, 2 and 3, respectively). The coefficient of correlation between the NT and the PoBI test for serotypes 1 and 2 was high in OPV-vaccinated blood donors and naturally immune persons (Table 2). However, the correlation coefficient was significantly lower for serotype 3 in OPV-vaccinated blood donors (0.73) and naturally immune persons (0.76) than in the IPV-vaccinated blood donors ($P < 0.01$) (Table 2). The correlation between PoBI and NT titers was similar for sera collected at different points in time after the vaccination of naturally immune persons vaccinated with IPV (Table 2).

Table 1. Cross-reactivity of sera from rabbits immunised with different enteroviruses in the Poliovirus-Binding Inhibition assay and neutralisation test.

Rabbit reference	**NT titer to**:			**PoBI titer to**:		
serum to:	Serotype 1	Serotype 2	Serotype 3	Serotype 1	Serotype 2	Serotype 3
Poliovirus						
Serotype 1	20,480	10	<10	4,096	16	4
Serotype 2	10	81,920	<10	64	16,384	4
Serotype 3	<10	<10	40,960	8	8	2,048
Coxsackievirus B						
Serotype 1-6	<2	<2	<2	<2	<2	<2
Echovirus						
Serotype 1-6, 15-19, 24-27, 29-33	<2	<2	<2	<2	<2	<2
Serotype 7-9, 11-14, 21-23	<2	<2	<2	<2	2	<2
Serotype 20	<2	<2	<2	<2	8	<2
Enterovirus						
Serotype 68, 71	<2	<2	<2	<2	4	<2
Serotype 69, 70	<2	<2	<2	<2	<2	<2

Table 2. Correlation of Poliovirus-Binding Inhibition assay and neutralisation test in groups of persons with vaccine-induced or natural immunity.

	Serotype 1	*Serotype 2*	*Serotype 3*	n=
Blood donors :				
IPV-vaccinated	0.97	0.95	0.94	26
OPV-vaccinated	0.91	0.95	0.73*	42
General population :	0.89*	0.89	0.84	747
Naturally immune :	0.90	0.87	0.76*	47
Weeks after IPV vaccination :				
1	0.80	0.79	0.84	47
4	0.79	0.76	0.84	47

NOTE * significantly different from IPV vaccinated blood donors at the 0.01 level. IPV= inactivated poliovirus vaccine, OPV= oral poliovirus vaccine.

Evaluation of immunity in the general (IPV-vaccinated) population

A total of 785, 763 and 771 sera were examined in PoBI and NT for serotypes 1, 2 and 3 respectively. In the general population, the correlation between NT and PoBI was 0.89, 0.89 and 0.84 for serotypes 1, 2 and 3, which was significantly lower than the correlation between NT and PoBI using sera from IPV vaccinees for serotype 1 (Table 2). No differences in the correlation between PoBI and NT were found between the different age groups (data not shown). From the regression line, the PoBI titer that corresponds with a titer in the NT of 8 was calculated to be 4 [Figure 1]. Therefore, sera with a PoBI titer < 4 were considered negative. The sensitivity of the PoBI test was high for all three serotypes: 98.6%, 97.4% and 92.1% for serotypes 1, 2 and 3 respectively. The positive predictive value of the PoBI test in the general population was 0.98, 0.97 and 0.97 for serotypes 1, 2 and 3 respectively. Specificity in this group was 80.3% for serotype 1, 82.0% for serotype 2 and 79.8% for serotype 3. The negative predictive value was 0.83 and 0.82 for type 1 and 2 respectively, and only 0.61 for type 3.

Both assays provided a normal distribution of titers in addition to a group of seronegatives (PoBI and NT titers of <4 and <8 respectively) for all three serotypes [Figure 2]. PoBI titers were generally two-fold lower than the standard NT titers.

The total number of seronegatives in the general population was estimated to be 7.5%, 12.4% and 17.4% with the PoBI and 7.8%, 12.5% and 13.3% with the NT test. These percentages were not significantly different (p-level <0.01). The PoBI was able to provide a good estimate of the total number of seronegatives for all three serotypes within the different age groups in the general population [Figure 3], and showed patterns of seroprevalence in the different age groups similar to the NT for all three serotypes.

Discussion

Previously described ELISAs used to measure protective antibodies to poliovirus could not compete with the very sensitive NT [6,7,10,17]. With a direct ELISA format for serotype 1, Simhon et al. [17] reached positive predictive values between 0.82 and 0.91, but found high numbers of false-negative results and low negative predictive values (between 0.29 and 0.55). Hagenaars et al. [7] described an inhibition ELISA in which serum antibodies and labelled bovine anti-poliovirus serotype 1 competed for binding places on the bound antigen. The assay correlated well with NT but the standard NT assay was more sensitive then the inhibition ELISA.

The present format of the PoBI test, in which inhibition of the signal depends on both reduction of virus-antigen binding to the capture antibody and reduction of binding of the indicator monoclonal antibody, was found to be a suitable replacement for the NT in large scale population studies. Although PoBI titers were generally (two-fold) lower than NT titers, neutralisation positive samples could easily be identified. Sensitivity and positive predictive values were high in both IPV- and OPV-vaccinated persons as well as in naturally exposed people. The specificity of the PoBI test was determined with selected sera from non-vaccinated subjects and was high (95-100%).

In the general population, persons with low NT titers (2 and 4) are considered to be at risk for poliovirus-induced disease. Therefore, for the purpose of this evaluation, sera with NT titers < 8 as well as sera with a corresponding cut-off titer in the PoBI of <4

Figure 1. Correlation between the NT and PoBI titers (expressed as 2log values) for the three poliovirus serotypes in a cross-section of different age groups in the general population.

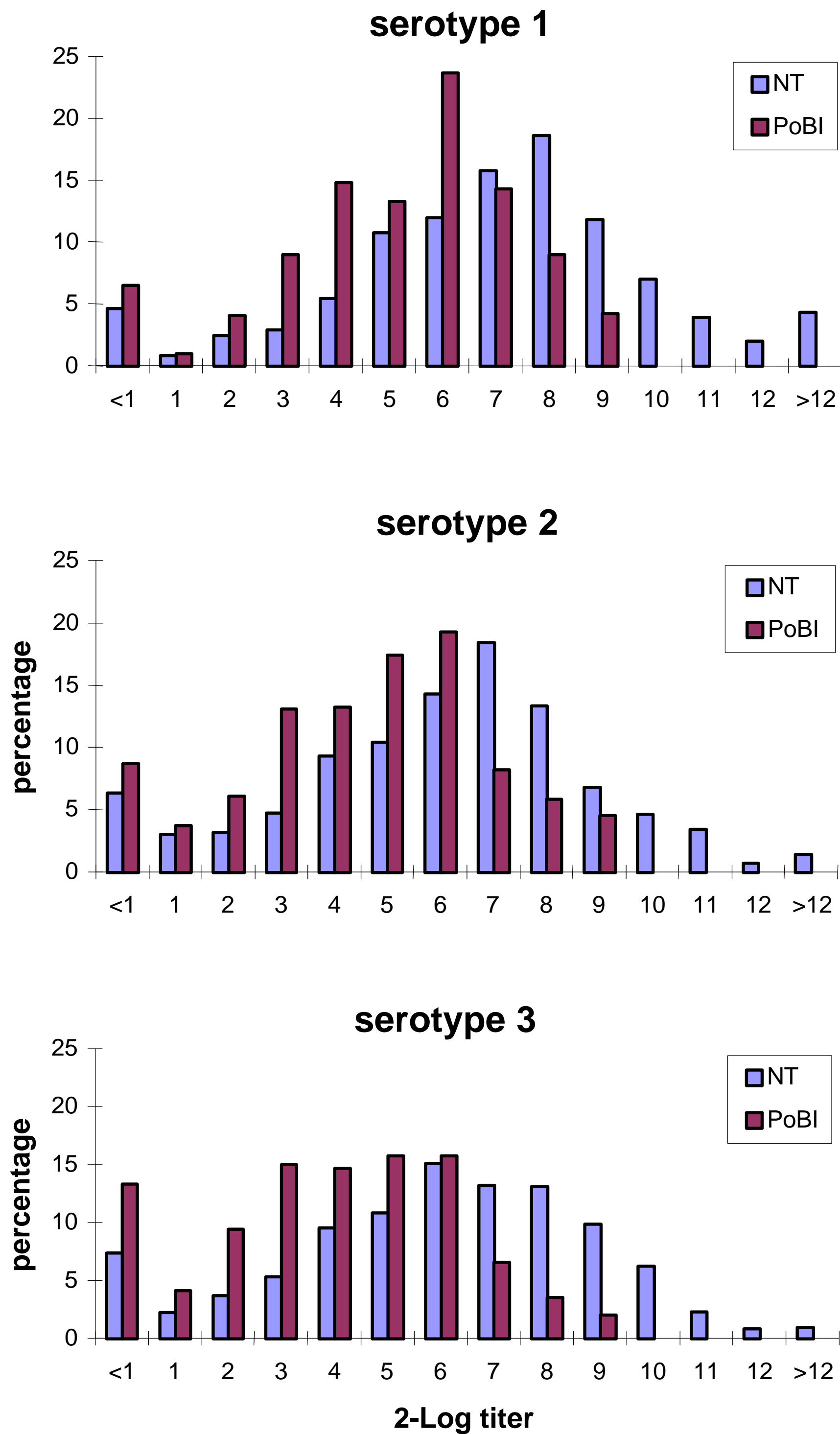

Figure 2. Frequency distribution of NT and PoBI titers (expressed as 2log values) in a cross-section of different age groups in the general population. Results are presented as percentage of persons positive per titer. The highest serum dilution tested in the PoBI was 1:256 (2log 8) and 1:4096 (2log 12) in the NT.

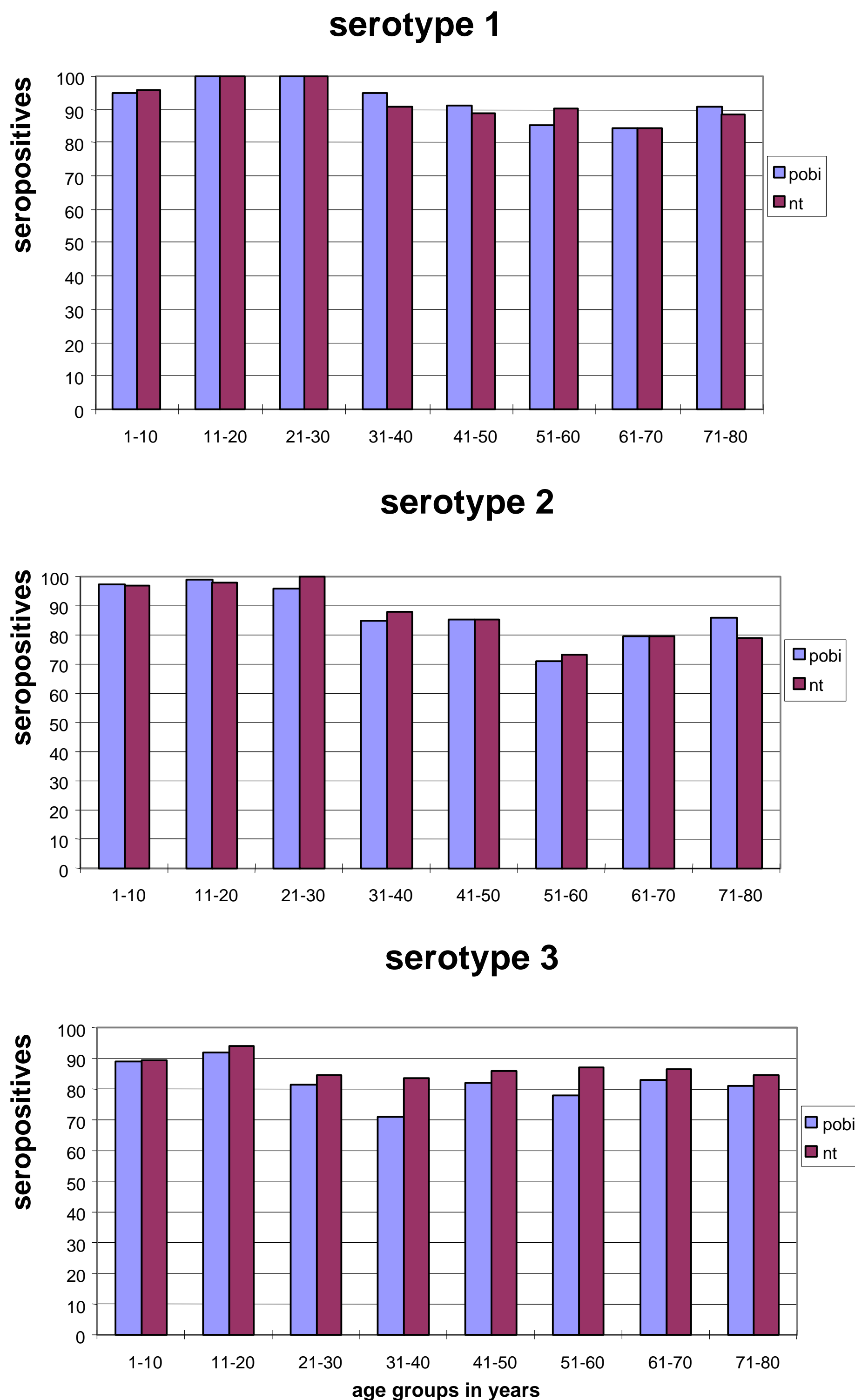

Figure 3. Seroprevalence of poliovirus antibodies in a cross-section of different age groups in the general population as determined by PoBI and NT assay. Results are expressed as percentage seropositives per age group. NT titers were considered positive if the titer was ≥ 8. PoBI titers were considered positive if the titer was ≥ 4.

were considered seronegative. The detection of these low NT titers in the PoBI is responsible for the lower specificity of the PoBI calculated from the comparative serology in the population (79.8-82.0%) as well as for the low negative predictive value for the serotype 3 assay.

The question remains whether NT titers of 2 and 4 are truly negative or whether they should be considered low but specific antibody levels. It is most likely that this group consists of both true negatives and low positive samples. Therefore, PoBI-negative sera should be re-tested by NT. The PoBI results with rabbit hyperimmune sera showed that cross-reactivity with other enteroviruses did not occur for serotype 1 and 3, and only at low levels for type 2. Since titers to enterovirus antigens as high as the levels found in the hyperimmune rabbit serum samples are not likely to be detected in the general population or in patients, this low level cross-reactivity is not a problem for serosurveys in which the PoBI is used.

The purpose of this seroprevalence study was to estimate the total number of seronegatives (as determined by NT and by PoBI) within the Dutch population. A high percentage of seronegatives in the population indicates a potential risk for poliomyelitis outbreaks and requires active re-vaccination of the (age) groups at risk. The PoBI test proved to be an excellent indicator of seroprevalence in all age groups from an IPV-vaccinated population. Both assays yielded similar estimates of the total number of seronegatives within the general population for all three serotypes.

We conclude that the PoBI is a suitable test for seroprevalence studies of poliovirus. It is a less labour intensive assay than the NT, it is easier to perform, it can be further automated and it is not dependent on a visual screening of the cytopathic effect as is the case in the standard NT. This assay could be used for population screening when combined with confirmatory testing of PoBI negatives by the NT. Given the current prevalence of poliovirus antibodies, this approach could reduce the total number of serum samples examined in the NT by 87.5%.

We reached a high correlation between PoBI and NT similar to that described by Edevag et al. [1] for IPV-vaccinated subjects. However, OPV vaccine recipients and naturally immune persons provided correlations between PoBI and NT titers against serotype 3 that were significantly lower. These correlations tended to be lower for the other serotypes as well. This may be explained in part by the use of IPV vaccine (and not OPV) as antigen in the PoBI. The greater difference observed in the serotype 3 PoBI test may be explained by a narrower immune response against serotype 3 poliovirus as compared with the other serotypes. In animals, site 1 of serotype 3 is extremely immunodominant [3,9]. Therefore, the polyclonal coat for the serotype 3 PoBI may consist mainly of (neutralising) antibodies to site 1, and as a result may be very sensitive to changes in immune response directed to site 1.

In this context, it is intriguing that differences in immune responses have been observed between infection by wild-type 3 poliovirus or OPV at the one end and IPV-induced immunity at the other. During infection with live viruses (wild-type or OPV), site 1 of serotype 3 is cleaved by trypsine during the passage through the gut lumen, thereby exposing other immunogenic sites on the viral capsid [14,15]. This trypsine effect will not occur in IPV-recipients because the vaccine is given by intramuscular injection. The trypsin-dependent immunogenic sites will therefore be less well exposed to the immune system. In contrast to serotype 3, the PoBI assays for serotypes 1 and 2 are probably reactive with antibodies to more than one antigenic site

so that differences between OPV- and IPV-vaccinees are not detected. Future work will focus on the site-specificity of the human antibody response to poliovirus.

In conclusion, the newly developed PoBI test can replace NT in large scale population studies for determining protective levels of antibodies to polioviruses. PoBI negative sera should be re-tested by NT for confirmation of seronegativity. One of the major advantages of the PoBI over the NT is that inactivated virus is used. In view of the ongoing eradication of poliovirus, the use of live wild-type poliovirus in diagnostic assays should be discouraged and eventually cease altogether in the near future.

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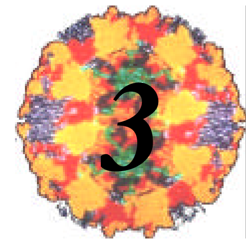

Differences in the antibody responses to antigenic sites 1 and 3 of serotype 3 poliovirus after OPV or IPV vaccination and after natural exposure

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Submitted for publication

Abstract

Three important antigenic sites involved in virus neutralisation have been identified on polioviruses in mouse experiments. These sites are located at the surface of the virion and have been designated antigenic sites 1, 2 and 3. The antibody response to antigenic site 1 of serotype 3 poliovirus is considered to be immunodominant in mice, but little is known about the immunogenicity of these sites in humans. It has been reported that trypsin, present in the intestinal fluids, can cleave serotype 3 polioviruses at precisely this immunologically important antigenic site, thereby altering its antigenic properties. In theory, therefore, the site-specific antibody response induced by intramuscularly-applied inactivated poliovaccine will differ from that induced by orally-administered live poliovirus that has been exposed to trypsin in the gut lumen. In the present study, we developed inhibition ELISA assays specific for (mouse) antigenic sites 1 and 3 in order to measure antibody responses to these sites in fully-vaccinated IPV (n=63) and OPV-recipients (n=63), and in naturally infected persons (n=25). We found that both sites are strongly immunogenic in humans. Similar levels of site-specific antibodies were found for sites 1 and 3 in naturally infected persons. Similar levels of antibodies to site 1 were detected in IPV and OPV vaccinees. However, significantly more OPV recipients (88.7%) had detectable antibodies to antigenic site 3 ($p<0.01$) when compared to IPV-vaccinated persons (63.1%). Both previously IPV- and OPV-vaccinated persons responded with a significant increase in antibodies to sites 1 and 3 after an IPV booster vaccination ($p<0.01$). We conclude that the immune response following natural infection with serotype 3 poliovirus in humans consists of both site 1- and site 3-specific antibodies, and that these responses can be induced by OPV or by recent IPV vaccination.

Introduction

The poliovirus capsid consists of 20 copies of each of the four structural virion proteins (VP1, VP2, VP3 and VP4) [9]. The epitopes responsible for inducing poliovirus-neutralising antibodies are located on surface-exposed loops in the three structural proteins: VP1, VP2 and VP3 [3]. VP4 is completely located on the inside of the viral capsid and plays no known role in the induction of poliovirus-neutralizing antibodies. VP1 is the most exposed surface protein and plays a major role in the induction of neutralizing antibodies for all three the poliovirus serotypes [27].

Three important antigenic sites (epitopes) involved in virus neutralisation have been identified on polioviruses and have been designated as site 1, 2 and 3 [9,15]. These sites have been identified through the isolation and characterisation of Sabin mutant strains resistant to neutralisation by poliovirus-specific antibodies and by epitope mapping using neutralizing monoclonal antibodies [15].

Antigenic site 1, composed of amino acids 89 to 100 of VP1, is a major immunogenic site for serotype 2 and 3 polioviruses as determined by neutralizing monoclonal antibodies induced in mice [15]. This site is usually immunorecessive in serotype 1 poliovirus [22]. Antigenic site 2 is a complex site including residues 220 to 222 of VP1 (site 2a) as well as residues 169 and 170 on VP2 (site 2b) [15]. Sites 2a and 2b have both been detected in serotype 1 poliovirus, while only site 2b has been detected in serotype 3 poliovirus. Site 3 is also a complex site and includes the residues 286 to 290 from VP1 (site 3a) as well as the residues 58 and 59 and others from VP3 (site 3b). Both sites 3a and 3b have been detected in serotype 3 poliovirus, while

neutralising antibodies to site 3b have been detected in serotype 1 poliovirus only, suggesting that site 3a is not immunogenic in serotype 1 poliovirus [22].

The location of the amino residues within the three dimensional structure of the virion indicates that the majority of these amino acids residues are highly exposed and located within prominent structural features of the viral surface [21]. A deep canyon or pit on the surface of poliovirus has been identified as the receptor binding site [5]. The neutralising epitopes themselves are not involved in receptor binding, but binding of antibodies to these spots probably causes steric hindrance with the actual receptor binding site within the canyon [5]. Whether all these sites are also antigenic for humans is not clear.

It has been reported that trypsin treatment can cleave both serotype 1 and serotype 3 polioviruses at antigenic site 1 at residue 98 (arginine) [4,11,23,25]. While the poliovirus retains its infectivity in both cases, its antigenic properties are drastically altered, and the trypsin-cleaved viruses are not neutralised or immunoprecipitated by monoclonal antibodies to site 1 of non-treated virions [11]. This trypsin effect will not occur in IPV recipients who have received the vaccine through intramuscular injection. If antigenic site 1 of poliovirus serotype 3 is also immunodominant in humans, vaccination with IPV could theoretically induce predominantly neutralizing antibodies to site 1, leaving a possible gap in the immune response to trypsin cleaved serotype 3 poliovirus [9,15].

This study compared the site-specific humoral immune responses of naturally infected and IPV- or OPV-vaccinated persons for poliovirus serotype 3. The effect of an IPV booster vaccination on the site-specific antibody titers was also examined.

Materials and methods

Serum samples

Negative control serum samples were used to test the specificity of the antigenic site 1- and 3-specific assays. These samples were obtained from non-vaccinated children (n=20) from a religious group in The Netherlands that refuses vaccination. The sera had been pre-screened by neutralisation test for absence of neutralising antibodies to poliovirus (titer, <2) [6]. The seroprevalence of antigenic site 1- and 3-specific antibodies after vaccination was determined for sera from IPV-vaccinated healthy blood donors (n=63) and from age-matched OPV-vaccinated blood donors from Belgium (n=63) who had received a complete series of vaccination as children [6].

The IPV-vaccinated (n=11) and OPV-vaccinated subjects (n=10) were given an IPV booster vaccination to determine the influence of IPV vaccination on the induction of antigenic site 1- and 3-specific responses for the different vaccine backgrounds. Blood samples were collected before booster vaccination and at 3, 7 and 28 days post-vaccination. Full details of this study have been described elsewhere [8]. Serum samples from poliomyelitis patients (n=25) from the 1992/1993 serotype 3 epidemic in The Netherlands were tested to determine antigenic site 1- and 3-specific responses after natural infection [19].

