

Cellular and humoral immune responses to poliovirus in mice: a role for helper T cells in heterotypic immunity to poliovirus

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Immunization of BALB/c mice with a single dose of the Sabin type 1, type 2 or type 3 poliovirus vaccine strains stimulated cross-reactive T helper cell responses detected by both *in vitro* proliferation and interleukin (IL)-2/IL-4 production. Although the polyclonal T cell responses were cross-reactive, the results also suggest that a proportion of the T cells were directed against serotype-specific determinants. In contrast, neutralizing antibodies, assayed in the serum from the same animals, were predominantly serotype-specific and only reached significant titres after secondary immunization. A comparison of the immunogenicity of

poliovirus administered subcutaneously in Freund's complete adjuvant or intraperitoneally as an alum precipitate or without adjuvant, showed that optimum responses were obtained by immunization with virus in the presence of alum. An examination of the effect of heterotypic priming showed that immunization with type 2 virus primed for a secondary antibody response to each of the three serotypes, whereas priming with type 1 or type 3 viruses could only generate a secondary antibody response to the homologous virus or to type 2 virus.

Introduction

The ability of poliovirus vaccines to protect the host against disease has been attributed directly to the generation of antiviral neutralizing antibodies (Glezen *et al.*, 1969). However, protection against disease is possible in the virtual absence of measurable circulating neutralizing antibodies, for example where the levels have waned after the last vaccination (Bottiger, 1973), or after a single immunization with the inactivated poliovirus vaccine. It has been suggested that immunological memory could account for this protection (Salk *et al.*, 1984). Although the nature of immunological memory to poliovirus has not been fully investigated, it seems probable that the role of circulating neutralizing antibody would be the immediate prevention of infection, whereas immunological memory would provide durable immunity in the long term.

Although the antigenic structure of poliovirus is well characterized, with at least four neutralizing epitopes identified (Minor, 1990) and located on the three-dimensional structure of the virus particle (Hogle *et al.*, 1985; Filman *et al.*, 1989; Page *et al.*, 1988), the role of T cells in the immune response to poliovirus has not been established. T cells are known to have important functions in immune protection against viral diseases. Major histocompatibility complex (MHC) class II-

restricted T helper (Th) cells proliferate after stimulation with antigen, secrete cytokines, such as interleukin (IL)-2, IL-4 and γ -interferon, and activate B cells to produce antibody (Reinherz & Schlossman, 1981; Schwartz, 1985; Paul, 1989); MHC class I cytotoxic T cells have the capacity to lyse virus-infected cells, thus directly inhibiting virus replication (Zinkernagel & Doherty, 1979). T cell responses have been demonstrated with a range of viruses including human immunodeficiency virus, measles virus, respiratory syncytial virus and influenza virus (Mills, 1989), hepatitis B virus (HBV) (Milich & McLachlan, 1988), coxsackie B4 and mumps viruses (Bruserud & Thorsby, 1985), foot-and-mouth disease virus (Collen *et al.*, 1989) and Theiler's murine encephalomyelitis virus (Welsh *et al.*, 1989).

In this report we examined the specificity of T cell proliferative and antibody responses following primary and secondary immunization of mice with poliovirus. We show that neutralizing antibody responses which only reached significant titres after secondary immunization were mainly serotype-specific, whereas T cell proliferative responses were cross-reactive. Furthermore, after secondary immunization with one virus serotype, antibody responses increased significantly in mice primed with another virus serotype, which suggests that cross-reactive Th cells may play a role in heterotypic priming with poliovirus.

Methods

Virus preparations. Attenuated live poliovirus vaccine strains Sabin type 1, 2 and 3 were used throughout. Viruses were propagated in Hep-2c cells and purified concentrates obtained as described by Minor (1985). Briefly, infected tissue culture fluid was clarified by low speed centrifugation (3000 g), precipitated with 0.4 g/ml ammonium sulphate and purified through a 15 to 45% sucrose gradient. Fractions containing virus were pooled, centrifuged at 30 000 g for 4 h and pellets were resuspended in phosphate-buffered saline. Virus infectivity was determined by plaque assay as described below and the concentrates were stored as aliquots at -70°C . Protein concentrations were estimated using the Bio-Rad Protein Assay Kit I.