Monoclonal antibodies

A panel of serotype 3-specific monoclonals with specificity for antigenic site 1, 2 or 3 was used to test the newly developed assays for specificity. The monoclonals 204 (site 1), 877 (site 2) and 889 (site 3) were kindly donated by Dr. Ferguson (NIBSC, UK) [14,15,22]. Monoclonals 2-13D9 (site 1), 2-15E4 (site 1) and 4E5E9 (site 2/3) were produced at the RIVM (Bilthoven, The Netherlands) [12,20].

Virus preparation

Sabin mutant virus strains 335 (site 1) and 4021 (site 3) (kindly provided by Dr Ferguson) were grown on Hep-2C cells in Eagle MEM supplemented with 10% FCS, until full cytopathic effect developed [14]. The culture supernatant was collected and centrifuged for 30 min at 3000 rpm to remove cell debris. The supernatant was extracted with 10% v/v arklone at 4°C for 45 min during constant shaking followed by centrifugation (30 min, 3000 rpm). The supernatant was concentrated by direct ultrafiltration using membranes with a molecular weight cut-off of 10kD (type PM10; Amicon) [24]. The optimal working dilution of the concentrated virus in the site-specific assay was determined by checkerboard titration.

Antigenic site 1-specific Poliovirus-Binding Inhibition assay (PoBI)

The site-specific PoBI assays were a modification of the previously described PoBI assay [6], except that monoclonal antibodies to specific sites were used as capture antibodies in the ELISA. The inhibition of the ELISA signal depends on both reduction of virus-antigen binding to the capture antibody due to the presence of competing (blocking) antibodies as well as reduction of binding to the indicator monoclonal antibody by the same mechanism. Briefly, two-fold dilutions of serum samples were made directly into the wells of microtiter cell culture plates (Greiner, Alphen aan de Rijn, The Netherlands) and poliovirus antigen was added to each well. The antigenic site 3 Sabin mutant strain 4021 (kindly provided by Dr. Ferguson) was used as antigen to prevent cross reactivity with this antigenic site [14]. Serum-virus mixtures were incubated for 2 hours at 37°C. After washing of the plates, the pre-incubated serum-virus mixture was transferred to ELISA plates, that had been coated overnight with the antigenic site 1-specific monoclonal 2-13D9 (IgG isotype) in a dilution of 1/8000 [12]. After the incubation, the homologous monoclonal antibody (2-13D9), labelled with horseradish peroxidase, was used to detect bound poliovirus [18]. TMB was used as a substrate, and colour development was stopped after 15 minutes by the addition of 2M H_2SO_4. The plates were read at 450nm. Serum samples were considered positive if a reduction in extinction of ≥50% was reached. The reciprocal of the first serum dilution that was positive in the inhibition test was taken as the titer of the test sample. For specificity testing, serum was replaced by serial dilution of monoclonals to site 1, 2 and 3. Optimal dilutions of reagents were determined by checkerboard titration.

Antigenic site 3-specific PoBI

The antigenic site 3-specific assay was conducted in a manner similar to the site 1 assay described above, using site 3-specific IgM monoclonal 889 as a capture antibody in the PoBI. These IgM molecules were first degraded into F(ab')2

fragments by pepsin according to the manufacturer's instructions [Pierce, Rockford, USA], since IgM antibodies cannot be coated efficiently [1]. The Sabin mutant strain 335 (kindly provided by Dr. Ferguson) was the antigen used to reduce cross-reactivity within the antigenic site 1 [14]. Biotin-labelled antigenic site-3 specific monoclonal 889 was used to detect bound antigen. Avidin conjugated with alkaline phosphatase (Sigma, Zwijndrecht, The Netherlands) was added and incubated for 1 hour at 37°C. The plates were washed, and 100 µl of p-nitrophenylphosphate at a concentration of 1mg/ml in 0.1 M glycine buffer was added to each well. The plates were read at 405 nm after incubation at room temperature for 30 minutes. Results were analysed as described for the site 1-specific assay.

Poliovirus-Binding Inhibition assay (PoBI)

A Poliovirus-Binding Inhibition assay (PoBI) was used to determine the total number of poliovirus-specific antibodies as an indicator of neutralizing antibodies, and was performed as described [6]. Serum samples were considered positive if a reduction in extinction of ≥50% was reached. The reciprocal of the first serum dilution that was positive in the inhibition test was taken as the titer of the test sample.

Statistical methods

An unpaired Student's t-test was used to evaluate the differences between the titers of site-specific antibodies to poliovirus between two groups. P values of <0.01 were considered significant.

Results

Specificity of the antigenic site 1- and site 3-specific PoBI serotype 3 assays

None of the negative control serum samples tested (n=20) showed any inhibition of the antigenic site 1 and site 3 PoBI signal (data not shown). A panel of monoclonals was used to test the site specificity in the site 1 and site 3 PoBI assay. The antigenic site 1-specific monoclonals 2-13D9 (titer, >12800; homologous), 2-15E4 (titer, 12800) and 204 (titer, >12800) were able to inhibit the antigenic site 1 PoBI signals, whereas none of the monoclonals (4E5E9, 877, 889) to antigenic sites 2 and 3 of serotype 3 poliovirus did so (titer, <100). In the site 3-specific assay, the homologous monoclonal was able to inhibit the PoBI signal at a high level (titer 6400), and only low level cross-reactivity was detected with site 1-specific monoclonals 204 (titer, 200), 2-15E4 (titer, 400) and 2-13D9 (titer, 400).

PoBI and antigenic site 1- and site 3-specific immune responses in poliomyelitis patients

Twenty-five patients from the 1992/93 serotype 3 outbreak in The Netherlands were tested using the site-specific assays [19]. A median antibody titer of $^{2}\log 6$ to both antigenic site 1 and 3 was detected in all patients [Figure 1].

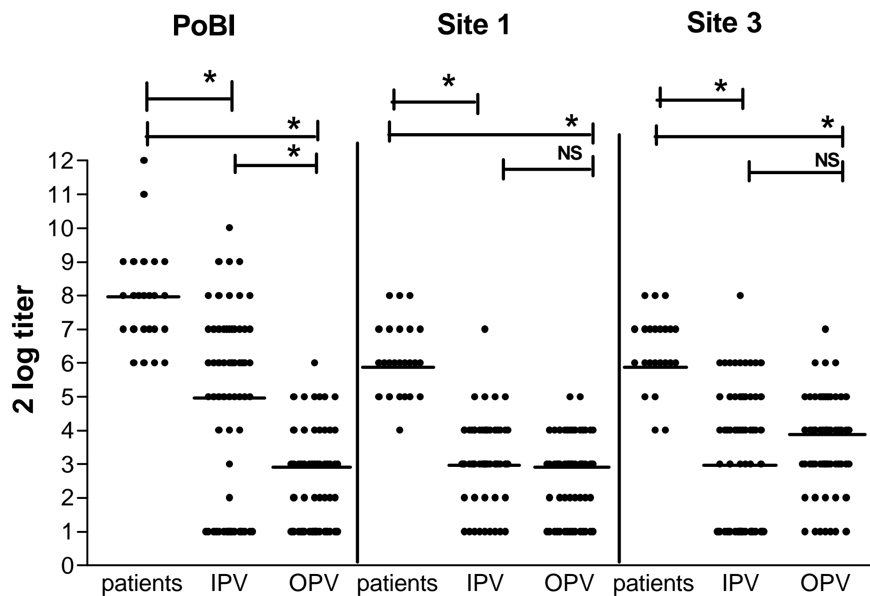

Figure 1. PoBI titers and, antigenic site 1 and 3-specific antibody titers in patients with paralytic poliomyelitis (n=25), IPV- (n=63) and OPV- (n=63) vaccinated blood donors. Results are expressed as ^{2}log titers. Horizontal lines indicate the median value. (*= p<0.01, NS =not significant).

PoBI and antigenic site 1- and site 3-specific immune responses in IPV- and OPV- vaccinated subjects

PoBI titers in IPV vaccinated persons were, on average, 8 fold lower than in naturally immune persons (p<0.01) and 4 fold higher than in OPV recipients (p<0.01). There was no significant difference in the median titers to antigenic site 1 and site 3 of serotype 3 poliovirus between the IPV- and OPV-vaccinated groups, although the median titer for site 3 was 1 log step higher in OPV-vaccinated persons [Figure 1]. However, a significantly higher proportion of OPV-vaccinated persons (88.7%) had site 3-specific antibodies compared to IPV-vaccinated persons (63.1%) (p<0.01) [Table 1]. No differences were observed in the proportions of positive IPV and OPV recipients in the PoBI assay (66.2% versus 75.8%) and the antigenic site 1-specific assay (72.3% versus 64.5%) [Table 1].

Table 1. Proportions of IPV and OPV vaccinated persons with detectable poliovirus-specific antibodies in the PoBI, site 1- and site 3-specific assays.

	IPV	*OPV*	*p-value*
PoBI	66.2%	75.8%	p=0.32
Site 1	72.3%	64.5%	p=0.45
Site 3	63.1%	88.7%	p<0.01

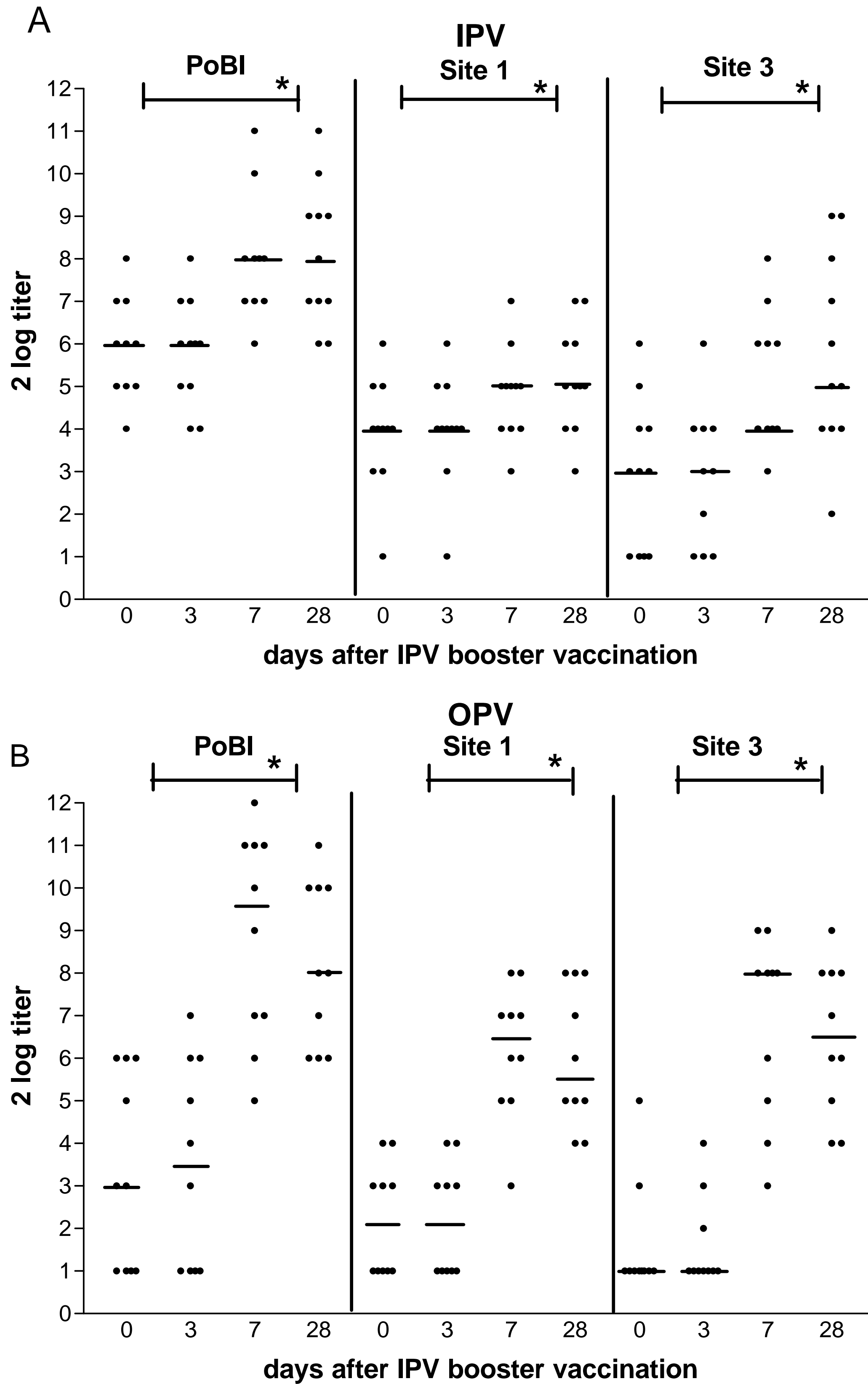

Figure 2. Antigenic site 1- and site 3-specific responses after IPV booster vaccination of previously IPV (n=11) and OPV (n=10) vaccinated persons. Results are expressed as ^{2}log titers. Horizontal lines indicate the median value. (*= p<0.01, NS =not significant).

PoBI and antigenic site 1- and site 3-specific immune responses after IPV booster vaccination in IPV and OPV recipients

A significant booster of the antigenic site 1- and site 3-specific antibodies and PoBI titers was detected in both IPV and OPV recipients at 7 and 28 days compared to day 0 and 3 after booster vaccination with IPV ($p<0.01$). However, the detected increase in titers was smaller in the IPV recipients compared to OPV recipients ($p<0.01$) [Figure 2].

Discussion

The data presented in this paper clearly indicate that both antigenic site 1 and site 3 are immunogenic in humans, as antibodies specific for these sites were detected after natural infection and in IPV- and OPV-vaccinated persons. These results differ from those in mice experiments [9,15], because site 1 does not appear to be as immunodominant in humans. Similar levels of both site 1- and site 3-specific antibodies were readily detected in those who had been naturally exposed as well as in IPV- and OPV-vaccinated persons.

We used site-specific inhibition ELISA assays to measure antibody levels to antigenic site 1 and site 3. The inhibition levels reached in the site 1- and site 3-specific assays with the homologous antibodies were very high (site 1: >12800; site 3: 6400). The site 1 assay proved to be completely site-specific. Some cross-reactivity was detected in the site 3-specific assay with monoclonals to the antigenic site 1, but titers were 64-fold lower. Titers to poliovirus antigen as high as the levels found in the homologous reactions with the monoclonals are not likely to be detected in the general population or in patients. For this reason, low level cross-reactivity is not considered to be a problem for the estimation of the level of antibodies to site 3 when human sera are tested.

No significant difference was observed in the immune response between OPV and IPV recipients with respect to antigenic site 1 of serotype 3 poliovirus. However, significantly more OPV-vaccinated persons had detectable antibodies to antigenic site 3 (88.7%) compared to IPV recipients (63.1%). It is conceivable that the lower number of site 3 seropositives in the IPV-vaccinated group (compared to the OPV vaccinated group) can be explained by trypsin cleavage of site 1 after passage of the OPV strains through the gut lumen [23,25]. Field trials using regular IPV or trypsin-treated serotype 3 Saukett strains (IPV) demonstrated that both vaccines induced an increase of neutralising antibody titers to intact and trypsin-treated virus, but the response was not studied at the level of individual antigenic sites [23]. Alternatively, the difference between the number of positives in the OPV-vaccinated group and the number in the IPV-vaccinated group may be explained by a longer lasting induction of site 3-specific antibodies. Site 3-specific antibodies were induced in previously IPV-vaccinated individuals after IPV booster vaccination. Tests using sera from people who had been vaccinated less recently, however, provided a lower number of positive results for site 3-specific antibodies.

It is conceivable that the site 3-specific antibodies in some of the IPV and OPV recipients are attributable to previous natural exposure to live poliovirus (wild-type or OPV strains). The high seroprevalence of IgA in the circulation of IPV-vaccinated persons probably indicates mucosal contact with poliovirus [7,8]. Poliovirus is no longer endemic in The Netherlands, but in 1992 a large outbreak of serotype 3

poliovirus occurred [19]. While widespread circulation during this epidemic was not demonstrated [2,16,26], part of the vaccinated population may have come into contact with the circulating wild-type strain or OPV strains that were used to control the epidemic. Similarly, the trypsin exposure of OPV in the gut may result in reduced immunogenicity of antigenic site 1, favouring the development of site 3-specific antibodies. However, 49.0% of the IPV recipients in this study had site 3-specific antibodies in the absence of an IgA response (data not shown). These results might indicate that site 3 responses are longer lasting than the serotype 3-specific IgA in the circulation after mucosal contact. Alternatively, the site 3-specific antibodies may have been induced by IPV only.

Persons with no neutralizing antibodies to antigenic site 3 might not have an effective response to polioviruses with an altered antigenic site 1. The importance of site 3 for protection against poliomyelitis is illustrated by the Finland epidemic of 1983 [10], in which the serotype 3 endemic poliovirus strain was altered in antigenic site 1 [13]. This outbreak was also influenced by low antibody titers to serotype 3 poliovirus in the general vaccinated population [13]. In addition, the significantly lower antibody titers to the altered virus reflect the possible dangers of a reintroduction of antigenic site 1 variants or their actual generation in persons with no site 3-specific antibodies. Recently, we described a serotype 1 Sabin virus with a 6 aminoacid deletion in antigenic site 1 [17]. Neutralisation of this mutant strain by an antigenic site 1 monoclonal was altered. While this strain was still effectively neutralised by sera from IPV and OPV vaccinees, our present findings suggest that neutralising activity should be investigated in persons with and without site 3-specific antibodies in order to estimate the risk of infection with mutant viruses.

In conclusion, we observed that the immune response following natural infection with serotype 3 poliovirus in humans consists of both site 1- and site 3-specific antibodies. No significant difference was observed between IPV and OPV recipients' immune responses to antigenic site 1. Responses to antigenic site 3 of serotype 3 poliovirus was detected more frequently in persons who had been vaccinated by OPV in their childhood than in those who had received IPV. However, site 3-specific antibodies were increased after IPV-booster vaccination in both groups. These results indicate that IPV recipients are likely to be sufficiently protected against trypsin treated serotype 3 poliovirus.

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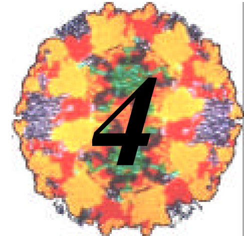

Poliovirus-specific IgA in persons vaccinated with inactivated poliovirus vaccine (IPV) in The Netherlands

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Abstract

The inactivated poliovirus vaccine (IPV) is used for protection against poliomyelitis in The Netherlands. It is not clear if parenteral IPV vaccination can lead to priming of the mucosal immune system. We developed and evaluated ELISA assays for the detection of poliovirus-serotype-specific IgA and secretory IgA antibodies. Using these assays, we examined the kinetics of the IgA response in sequential serum samples from 15 poliomyelitis patients after natural infection with serotype 3 poliovirus. IgA remained present in 36% of the patients for up to five months post infection. Furthermore, the presence of IgA antibodies was examined in an IPV-vaccinated population using sera from young children (4-12 years of age, n=177), older children (13-15 years of age, n=123), healthy blood donors (n=66) and naturally immune elderly persons (n=54). Seroprevalence of IgA was low in young vaccinated children for all three serotypes (5%-7%), and in older vaccinated children for types 2 and 3 (2%-3%). Seroprevalence for type 1 was significantly higher (18%) in older children than in younger children. This higher seroprevalence is most likely explained by the persistence of IgA following infection with the serotype 1 wild type poliovirus strain during the 1978 epidemic. The seroprevalence of type 1- and 2-specific IgA was significantly higher in healthy adults than in young children. These results suggest that at least part of the IgA found in the older population is induced by infections unrelated to the IPV vaccination schedule. Finally, we found that parenteral IPV vaccination was able to boost (secretory) IgA responses in 74%-87% of a naturally exposed elderly population (n=54). While the presence of (s)IgA in IPV-vaccinated persons has been previously documented, our findings suggest that mucosal priming with live virus is necessary to obtain an IgA response after IPV booster vaccination.

Introduction

Systemic antibody responses to poliovirus infection and vaccination (with live or inactivated virus) have been extensively studied. The presence of circulating neutralizing antibodies is sufficient for protection against paralytic disease [13]. In contrast, less is known about the induction of mucosal immunity, which is important for the limitation of virus circulation in the community as well as for protection from infection [7,13].

An important component of mucosal immunity is secretory IgA (sIgA). The presence of sIgA on mucosal surfaces reduces viral excretion after oral poliovirus vaccine (OPV) challenge [12]. In theory, intramuscular vaccination by inactivated poliovirus (IPV) is expected to induce little or no sIgA. However, several studies have measured some degree of local immunity in IPV vaccinees, albeit less effective immunity than in people vaccinated orally with OPV or infected with wild virus [3,4,7,8,14].

An inherent difficulty in the comparison of results from different studies lies in the fact that vaccination schedules and dosages have not been standardised between countries. It therefore remains unclear whether the superiority of OPV vaccination over enhanced potency IPV (eIPV) with respect to mucosal immunity applies to the situation in The Netherlands, where a total of six IPV vaccinations are given at 3, 4, 5 and 12 months, and subsequently at 4 and 9 years of age. In addition, most studies of mucosal immunity have been conducted with recently vaccinated individuals. This may not reflect the situation as it applies to older age groups.

The issue at hand is whether IPV vaccination confers sufficient mucosal immunity to prohibit virus circulation following introduction in the community. This is particularly important in The Netherlands, where pockets of religious communities with low vaccination coverage exist. Epidemics of poliomyelitis occurred in these groups in 1978 and 1992 [5,16,17].

The purpose of this work was to develop poliovirus-specific IgA assays and to study the kinetics of IgA responses after infection and in an IPV vaccinated population.

Materials and Methods

Clinical samples

The specificity of the poliovirus-specific IgA-ELISAs was tested using serum samples from persons negative for neutralising antibodies to poliovirus (n=114). These samples were obtained from non-vaccinated children from a segment of the population that refuses vaccination for religious reasons. Samples that had titers of neutralising antibodies of < 1:2 for all three serotypes of poliovirus and that were negative for antibodies to other components of the vaccine cocktail (diphtheria and tetanus toxoid) used in the routine immunisation of children in The Netherlands were considered as true negatives. The sensitivity of the assays was determined using a panel of sera collected from patients with a proven poliovirus infection within 2.5 months after the onset of paralysis. Patients were from the 1992-1993 serotype 3 outbreak in The Netherlands (n=54) and from the outbreak in Pakistan during 1991-1995 involving all three serotypes (n=98). Infection was confirmed in all patients by the isolation of wild-type poliovirus from stool samples and by the detection of poliovirus-specific IgM in serum by ELISA [11]. Isolated wild-type polioviruses were discriminated from vaccine-derived viruses by an ELISA using type-specific cross-absorbed antisera as described previously [20].

Sequential serum samples from 15 patients (age range 1-36 years) from the 1992-1993 serotype 3 epidemic in The Netherlands were tested for the presence of IgM and IgA antibodies in order to determine the kinetics of the IgA response after natural infection. Poliovirus-specific IgM levels in serum samples were determined to confirm primary infection. Sera from the following groups were investigated to determine the seroprevalence of poliovirus-specific IgA in an IPV-vaccinated population:

1] Sera from schoolchildren between 4 and 12 years of age, collected shortly after IPV vaccination (n=177), or between 13 and 15 years of age (n=123). These serum samples had been collected during the 1992-1993 outbreak in The Netherlands in order to check the seroprevalence of neutralising antibodies to poliovirus and to test for evidence of poliovirus infection within this group. All children had been vaccinated with IPV, had protective levels of neutralising antibodies to all three serotypes, and were positive for antibodies to diphtheria and tetanus toxoid (also included in the vaccine). The younger children (4-12 years) had received four to six doses of IPV and the older schoolchildren (13-15 years) had completed the IPV vaccination schedule several years previously.

2] Sera from fully IPV-vaccinated healthy blood donors were tested to determine the long term persistence of IgA after vaccination (n=66). This latter group was compared

with age-matched blood donors from Belgium (n=66) where OPV is used in the national vaccination program (OPV at 3, 4, 5 12 months, and at 6 and 12 years of age). The age range of both groups of blood donors was between 18 and 65 years (average 39 years).

3] Sera from a group of non-vaccinated older persons from The Netherlands (52-85 years, n=54) who had been given a single dose of IPV were examined for the presence of neutralising antibodies and poliovirus-specific IgA. This group was expected to be naturally exposed at a young age when poliovirus was endemic in The Netherlands. Sera were collected at the time of IPV vaccination and at 1 and 4 weeks thereafter.

IgA-ELISA

Wells of microtiter plates (Nunc, Maxisorb) were coated overnight at + 4°C with serotype-specific monoclonal antibody to poliovirus at a concentration of 0.6 to 1.2 µg/ml in 0.04 M carbonate-bicarbonate buffer (pH 9.6). The monoclonal antibodies used were 5-18D8 for poliovirus type 1, 1-10C9E6 for type 2, and 2-13D9 for type 3 [15]. Negative control wells were coated with a monoclonal antibody to influenza A virus (6-21/19-6, A/Singapore/6/86 (H1N1) strain-specific). After blocking for 1 hour at 37°C with 5 % Blotto (Pierce, Oud Beijerland, The Netherlands) in PBS containing 0.05 % Tween 20, 40 to 70 D-antigen units (the form found in the infectious virus) of formaldehyde-inactivated poliovirus were added to each well. We used the inactivated Mahoney strain for the type 1, MEF for the type 2, and Saukett for the type 3 assay, all from the vaccine production facility of the National Institute of Public Health and the Environment (RIVM). Poliovirus strains were originally derived from the American Type Culture Collection (ATCC). The plates were incubated for two hours at 37°C. Phosphate-buffered saline containing 0.5% Tween 20 and 2% Blotto was used as a dilution buffer. Volumes of 100µl were used, and plates were washed four times in PBS with 0.05% Tween 20 between each incubation step. In parallel wells, dilution buffer without virus was added to control for non-specific binding of sera. Prior to testing, sera were depleted of IgG with Quik-Sep (Isolab, Mechelen, Belgium) according to the manufacturer's instructions to prevent possible inter-isotype competition. Serum dilutions of 1/50 were added to the plates and incubated overnight at + 4°C. After washing, an optimal dilution (1/8000) of goat-anti-human IgA labelled with alkaline phosphatase (alfa-chain specific, Sigma, Zwijndrecht, The Netherlands) was added and incubated for 1.5 hours at 37°C. The plates were washed and 100 µl per well of P-nitrophenylphosphate was added at a concentration of 1 mg/ml in 0.1 M glycine buffer (pH 10.4). After incubation at room temperature for 30 minutes the plates were read at 405 nm by use of an Organon Teknika microwell system 510 spectrophotometer. A serum sample was considered positive if the optical density (OD) was above the cut-off level, defined as the average OD + 3 standard deviations of results obtained with negative control sera from non-vaccinated persons (n=114). At least one IgA positive (derived from an OPV-vaccinated subject) and one IgA negative control serum was included on each plate. Optimal dilutions of monoclonal antibodies, viral antigen, sera, and detector antibodies were established by checkerboard titrations. The reagent dilutions chosen for this test provided the highest OD differences between signal and background levels. Specificity of positive signals was confirmed by blocking experiments in which serum samples were pre-incubated with homologous poliovirus (± 120 D-antigen units) for 2 hours at 37°C,

and centrifuged for 3 minutes at 10.000 rpm to remove immune complexes prior to testing in ELISA. A reduction of the signal of >50% was considered confirmative.

Secretory IgA capture ELISA

A capture ELISA was used to investigate the sera of the older population to determine if IgA detected after IPV vaccination was also present in its secretory form. The assay was a modification of the IgM ELISA that has been described previously [11]. Briefly, microtiter plates were coated with monoclonal antibody against secretory component (Sigma, Zwijndrecht, The Netherlands) overnight at 4°C in carbonate buffer. Plates were blocked with 5% normal goat serum, serum dilutions (1:50) were added, and the plates were incubated for 1.5 hours at 37°C. Formaldehyde-inactivated poliovirus type 1, 2 or 3 was added as described, and bound antigen was detected with horseradish-peroxidase-labelled serotype-specific monoclonal antibody (1 hour, 37°C). Tetramethylbenzidine (TMB) was used as a substrate (0.1 mg/ml) in 0.11 M sodiumacetate buffer, and the reaction was stopped after 30 minutes with 2M H2SO4. Serum samples were considered positive if responses were above the cut-off level, defined as the average optical density (OD) + 3 standard deviations of results obtained with negative control sera from non-vaccinated persons (n=114). At least one sIgA and one sIgA negative control serum was included on each plate.

Poliovirus type-specific IgM-antibody capture ELISA

The IgM-ELISA was performed as described previously [11]. In brief, wells of microtiterplates were coated overnight at 4°C with 100μl of μ-chain-specific monoclonal antibody to human IgM (Sanbio BV, Uden, The Netherlands) at a dilution of 1:100 in PBS, supplemented with 0.5% Tween 20, and 5% fetal calf serum. Serum dilutions (1:50) were added and were incubated overnight at 4°C. Inactivated poliovirus suspension containing between 40 and 70 D-antigen units was added and incubated for 2 hours at 37°C. Bound antigen was detected by horseradish-peroxidase-labelled serotype-specific monoclonal antibodies (1 hour, 37°C). TMB was used as substrate, and colour development was stopped after 12 minutes by the addition of 2M H2SO4. A positive and a negative control serum and a positive/negative cut-off control serum were examined in each assay. The positive/negative cut-off serum was prepared on the basis of comparison with the distribution of the OD450 values obtained with sera from both patients and healthy controls. A ratio of >1 between the sample OD450 and the cut-off serum OD450 was considered to indicate the presence of poliovirus-specific IgM in the sample.

Neutralisation assay

Poliovirus neutralising antibody titers (NT) of sera were determined in the standard microneutralisation test as recommended by the WHO [21], using Mahoney (serotype 1), MEF (serotype 2) and Saukett (serotype 3) virus strains as challenge viruses.

Statistical Methods

A Chi-square analysis was performed to determine the significance of the difference in seroprevalence between two groups. P values <0.05 were considered significant.

Results

Specificity and sensitivity of the poliovirus-specific IgA ELISAs

The specificity of the poliovirus-specific IgA-ELISAs was 100%, 99%, and 99% for poliovirus serotypes 1, 2 and 3 respectively. Poliovirus-specific IgA was detected in 89%, 81% and 90% of the serotype 1, 2 and 3 samples from infected patients. Positive signals in the ELISA could only be blocked by pre-incubation with the homologous poliovirus and not with heterologous virus (results not shown).

Kinetics of IgA production in patients infected with wild type poliovirus

All type 3-infected poliomyelitis patients (n=15) had poliovirus serotype 3-specific IgA and IgM antibodies in at least one of the serum samples. Four patients had IgA antibodies to all three serotypes, and one patient had IgA against type 1 and 3. Poliovirus serotype 3-specific IgA reached a peak at three to four weeks after onset of paralysis [Figure 1] and decreased to low levels within three months. Thirty-six percent of the patients remained positive for IgA for up to five months post infection. Maximum levels of IgA were found at a later date post-infection than the maximum levels of IgM in all patients (data not shown).

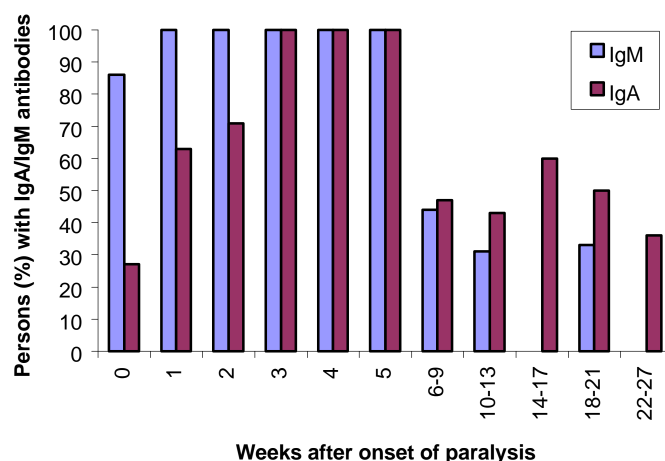

Figure 1. Positivity rates of IgM and IgA antibodies to poliovirus type 3 in poliomyelitis patients. Results are from consecutive serum samples from 15 patients with poliomyelitis collected during the 1992-1993 outbreak in The Netherlands.

IgA in IPV-vaccinated children

Poliovirus-specific-IgA was found in 4.5%, 7.3% and 5.6% of young children (4-12 years) for serotypes 1, 2 and 3 [Figure 2]. No correlation was found between the total number of IPV doses received and the number of children with detectable IgA levels (results not shown). Eighteen percent of the older schoolchildren (13-15 years) had IgA antibodies to serotype 1, whereas the seroprevalence of IgA to serotypes 2 and 3 was low (3.3% and 2.3 % respectively) [Figure 2].

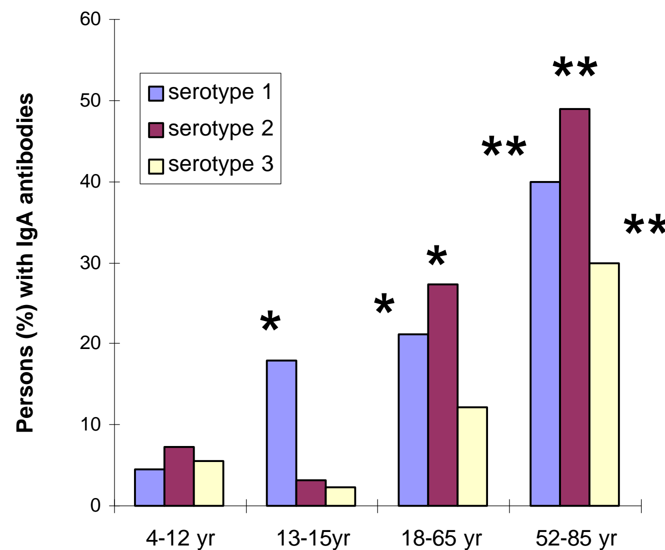

Figure 2. Seroprevalence of IgA antibodies to poliovirus type 1, 2 and 3 in different age groups of IPV vaccinated persons in The Netherlands (group I-III) and in persons born before the vaccination programme (group IV). The populations were I) 4-12 year old school children (n=177), II) 13-15 year old school children (n=123), III) IPV vaccinated blood donors (n=66, labelled 18-65 years) and IV) 52-85 year old persons without previous vaccination (n=54). *= significant (p<0.05) compared to 4-12 year old schoolchildren, **= significant (p<0.01) compared to schoolchildren and blood donors.

IgA levels in IPV-vaccinated healthy adults

IgA antibodies were found in 21.2%, 27.3% and 12.1% of healthy adult blood donors for serotypes 1, 2 and 3 respectively [Figure 2]. The seroprevalence of poliovirus serotype 1- and serotype 2-specific IgA was significantly higher (χ^2, $p<0.05$) in adults than in the young schoolchildren (4-12 years). Antibodies to serotypes 2 and 3 were significantly more prevalent (χ^2, $p<0.05$) in the blood donors than in the older children (13-15 years).

IgA levels in OPV-vaccinated healthy adults

IgA was found more frequently in the OPV-vaccinated Belgian blood donors (33.8%, 32.3% and 32.3% for serotypes 1, 2 and 3 respectively) than in age-matched IPV-vaccinated donors from The Netherlands as mentioned (21.2%, 27.3% and 12.1% for serotypes 1, 2 and 3 respectively). The difference was only significant for serotype 3 (χ^2, $p<0.05$).

IgA antibodies in elderly adults pre- and post-IPV vaccination

Before IPV vaccination, the group of older subjects (52-85 years old, n=53) had neutralisation titers (≥ 1:8) to serotypes 1, 2 and 3 (87%, 83% and 79% respectively). Four weeks after vaccination, 96% of these subjects had protective levels of neutralising antibodies for all three serotypes. Only two subjects did not develop neutralising antibodies to serotype 3 after the IPV booster. Before IPV vaccination,

40%, 49% and 30% of the subjects had detectable IgA against serotype 1, 2 and 3 respectively [Figure 2], In most cases the ELISA absorbance values were low, and median values were below cut off levels for all three serotypes [Figure 3].

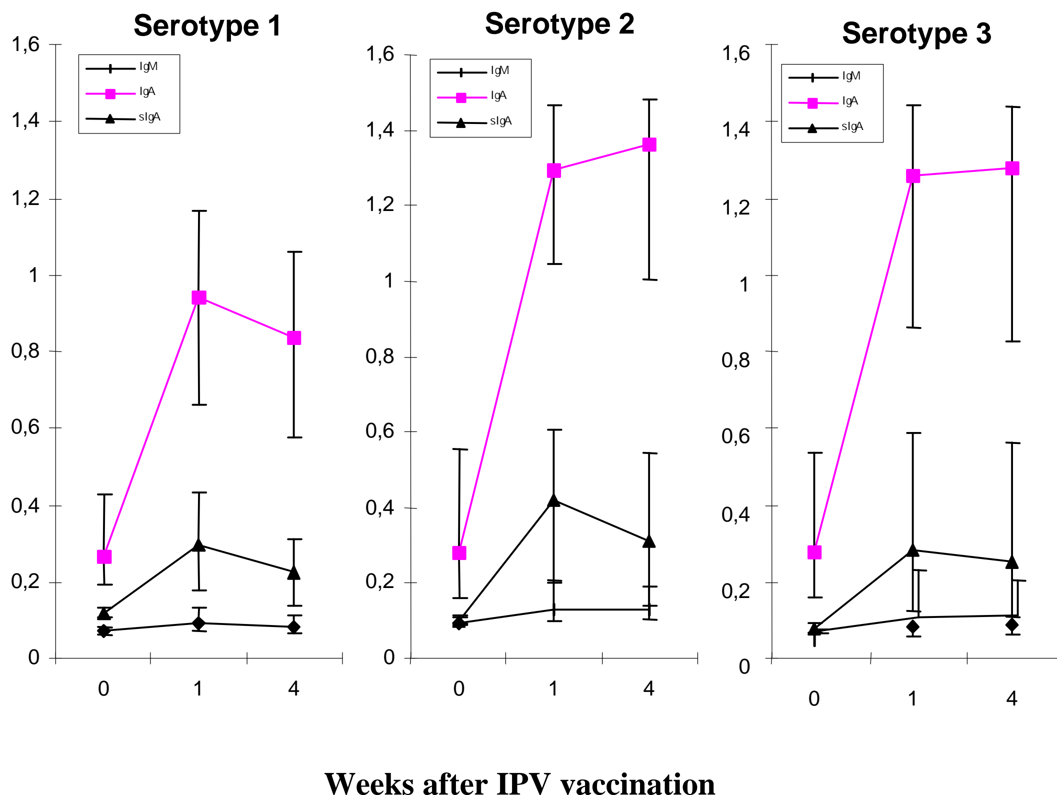

Figure 3. Levels of IgM, IgA and sIgA before and after vaccination with IPV in an older population. Dots indicate median values in each group. Error bars indicate upper (75 %) and lower (25 %) quartiles.

After the IPV booster, a strong increase in IgA levels occurred within one week for serotype 1 (93% of the subjects), serotype 2 (94% of the subjects) and serotype 3 (83% of the subjects). A booster response to all three serotypes was found in 77% of the subjects [Figure 3]. Four weeks following IPV vaccination, three subjects (6%) had no detectable IgA antibody to serotype 1 poliovirus, one subject (2%) had no detectable IgA antibody to serotype 2 and seven subjects (14%) had no detectable IgA antibody to serotype 3. All IgA positive serum samples were also positive in the neutralisation assay.

Secretory IgA levels in elderly adults pre- and post-IPV vaccination

Only a small fraction of the older subjects had detectable secretory-IgA against poliovirus serotype 1 (8.3%), serotype 2 (14.6%) and serotype 3 (10.4%) before IPV vaccination. In all cases except one (serotype 2), OD values were low (< 2 times the cut-off value). One week after the administration of one dose of IPV, 75%, 87%, and 74% of the subjects reacted with a rapid increase in sIgA in serum for serotype 1, 2 and 3 respectively [Figure 3]. Levels of sIgA remained high until at least four weeks after the administration of IPV. All serum samples tested in the sIgA assay were IgM-negative.

Discussion

We studied serum IgA responses after poliovirus infection or vaccination in different age groups of persons vaccinated with IPV in The Netherlands. We developed ELISA assays to measure poliovirus-specific (secretory) IgA circulating antibodies. The specificity of both IgA ELISAs was high for all three serotypes (99% to 100%). A positive reaction could only be blocked with homologous virus indicating that cross-reactivity with other poliovirus serotypes, and probably other enteroviruses as well, did not occur.

IgA antibodies to the other two serotypes were also detected in four out of the 15 persons from the group of poliomyelitis patients from the 1992/1993 serotype 3 epidemic in The Netherlands, suggesting cross-reactivity. A more likely explanation, however, is that this was caused by contact with the OPV virus offered during the epidemic. The sensitivity was determined by testing a group of poliomyelitis patients, and was rather low (81% to 90%). This may be explained by a short duration of the IgA response that may have been missed during the infrequent sampling schedule. The sensitivity of the ELISAs appears sufficient for the study of poliovirus immunity in well-defined populations.

IgA in the group of poliomyelitis patients peaked, on average, at four weeks after the onset of paralysis, and was back at low levels in the majority of persons after three months. It was striking that the poliovirus serotype 3-specific IgA antibodies persisted in approximately one third of the patients beyond five months post infection, confirming data from the literature [12]. Persisting IgA levels suggest a continuous or repeated antigenic stimulus of the immune system, however, there is little or no evidence of persistent poliovirus infection in contrast to other enterovirus infections. An alternative explanation might be the retention of viral antigen in dendritic cells in the bone marrow [1,18].

We examined sera from different age groups for IgA antibodies. The seroprevalence of poliovirus-specific IgA was low (5%-7%) in young schoolchildren (4-12 years), and we found no increase after more dosages of IPV. A drawback of this study is that the sera used for these experiments were not collected immediately after vaccination, and IgA induced by IPV could have disappeared from the circulation at the time of testing. The low number of fully IPV-vaccinated children that tested positive for IgA shows that IPV may induce IgA antibodies, but only in a small proportion (5%-7%), and that IgA may not be present in serum for an extended period of time. In keeping with our findings, it has been described that IPV vaccination in children can induce sIgA in saliva after at least three dosages, but only in a minority of vaccinated children (9%) [3,18,19]. This is not a result of the incapability of children to produce sIgA, since children at six months of age in Pakistan produced levels of salivary IgA similar to those in adults [3].

Serotype 2- and serotype 3-specific IgA was less prevalent in the older children than in the group of younger children, a finding that may reflect waning immunity. The higher seroprevalence rates of IgA to serotype 1 poliovirus in the older schoolchildren is most likely explained by the persistence of IgA following infection with the serotype 1 wild-type poliovirus strain during the 1978 epidemic [17]. After 1978, wild poliovirus type 1 was not found in this community, and any vaccine would have included all three serotypes. The older schoolchildren may have been infected in their first few years of life. In addition, healthy adults had a significantly higher seroprevalence of IgA for serotypes 1 and 2 than did IPV vaccinated children. This

finding is not merely explained by cohort effect, as the difference was also seen when blood donors born before the start of the national vaccination program were excluded from the data analysis.

This finding strongly suggests that additional stimulation of the immune response to poliovirus with wild-type poliovirus or live vaccine strains has occurred. Similarly, an unvaccinated population of 52 to 85 year old persons had the highest seroprevalence of IgA to all three serotypes compared to all other age groups. All IgA levels found in this group must have been induced by infection with live virus (vaccine or wild type). This may have occurred during childhood when poliovirus was still endemic in The Netherlands, during holidays in OPV-countries, or through the importation of wild or vaccine-derived polioviruses into The Netherlands. However, there is no evidence for the endemic circulation of wild or vaccine virus in The Netherlands during the period 1979-1991 [16].

Interestingly, parenterally administered IPV in elderly, non-immunized persons was able to induce strong memory IgA and secretory IgA responses. Induction of memory secretory IgA responses by a parenterally administered inactivated vaccine has also been described for the influenza virus [2,10], pseudomonas aeruginosa and meningococci [6,9]. The authors postulated that the cause of these responses is most likely to be previous mucosal priming with wild type infection (e.g. with the influenza virus), as may have been the case for live poliovirus in the population that we studied.

The rapid immune response in our own experiments combined with the lack of induction of poliovirus-specific IgM also suggests a secondary response and shows that parenteral poliovirus vaccines can induce a secretory IgA response in persons previously exposed to live poliovirus. It remains to be determined how sIgA responses in serum correlate to sIgA at mucosal sites and to protection, and whether IPV can also boost sIgA in persons without previous mucosal infection. Experiments are underway to examine these relations.

In summary, we have shown that IgA antibodies to poliovirus are present in fully IPV-vaccinated children, but only in a small proportion, whereas the seroprevalence is significantly higher in adult IPV vaccinees. This suggests that most of the IgA present later in life in an IPV-vaccinated population was induced by continuous or additional exposure to live virus strains (wild or vaccine) and/or due to the persistence of IgA. In addition, we have shown that parenteral IPV was able to boost secretory IgA responses in a naturally exposed elderly population, suggesting a link between the systemic and mucosal immune system.

In the near future, poliomyelitis due to wild-type infection will be eradicated and the circulation of live polioviruses will decrease. An important question remains: Will an IPV-vaccinated population be able to mount a mucosal (booster) immune response under these circumstances? Our future work will focus on the capacity of parenteral IPV to prime for mucosal memory responses and to provide protection against virus excretion.

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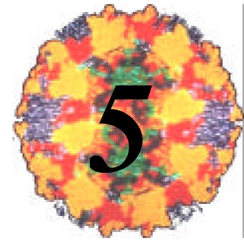

Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus

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Abstract

The inactivated poliovirus vaccine (IPV) is used for protection against poliomyelitis in The Netherlands. It is not clear, however, whether IPV vaccination can lead to priming of the mucosal immune system and the induction of IgA. We have previously shown that significantly fewer children than adults have circulating IgA in this IPV-vaccinated population, suggesting that at least part of the IgA found in the older population in The Netherlands is induced by infections and is unrelated to IPV vaccination. It has also been demonstrated that IPV vaccination is able to induce strong memory IgA responses in the serum of persons who have been naturally exposed to live (wild-type) poliovirus. This has led to the hypothesis that IPV vaccination is able to induce poliovirus-specific IgA at mucosal sites in persons who have been previously primed with live (wild or vaccine) poliovirus at mucosal sites. To test this hypothesis, the kinetics of the IgA response in serum and saliva after IPV vaccination were examined in persons previously vaccinated with oral poliovirus vaccine (OPV) or IPV. ELISA and ELISPOT-assays were used for the detection of poliovirus-specific IgA responses. In addition, B cell populations were separated on the basis of the expression of mucosal (α4β7 integrin) and peripheral (L-selectin) homing receptors. Parenteral IPV vaccination was able to boost systemic and mucosal IgA responses in previously OPV-vaccinated persons only. None of the previously-vaccinated IPV recipients responded with the production of IgA in saliva. In agreement with this finding, a large percentage of the poliovirus-specific IgA-producing lymphocytes detected in previous OPV recipients expressed the α4β7 integrin. It is concluded that IPV vaccination alone is insufficient to induce an effective mucosal IgA response against poliovirus and that, conversely, mucosal IgA responses measured after IPV vaccination are most likely the result of previous mucosal priming with live poliovirus. In mucosally- (OPV-) primed individuals, however, booster vaccination with IPV leads to a strong mucosal IgA response.