Microneutralization assay. Dilutions of sera in MEM supplemented with 1% foetal calf serum (FCS), 1.4 $\mu\text{g/ml}$ penicillin, 1 $\mu\text{g/ml}$ streptomycin and 10 $\mu\text{g/ml}$ Fungizone were incubated with 100 TCID₅₀ of virus in 96-well microtitre plates (Falcon Plastics) for 3 h at 35 $^{\circ}\text{C}$. Hep-2c cells in suspension were then added at approximately 1×10^4 cells per well and the plates were re-incubated for 3 days at 35 $^{\circ}\text{C}$ in a humidified incubator with 5% CO₂. Each test was performed in quadruplicate and the results expressed as the reciprocal log₂ or log₁₀ of the final dilution of serum that totally inhibited the viral c.p.e. Results are expressed as geometric mean titres (GMT) of duplicate titrations.

In the context of this study, a primary response has been defined as an antibody titre detected 14 days after the first immunization, whereas a secondary response is an increase in the antibody titre of fourfold or greater than the primary response measured 14 days after the booster immunization.

Plaque assay. Serial log₁₀ dilutions of virus were allowed to adsorb on Hep-2c cell monolayers in FB-6 plates (Flow Laboratories) for 1 h at room temperature with occasional shaking. Excess fluid was removed and wells were covered with an agarose overlay containing MEM supplemented with serum and antibiotics. After 3 days incubation at 35 $^{\circ}\text{C}$ in a humidified incubator with 5% CO₂, the overlay was flicked off and the cell monolayer stained with 1% naphthalene black to visualize plaques. Results were expressed as p.f.u./ml.

Mouse immunizations. Female BALB/c mice (between 8 and 10 weeks old), maintained as an inbred colony at NIBSC, were used for all experiments. Virus emulsified in Freund's complete adjuvant (CFA) was inoculated subcutaneously (s.c.) at the base of the neck, whereas virus without adjuvant or virus precipitated with alum was administered intraperitoneally (i.p.). Each mouse received approximately 10⁸ p.f.u. purified virus (equivalent to approximately 1 μg of protein). Serum samples prepared from peripheral blood were collected 14 days after either primary or secondary immunization, immediately before removal of spleens, which were used in T cell assays.

Proliferation assay. Spleens from immunized BALB/c mice were individually homogenized and suspended in RPMI 1640 supplemented with either 8% heat-inactivated FCS or 2% normal mouse serum. Cells (4×10^5 /well) were cultured in 96-well flat-bottomed microtitre plates in the presence of either poliovirus (at concentrations between 4×10^6 and 1×10^9 p.f.u./ml) or medium alone. Cultures were incubated for 3 to 4 days and pulse-labelled with 0.5 μCi [³H]thymidine for the final 4 to 6 hours. [³H]thymidine incorporation was measured by liquid scintillation counting after harvesting cultures on to filter paper. Results were expressed as mean c.p.m. in triplicate cultures, or as Δ c.p.m. after subtracting the background c.p.m. measured in the absence of antigen.

Cytokine assay. Cytokine release was assessed by testing the ability of supernatants to support the proliferation of the IL-2/IL-4-dependent cell line CTLL-2 (Gillis *et al.*, 1978). The CTLL-2 cell line responded to

recombinant IL-2 and IL-4, but these responses were abolished by monoclonal antibodies to the IL-2 receptor or IL-4 respectively. Supernatant (50 μl), removed after 24 h of culture, was added to 50 μl (1×10^4) CTLL-2 cells that had been washed free of residual IL-2 for 2 h before the assay. CTLL-2 proliferation was determined after 24 h by [³H]thymidine incorporation. Results were expressed as the mean c.p.m. for triplicate assays after subtraction of background controls.

Results

Specificity of neutralizing antibody responses to poliovirus after primary and secondary immunization

Sera from mice immunized with one or two doses of type 1 or type 3 virus in alum, CFA or no adjuvant, were tested for neutralizing activity against each of the three serotypes. Neutralization titres of pooled sera from groups of five mice are shown in Fig. 1.