Introduction

Poliomyelitis has been effectively controlled through the use of two different vaccines; the inactivated poliovirus vaccine (IPV) and the attenuated poliovirus vaccine (OPV) [26]. Mucosal immunity protects from (re)infection and is essential for the reduction of poliovirus circulation in the population [9,21,22]. The induction of mucosal immunity is, therefore, of particular importance for the poliomyelitis eradication program because both poliovirus-induced paralysis and poliovirus circulation must cease completely in order to reach the target of a polio-free world.

Whether wild-type poliovirus can remain circulating in vaccinated populations (silent circulation) is an important question for the eradication program. In theory, silent circulation is possible in IPV-vaccinated populations because intramuscular vaccination with inactivated poliovirus (IPV) probably induces little or no secretory IgA (sIgA) at mucosal sites. Several studies, however, indicate that some degree of mucosal immunity can be measured in IPV vaccinees, albeit less than in people who have been vaccinated with the oral poliovirus vaccine (OPV) or infected with wild-type virus [4,5,9,11,23]. Most information comes from studies that were conducted during periods when poliovirus was still endemic or in regions where OPV was also used. The results of these studies, therefore, are likely to be confounded by additional priming of the (mucosal) immune system through infection with live poliovirus (vaccine or wild-type). Some of the more recent studies have also included IPV-

vaccinated subjects recruited from endemic regions [5,16]. It is still unclear whether IPV vaccination alone is able to induce mucosal immunity and is responsible for the induction of secretory IgA in saliva and/or stool samples.

We have previously shown that IPV vaccination can induce strong memory IgA responses in the serum of persons who have been naturally exposed to live (wild-type) poliovirus [12]. An age-dependent increase in the presence of IgA in the circulation of the IPV-vaccinated population in The Netherlands has also been described, and this increase can not be explained by IPV vaccination alone [12]. Based on these results, we have postulated that a memory IgA response after IPV vaccination is dependent on previous mucosal infection with live poliovirus (vaccine or wild-type).

To test this hypothesis, both IPV- and OPV-recipients were given a booster vaccination with one dose of IPV. The group of OPV recipients served as a model for previous mucosal priming with live poliovirus. The induction of poliovirus-specific IgA was measured in the plasma, saliva and stool samples of the volunteers. Poliovirus-specific IgG and IgA antibody-producing cells isolated from the circulation were enumerated by ELISPOT assays. The homing potentials of the poliovirus-specific IgG- and IgA-producing lymphocytes found in the circulation were also examined to determine their final destination.

Materials and Methods

Vaccine recipients and booster immunisation

Fourteen IPV-vaccinated volunteers from The Netherlands (average age 25.8, range 20-41 years) and 11 OPV-vaccinated volunteers (average age 32.5, range 25-44 years) from various countries using OPV in their national programs were enrolled in the study. Most OPV recipients were from countries in which circulation of wild-type poliovirus has been absent or at low levels for some time (Canada, Germany, Belgium, Italy, New Zealand, Austria, Spain and Curaçao). One OPV recipient was from Morocco, where wild-type poliovirus has been detected as recently as 1995.

All volunteers were injected intramuscularly with a standard dose of the inactivated poliovirus vaccine (Diphtheria-, Tetanus-, Poliomyelitis vaccine, RIVM, Bilthoven, The Netherlands) containing 40, 4 and 7.5 D-antigen units for serotypes 1, 2 and 3 respectively. This vaccine is also used in the regular immunisation program in The Netherlands, where a total of six IPV vaccinations are given at 3, 4, 5 and 12 months, and at 4 and 9 years of age. Blood specimens were collected before booster vaccination and at 3, 7 and 28 days post-vaccination and were immediately processed. Saliva samples were collected at each of the first 10 days after vaccination, and every week thereafter until 8 weeks post-immunisation. Three stool specimens (week 0, 1 and 2) were examined for poliovirus-specific antibodies. Stool and saliva samples were stored at –20°C until use.

This study was reviewed and approved by the Ethical Review Committee of TNO (Zeist, The Netherlands). All volunteers signed an informed consent form at the start of the study.

Isolation of lymphocytes

Blood samples were collected in containers using EDTA as an anticoagulant. The blood samples were layered on an equal volume of ficoll (Histopaque, Sigma, Zwijndrecht, The Netherlands). After centrifugation (30 min, 400 x g) the lymphocyte-rich interphase was removed by pipette. The plasma was collected and stored at –20°C until testing in the ELISA assays was done. Cells were washed twice in RPMI/10% FCS (10 min, 250 x g), counted and adjusted to the required concentration.

Separation of homing receptor -positive and -negative cell populations

The separation of the lymphocytes into homing receptor-positive and -negative populations has been described by Kantele et al. [15]. Cells were separated on the basis of the expression of the integrin $\alpha4\beta7$ which mediates trafficking to the intestine and intestinal lymphoid tissues, and L-selectin (Chemicon, Temecula, USA), which mainly mediates trafficking to the peripheral lymph nodes [1,10,28]. Cells (107 cells/ml) were incubated with 1 μg/ml monoclonal antibody to L-selectin or with 2 μg/ml monoclonal antibody to $\alpha4\beta7$ (Act-1, kindly provided by Leukosite, Ambridge, USA, and Dr. Lazarovitz) for 30 minutes at + 4°C under rotation in a volume of 1 ml medium. Cells were washed three times and incubated with $2*10^7$ magnetic beads coated with sheep-anti-mouse IgG (Dynal M-450, Oslo, Norway). The beads with the attached cells were separated from the receptor-negative population through the application of a magnet. The beads were washed once and the separation was repeated. The receptor positive cells attached to the beads were suspended in medium. Both positively- and negatively-selected cell populations were used in ELISPOT assays.

FACS analysis

The composition of the negatively-selected cell populations was examined after cell separation by FACS analysis. Cells were incubated on ice for 30 minutes with primary antibody to L-selectin or $\alpha4\beta7$-integrin (Act-1). After incubation, the cells were washed 3 times with 1% BSA in PBS and incubated on ice for 30 minutes with FITC-conjugated goat anti-mouse conjugate (Cappel, Aurora, USA). Cells were washed and analysed using FACScan (Becton Dickinson, San Jose, USA). The average purity of the negatively-selected cell population after separation was 95% and 97% for L-selectin, and $\alpha4\beta7$ respectively.

ELISPOT-assay

Microtiter plates were coated with an optimal dilution in carbonate buffer of bovine anti-poliovirus serotype 1, 2 or 3 (RIVM, Bilthoven, The Netherlands) and were incubated overnight at + 4°C. The wells were then saturated with 10% fetal calf serum (FCS) in RPMI for 1 hour at 37°C. Antigen was added in a concentration of 40 to 120 DU/ml inactivated poliovirus and incubated for 2 hours at 37°C. Plates were washed four times with PBS supplemented with 0.5% Tween. Two-fold serial dilutions of the PBMC in a volume of 100 μl starting at 106 cells/ml were incubated for 4 hours, allowing the lymphocytes to secrete antibodies. Plates were washed and the antibodies

bound to the viral antigen on the plate were detected by alkaline phosphatase-conjugated IgG or IgA class-specific immunoglobulins (Sigma, Zwijndrecht, The Netherlands). Plates were incubated for 2 hours at 37°C. After washing the plates, substrate (5-bromo-4-chloro-3-indolyl phosphate) in a concentration of 0.65 mg/ml was diluted in 2-amino-2methyl-1-propanol substrate buffer with agarose of 40°C, then added to the wells and allowed to harden. Antibody-producing cells were visible as blue spots and were enumerated under a microscope allowing the total number of antibody-producing cells per 10^6 cells to be calculated. Cells were cultured in the absence of the poliovirus antigen as a control.

Poliovirus-specific total IgA, IgA1 and IgA2-ELISAs (plasma, saliva and stool)

The IgA ELISA was performed as described [12]. The presence of poliovirus-specific IgA was determined in plasma, saliva and stool samples. Plasma samples were inactivated (30 minutes at 56°C) before use in the IgA-ELISA, and were depleted of IgG antibodies with Quik-Sep (Isolab, Mechelen, Belgium) according to the manufacturer's instructions to prevent possible inter-isotype competition. Saliva samples were centrifuged (10 minutes, 3500 rpm) and inactivated for 30 minutes at 56°C. A 10% w/v suspension of the stool samples was added to the IgA ELISA at a 1:2 dilution. ELISA assays were performed with IgA1- and IgA2-specific conjugates (Southern Biotechnology Associates, Uithoorn, The Netherlands) to determine the subclasses of poliovirus-specific IgA. Optimal dilutions of reagents were determined by checkerboard titration. Positive and negative control serum samples were included in all IgA assays.

Poliovirus-specific secretory antibody capture-ELISA

A capture-ELISA was used as described to determine whether IgA detected in plasma samples after IPV vaccination was also present in its secretory form [12]. Briefly, microtiter plates were coated with a monoclonal antibody against the secretory component (Sigma, Zwijndrecht, The Netherlands) by overnight incubation at 4°C in carbonate buffer. Plates were blocked with 5% Blotto (Pierce, Oud Beijerland, The Netherlands). Plasma dilutions (1:50) were added and the plates were incubated for 1.5 hours at 37°C. Inactivated poliovirus was added and bound antigen was detected with horseradish-peroxidase-labelled serotype-specific monoclonal antibody (1 hour, 37°C). Tetramethylbenzidine (TMB) was used as a substrate (0.1 mg/ml) in 0.11 M sodiumacetate buffer, and the reaction was stopped after 30 minutes with 2 M H_2SO_4.

Poliovirus-specific subclass and total IgG ELISA (saliva and plasma)

Saliva and serum samples were tested for the presence of poliovirus-specific IgG antibodies. Assays were performed as described for the IgA ELISA but with anti-human-IgG-alkaline phosphatase labelled conjugate or with biotin-labelled antibodies to the different subclasses of IgG (IgG1, 2, 3 and 4; Sigma, Zwijndrecht, The Netherlands). Optimal dilutions of reagents were obtained by checkerboard titration. Avidin conjugated with alkaline phosphatase was added to the plates which were then incubated for 1 hour at 37°C. The plates were washed, and 100 µl of p-nitrophenylphosphate at a concentration of 1 mg/ml in 0.1 M glycine buffer was

added to each well. The plates were read at 405nm after incubation at room temperature for 30 minutes.

Poliovirus-Binding Inhibition assay (PoBI)

The PoBI was performed as described to determine the poliovirus-specific antibody titer in the plasma samples [13]. The reciprocal of the first serum dilution that was positive in the inhibition test was taken as the titer of the test sample.

Poliovirus-specific IgM capture-ELISA

The IgM-ELISA was performed as described [20]. A positive and a negative control serum were examined in each assay.

Statistical Methods

Student's t-tests were performed to determine the significance of the difference between IPV and OPV recipients. P-values of <0.01 were considered significant.

Results

Poliovirus-specific IgA- and IgG-producing cells in volunteers before and after IPV booster vaccination.

The number of poliovirus-specific IgA-and IgG-producing cells in the circulation was determined for all three serotypes of poliovirus at 0, 3, 7 and 28 days [Figure 1]. Both IPV- and OPV-vaccinated subjects responded with IgG-producing cells that were only detectable at day 7 after booster vaccination with IPV [Figures 1A and 1B]. High numbers of IgA-producing cells were detected in OPV-vaccinated persons 7 days after vaccination [Figure 1D]. In contrast, none of the IPV recipients had IgA-producing cells to serotypes 1 and 2 and only one IPV-vaccinated subject responded with 230 serotype 3-specific IgA-producing cells/106 cells at day 7 [Figure 1C]. No poliovirus-specific IgG- and IgA-producing cells were detected in either group at 0, 3 or 28 days after booster vaccination.

The L-selectin and α4β7 positive and negative cell populations were tested in serotype 3-specific ELISPOT assays [Figure 2]. The majority (77.3%) of the poliovirus-specific IgA-producing cells detected 7 days after booster vaccination in the OPV-recipients expressed the α4β7-integrin on their surface [Figure 2A]. A median level of 2744 and 808 poliovirus-specific IgA-producing cells/106 cells were measured for α4β7 integrin-expressing and non-expressing cells respectively. Poliovirus-specific IgA-producing cells were detected in both the L-selectin-positive and -negative populations in the OPV vaccinated group [Figure 2B]. Thirty-nine percent of the poliovirus-specific IgA-producing cells expressed L-selectin on their surface. There was no significant difference between the IPV and OPV recipients in the proportion of poliovirus-specific IgG-producing cells expressing the α4β7 integrin (72.3% versus 72.6%, data not shown). However, 80.9% of the poliovirus-specific

IgG producing cells expressed L-selectin in the IPV-vaccinated group while only 46.5% tested positive for L-selectin in the OPV recipients (data not shown).

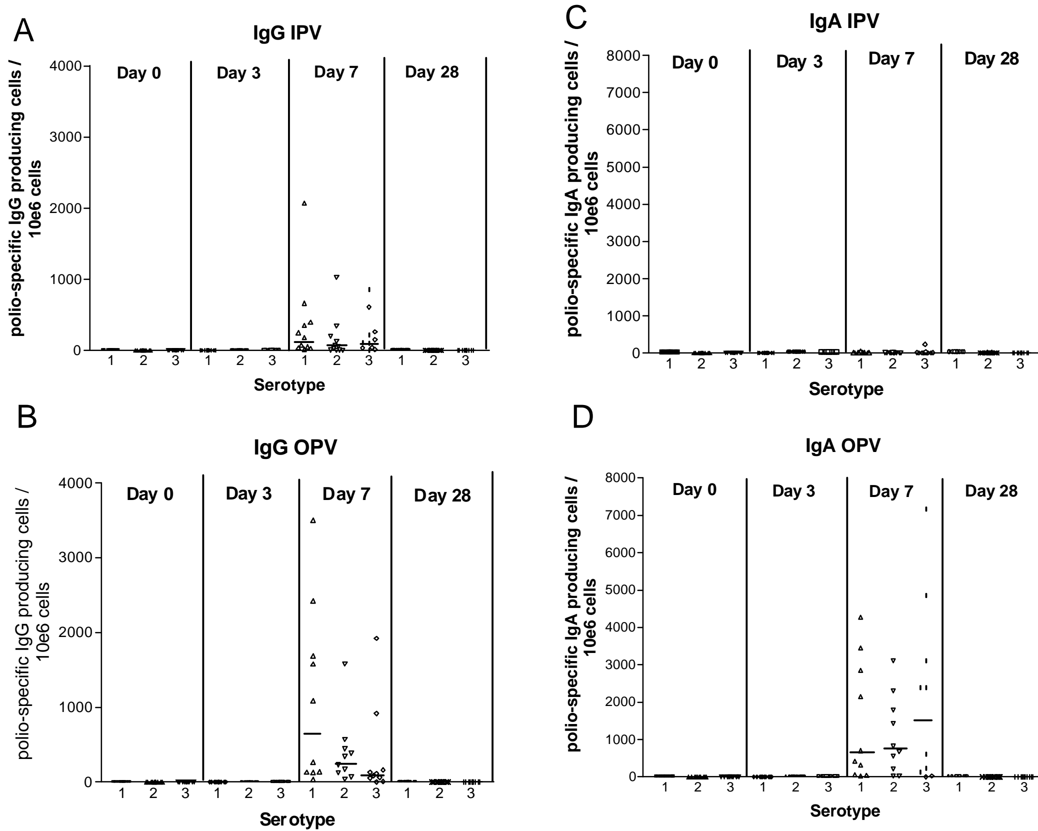

Figure 1. Poliovirus-specific IgA and IgG producing cells in the circulation at 0, 3, 7 and 28 days after an IPV booster vaccination in previously IPV-and OPV-vaccinated individuals determined by ELISPOT assays. Horizontal lines indicate the median values.

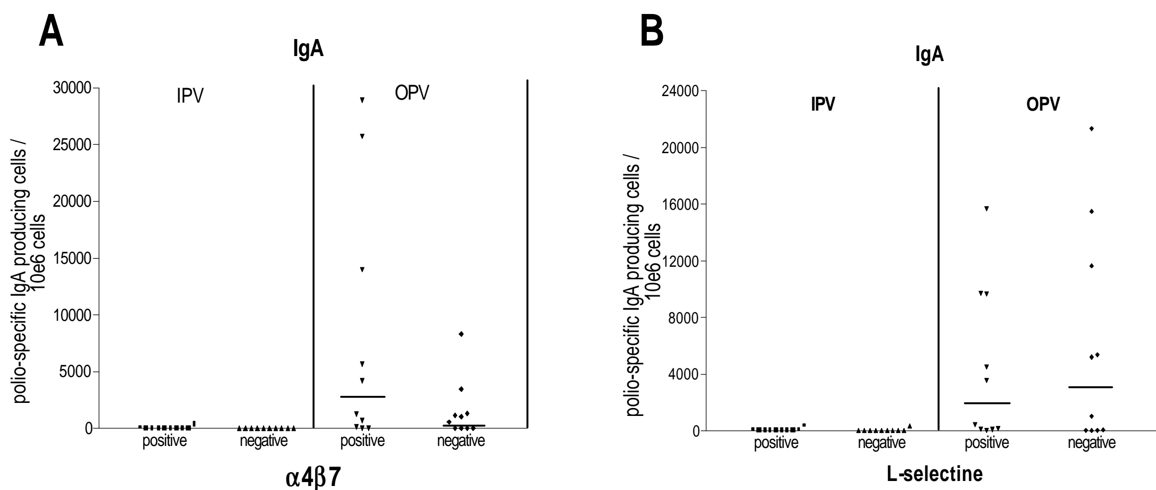

Figure 2. Expression of the L-selectin and α4β7 integrin homing-receptors on the poliovirus-specific IgA-producing cells in the circulation 7 days after an IPV booster vaccination in previously IPV- and OPV-vaccinated individuals. Horizontal lines indicate the median values.

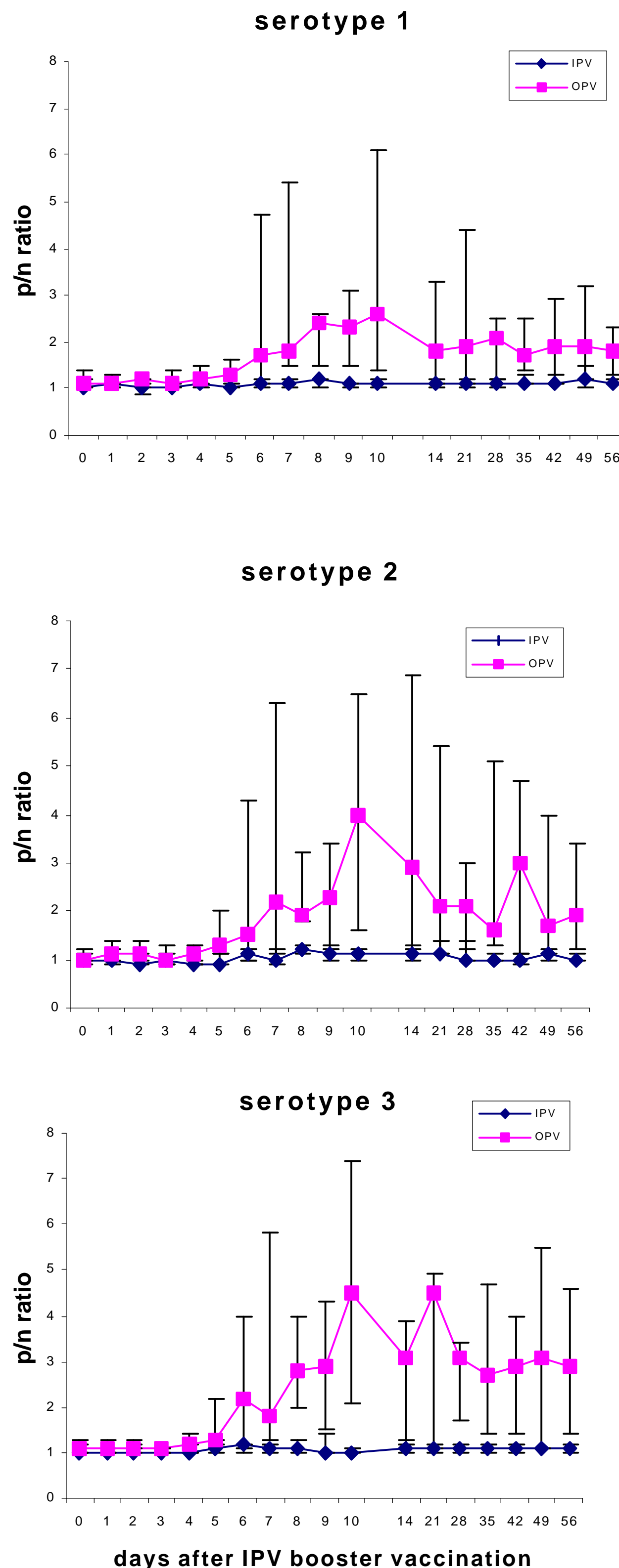

Figure 3. Poliovirus-specific IgA in the saliva after an IPV booster vaccination of previously IPV-and OPV-vaccinated individuals. Results are expressed in median values and the 25% and 75% percentiles of the ratio of positive reaction in ELISA readings in the presence of antigen divided by the ELISA readings in the absence of antigen (P/N ratio).

Poliovirus-specific IgA in saliva

A significant difference (p=<0.01) was seen between OPV and IPV recipients in the poliovirus-specific salivary IgA response for the three serotypes after the IPV booster vaccination [Figure 3]. Nine out of eleven OPV recipients developed a salivary IgA response to all three serotypes of poliovirus after the IPV booster vaccination. The poliovirus-specific IgA appeared in the saliva within five to six days after the booster vaccination. None of the IPV-vaccinated volunteers (n=14) responded with poliovirus-specific IgA in the saliva [Figure 3].

Poliovirus-specific IgA in stool

Poliovirus-specific IgA to all three serotypes was detected in the stool samples of three out of nine of the OPV-vaccinated subjects. A mucosal IgA response was not detected in any of the IPV-vaccinated subjects' stool samples. This difference was not significant (data not shown).

Poliovirus-specific IgA in plasma

Two subjects in the OPV group had detectable IgA to all three serotypes in their circulation before the IPV booster vaccination was given [Figure 4B], and one subject in the IPV group had detectable poliovirus-specific IgA to serotypes 2 and 3 at day 0. There was a clear increase in levels of circulating plasma IgA to all three serotypes in the OPV-vaccinated group at day 7 after the IPV booster vaccination, and the response remained elevated up to day 28 [Figure 4]. IgA responses to all three serotypes of poliovirus were also detected in the IPV-vaccinated group, but the levels were significantly lower than those observed in the OPV recipients (p= <0.01).

Poliovirus-specific IgA1 and IgA2 in plasma

Poliovirus-specific antibodies were clearly present in both IgA1 and IgA2 subclasses in the OPV recipients [Figure 4D & 4F]. IgA responses in the IPV recipients were seen at very low levels and appeared to be mainly of the IgA1 subclass. No poliovirus-specific IgA2 was detected in the majority of IPV recipients.