Primary immunization of mice with type 1 virus generated serotype-specific neutralizing antibodies with titres between 1:160 and 1:640 obtained on day 14 post-immunization. The maximum titre of 1:640 was detected in mice immunized i.p. with type 1 virus in the presence of alum (Fig. 1c). A booster immunization with homotypic virus produced a significant increase in secondary antibody titre, especially in the presence of alum (1:10240). Mice immunized with type 1 virus did not generate cross-neutralizing antibodies to type 3 virus (Fig. 1), even after four immunizations (data not shown). However, low levels of cross-reactive antibody were detected against type 2 virus (Fig. 1a and c). The addition of adjuvants did not augment neutralizing antibody titres to type 3 virus over that obtained by immunization with virus alone. Primary antibody titres to type 3 virus were consistently lower than those generated by type 1 virus. However, secondary immunization with homotypic virus produced a significant boost in antibody titre (1:160), indicating that mice were successfully primed after the first immunization. Additionally, mice immunized with type 3 virus did not generate cross-neutralizing antibodies to type 1 virus, even after four immunizations (data not shown).

Specificity of proliferative T cell responses to poliovirus after primary and secondary immunization

The ability of polioviruses to stimulate virus-specific T cell responses was initially studied by immunizing mice with either one or two doses of type 1 and type 3 viruses, in the presence or absence of adjuvants. Low levels of cross-reactive proliferative responses were detected in pooled spleen cells of mice immunized with either serotype (Fig. 2). The use of CFA (s.c.) or alum (i.p.) did not significantly enhance the proliferative responses after primary immunization, indeed, a slight reduction

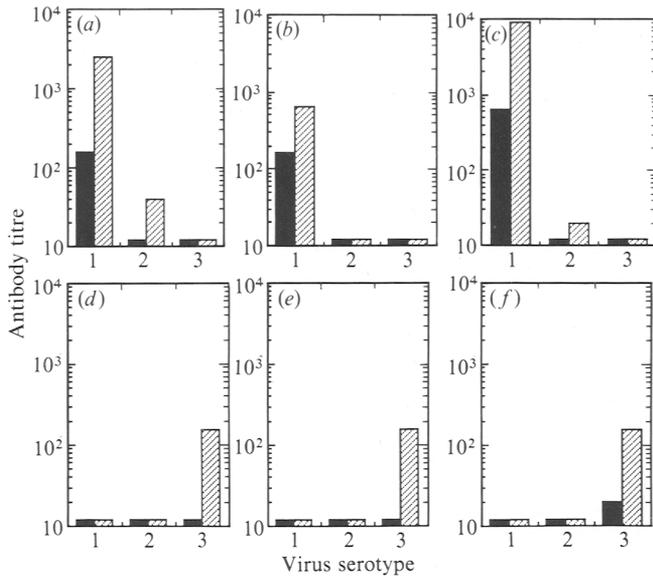


Fig. 1. Serotype specificity of neutralizing antibody responses to poliovirus. Antisera were tested 14 days after primary (filled) or secondary (shaded) immunization of mice with type 1 (*a* to *c*) or type 3 (*d* to *f*) poliovirus, without adjuvant (*a* and *d*), with CFA (*b* and *e*) or with alum (*c* and *f*). Each bar represents the titre of an equal volume pool of five sera and is expressed as the reciprocal of the log₁₀ dilution which neutralized type 1 (1), type 2 (2) and type 3 (3) viruses.

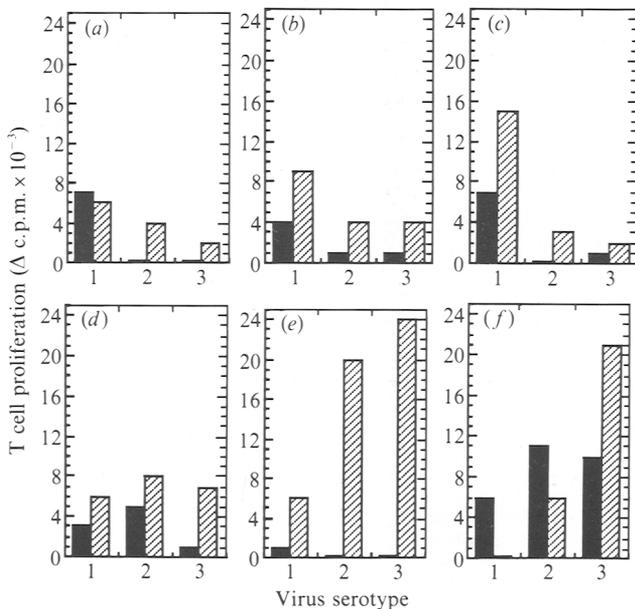


Fig. 2. Specificity of proliferative T cell responses following poliovirus immunization. Pools of spleen cells from groups of five mice were isolated 14 days after primary (filled) and secondary (shaded) immunization with type 1 (*a* to *c*) or type 3 (*d* to *f*) viruses, in the absence of adjuvant (*a* and *d*), with CFA (*b* and *e*) or with alum (*c* and *f*). Proliferation was measured 3 days after stimulation with 5×10^8 p.f.u./ml of type 1 (1), type 2 (2) or type 3 (3) polioviruses. Results are expressed as Δ c.p.m. after subtraction of background responses to medium alone, which ranged from 5000 to 10000 c.p.m.

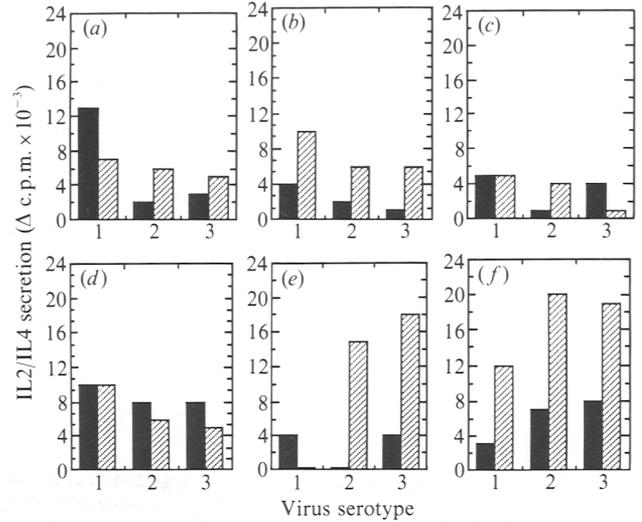


Fig. 3. IL2/IL4 secretion by immune spleen cells following stimulation by poliovirus types 1, 2 and 3. Primary and secondary immunizations and measurement of proliferation are described in the legend to Fig. 2.

was detected in mice immunized s.c. with virus emulsified in CFA (Fig. 2*b* and *e*). After secondary immunization with homotypic virus, the *in vitro* T cell responses increased, especially in spleen cells obtained from mice immunized with virus in the presence of adjuvant (Fig. 2, 3). For example, spleen cells from mice immunized with type 3 virus in alum (Fig. 2*f*) showed higher levels of proliferation than in the absence of adjuvant (Fig. 2*d*). However, in contrast with neutralizing antibody production, this effect was not seen with type 1 virus (Fig. 2*a* to *c*).

A cytokine release assay, which measures the amount of IL-2/IL-4 released into the supernatant fluid by antigen-stimulated T cells (Fig. 3), largely confirmed the results shown in Fig. 2 and showed that proliferative activity of spleen cells from poliovirus-immunized mice was probably a function of the Th cell population.