Poliovirus-specific secretory antibodies in plasma

The IPV and OPV recipients also differed in the induction of poliovirus-specific antibodies bound to the secretory component (SC) in their circulation [Figure 5]. Poliovirus-specific secretory antibodies appeared in 7 out of 10 OPV recipients for all three serotypes. Such responses were absent in the IPV recipients for serotype 1, and only 2 out of 11 IPV recipients had detectable poliovirus-specific secretory antibodies for serotype 2 and 3 [Figure 5A]. In all cases, the secretory antibody responses were at low levels and of short duration, with an apparent peak at day 7.

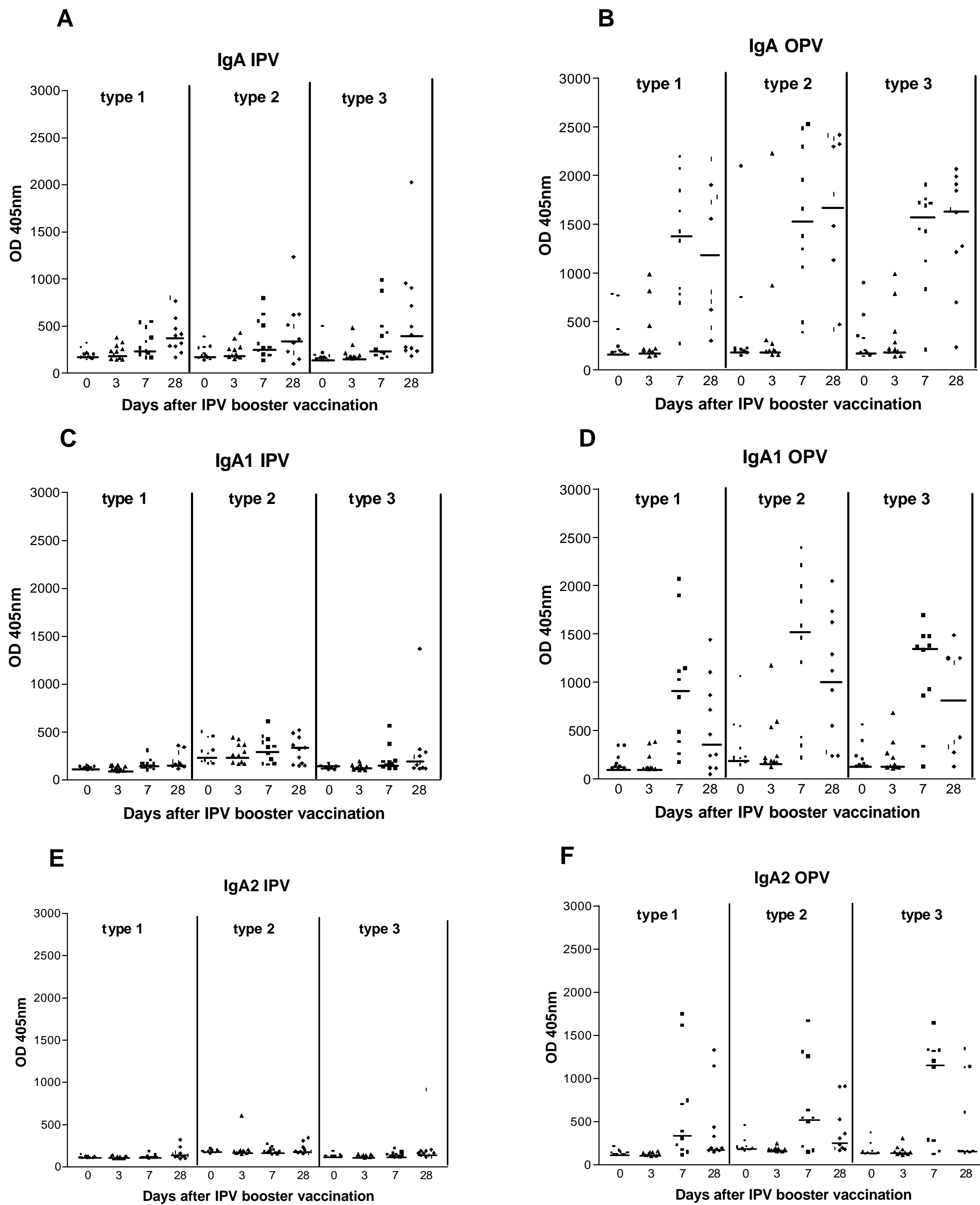

Figure 4. The induction of poliovirus-specific IgA and subclass IgA1 and IgA2 in the plasma samples after an IPV booster vaccination in previously IPV- and OPV-vaccinated individuals at 0, 3, 7 and 28 days after IPV booster vaccination. Horizontal lines indicate the median values.

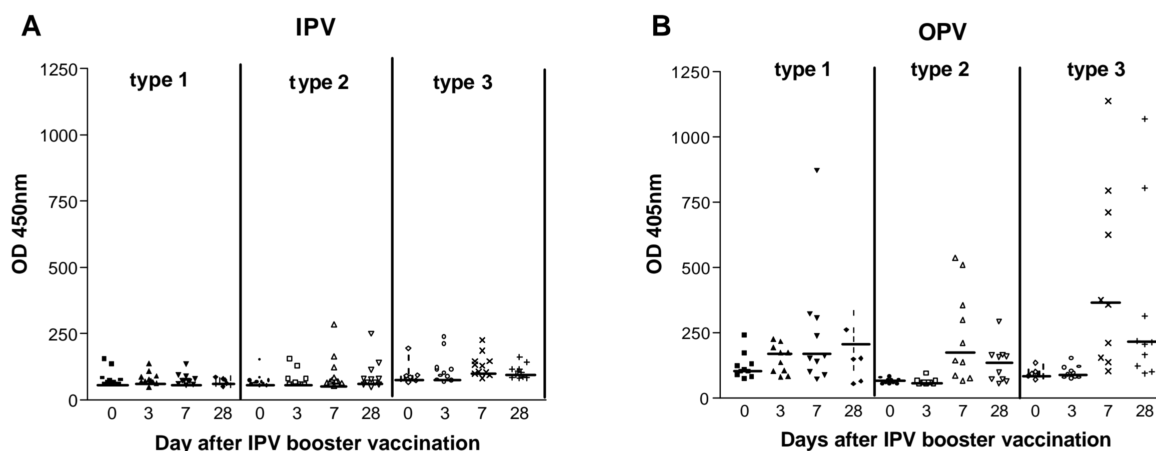

Figure 5. Induction of poliovirus-specific antibodies in association with secretory component after an IPV booster vaccination in previously IPV- and OPV-vaccinated individuals at 0, 3, 7 and 28 days after IPV booster vaccination. Horizontal lines indicate the median values.

Poliovirus-specific (subclass) IgG in plasma and saliva

In general, the total IgG response in the plasma samples from both groups was not significantly different and consisted mainly of the IgG1 subclass [Figure 6]. However, the IgG3 subclass response was more prominent in the IPV group compared to the OPV recipients and this difference was significant ($p<0.05$) for serotypes 1 and 3 at day 7 and day 28 after vaccination [Figure 6E and 6F]. A low level IgG2 response was induced by the IPV booster vaccination in several individuals from both groups (data not shown). No clear response was seen in IPV and OPV recipients for IgG4 for all serotypes (data not shown). No poliovirus-specific IgG was seen in the saliva after IPV booster vaccination in most of the IPV and OPV recipient groups. However, two IPV recipients and one volunteer from the OPV group responded with an IgG response in the saliva to all three serotypes (data not shown).

Poliovirus-Binding Inhibition assay (PoBI)

At the start of the study, the median levels of PoBI titers were generally higher in the IPV-vaccinated group than in the OPV recipients. This difference was significant for serotype 3 ($p<0.01$). However, the OPV recipients responded with a similar increase in PoBI titers after IPV vaccination and reached the same levels at day 28 (data not shown).

Polio-specific IgM in plasma.

Low positive IgM responses only were detected in recipients from both study groups at 7 and 28 days after booster vaccination. Three, two and four out of 11 volunteers in the IPV-vaccinated group had an IgM response to serotype 1, 2, and 3 respectively. Three, five and five persons out of 10 volunteers in the OPV-vaccinated group had an IgM response for serotype 1, 2 and 3 respectively. No significant differences were detected in IgM responses between the OPV recipients and IPV recipients (data not shown).

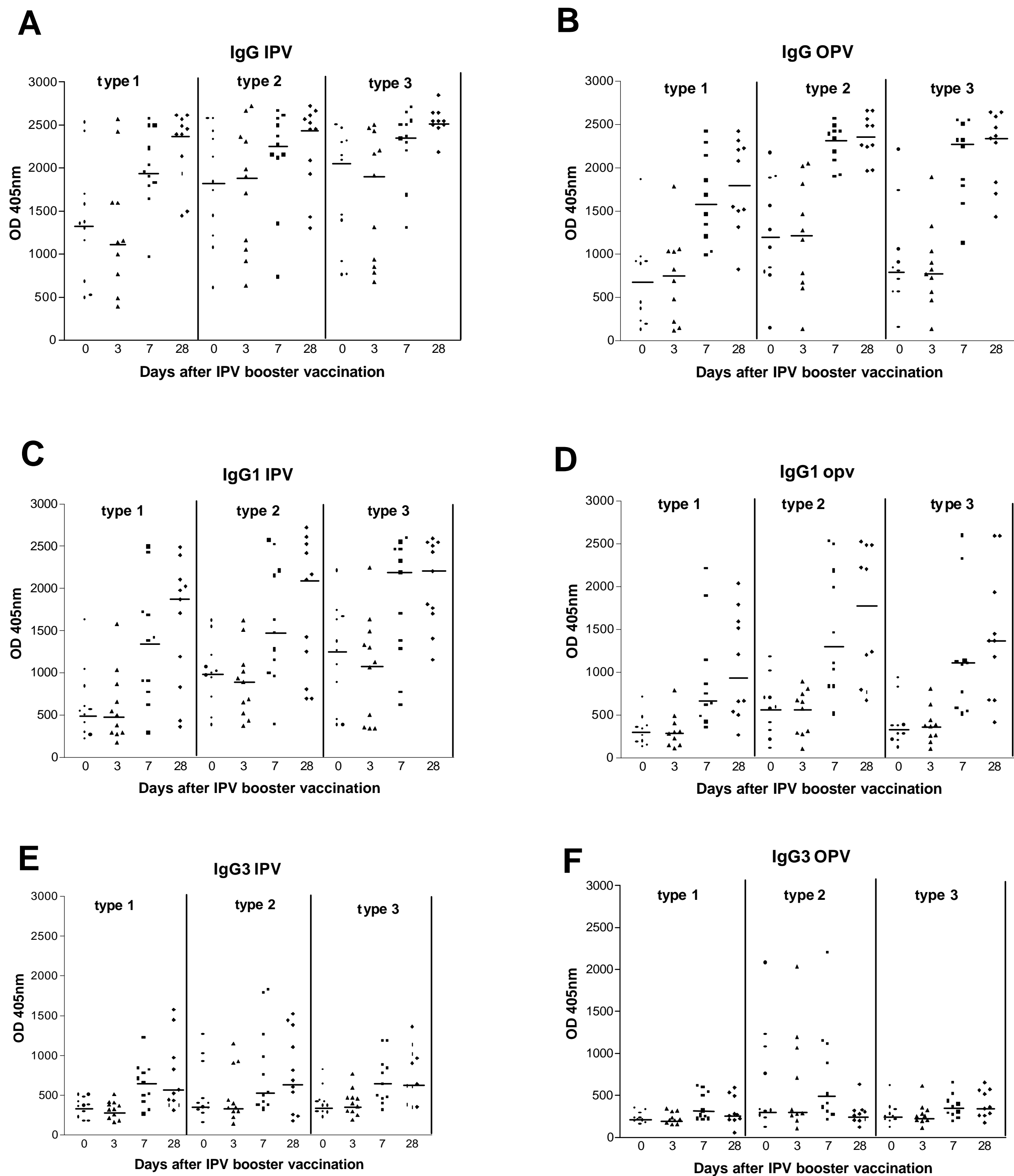

Figure 6. Induction of total IgG, IgG1, and IgG3 after an IPV booster vaccination of previously IPV and OPV recipients at 0, 3, 7, and 28 days after IPV booster vaccination. Horizontal lines indicate the median values.

Discussion

Although low levels of mucosal immunity have been found after IPV vaccination in previous studies, these responses have been less effective in reducing viral shedding after a challenge with OPV than those observed after OPV vaccination [4,5,9,11,16,23]. This study was conducted in order to determine mucosal immune

responses following IPV vaccination in a country with almost no circulating poliovirus. Under these circumstances, it is clear that IPV vaccination alone is not sufficient to induce mucosal IgA. From this and previous work we conclude that the previously reported mucosal responses after IPV vaccination are more likely to be the effect of previous mucosal priming with live viruses than the effect of IPV vaccination alone. It remains to be determined whether or not IPV vaccinees (in the total absence of a mucosal IgA response) are partially protected in challenge experiments. Reduced virus shedding in IPV recipients after challenge with OPV has been reported previously, but again, it is unclear in these experiments whether poliovirus immunity was solely induced by IPV vaccination as opposed to a mixed immunisation of IPV combined with mucosal infection [11,21,23].

The poliovirus-specific IgG levels detected in the saliva of IPV recipients were low or absent, suggesting that salivary IgG does not play a role in protection from mucosal infection with poliovirus. IgG is thought to enter the mucosal secretions non-specifically through paracellular transport. It remains to be investigated whether or not circulating IgG is able to exert an influence on protection to a mucosal poliovirus challenge. It has been postulated that a critical level of specific serum IgG may be sufficient to protect against infectious diseases by inactivating the inoculum of the pathogen [25].

Assuming that the absence of mucosal IgA reflects a lack of mucosal protection, our observation may have implications for the poliomyelitis eradication program in The Netherlands. In the absence of an efficient mucosal barrier, IPV recipients will remain sensitive to poliovirus infection. These infections will go unnoticed because fully-vaccinated persons will not develop any symptoms of disease. Under these circumstances, IPV recipients will contribute to the (continuous) circulation of poliovirus. This poses a special risk to the religious communities with low vaccine coverage that presently exist in The Netherlands. Despite high national vaccine coverage, epidemics of poliomyelitis within these groups occurred in 1978 and 1992 [2,6,24,27]. During the advancing stages of poliomyelitis eradication, and taking into account a decrease in the incidence of the mucosal infection of IPV recipients by live poliovirus, this effect is likely to be even more pronounced, resulting in the waning of presently existing mucosal immunity in the general population.

Parenteral IPV vaccination induced a strong and rapid IgA response in previously OPV-vaccinated persons both at mucosal sites and in the circulation. Similar memory IgA responses in the circulation after IPV vaccination were detected in a group of non-vaccinated but naturally exposed persons [12]. Induction of memory sIgA responses by parenterally-administered inactivated vaccines has also been described for influenza virus [3,19], Pseudomonas aeruginosa, and meningococci [8,18]. Natural infection with these agents is very common in such cases, and the authors postulate that the memory IgA response most likely resulted from previous mucosal infection with the wild-type virus or bacteria, based on the dogma that mucosal antigen presentation is required for an effective mucosal immune response.

The mechanism by which inactivated parenteral vaccination can re-stimulate the mucosal IgA responses is unknown. The memory IgA-producing cells detected in our experiments have most likely been originally primed at mucosal sites. IPV is applied intramuscularly, therefore the peripheral lymph nodes are theoretically the first lymphoid location for antigen presentation to memory cells [29]. Expression of L-selectin (indicating homing to peripheral lymph nodes) on poliovirus-specific IgG-

and IgA-producing cells was demonstrated in this study. The memory lymphocytes will proliferate after re-stimulation and leave the peripheral lymph nodes. A large proportion (73%) of the circulating poliovirus-specific IgA-producing cells expressed the $\alpha 4\beta 7$ mucosal homing receptor, indicating a preference for homing to mucosal surfaces. This was underscored by the induction of poliovirus-specific IgA in saliva.

At least a proportion of the poliovirus-specific IgA-producing cells in this experiment expressed both the $\alpha 4\beta 7$ integrin and L-selectin on their surface. It is known that some homing receptors are expressed continuously while others are induced by local activating signals acting on both circulating and local cells [1]. The final combination of homing receptors on the cells' surface is likely to account for the regional preference of the activated cells. The presence of memory cells that express both the L-selectin and $\alpha 4\beta 7$ homing receptors are an ideal immune surveillance mechanism for the control of infections both at the systemic and mucosal level.

The poliovirus-specific subclass responses of IgA and IgG were determined in order to investigate whether there was a qualitative and quantitative difference in the humoral immune response after IPV booster vaccination in previously IPV- and OPV-vaccinated persons. Poliovirus-specific IgA2 in the circulation was clearly present in the OPV recipients and might be derived from lymphocytes that were originally primed at mucosal sites. The presence of poliovirus-specific IgA2 in the plasma might serve as a systemic marker for mucosal memory rather than the presence of total poliovirus-specific IgA, since an IgA1 response (albeit a small one) was also seen in some of the IPV recipients.

We detected an IgG1 and IgG3 subclass response before and after IPV booster vaccination in both the OPV and IPV recipients [Figure 7]. Similar results were reported in poliomyelitis patients and for other enteroviral antigens [14,30]. However, the IgG3 response in the OPV recipients was significantly lower than in IPV recipients in this study. These results might indicate a difference in preference for an IgG1 switch over an IgG3 switch after mucosal priming with OPV compared to systemic vaccination with IPV. No clear IgG4 response was observed and only a few individuals responded with IgG2 poliovirus-specific antibodies. Mechanisms for the observed differences remain to be investigated.

Not much is known about the induction and presence of circulating secretory IgA. Immunoassays indicate that secretory IgA can be detected at relatively low levels (+/- 10μg/ml) in serum [7,17]. This study detected poliovirus-specific secretory IgA after booster vaccination with IPV in previously OPV-vaccinated recipients. The response was at low levels and decreased rapidly. The biological relevance of this finding is speculative and remains to be investigated, although we speculate that it represents an overload of the secretory system.

Vaccination with OPV introduces live virus into the environment and must therefore cease completely in the future. This study has demonstrated that IPV booster vaccinations are able to maintain a high level of mucosal IgA response for years after initial vaccination with OPV. A combination schedule of OPV and IPV vaccination could serve as a powerful tool in the final stages of the eradication program.

Acknowledgements

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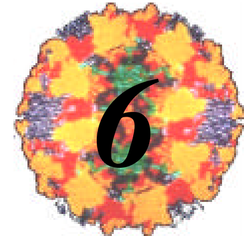

Lessons from diagnostic investigations of poliomyelitis patients and their direct contacts for the present surveillance of acute flaccid paralysis

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Submitted for publication

Abstract

One of the key strategies for the global eradication of poliomyelitis is the virological investigation of stool samples from all cases of acute flaccid paralysis (AFP) to exclude poliovirus as a possible cause. However, adequate virological investigation of the stool specimens from these patients is carried out in fewer than the required number of cases, especially in industrialised countries. Poliomyelitis cannot be excluded in all of these cases. Other diagnostic assays using both immunological and molecular biological methods have been developed within the last decade for the detection of poliovirus infection. Clinical specimens from the 1992/93 poliovirus serotype 3 outbreak in The Netherlands provided an opportunity to examine the potential of the various methods for the diagnosis of poliomyelitis. The virus isolation rate in poliomyelitis patients was maximal (89.6%) during the first two weeks after the onset of paralysis and then dropped sharply to 18.6%. In contrast, a high percentage of the patients tested positive for serotype 3-specific IgM (93.3%) and IgA (87.1%) in the early phase of the infection and remained positive up to eight weeks after the onset of paralysis. While the serotype 3-specific IgM rate dropped to 20% at 8 weeks after infection, the poliovirus-specific IgA persisted in many patients (56.7%) for more than 8 weeks after infection and is therefore less suitable for the diagnosis of recent infections. The use of virus isolation would have correctly identified only 54.9% of AFP cases in the theoretical event that all of these cases were due to poliovirus infection. The identification rate would have been increased to 92% through the use of the poliovirus-specific IgM ELISA. Virus isolation from stool specimens will remain the standard diagnostic method. The speed and ease of performance of the IgM ELISA, however, could allow it to serve as an important additional tool for the rapid diagnosis of poliomyelitis. Moreover, the IgM ELISA may facilitate the resolution of AFP cases when stool investigation has been less than adequate or in cases analysed late after the onset of disease.

Introduction

In 1988, the World Health Organisation (WHO) launched an initiative to eradicate poliomyelitis by the year 2000 [10]. One of the key strategies for the global eradication of poliomyelitis is the virological investigation of stool samples from all cases of acute flaccid paralysis (AFP) to identify poliovirus as a possible cause [29]. Acute flaccid paralysis (AFP) is characterised by the rapid onset of muscle weakness in the extremities, or in some cases the muscles involved in respiration and swallowing. The maximum severity of symptoms is apparent within one to ten days post-onset [30]. AFP can have numerous other causes apart from poliovirus infection, including infections with other enteroviruses, acute myelopathy, peripheral neuropathy (such as the Guillain-Barré Syndrome) and neuromuscular transmission disorders [30].

The incidence of AFP in the absence of poliomyelitis is estimated to be at least one case per 100,000 children younger than 15 years of age [3]. The timely collection of adequate stool samples from AFP cases is considered crucial for viral detection, and the collection of two samples 48 hours apart within 14 days after onset of paralysis is used by the WHO as performance criteria for the quality of the AFP surveillance [30]. However, compliance with these criteria is limited. This is especially true in industrialised countries where, because of the absence of poliomyelitis cases, clinicians do not always recognise the need for intensive AFP surveillance [10].

Both our laboratory [15,20] and other groups [11,14,21,22,25,32] have developed poliovirus serotype-specific IgM- and IgA-enzyme-linked immunosorbent assays (ELISAs) and methods for the detection of poliovirus based on RT-PCR. While most of these assays have been used in seroprevalence or epidemiological studies, they may also be useful for the rapid diagnosis of poliovirus-related illness as well as for a resolution of cases reported late after the onset of disease in the AFP surveillance system. Therefore, this study re-examines specimens that have been collected from patients and their contacts during a poliovirus serotype 3 outbreak in The Netherlands in 1992/1993 [7,23,27].

Stool samples, sera, cerebrospinal fluids (CSF) and/or throat swabs were examined from most of the 71 patients from this outbreak. In addition, stool specimens and sera from 86 family members or close contacts of the poliomyelitis patients were examined for poliovirus excretion and poliovirus-specific antibodies. This article describes the application of this broader panel of diagnostic assays in order to determine whether these tests may provide an added value for the surveillance of AFP as a crucial step in the poliomyelitis eradication program.

Material and methods

Patients and clinical samples

Seventy-one patients were notified during the outbreak of type 3 poliomyelitis in The Netherlands in 1992/1993 [23]. Clinical samples from 69 poliomyelitis patients were available for analysis in the laboratory. One or more serum samples were collected from 69 cases (97.2%), one or more stool samples were available from 58 patients (81.7%), a CSF sample was obtained from 44 poliomyelitis cases (62.0%) and throat-swabs were collected from 25 patients (35.2%).

Contacts of poliomyelitis patients

Stool and serum samples were obtained from 86 contacts of 25 poliomyelitis patients. These specimens were examined for the presence of poliovirus in the stool samples as well as for the presence of poliovirus-specific IgM and IgA antibodies in the serum samples. Poliovirus-Binding Inhibition assays were performed to determine levels of poliovirus type 3-specific binding antibodies [16].