The results of the T cell responses taken together with neutralization data suggested that i.p. immunization with poliovirus adsorbed with alum gave the most satisfactory immune responses. Therefore all subsequent immunization studies were performed using alum as adjuvant. In order to study T cell responses in more detail, we examined the responses of mice primed with type 1, 2 and 3 viruses. In these proliferation experiments, normal mouse serum was substituted for FCS in order to reduce the high background levels shown in Fig. 2. The data shown in Fig. 4 clearly demonstrate that proliferation of spleen cells from individual mice was antigen dose-dependent and cross-reactive between type 1 and type 3 viruses, following primary immunization with either virus serotype. In addition, spleen cells derived from individual mice immunized with one dose

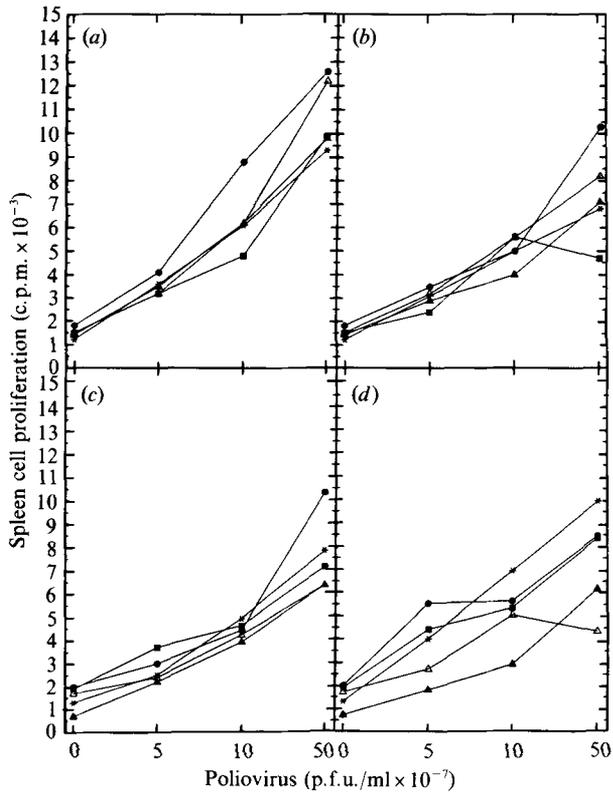


Fig. 4. Antigen dose-dependent proliferation of immune spleen cells *in vitro*. Proliferative response of spleen cells from mice immunized with either type 1 (*a* and *b*) or type 3 (*c* and *d*) viruses were tested against a range of concentrations of type 1 (*a* and *c*) or type 3 (*b* and *d*) polioviruses. Each curve represents the proliferative response of spleen cells obtained from a single mouse.

(Fig. 5*a, c* and *e*) or two doses (Fig. 5*b, d* and *f*) of type 2 virus showed that proliferation was cross-reactive and dose-dependent but not significantly increased after secondary immunization with homologous virus.

Heterotypic priming and its effect on neutralizing antibody production and T cell responses

As shown in Fig. 1, secondary immunization with homotypic virus produced a boost in neutralizing antibody titres against both type 1 and type 3 viruses which were serotype-specific and cross-reacted weakly only with type 2 virus. However, as spleen cells from the same groups of mice showed broadly cross-reactive T cell proliferative activity, it is possible that such cross-reactive T cells may be capable of establishing heterotypic memory *in vivo*. We therefore examined the effect of heterotypic priming on antibody and T cell responses. The results shown in Fig. 6 demonstrate that the cross-reactive component of the T cell response against type 1 or type 3 viruses observed after primary immunization