Acute Flaccid Paralysis (AFP) surveillance

Recent AFP surveillance data from The Netherlands were used to calculate the added value of the new diagnostic methods in the present AFP surveillance. Surveillance of AFP among children younger than 15 years of age in The Netherlands started in 1992. The measured incidence of non-polio AFP was between 0.39 to 0.87 per 100,000 children [8,24]. This was lower than the required non-polio AFP incidence of 1:100,000, indicating inadequate surveillance [8,24]. The median reporting time of AFP cases in The Netherlands was 91 days [8]. None of the non-polio AFP cases met the required stool sampling criterion of two samples taken 24-48 hours apart within the first 14 days after onset of paralysis. Virus isolation was carried out in only a low percentage (31%) of the AFP cases [8].

Isolation of poliovirus from stool and throat swabs

Virus isolation and typing was carried out on HEp-2C and RD cells as recommended by the WHO [29]. Poliovirus strains were differentiated as wild- or vaccine-derived strains in an enzyme immuno assay using intra-typic cross-absorbed rabbit antibodies [28]. The wild-type character of all of these isolates was confirmed by RNA sequencing [19].

Poliovirus serotype-specific IgM and IgA ELISAs

The ELISAs for poliovirus serotype-specific IgM or IgA antibodies in serum from patients and contacts were performed as described [15,20]. Poliovirus-specific IgM was also determined in patients' CSF [20]. Inactivated poliovirus was used as the antigen in both assays. A positive and a negative control serum sample were included in each assay.

Reverse Transcriptase (RT)-PCR for the detection of poliovirus in CSF

A generic enterovirus-specific RT-PCR was used to examine CSF for the presence of polioviruses as described [26]. Viral RNA was purified from the CSF samples by binding to silica particles in the presence of GuSCN [5].

Poliovirus-Binding Inhibition assay (PoBI)

A Poliovirus-Binding Inhibition assay was used to determine the poliovirus-specific antibody titers as an indicator of neutralising antibodies. The PoBI assay was performed as described [16]. The reciprocal of the first serum dilution that was positive in the inhibition test was taken as the titer of the test sample.

Results

Virus isolation from poliomyelitis patients

Stool samples from 58 poliomyelitis patients were examined for the presence of serotype 3 poliovirus at different times after the onset of paralysis. Stool samples provided during the first two weeks after the onset of paralysis were most frequently positive (89.6%) [Figure 1, Table 1]. The virus isolation rate dropped sharply to approximately 20% in samples provided between three to ten weeks after the onset of paralysis. No virus was isolated from any of the stool samples collected more than ten weeks after the onset of disease [Figure 1, Table 1]. The first stool sample examined was poliovirus serotype 3 positive in 50 of 54 polio patients (84.7%); the accumulating positivity rate increased to 93.2% and 94.8% after examination of a second and third specimen respectively. Throat swabs were collected during the first two weeks after the onset of paralysis only (n=25). Virus was isolated from the throat of 32% of the patients during this period [Figure 1, Table 1].

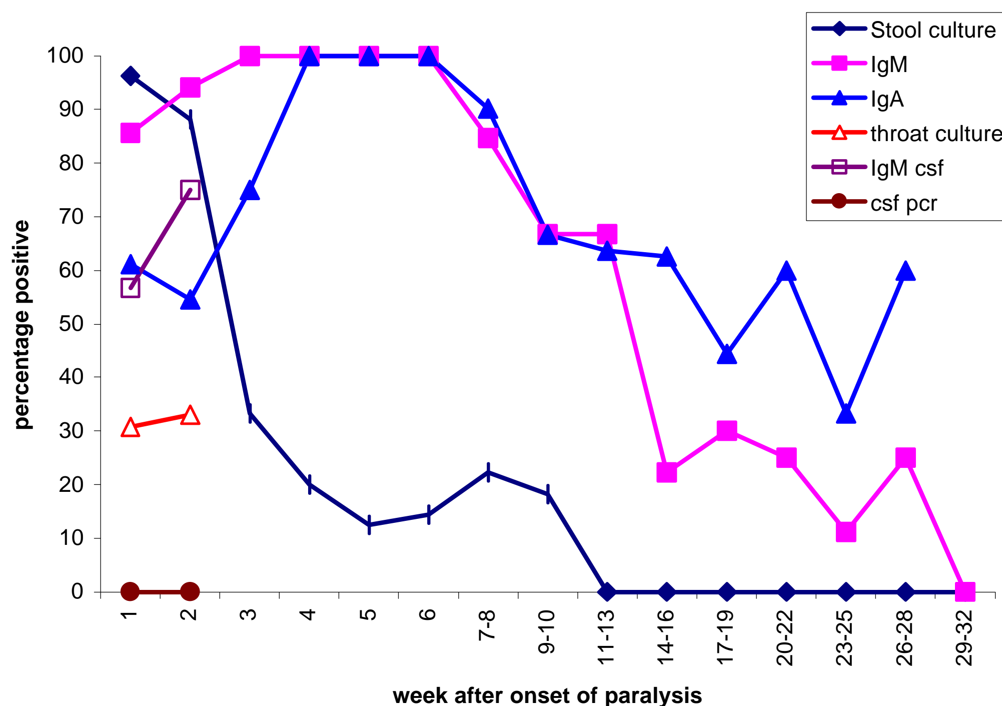

Figure 1. Positivity rates of virus isolation from stool, and of serum IgM and IgA antibodies to poliovirus type 3 in poliomyelitis patients. Results are from consecutive serum samples collected during the 1992/1993 outbreak in The Netherlands.

Table 1. Sensitivity of assays per time period after onset of paralysis

Assays	0-2 Weeks (%)	2-8 Weeks (%)	> 8 Weeks (%)
Culture			
Stool	89.6	18.6	2.0
Throat	31.0	nd	nd
Serology			
IgM	89.5	93.9	33.3
IgA	60.7	87.1	56.7
CSF			
IgM	63.6	nd	nd
RT-PCR	0.0	nd	nd

Detection of poliovirus-specific IgM in poliomyelitis patients

Serotype 3-specific IgM was detectable in all serum samples from patients obtained between three to six weeks after the onset of paralysis (n=24); a high percentage was also positive before this period (89.5%) [Figure 1, Table 1]. Twenty percent of the patients had low but detectable levels of virus-specific IgM three months after the onset of paralysis.

Detection of poliovirus-specific IgA in the serum of poliomyelitis patients

Sera from 49 patients were available for the determination of poliovirus-specific IgA. The kinetics of the IgA response in patients have been reported elsewhere [15] and show that the IgA peak is maximal between three to eight weeks after the onset of paralysis. Five months after the onset of paralysis, 33 (60%) of the patients still had detectable type 3 specific IgA in their circulation [Figure 1, Table 1].

Examination of the CSF of poliomyelitis patients

A CSF sample collected during the first two weeks after the onset of paralysis was available from 44 patients. Poliovirus type 3-specific IgM was present in 28 (63.6%) of these CSF samples [Figure 1, Table 1]. All matching serum samples were also positive for serotype 3-specific IgM. Sera from fourteen patients (31.8%) without IgM in their CSF was positive for serotype 3-specific IgM.

Twenty-one CSF samples from poliomyelitis patients were examined by RT-PCR for the presence of poliovirus RNA [Figure 1, Table 1]. Viral RNA was not detected in any of these samples, whereas the same extracts yielded positive RT-PCR signals after spiking with poliovirus.

Comparison of IgM, IgA and virus culture in contacts

A comparison of assays was possible for 86 contacts of poliomyelitis patients with a complete diagnostic data set (virus isolation, IgM, IgA and PoBI). A total of 68 stool samples were positive for the epidemic wild-type serotype 3 strain in culture (79%). Fifty-nine (68.6%) and 46 (53.5%) contacts were positive for serotype 3-specific IgM and IgA respectively. Eighty-five (67.5%) contacts were positive in the IgM-ELISA and secreted poliovirus. Only ten (11.6%) contacts were excreting poliovirus in the absence of an IgM response. Seventy-seven (89.5%) contacts had PoBI titers to serotype 3.

The prevalence of serotype 3-specific IgM antibodies was high (86.1%) in the young age groups (three to ten years) and declined with age [Figure 2]. The prevalence of serotype 1- and 2-specific IgM was below 10% in all age groups. Virus excretion in the stool was high (96.7%) within the age group ranging from three to 15 years, and followed the same decreasing pattern with age as did the IgM seroprevalence for serotype 3 [Figure 2C]. In contrast, poliovirus-specific IgA followed the same patterns as the poliovirus-specific antibodies for all three serotypes [Figures 2A and 2C]. The presence of serotype 1-specific IgA, serotype 2-specific IgA and poliovirus-binding antibodies (PoBI) increased with age and was highest in the oldest (31-80 years) age group (46.2-53.8%) [Figures 2A and 2B].

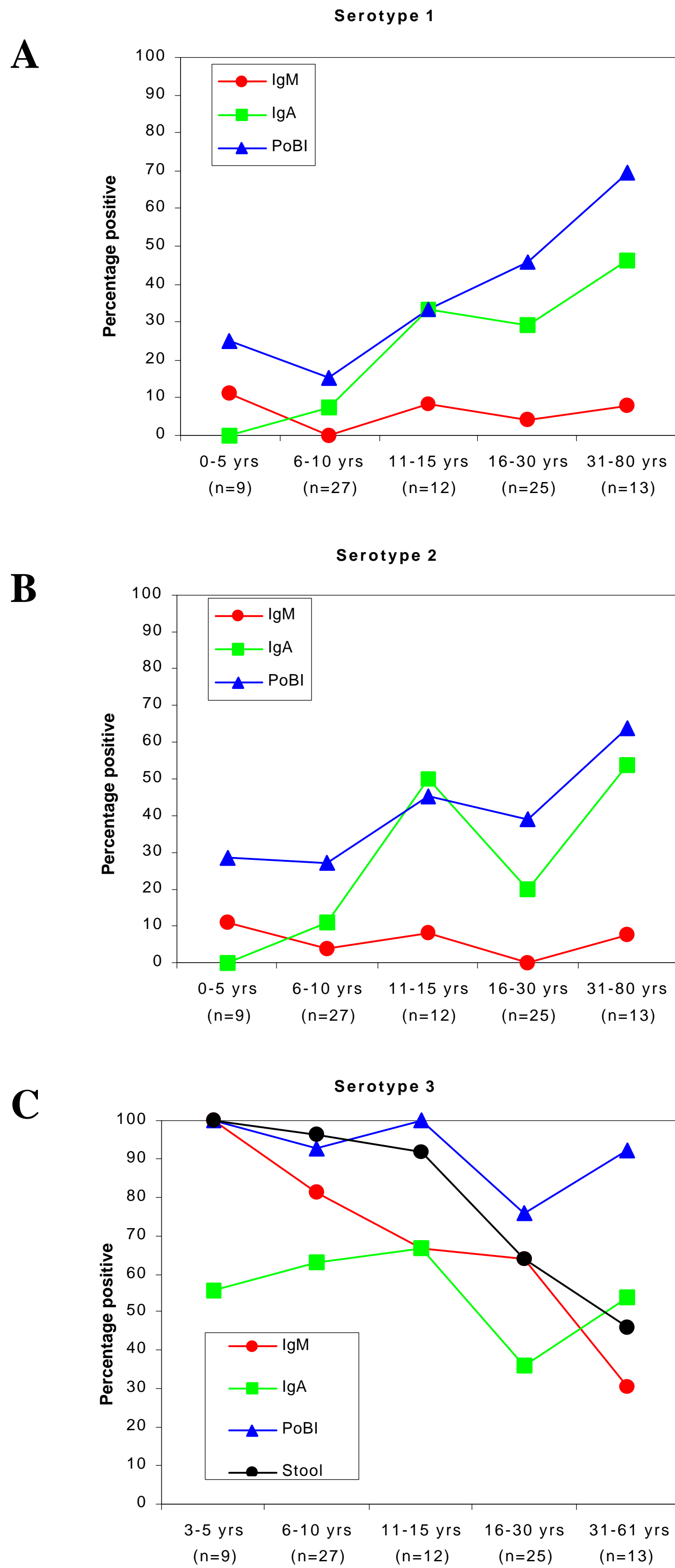

Figure 2. Prevalence of IgM, IgA and neutralizing antibodies to poliovirus type 1 [Fig 2A], type 2 [Fig 2B] and type 3 [Fig 2C] in contacts of poliomyelitis patients of different ages.

Calculation of the theoretical benefit of the application of IgM and IgA ELISAs for AFP surveillance in The Netherlands

Virus isolation was attempted during the first to the seventh week after the onset of paralysis in 31% of all AFP cases reported in The Netherlands in 1995 and 1996. This virus isolation was carried out within the recommended time period of two weeks after the onset of disease in only 51% of these cases. The sensitivity of virus isolation is 89.6% during this time period [Figure 1], which theoretically leads to 45.8% correctly diagnosed AFP cases in the virologically investigated group [Figure 3A]. Stool samples from 49% of these cases were examined between three to seven weeks after the onset of paralysis. The chance of successful isolation during this period is 18.6%, leading to a calculated percentage of correct poliovirus diagnoses in the virologically-examined AFP patients of only 9.1% [Figure 3A]. Theoretically then, the overall virological investigation of these AFP cases could only yield a correct diagnosis in 54.9% of the virologically examined cases, or 17% of all reported AFP cases.

A similar calculation was performed for the IgM ELISA [Figure 3B]. Only 15% of the reported AFP cases were serologically examined by IgM ELISA. In the first two weeks after the onset of paralysis, IgM-expected positivity rates were almost identical to the rates of virus isolation from the stool (89.5%). Expected rates remained high (93.9%) three to seven weeks later, leading to an additional 46.0% of theoretically correctly diagnosed cases. The overall percentage of theoretically correctly diagnosed AFP cases with the IgM ELISA was 92.0%.

Discussion

Historically, the diagnosis of poliomyelitis has relied on poliovirus isolation. Virus isolation is still the diagnostic method of choice and was, until recently, the only method that could reliably distinguish between wild or vaccine-virus infection. However, several new diagnostic methods have been developed in recent years that have not been previously evaluated under field conditions due to the lack of poliomyelitis cases. An epidemic of serotype 3 poliovirus in The Netherlands in 1992/1993 provided the opportunity to examine the potential of a number of these new methods [7,23,27].

Serotype 3 poliovirus from the outbreak was isolated most frequently from the stool of poliomyelitis patients at one to two weeks after the onset of paralysis (89.6%) but positivity rates dropped sharply thereafter to a level of less than 20%. As has been previously described, 63% to 93% of serotype 1 stool samples test positive during the first two weeks after the onset of disease [2]. During the third and fourth weeks after onset, isolation frequencies for serotype 1 generally decline to a range of 35% to 75%. Specimen positivity is usually below 50% after four weeks [2]. There is limited data on the duration of excretion of wild strains of poliovirus types 2 and 3 [2]. However, our study shows that poliovirus type 3 excretion seems to be about two weeks shorter than that of serotype 1 [2].

In this study, poliovirus was isolated from the throat in only 32% of patients during the first two weeks after the onset of disease. Because poliovirus is excreted from the intestine for several weeks after infection, it is most frequently isolated from the stool samples. These stool samples are, therefore, the most suitable materials for isolation of poliovirus, confirming WHO criteria [29].

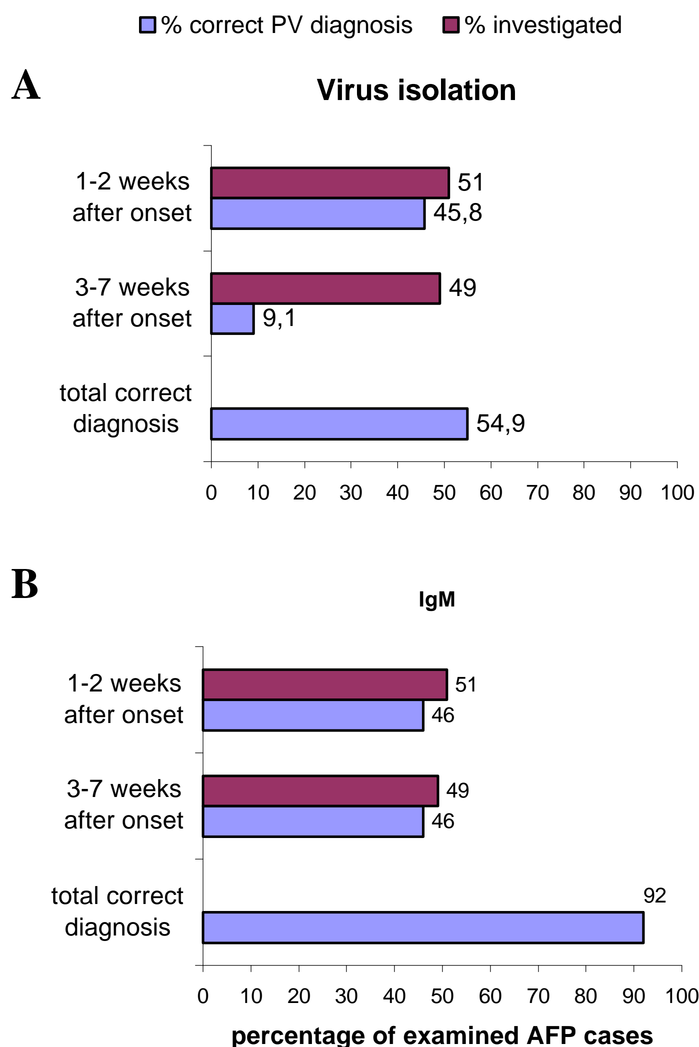

Figure 3. Calculations of the number of correct exclusions of poliomyelitis cases by A] virus isolation and B] IgM-ELISA.

The poliovirus-specific IgM ELISA does not discriminate between vaccine and wild-type poliovirus-induced antibodies. However, our data show that in addition to virus isolation from the stool, the detection of poliovirus serotype-specific IgM in AFP patients may facilitate the laboratory diagnosis of poliomyelitis and may help to exclude poliovirus as the causative agent. The detection of poliovirus-specific IgM has the additional value of indicating proof of a recent infection with poliovirus even in the absence of virus isolation. Seven cases (10%) of poliomyelitis were diagnosed solely on the basis of IgM serology during the 1992/1993 outbreak in The Netherlands. Virus-specific IgM in poliomyelitis patients could be detected for at least six weeks longer than virus could be isolated from the stool [Figure 1]. An additional advantage of the IgM-ELISA lies in the fact that it provides a result within 24 hours compared to the five to seven days required for routine virus isolation and typing. Poliovirus serotype 1- and 2-specific IgM antibodies were detected in a small number (<10%) of patient contacts. The presence of these IgM antibodies is probably due to circulating OPV vaccine strains that were offered to control the epidemic.

The percentage of samples with serotype 3-specific IgM positive results was remarkably lower in CSF (63.0%) than in serum samples (89.5%). In addition, all patients with serotype 3-specific IgM-positive CSF had virus-specific IgM in their serum. Similar results were described by Roivainen et al [25] who reported that 58% of the patients in their study had detectable poliovirus-specific IgM in their CSF, while 94% tested positive for poliovirus-IgM in serum. These results indicate that there is no additional diagnostic benefit of poliovirus-specific IgM detection in CSF.

Poliovirus RNA could not be detected with an enterovirus-specific RT-PCR in the CSF samples of poliomyelitis patients. This is in contrast to results obtained in the diagnosis of infections with other enteroviruses, where infection can easily be confirmed in CSF by PCR and/or virus culture [1,6,9,18]. In conclusion, laboratory examination of CSF either by PCR or by IgM assay is not recommended for the laboratory confirmation of poliomyelitis, although it may be useful for the determination of other causes of paralytic disease.

Not much is known about how polioviruses are able to cross the blood brain barrier. Data from transgenic mouse experiments shows that polioviruses permeate through the blood brain barrier at a high rate, independently of the poliovirus receptor [31]. It has been proposed that poliovirus-infected monocytes that cross the blood brain barrier into the CNS are a possible route [12,17].

As for IgM, the detection of serotype 3-specific IgA antibodies was much more sensitive than was virus isolation from the stool for diagnosis of poliovirus infection between two to eight weeks after the onset of illness. However, the presence of IgA to serotype 3 might also reflect past rather than recent infection because of the persistence of the IgA isotype in the bloodstream after infection. For this reason, the detection of poliovirus-specific IgA is less suitable for the detection of recent poliovirus infection.

Previously, we described that the seroprevalence of poliovirus-specific IgA was significantly lower in young IPV-vaccinated children compared to older IPV-vaccinated individuals and to a group of naturally exposed persons. These results could not be explained by the IPV vaccination schedule [15] and we therefore hypothesised that the presence of circulating IgA in the population of The Netherlands reflects past contact with live poliovirus (wild poliovirus or OPV vaccine strains) rather than vaccination with IPV [15]. In the present study, we found a similar age-related increase of poliovirus-specific antibodies to serotype 1 and 2 in these contacts (determined with the PoBI) and poliovirus-specific IgA for serotype 1 and 2 in up to 53.8% of the contacts of poliomyelitis patients. Because these contacts belong to the same community that rejects vaccination, these IgA levels probably indicate natural exposure at a level similar to that observed in the general Dutch population. The serotype 1 outbreak that occurred within the risk group in 1978 [4,13] can explain the presence of antibodies to serotype 1 poliovirus. During this outbreak, serotype 1 circulated extensively, while at the same time, monovalent OPV type 1 was offered to the community at risk [4].

One of the strategies used for the eradication of poliomyelitis is the surveillance of patients with AFP. Performance criteria have been established to provide compelling evidence for high quality AFP surveillance. Adequate AFP case investigation includes the collection and laboratory investigation of two stool samples within 14 days after the onset of paralysis [30]. There is a long delay in reporting AFP cases in The Netherlands, which makes AFP surveillance in its present form insufficient as a tool

for the documentation of the absence of poliovirus. Only 31% of the reported AFP cases were virologically examined in The Netherlands [8], implying that poliovirus infection could not be excluded with certainty in 69% of these cases [24]. In addition, a further 12.4% of the virologically examined group could not be successfully diagnosed due to late sampling. When serum samples are available, the IgM ELISA may be helpful in resolving those cases of AFP that cannot be definitely classified as poliomyelitis retrospectively.

Reliable diagnostic virology depends upon the timely and correct collection of clinical specimens. Although stool samples should be collected as soon as possible, preferably within the first two weeks after the onset of paralysis, this does not always occur. Therefore, other methods such as the poliovirus serotype-specific IgM ELISA could serve as an important complementary tool for the diagnosis or exclusion of poliomyelitis in AFP patients, especially in the post-eradication era. Although the poliovirus-specific IgM assay cannot replace virus isolation from a clinical case, the method has proved to be very helpful for the rapid diagnosis of poliomyelitis and for the exclusion of poliovirus infection as the cause of AFP.

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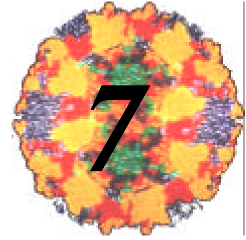

General Discussion

General Discussion

Both the (enhanced) inactivated poliovirus vaccine (e)IPV and the oral poliovirus vaccine (OPV) are capable of inducing high levels of circulating neutralising antibodies and both provide excellent protection against poliovirus-induced disease. The introduction of these vaccines has resulted in the elimination of poliomyelitis from most parts of the world and represents one of the greatest achievements of medical science. However, global eradication of poliovirus can only be considered complete when: a) no more cases of poliomyelitis caused by wild-type poliovirus occur and b) poliovirus no longer circulates in (vaccinated or non-vaccinated) humans and is no longer present in the environment [82].

It is essential that vaccination be continued even after paralytic disease has been eliminated so that the complete absence of wild-type poliovirus in the population and in the environment can be achieved. Mucosal immunity is considered to be of great importance for the interruption of poliovirus transmission and consequently for a reduction of the spread of poliovirus within the population. The experiments described in this thesis have been conducted in order to study the contribution of IPV vaccination to immunity from and protection against poliovirus infection. Our data may shed new light in the use of IPV in the course of the eradication programme.

The mechanism of induction of poliovirus-specific IgA after vaccination with IPV

The mechanism by which IPV (booster) vaccination can induce poliovirus-specific IgA after previous mucosal priming is unknown. IPV is applied intramuscularly in a clear peripheral tissue site. Lymph nodes are strategically located in the body to drain specific tissues and to process antigens deposited in these tissues, and antigens are transported to lymph nodes by antigen presenting cells through the afferent lymphatic system [59]. These antigens probably stay localised due to the fact that IPV does not replicate in the host and, as a consequence, the generation of a specific immune response will be initiated within the organised lymphoid structures of the draining lymph nodes [59].

Expanded populations of antigen-specific effector lymphocytes and memory lymphocytes leave the lymph node through the efferent lymphatics to find their way into the peripheral blood circulation via the thoratic duct system. In our experiments, poliovirus-specific IgG- and IgA-producing cells were detected at 7 days after an IPV booster vaccination [Chapter 5]. Lymphocytes migrate from the blood into tissues and lymph nodes via specialised capillary vessels. This occurs through the interaction of the cellular homing receptors that are expressed on the activated lymphocytes and their ligands on the vessel walls [9]. The final combination of homing receptors on the cells' surface is likely to account for the regional preference of the activated cells.

Lymphocytes activated in peripheral or mucosal lymphoid compartments are thought to migrate preferentially back to peripheral and mucosal tissue sites respectively [9,68]. However, IPV vaccination induced a strong memory IgA response at peripheral and mucosal levels under the condition of previous mucosal priming [Chapter 5]. These results indicate that memory B cells spread to both the peripheral and mucosal compartments after OPV vaccination or natural infection.

We have described how at least part of the poliovirus-specific IgA producing cells re-activated by IPV expressed both L-selectin (indicating homing to peripheral lymph

nodes) and the mucosal homing receptor α4β7 integrin on their surfaces (Chapter 5). This indicates homing to both the mucosal and peripheral compartments. These primed B cells may encounter IPV-derived antigen-specific peptides in the peripheral lymph nodes, resulting in the production of IgA following IPV booster in mucosally primed persons.

There is no information at present regarding the induction and homing potentials of memory T cells after vaccination in humans. Premier (et al.) used *in vitro* proliferation assays to conduct an interesting study on the distribution of ovalbumin-specific memory T cells to the different peripheral and mucosal lymph nodes in sheep after previous peripheral and mucosal immunisation [59]. Their results suggested that antigen-specific proliferative T cells preferentially migrate to peripheral lymph nodes (independent of their site of induction) where they may modulate the immune response following repeated antigen exposure [59].

A possible role for bone marrow in memory IgA response after IPV vaccination

The majority of Ig-secreting cells in mammals are localised in bone marrow [2,28]. Furthermore, it is well established that after systemic immunization (such as IPV), bone marrow is the major site of antibody production during a secondary immune response [2,3]. The induction of this immune response actually occurs in lymphoid tissues. Stimulated cells then migrate from the secondary lymphoid organs to the bone marrow [1,38]. Bone marrow has also been described as an important site for the production of IgG and IgA antigen-specific antibodies after mucosal immunisation (OPV) [5,66]. The induced poliovirus-specific IgA antibody producing cells originate as precursors from the Peyer's patches. After OPV vaccination, they migrate through the lymphatic ducts that drain the gut-associated lymphoid tissue (GALT) and the mesenteric lymph node. They then enter the blood circulation via the intestinal and thoratic duct lymph. These activated lymphocytes leave the circulation in the lamina propria, other mucosal sites and the bone marrow, where they reside as IgA producing plasma cells [5,10,17,33].

The importance of bone marrow as an effector site of the immune response induced in the GALT is suggested by the fact that IgG- and IgA-secreting cells are deficient in the bone marrow of germ-free mice [4]. Antigenic peptides may also reach the bone marrow after renewed antigenic stimulation (by IPV), resulting in a strong recall response of the resident blasts. In previously OPV-vaccinated persons, this will include B cells differentiated to produce poliovirus-specific IgA. The exact role of bone marrow in poliovirus immunity induced by IPV and OPV vaccination remains to be investigated.

The importance of T cells in poliovirus immunity

The exact importance of T cell mediated immunity to poliovirus and its role in viral clearance and protection from re-infection is not known. Neutralising antibodies are thought to be important for clearing poliovirus infections and for protecting against paralytic disease, because children with agammaglobulinemia often develop persistent poliovirus infections [80]. However, it is impossible to cure these patients or clear the poliovirus from the CNS even with infusion of high titered antibodies into the cerebrospinal fluid [45,61]. In addition, poliovirus persistence has been described in people with pure T cell deficiencies but with normal immunoglobulin levels and

normal antibody responses [29]. Thus, T cells and/or other cellular immune mechanisms play at least a partial role in the clearance of poliovirus.

The immune response to picornaviruses, including the development of a neutralising antibody response, is generally T cell dependent as shown in experimental studies with foot-and-mouth disease virus [14,15]. T cell-mediated cytotoxicity has been described as crucial for the resolution of infections with non-cytopathic viruses, whereas infections with cytopathic viruses (like poliovirus) are mainly cleared by soluble mediators such as neutralising antibodies and interferons that stimulate the aspecific cellular immunity [37].

T cells recognise linear peptide fragments of virus-encoded proteins associated with major histocompatibility complex class I (HLA-A, -B and -C determinants [CD8+ cells] or MHC class II (HLA-DR,-DP, and –DQ [CD4+ cells]) [26]. CD4+ T helper (Th) cells have been divided into at least two different subsets (Th1 and Th2) based on the cytokines that they produce upon antigen stimulation [47]. Th1 cells secrete interleukin 2 (Il-2), interferon gamma (IFN-γ) and tumour necrosis factor beta (TNF-β). These cells stimulate cell-mediated cytotoxicity, whereas Th2 lymphocytes produce Il-4, Il-5 and Il-10 and help B cells to produce neutralising antibodies [78]. Although these functionally distinct T cells were originally described in the mouse, human T cell clones possess similar but not identical restricted cytokine profiles [27]. Because humoral immunity appears to be of primary importance in the protection against poliovirus disease, one might expect a predominance of Th2 cells that are best adapted to helping B cells produce antibodies [11,14,15]. However, complement depletion and monoclonal antibody inhibition studies with humans have shown that the effector population after OPV vaccination was of the Th1 subclass [65]. This and other examples have made it clear that the Th1/Th2 dichotomy may not be as clear in humans as it is in mice [27].

Cytotoxic CD8+ T cells specific for picornaviruses have been described [32,73], and CD8+ MHC class I restricted T cells have been largely associated with the cytotoxic T-lymphocytes (CTL) function [84]. Recent evidence from cloned murine T cells suggests that CD4 positive Th1 cells are also involved in direct cell-mediated immunity, including the lysis of virally infected target cells [13,47,56].

Results from (transgenic) mouse experiments are consistent with results obtained using human participants [43,44,78,79]. CD4+ T cells mediated delayed type hypersensitivity (DTH) and T cell proliferation responses to poliovirus in mice, after systemic vaccination with UV-inactivated poliovirus [78,79]. These CD4+ cells included Th1 cells mediating delayed type hypersensitivity (DTH) responses and cytotoxicity as well as Th2 cells that stimulate B cells to produce neutralising antibodies [78]. Other studies in mice have identified poliovirus-specific T cell epitopes recognised by both MHC class I-restricted cytotoxic T cells and class-II restricted T helper cells [39,40,41,44].

No virus replication takes place in the cells of vaccine recipients after vaccination. After intramuscular injection, therefore, poliovirus antigens will be presented mainly through specialised antigen presenting cells such as macrophages and dendritic cells [67]. Although data on cellular immunity following IPV vaccination are lacking, it is likely that the presentation of antigen peptides will occur by the MHC class-II molecules to predominantly CD4+ T cells [26]. CD4+ T cells are important for the B cells' production of virus-specific antibodies, and this may explain the high induction

of neutralising antibodies and high seroconversion rates after only one dose of IPV (>90%) compared to OPV vaccination (40%) [7,57].

OPV viruses are able to replicate within the host. Therefore, viral antigen will also be presented to the immune system through the MHC-I molecules present on infected cells, which will consequently give rise to the stimulation of cytotoxic CD8+ T cells [67]. The stimulation of CD8+ T cells may in turn result in the clearance of poliovirus infections. CD8+ T cell responses are also detected after OPV vaccination, probably following contact between antibody presenting cells and debris from poliovirus lysed cells and intact poliovirus particles [65].

T cell responses after poliovirus infection and vaccination in humans have not been studied extensively and their capacity to evoke memory responses is largely unknown. In general, T cells play an important role in protection against re-infection by inducing rapid memory responses through the activation of memory B cells [72]. UytdeHaag (et al.) showed that the production of neutralising antibodies to poliovirus *in vitro* could be generated by the re-stimulation of cultured human lymphocytes with antigen, but only in the presence of Th2 cells [72].

Other unanswered questions pertaining to poliovirus-induced immunity relate to the possible role of other cellular factors such as natural killer (NK) cells and the genetic constitution of the host. It has been demonstrated that NK cells provide protection against enterovirus infection by limiting virus replication [25]. Most NK cells have Fc receptors and may induce antibody-dependent cell-mediated cytotoxicity [30]. However, the role that NK cells play during poliovirus infection is not known.

Studies on the influence of MHC restriction (HLA) on susceptibility to paralytic poliomyelitis have yielded conflicting results [19,58,83]. More recently, resistance to paralytic poliomyelitis has been suggested to be HLA-related [76]. Thus, while susceptibility to paralytic poliomyelitis in humans may be genetically influenced, it is unclear at this time what these influencing factors are [79].

Further research is needed to understand the precise role of cellular immunity in the protection against re-infection with polioviruses.

Circulation of poliovirus during the 1992-1993 epidemic in The Netherlands

The use of IPV vaccination combined with the existence of communities refusing vaccination on religious grounds makes The Netherlands unique. Epidemics within these groups have revealed poliovirus circulation within The Netherlands that would otherwise have gone undetected [8,54]. It was uncertain whether poliovirus circulation would be restricted to the unvaccinated risk groups during the 1992-1993 epidemic, and studies were conducted during this outbreak [16,74] in which stool and sewage samples were examined in and outside the risk area to determine the spread of poliovirus circulation. Although over 3000 stool samples were investigated, virus was isolated only from 8 persons (all in the risk area).

The lack of poliovirus circulation outside the risk groups supported the hypothesis of the existence of sufficient herd immunity in the IPV-vaccinated population. However, it is known that the chance of virus isolation in susceptible non-vaccinated subjects is (in most cases) limited to the first two to three weeks after infection [Chapter 6]. A cross-sectional study conducted during an epidemic can therefore be described as a search for the proverbial ‘needle in a haystack’.

The widespread OPV vaccination during the outbreak could have had a negative effect on the sensitivity of wild-type isolation. Interference between Sabin and wild-type strains in *in vitro* cultures has been demonstrated [75].

Poliovirus was also isolated from sewage samples during the epidemic. None of the samples collected outside the risk area and only 4.5% of the samples within the risk area tested positive for the endemic strain, illustrating the low sensitivity of sewage testing for wild-type poliovirus [74]. Further, OPV circulation may have interfered with the successful isolation of wild-type virus, as OPV was used to control the epidemic [75]. In conclusion, both the population and the sewage surveillance studies indicate that it is difficult to detect circulation within the population and in the environment even under 'ideal' (epidemic) conditions with proven widespread circulation of wild-type poliovirus [55]. The low negative predictive value of virus isolation under these circumstances implies that populations can not be declared free from poliovirus circulation based on these methods alone.

Implications of the lack of mucosal IgA induction by IPV vaccination for the transmission of poliovirus

Mucosal immunity is one of the most important factors influencing protection against re-infection and is essential for the reduction of poliovirus circulation in the population [24,51,52]. The induction of mucosal IgA may therefore be of particular importance for the poliomyelitis eradication program. We have shown that IPV-vaccination alone is insufficient to induce mucosal IgA [Chapter 5]. This finding is a clear indication of the remaining susceptibility of IPV recipients to poliovirus infection, even though they are protected against disease.

One has to keep in mind that there is a clear difference between protection against disease and protection against infection. Complete protection against disease is achieved in all fully IPV- and OPV-vaccinated persons. However, it is sometimes mistakenly believed that the absence of clinical cases induced by poliovirus is evidence for a break in the chain of transmission or even the (complete) absence of poliovirus circulation.

It has been shown that both IPV and OPV recipients can be re-infected with polioviruses, thereby contributing to the chain of transmission [31,54,70]. Outbreaks of poliomyelitis in Finland [31], The Netherlands [54] and Oman [70] have demonstrated that wild-type poliovirus can replicate in vaccine recipients (IPV or OPV) without clinical disease, and that it can then spread to other (susceptible) persons.

Possible silent circulation of poliovirus in The Netherlands?

Poliovirus infection can spread unnoticed within a population because it may take several hundred infections among non-vaccinated or inadequately vaccinated subjects to produce one paralytic case. This ratio varies according to serotype and is highest for serotype 3 (estimated at between 4000:1 and 500:1 [20,46,63]). The lowest ratio is detected for serotype 1 (between 60:1 to 175:1 [46]) and intermediate values are found for serotype 2 (1000:1 or higher [71]). No clinical cases emerge at all in fully vaccinated persons.

If silent circulation does occur, it must come to a complete stop before vaccination can end. Unfortunately, poliovirus circulation within The Netherlands is not being studied extensively at present. However, all poliovirus strains isolated in clinical virology laboratories in The Netherlands are sent to the RIVM for further analysis [62]. Wild-type polioviruses were isolated from three imported cases of poliomyelitis during the time period between the 1978 and 1992/1993 outbreaks [62]. Between 1979 and 1989, wild-type strains were isolated on 44 occasions from 5868 stool samples, all from adopted children who had recently entered the country (11 x serotype 1, 13 x serotype 2 and 20 x serotype 3) [62]. Twenty-one wild-type strains (15 x serotype 1 and 6 x serotype 3) were reported between 1979 and 1990, all isolated from persons with a history of international travel [62]. These results indicate a continued importation of wild-type polioviruses into the general population of The Netherlands and the continued threat of exposure to wild-type poliovirus into the (non-) vaccinated community, but provide no evidence for continued circulation in The Netherlands.

However, indications for silent circulation in The Netherlands do exist. For example, wild-type poliovirus strains were detected 13 times in 58 water samples from the Rhine and Meuse rivers during the period between 1979 to 1989 (11 x serotype 1, 1 x serotype 2 and 1 x serotype 3) [62]. Interestingly, researchers repeatedly isolated of viruses identical or closely related to the epidemic serotype 1 strain from 1978 (<3% sequence divergence) up to 5 years after the epidemic [48]. These viruses were found both in clinical and environmental specimens until 1983 [48]. This continued isolation is more likely explained by continued circulation in the population rather than a repeated introduction of exactly the same wild-type poliovirus strain into The Netherlands.

Another indication that silent circulation may exist comes from a serosurvey among non-vaccinated schoolchildren born between 1962 and 1968 [62]. These children developed antibodies to the serotype 1 strain during the 1978 outbreak [48]. The children also demonstrated antibodies to serotype 3, indicating that they had already been exposed to this serotype [62]. This finding led to the conclusion that serotype 3 must have circulated between 1968 and 1978 [62].

As in the 1978 type 1 poliovirus epidemic in The Netherlands, the type 3 epidemic that occurred in Finland in 1984 seemed to have had its source in the Mediterranean region [31,48]. Genetically similar viruses (<4% sequence divergence) were isolated on three separate occasions in The Netherlands between 1980 and 1982. Therefore, the theory that the Finland epidemic strain might have originated in The Netherlands cannot be excluded.

Finally, mucosal contact with live poliovirus (wild-type or OPV) is suggested by our own data: we demonstrated that IPV alone is incapable of inducing IgA and yet we found a high seroprevalence (12.1 to 27.3%) of IgA in healthy blood donors [Chapter 4]. The findings described above do not prove the local circulation of wild-type poliovirus, but can not solely be explained by virus importation. More sensitive surveillance tools must be developed to ensure that poliovirus transmission is halted.

When can we be sure that poliovirus has been eradicated?

Eichner and Dietz [22] calculated that a case-free period of over three years must be observed before one can be 95% certain that wild-type poliovirus is no longer present

in the population. These calculations were based on the assumptions that 80% of the population were immunised, that IPV recipients were partly protected from infection (with a simulated 50% reduction in virus excretion) and that previously infected persons were fully protected against re-infection. The calculations did not take the importation of virus strains from other countries into consideration [22].

Vaccination acceptance in The Netherlands is high (>97%) [77]. Under these circumstances, the probability of polio-like symptoms in the general population after re-infection is very low [16,62]. The presence of poliovirus-specific IgA at mucosal sites is important for the reduction of viral excretion [51-53]. Our finding that no poliovirus-specific IgA is induced at mucosal sites after IPV vaccination [Chapter 5] makes Eichner and Dietz's assumption that IPV recipients were partially protected less likely [22]. In addition, several cases of wild-type poliovirus importation into The Netherlands have been reported [48,62]. Together, these findings challenge the idea that the virus will be eliminated after a three-year polio-free period in IPV-vaccinated populations. Studies investigating resistance to an OPV challenge in IPV-vaccinated persons with and without poliovirus-specific mucosal IgA are currently underway.

Vaccination with OPV results in the release of a wide variety of live mutant viruses in the stool [12], some of which are highly virulent (even in countries declared to be 'polio free') [60]. A stricter definition of eradication should include the elimination of all polioviruses (including OPV) from the population and the environment. This goal cannot be accomplished using the current protocol of OPV vaccination, and will require the exclusive use of IPV in the final stages of poliovirus eradication combined with intensive surveillance for wild-type poliovirus.

Our data indicate that IPV can serve as a strong inducer of mucosal immunity after previous mucosal priming with live viruses [Chapter 5]. This finding indicates that a combination schedule of OPV and IPV vaccination could serve as a powerful tool in the final stages of the eradication program. However, this may also lead to complicated vaccination schedules. Another option is to stop 'cold-turkey', meaning a complete cessation of polio vaccination upon reaching a certain polio-free period after the last clinical case of poliomyelitis. However, this might be a risky proposition in the absence of sensitive tools to check for wild-type poliovirus circulation in the vaccinated population.

The detection of poliovirus-specific IgA as a marker for previous mucosal priming and poliovirus circulation

Poliovirus-specific IgA is readily detected in the serum after wild-type infection [51,52]. No mucosal poliovirus-specific IgA was produced after an IPV booster vaccination of IPV-vaccinated adult volunteers, and serum IgA was only detected in some volunteers at a low level [Chapter 5]. Because these volunteers had been vaccinated years ago, a recent study involving a group of nine year-old children from The Netherlands was conducted to search for the presence of poliovirus-specific IgA in serum four months after a sixth IPV vaccination [62]. None of the children in this group had serotype 1- or 3-specific IgA and only 5.8% were found positive at low levels for serotype 2-specific IgA [Herremans et al., unpublished results]. This confirmed the results from the study described in Chapter 5. The absence of poliovirus-specific IgA after IPV vaccination may enable its use as a marker for the detection of poliovirus circulation within an IPV vaccinated population. Both saliva

and serum samples could be used in a large scale screening of the population for the presence of poliovirus-specific IgA.

Although not much is known about the persistence of poliovirus-specific IgA after exposure to live polioviruses, 36% of a group of poliomyelitis patients were still positive five months post-infection [Chapter 4]. Further investigations examining the possibilities and sensitivity of applying the IgA ELISA for this purpose are currently underway.

The detection of antibodies to the non-structural proteins of poliovirus as a marker for circulation

Another method that may be used to discriminate between antibodies induced by IPV vaccination and those induced through mucosal contact with a replicating poliovirus (wild-type or OPV strain) is the detection of antibodies against the non-structural proteins of poliovirus. Poliovirus has seven different non-structural proteins (2A to 2C and 3A to 3D). These proteins are expressed during virus replication in the host and are subsequently presented to the immune system. No virus replication occurs after vaccination with inactivated poliovirus, and therefore no induction of antibodies specific for the non-structural proteins will occur.

While this test cannot discriminate between OPV vaccination and wild-type infection, such an assay may be very useful in IPV-vaccinated populations. The 3ABC and 3D non-structural proteins have been successfully used in the surveillance of foot and mouth disease [6,21]. The 3C non-structural protein has been tested with success for another (human) picornavirus (hepatitis A) [69]. Both direct ELISA, immunoprecipitation and immunoblotting were used to detect antibodies to viral non-structural proteins [6,21,69]. The literature indicates that 3C (protease) has the largest antigenic variation in non-structural proteins of picornaviruses [57]. Cross reactivity between polioviruses and coxsackie A12 has also been described [57]. Coxsackie A12, A24 and polioviruses belong to the same genetic group based on alignment of the coding regions of picorna viruses. Further, other epitopes on the non-structural proteins might be shared with other picornaviruses and might cause cross-reactions.

We will conduct future studies to develop and evaluate assays for the determination of antibodies to recombinant non-structural proteins of poliovirus. This will assist efforts to monitor the absence of poliovirus circulation in The Netherlands. However, there are some reports describing the presence of non-structural proteins (2C, 3CD, 3C and 3D) in the viral capsid of (inactivated) poliovirus [49,50]. Therefore, it remains to be seen whether infection-specific assays can be developed.

Possible side effects of poliovirus eradication

All countries will benefit from ending polio vaccination once global eradication has been achieved. Human and financial resources will become available for other health priorities. Despite the great benefits of polio eradication, however, some side effects may occur. Cross-reacting T cells and neutralising antibodies between poliovirus and other enteroviruses have been described [11,18,43,64]. Samuelson et al. [64] demonstrated that the essential residues for the binding of cross-reactive antibodies are well conserved within the enterovirus family. Most cross-reacting neutralising antibodies are directed against VP1 [64], while cross-reacting T cells are thought to be

directed mainly against VP4 [11]. In addition, cross-reacting antibodies and T cells directed against NSPs have been found [42].

It is possible, therefore, that poliovirus vaccination also induces some level of protection against other enterovirus infections. Enterovirus infections are very common during infancy, and most children experience at least one enterovirus infection during their first year of life [11,35]. Enteroviral infections have been linked to the pathogenesis of auto-immune diseases such as chronic cardiomyopathies and insulin-dependent diabetes mellitus [34,81]. There are some indications that cross-reacting T cells induced by polio vaccination might have a protective effect against the induction of insulin-dependent diabetes mellitus in young children, thought to be related to coxsackie B virus infections [36].

Time will reveal the specific consequences that polio eradication will have on the incidence of other enteroviral infections (most of which are now asymptomatic) [11,35]. Further investigation is necessary to determine the effects on enterovirus infections if polio-vaccination is stopped.

Final considerations

Despite all of the problems discussed above, we are well on our way to the world-wide eradication of poliovirus through the use of the currently available IPV and OPV vaccines. Before vaccination stops, however, we must ensure that all (silent) circulation of poliovirus within vaccine recipients is terminated. Poliovirus infections in vaccinated recipients are hard to detect, since none of these people will develop any clinical signs. It is for this reason that the absence of clinical cases induced by poliovirus in a vaccinated population can never serve as compelling evidence of poliovirus eradication. More sensitive tools must be developed to ensure that poliovirus transmission is halted in the vaccinated population.

To this end, IPV vaccination may assume an important role in the final stages of polio eradication. It does not introduce live poliovirus into the environment, and it also allows the development of markers other than direct poliovirus isolation in the general population.