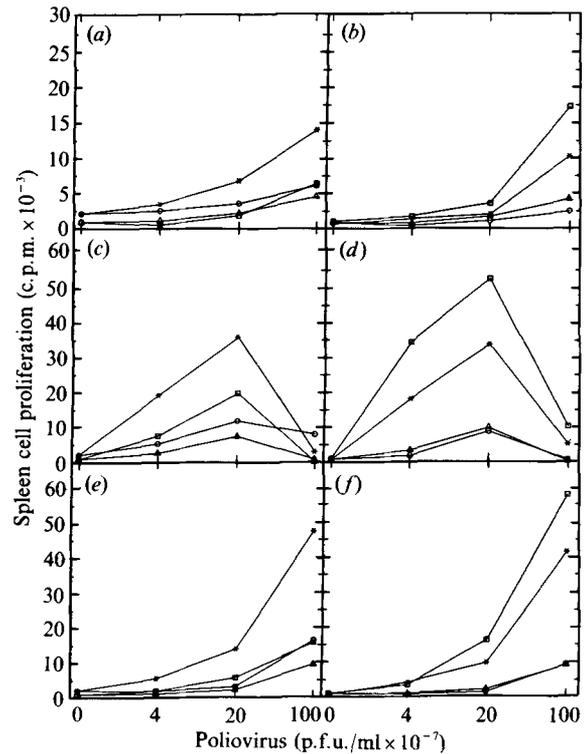


Fig. 5. Antigen-dependent proliferation of spleen cells *in vitro* in response to type 1 (*a* and *b*), 2 (*c* and *d*) or 3 (*e* and *f*) viruses after primary (*a, c* and *e*) and secondary (*b, d* and *f*) immunization with type 2 virus in alum. Each curve represents the proliferative response of an individual mouse.

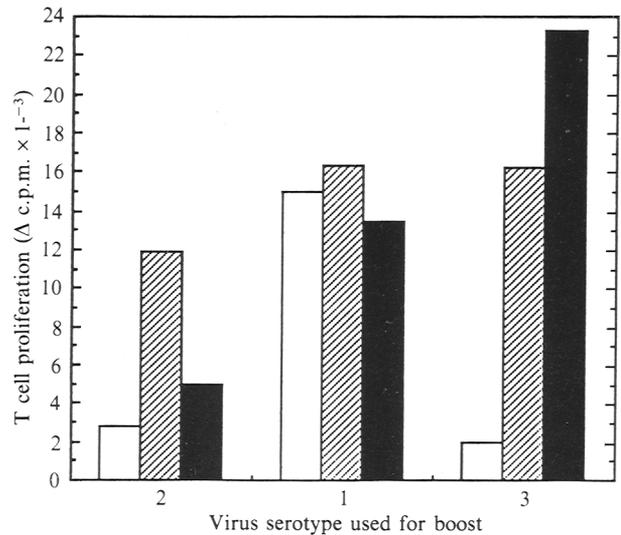


Fig. 6. Specificity of T cell proliferative responses after heterotypic priming. Mice primed with type 2 virus were boosted with either type 1, type 2 or type 3 viruses. Proliferation was measured 3 days after stimulation with 10^8 p.f.u. of type 1, 2 or 3 viruses. Each bar represents the mean c.p.m. of groups of three mice. Results are expressed as Δ c.p.m. after subtraction of background responses to medium alone, which ranged from 500 to 2000 c.p.m. □, Type 1 virus; ▨, type 2 virus; ■, type 3 virus.

Table 1. Effect of heterologous priming on neutralizing antibody specificity

First immunization	Antibody titre*	Second immunization	Neutralizing antibody titre after second immunization		
			Type 1	Type 2	Type 3
-	< †	Type 1	56	<	<
-	<	Type 2	<	538	<
-	<	Type 3	<	<	47
Type 1	80	Type 1	1810	<	<
Type 1	16	Type 2	20	1076	<
Type 1	14	Type 3	28	<	40
Type 2	226	Type 1	226	1076	<
Type 2	269	Type 2	<	4063	<
Type 2	226	Type 3	<	403	201
Type 3	<	Type 1	67	<	<
Type 3	<	Type 2	<	1522	<
Type 3	<	Type 3	<	<	269

* Neutralizing antibody titres tested 30 days post-primary immunization, immediately before second immunization.

† Indicates GMT below 1:10 dilution.

with type 2 virus is boosted considerably after secondary immunization with the heterologous virus. Generally, the highest proliferative response was obtained against the serotype used for the second immunization, although the response to type 1 virus was consistently lower than that to type 2 or 3 viruses (Fig. 6). Although these results clearly demonstrate cross-reactivity between the three serotypes, they also suggest that a component of the T cell response to the type 2 virus is serotype-specific.