We were able to detect poliovirus-specific IgA in young IPV-vaccinated children, indicating that they have never been in contact with live poliovirus. This is a clear indication that we are on the right track towards the elimination of poliovirus from The Netherlands. We are only a short time away from a complete absence of poliomyelitis outbreaks.

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Summary

Summary

The aim of this thesis was defined as the study of the contribution of IPV vaccination to the induction of a) protection against poliovirus infection and b) mucosal immunity. We have described the development of new immunological tools for the rapid detection of poliovirus-specific antibodies and have investigated the induction of mucosal immunity after IPV vaccination. Our studies compared the immunity induced by IPV vaccination to the immune responses after OPV vaccination and/or exposure to wild-type poliovirus.

The presence of antibodies that protect individuals from poliomyelitis is usually determined by a neutralisation assay using cell cultures. Cell culture assays, however, are technically demanding. Disadvantages of the serum neutralisation test (NT) include its long duration and the need for a manual screening of the test results, making this assay labour intensive, difficult to standardise and less suitable for the screening of large populations. Other assays able to detect poliovirus-specific antibodies have been developed within the last decade [6,7]. However, the new assays estimating immunity to polioviruses measure both neutralising and non-neutralising antibodies, whereas it is the presence of neutralising antibodies that is correlated with protection from (re)infection.

A newly developed inhibition ELISA known as the PoBI test (Chapter 2) can replace the NT for the determination of protective levels of antibodies to polioviruses in large-scale population studies. Correlations between the PoBI test and the NT were high: 0.89, 0.89 and 0.84 for serotypes 1, 2 and 3 respectively. The sensitivity of the inhibition ELISA was 98.6%, 97.4% and 92.1% for serotypes 1, 2 and 3 respectively. The specificity of the PoBI test as determined with sera from non-vaccinated persons was also high for all three serotypes (99.0%, 95.8% and 100% for serotypes 1, 2 and 3 respectively). One of the major advantages of the PoBI test over the NT is the use of inactivated virus as the antigen. In view of the ongoing eradication of poliovirus, the use of live poliovirus in diagnostic assays should be discouraged and must cease altogether in the near future. Under these circumstances, the PoBI assay is an excellent replacement for the standard NT.

Three important antigenic sites (epitopes) involved in virus neutralisation have been identified on polioviruses in mouse experiments [13]. It has been reported that trypsin, present in the intestinal fluids, can cleave serotype 3 polioviruses at antigenic site 1 [14]. Trypsin cleavage of poliovirus results in drastically altered antigenic properties, and trypsin-cleaved viruses may escape neutralisation by monoclonal antibodies to antigenic site 1 [9].

Antibody responses to antigenic sites 1 and 3 were determined in fully IPV- or OPV-vaccinated recipients and in individuals who had been naturally infected (Chapter 3) in order to study the immunogenicity of these sites in humans and the effect of trypsin exposure *in vivo*. Both sites were immunogenic in naturally infected humans. No significant differences were detected in the responses to antigenic site 1 between IPV- and OPV-recipients. However, significantly more OPV recipients (88.7%) had detectable antibodies to antigenic site 3 ($p<0.01$) when compared to IPV-vaccinated persons (63.1%).

While there are no major differences in the systemic humoral immune response between IPV- and OPV-vaccinated persons, it is not clear whether parenteral vaccination with IPV can lead to priming of the mucosal immune system. We

developed and evaluated ELISAs for the detection of poliovirus serotype-specific IgA and secretory IgA antibodies, and used these assays to examine IgA responses after wild-type infection or vaccination (described in Chapter 4). All of the examined poliomyelitis patients developed a humoral poliovirus-specific IgA response after infection with wild-type poliovirus. In addition, poliovirus-specific IgA was found more frequently in OPV-vaccinated persons than in IPV-vaccinated persons.

We observed an age-related increase in the seroprevalence of IgA in the IPV-vaccinated population of The Netherlands. These results may be explained by the assumption that IgA is induced by infection with live poliovirus (wild-type or OPV strains) in the older population, and is unrelated to the IPV vaccination schedule. This is best illustrated by the finding that children between the ages of 13 and 15, born prior to the serotype 1 outbreak of 1978, had significantly more serotype 1-specific IgA in their serum than serotype 2- or 3-specific IgA. We also found that parenteral vaccination with IPV was able to boost IgA responses in 74% to 87% of a naturally exposed population. While the presence of IgA in IPV-recipients has been previously documented, our findings support the hypothesis that mucosal priming with live virus is necessary to obtain an IgA response after IPV booster vaccination.

A group of fully OPV- or IPV-vaccinated recipients were given a booster vaccination with IPV to investigate the effect of IPV vaccination on the mucosal IgA response (described in Chapter 5). ELISA and ELISPOT-assays were used for the detection of poliovirus-specific IgA responses. No induction of poliovirus-specific IgA was detected in either saliva or stool samples from individuals in the IPV-vaccinated group, and no IgA-producing cells could be detected in their blood. These findings led to the conclusion that IPV vaccination is unable to induce a response to poliovirus at the mucosal level, indicating the possibility of a lower level of protection against (re)infection in IPV recipients.

However, IPV did induce high levels of circulating IgA in fully OPV-vaccinated subjects at both the humoral and the mucosal level. When B cell populations were separated on the basis of the expression of mucosal ($\alpha4\beta7$ integrin) or peripheral (L-selectin) homing receptors, a large percentage (77.3%) of the poliovirus-specific IgA-producing cells in the previously OPV-vaccinated group expressed the $\alpha4\beta7$ integrin. It was concluded that IPV vaccination alone is insufficient to induce a mucosal IgA response against poliovirus. Our results did indicate, however, that IPV vaccination can serve as an excellent stimulator of mucosal immunity in mucosally (OPV) primed individuals. These observations indicate that the interpretation of findings from challenge studies using IPV recipients must take into account subjects' possible previous contact with live poliovirus. Subjects from endemic regions, for example, may have had previous exposure to live poliovirus, and this may explain the reported induction of mucosal IgA by IPV vaccination in the past [2,5,8,10,16,17,19].

Cases of poliomyelitis in which paralysis occurs are very difficult to distinguish clinically from other cases of acute flaccid paralysis (AFP). Several new diagnostic methods have been developed in recent years (in our laboratory and elsewhere) that have not been evaluated under field conditions [3,4,10,11,15,18]. While the virological investigation of stool samples is important, it is a laborious procedure [1]. The detection of poliovirus serotype specific-IgM in AFP patients facilitates the laboratory diagnosis of poliomyelitis and helps to exclude poliovirus as the causative agent (Chapter 6). In fact, virus-specific IgM was detected in the blood for six weeks longer than virus was able to be isolated from stool samples. Poliovirus-specific IgA

persisted in many patients for more than eight weeks after infection and may therefore reflect past exposure rather than a recently acquired infection. For this reason, poliovirus-specific IgA is less suitable for the diagnosis of recent infections.

Reports of AFP cases in The Netherlands often succumb to serious delays. As a result, AFP surveillance (in its present form) is not an adequate tool with which to document the absence of poliovirus. To make matters worse, only 18.6% of reported AFP cases are virologically examined in The Netherlands (according to WHO guidelines [1,12]). This implies that poliovirus infection can not be excluded with certainty in 69% of these cases. The IgM ELISA will be helpful in resolving cases of AFP that cannot be retrospectively classified as poliomyelitis and for which serum samples are available.

Despite all of the problems discussed above, we are well on our way to the world-wide eradication of poliovirus through the use of the currently available IPV and OPV vaccines. Before vaccination stops, however, we must ensure that all (silent) circulation of poliovirus within vaccine recipients is terminated. Poliovirus infections in vaccinated recipients are hard to detect, since none of these people will develop any clinical signs. It is for this reason that the absence of clinical cases induced by poliovirus in a vaccinated population can never serve as compelling evidence of poliovirus eradication. More sensitive tools must be developed to ensure that poliovirus transmission is halted in the vaccinated population.

We were able to detect poliovirus-specific IgA in young IPV-vaccinated children, indicating that they have never been in contact with live poliovirus. This is a clear indication that we are on the right track towards the elimination of poliovirus from The Netherlands. We are only a short time away from a complete absence of poliomyelitis outbreaks.

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Nederlandse Samenvatting

Tijdens de laatste polioepidemie in Nederland in 1992/93, waarbij 71 slachtoffers vielen onder principieel niet-gevaccineerden, rees de vraag of het poliovirus ook nog bij het gevaccineerde deel van de bevolking voorkomt. Het zou kunnen zijn dat gevaccineerden het virus bij zich kunnen hebben zonder ziek te worden. Als dat het geval zou zijn zouden gevaccineerden kunnen bijdragen aan het besmetten van principieel ongevaccineerden. Deze vraag is bovendien belangrijk omdat onder leiding van de wereldgezondheids- organisatie getracht wordt het poliovirus uit de wereld te bannen. Pas nadat men zich er van verzekerd heeft dat nergens meer poliovirus circuleert, kan worden gestopt met vaccineren. Dan is de uitroeiing van polio een feit. De huidige poliovirus vaccins geven een uitstekende bescherming tegen de ziekte, dat wil zeggen tegen het ontstaan van verlammingen nadat poliovirus is binnen gedrongen in het lichaam. Voor het uitroeiings- programma is het belangrijk dat vaccins nog een stapje eerder hun werk doen, namelijk door te voorkomen dat poliovirus het lichaam binnenkomt. Immers, wanneer dit het geval is, heeft het virus geen mogenlijkheid meer om zich te handhaven. Dit is alleen het geval als er ook een mucosale immuniteit bestaat. Dat betekent dat op de slijmvliezen antistoffen tegen poliovirus aanwezig is die er voor zorgen dat het virus zich niet kan nestelen op de slijmvliezen.

Het doel van dit proefschrift was het bestuderen van de bijdrage van vaccinatie met geinactiveerd poliovaccin (IPV) bij het induceren van bescherming tegen poliovirus infecties en het induceren van mucosale immuniteit. IPV wordt in Nederland gebruikt in het rijks vaccinatie programma. Wij hebben nieuwe methoden ontwikkeld om op een eenvoudige en snelle manier poliovirus-specifieke antistoffen aan te kunnen tonen als mede ook assays om de mucosale immuniteit te kunnen bestuderen. Wij hebben vaccinatie met IPV vergeleken met het levend/verzwakt poliovirus vaccin (OPV) dat in de meeste andere landen wordt gebruikt. Verder werd ook de afweer na een natuurlijke poliovirus infectie onderzocht.

Bescherming tegen poliomyelitis (kinderverlamming) wordt afgelezen aan de aanwezigheid van poliovirus neutralizerende antistoffen in het bloed die het virus onschadelijk maken. Deze antistoffen worden bepaald met behulp van een standaard virus-neutralisatietest. Nadelen van deze test zijn echter o.a. de lange duur, en het visueel moeten screenen van de uitslagen. Deze eigenschappen maken deze test erg arbeidsintensief en onhandig om grote hoeveelheden te testen. Bovendien moet in de neutralisatietest gebruik gemaakt worden van levend poliovirus. Pogingen om eenvoudigere testen op te zetten hebben tot nu toe gefaald omdat met deze testen geen onderscheid kon worden gemaakt tussen poliovirus neutraliserende en niet neutraliserende antistoffen. Alleen de neutraliserende antistoffen zijn van invloed op de bescherming tegen verlammingen veroorzaakt door poliovirus.

Met een nieuw opgezette Poliovirus Binding Inhibition test, afgekort PoBI (zie hoofdstuk 2) is gebleken dat de resultaten van deze test sterk overeenkomen met de standaard virus-neutralisatietest. De PoBI test is met name geschikt voor een snelle screening van grote hoeveelheden sera zoals bijvoorbeeld nodig is voor het evalueren van het Nederlandse vaccinatieprogramma. Bij dit soort studies wordt de aanwezigheid bepaald van poliovirus immuniteit binnen de Nederlandse bevolking en eventuele risicogroepen worden zo in kaart gebracht. Een belangrijk voordeel van de PoBI test is dat niet langer gewerkt hoeft te worden met levend poliovirus. Dit is dus

veel veiliger. Dit is met name belangrijk voor het uitroeiings programma waarbij in de toekomst het werken met levend poliovirus zal worden ontmoedigd of zelfs verboden. Onder deze omstandigheden is de PoBI test een uitstekend alternatief voor de nu nog veel toegepaste neutralisatie test.

Binding van de eerder beproken virus-neutraliserende antistoffen vinden plaats op heel specifieke plaatsen op de buitenkant van het poliovirusdeeltje. Binding van deze antistoffen voorkomt o.a. aanhechting van het virus aan gevoelige cellen zodat deze niet meer kunnen worden geinfecteerd. Drie belangrijke bindingsplaatsen betrokken bij de poliovirus neutralisatie zijn geidentificeerd op het oppervlak van het poliovirusdeeltje. In het maag-darmkanaal waar het poliovirus normaal gesproken het lichaam binnenkomt bevindt zich het enzym trypsine, dat de eigenschap heeft de buitenkant van het poliovirus te kunnen veranderen dat bepaalde virus-neutraliserende antistoffen niet meer werken. Bij contact met poliovirus via het maag-darmkanaal zouden er dus problemen op kunnen treden omdat na vaccinatie met IPV niet de juiste antistoffen zijn aangemaakt en het virus tijdelijk kan ontsnappen aan het immuunsysteem. Dit komt omdat personen die gevaccineerd worden met geinactiveerd virus het vaccin rechtstreeks in de spieren krijgen geinjecteerd en het virus dus niet in aanraking komt met trypsine uit het maag-darmkanaal. Hierdoor maakt het immuunsysteem alleen maar antistoffen die een intact virusdeeltje kunnen neutraliseren.

Om dit mogelijke gevaar te bestuderen hebben we testen opgezet om antistoffen tegen de verschillende plaatsen op het virusdeeltje apart te kunnen meten (zie hoofdstuk 3). Zowel in het bloed van personen, gevaccineerd met IPV als personen die in contact zijn geweest met poliovirus via het maag-darmkanaal (OPV en patienten) werden naast antistoffen tegen de trypsine gevoelige plaatsen ook antistoffen gevonden tegen de ongevoelige plaatsen. Er is dus geen reden om aan te nemen dat IPV vaccinatie een mindere bescherming geeft tegen poliovirus dat via het maag-darmkanaal binnenkomt.

Er zijn dus geen benoemenswaardige verschillen tussen de antistof response na vaccinatie met geinactiveerd virus of met levend verzwakt virus. In beide gevallen werkt de vaccinatie uitstekend tegen het voorkomen van verlammingen omdat het poliovirus voordat het het centrale zenuwstelsel kan bereiken al wordt uitgeschakeld. Het is echter nog niet duidelijk of IPV vaccinatie ook een bijdrage kan leveren aan bescherming tegen een (her-)infectie met poliovirus. Dit komt omdat IPV in de spieren wordt geinjecteerd en door deze route waarschijnlijk geen effect heeft op de locale afweer in het maag-darmkanaal. Bescherming op de slijmvliezen van het maag-darmkanaal is zo belangrijk omdat daar het poliovirus normaal gesproken het eerste binnenkomt. Wij hebben testen opgezet om polio antistoffen op slijmvliezen (zg IgA) te kunnen meten (zie hoofdstuk 4). Na een natuurlijke infectie ontwikkelen alle patienten dit type antistof in hun bloed. IgA gericht tegen poliovirus werd ook vaker teruggevonden bij OPV gevaccineerden (zijn geinfecteerd geweest via het maag-darmkanaal). Wij vonden een leeftijdsafhankelijke toename van poliovirus-specifiek IgA in het bloed van de Nederlandse bevolking. Deze resultaten kunnen alleen goed verklaard worden door een natuurlijke blootstelling met levend poliovirus uit de omgeving en kan niet verklaard worden door de IPV vaccinatie zelf. Ook bleek dat na een her-vaccinatie van personen die in het verleden op natuurlijke wijze geinfecteerd waren met poliovirus een sterke toename hadden van polio-specifiek IgA in het bloed.

Deze resultaten ondersteunen de hypothese dat IPV alleen IgA kan induceren na een eerder contact met poliovirus via het maag-darmkanaal.

Om verder uit te zoeken of IPV een bijdrage kan leveren aan afweer op de slijmvliezen werd een groep van volwassen IPV en OPV gevaccineerden opnieuw gevaccineerd met IPV (zie hoofdstuk 5). IgA antistoffen werden zowel bepaald in het bloed als in het speeksel. In de IPV gevaccineerde groep werd in geen enkel geval IgA terug gevonden op de slijmvliezen. Deze resultaten laten zien dat vaccinatie met alleen IPV niet voldoende is om mucosale immuniteit te induceren. Dit betekent een mogelijke mindere bescherming tegen een herinfectie met poliovirus en dat deze groep eventueel kan bijdrage aan een stille circulatie van polio in Nederland. Her-vaccinatie met IPV gaf echter in de eerder OPV gevaccineerde groep een heel sterke IgA respons tezien, zowel in het bloed als op de slijmvliezen. Veel van de IgA producerende cellen uit het bloed bleken ook onderweg te zijn naar het maag-darmkanaal om daar voor de nodige bescherming te zorgen. IPV bleek dus een uitstekende stimultor te zijn van het mucosale immuun systeem mits men eerder met poliovirus in aanraking was geweest via het maag- darmkanaal. Deze bevinding geeft aan dat voor een juiste interpretatie van resultaten uit het verleden, waarin mucosal immuniteit na IPV vaccinatie wordt beschreven, waarschijnlijk te maken heeft met eerdere natuurlijke blootstelling aan poliovirus.

Poliomyelitis is klinisch heel moeilijk te onderscheiden van andere oorzaken van acuut optredende verlammingen. In veel gevallen wordt door artsen niet meteen gedacht aan een poliovirus-infectie als een mogelijke oorzaak, zeker niet in landen waar een hoge vaccinatiegraad is bereikt zoals in Nederland. Hierdoor wordt in veel gevallen de patienten niet of veel te laat virologisch onderzocht. Toch is het uitsluiten van poliovirus als mogelijke oorzaak van belang voor het eradicatie programma, omdat deze cijfers een beeld kunnen geven in hoevere poliomyelitis is uitgeroeid en wanneer men voorzichtig kan gaan denken aan het stoppen met vaccineren. In hoofdstuk 6 beschrijven we verschillende methoden die kunnen bijdragen aan de diagnostiek van poliovirusinfecties. Met name door het bepalen van antistoffen, die alleen worden aangemaakt bij een eerste contact met poliovirus en in de acute fase (zg IgM), kan gedurende een langere periode na de eerste ziektedag nog betrouwbaar uitsluitsel geven worden over een mogelijke poliovirus infectie.

Ondanks de nog aanwezige problemen van het eradicatie programma van polio worden er toch grote vorderingen gemaakt. Maar voordat we kunnen spreken over een poliovrije wereld moeten we er ons eerst van verzekerd hebben dat poliovirus nergens meer kan voorkomen, ook niet bij de gevaccineerde bevolking. Poliovirusinfecties bij gevaccineerden zijn heel moeilijk op te sporen omdat deze personen nooit ziek worden van een infectie en er dus geen enkele reden is om deze personen virologisch te onderzoeken. Vandaar dat de afwezigheid van klinische gevallen van kinderverlamming in een gevaccineerde populatie nooit voldoende bewijs is voor de afwezigheid van poliovirus. Nieuwe eenvoudige methoden om eventuele poliovirusinfecties bij de gevaccineerde bevolking te kunnen vaststellen zullen moeten worden ontwikkeld om deze vraag te kunnen beantwoorden.

Wij vonden echter bij jonge IPV gevaccineerde kinderen geen mucosale antistoffen wat dus hoogst waarschijnlijk betekent dat deze kinderen nooit in aanraking zijn geweest met het poliovirus. Deze resultaten zijn een duidelijke aanwijzing dat de

jonge generatie polio-vrij is gebleven ondanks dat vaccinatie met IPV niet leidt tot een mucosale afweer. Dit geeft aan dat we op de goede weg zijn naar de volledige uitroeiing van polio in Nederland. De polioepidemieen in Nederland zullen dan werkelijk tot het verleden gaan behoren.

Abbreviations

AFP	acute flaccid paralysis
BSA	bovine serum albumin
CNS	central nervous system
CSF	cerebrospinal fluid
CPE	cytopathic effect
eIPV	enhanced inactivated poliovaccine
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
GALT	gut-associated lymphoid tissue
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IPV	inactivated poliovaccine
MHC	major histocompatibility complex
NT	neutralisation test
OD	optical density
OPV	oral poliovaccine
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
pNPP	p-Nitrophenyl Phosphate
PoBI	Poliovirus binding inhibition assay
RIVM	Rijksinstituut voor de Volksgezondheid en het Milieu (National Institute of Public Health and the Environment)
rpm	rotation per minute
SC	secretory component
sIgA	secretory immunoglobulin A
SVM	Stichting voor de Volksgezondheid en het Milieu (Foundation for the advancement of Public Health and Environment)
TCID	tissue culture infective dose
TMB	tetramethylbenzidine
VAPP	vaccine-associated paralytic poliomyelitis
VP	virion protein
WHO	World Health Organization

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Tineke

Curriculum Vitae

Tineke Herremans werd geboren op 21 januari 1970 te 's-Hertogenbosch. Na het behalen van het VWO diploma aan het St. Jans lyceum te 's-Hertogenbosch begon zij in 1988 met de studie medische biologie aan de Universiteit Utrecht. Tijdens deze studie heeft zij stages gelopen bij de afdeling medische enzymologie (vakgroep haematologie) van het AZU, en vervolgens bij de toenmalige afdeling pathologie van het RIVM te Bilthoven bij de afdeling immunotoxicologie waar de schadelijke effecten van UV-B straling op de T cell immuniteit werd bestudeerd. Daarna werkte zij gedurende 6 maanden bij het virological research laboratory van de University of Edinburgh aan immuniteit tegen het Humaan Papiloma Virus. Eind 1994 studeerde zij af waarna zij is gestart als AIO bij het laboratorium voor Infectieziektenonderzoek (voorheen afdeling virologie) van het RIVM, waar de immuniteit tegen poliovirus in patienten en gevaccineerden personen is bestudeerd. Op dit moment is zij hier nog steeds werkzaam als onderzoeker, dit keer met de opdracht om immunologisch discriminerende assays op te zetten om het verschil tussen vaccinatie en infectie met poliovirus te kunnen aantonen.

List of Publications

- **Goettsch W, Garssen J, de Klerk A, Herremans MMPT, Dortant P, de Gruijl FR, van Loveren H.** Effects of ultraviolet-B exposure on the resistance to Listeria monocytogenes in the rat. *Photochemistry and Photobiology. 1996;63:672-679.*
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- **Herremans MMPT, Reimerink JHJ, Kimman TG, Koopmans MPG.** Induction of virus-specific IgA in persons vaccinated with inactivated poliovirus and life attenuated oral vaccine. *Nederlands tijdschrift voor Medische Microbiologie. 1998;6:S17.*
- **Herremans MMPT, Reimerink JHJ, Buisman AM, Kimman TG, Koopmans MPG.** Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus. *Journal of Immunology in press.*
- **Herremans MMPT, Koopmans MPG, van der Avoort HGAM, van Loon AM.** Lessons from diagnostic investigations of poliomyelitis patients and their direct contacts for the present surveillance of Acute Flaccid Paralysis. *Submitted.*
- **Herremans T, Reimerink J, Kimman T, van der Avoort H, Koopmans M.** Differences in the antibody responses to antigenic sites 1 and 3 of serotype 3 poliovirus after vaccination with oral live attenuated or inactivated poliovirus vaccine, and after natural exposure. *Submitted.*