As previously observed (Fig. 1), significant increases in serotype-specific neutralizing antibody responses were generated in mice primed with type 1 or type 3 viruses after boosting with homotypic virus (Table 1). However, neutralizing antibody titres were also augmented in mice primed with type 2 virus and boosted with either homotypic or heterotypic virus (Table 1). For example, mice primed with type 2 virus generated anti-type 2 virus titres of 1:538 which increased to 1:4063 after boosting with type 2 virus. Furthermore, mice primed with type 2 virus followed by boosting with type 1 virus generated titres of 1:226 and 1:1076 against type 1 and type 2 viruses respectively, whereas boosting with type 3 virus generated titres of 1:403 and 1:201 against type 2 and type 3 viruses respectively. Heterotypic priming was also observed in mice primed with either type 1 or type 3 viruses and boosted with type 2 (Table 1). For example, secondary antibody titres against type 2 virus of 1:1522 and 1:1076 were detected in mice primed with type 3 and type 1 virus respectively, although these levels show only a two- to threefold increase in primary antibody titres. In contrast, heterotypic priming between type 1 and type 3 viruses was not observed (Table 1).

Table 2. Summary of T and B cell responses to poliovirus

Response	Recognition of poliovirus <i>in vitro</i>	Immunization with poliovirus		
		Type 1	Type 2	Type 3
T cell	Type 1	++*	+	+
	Type 2	+	+++	++
	Type 3	+	++	++
Neutralizing antibody	Type 1	+++	-	-
	Type 2	+/-	+++	-
	Type 3	-	-	++

* The results represent data from at least five independent experiments and are presented as positive (+), negative (-) or inconclusive (+/-).

Discussion

In a study of poliovirus immunity in mice, we have identified immunization protocols capable of generating consistent humoral and cell-mediated immune responses and shown that a cross-reactive T cell response can be detected after a single immunization. In contrast, neutralizing antibodies assayed in serum from the same animals were mainly serotype-specific (summarized in Table 2). Furthermore, immunization with type 2 poliovirus was found to prime for a secondary antibody response to each of the three serotypes, whereas priming with type 1 or type 3 virus could only generate a secondary response to the homologous virus or to type 2 virus. Although there is evidence for some cross-reactivity at the antibody level between type 1 and type 2 viruses, our results raise the possibility that heterotypic memory at the Th cell level may play a role in the stimulation of cross-reactive immune response to poliovirus and thus in immune protection against poliomyelitis.

The cooperation of Th cells with B cells to generate neutralizing antibody is a well established phenomenon. In a study by UytdeHaag *et al.* (1985), the production of neutralizing antibody to poliovirus *in vitro* could be generated by restimulation of cultured human lymphocytes with antigen, but only in the presence of Th cells. Our studies show that in mice immunized with poliovirus, neutralizing antibody responses were generated concomitantly with cross-reactive Th cell responses. Cross-reactive T cell proliferative responses were also reported by Wang *et al.* (1989) after immunization of mice with u.v.-inactivated wild-type poliovirus strains. However, in their studies, lymph node cells from mice immunized with type 3 (Leon) virus did not cross-proliferate with type 1 (LS-a) virus, which may suggest greater heterogeneity between wild-type polioviruses than between the Sabin strains used in our study.

Although we have demonstrated intertypic cross-reactivity following immunization with each of the three virus serotypes, there is also evidence that a component of the T cell response was serotype-specific. In general, the strongest T cell responses were demonstrated against the immunizing subtype (Table 2). Furthermore, following heterotypic priming we detected an amplification in the cross-reactive response to the virus serotype used for secondary immunization of mice primed with virus of another serotype.

It has been shown that Th cells specific for the internal nucleoprotein or matrix protein of influenza virus and the nucleoprotein of HBV, can provide help to B cell production of neutralizing antibody against the more variable influenza virus haemagglutinin and HBV surface proteins respectively (Scherle & Gerhard, 1986; Milich & McLachlan, 1988). The production of anti-poliovirus neutralizing antibodies, against the generally variable exposed loop regions of the capsid proteins, may thus be mediated through helper functions provided by Th cells which recognize regions conserved between the three virus serotypes. Our data suggest that immunization with type 2 virus could prime for a secondary response to all three virus serotypes. However, immunization with type 1 or type 3 viruses did not prime for a significant increase in antibody levels after secondary immunization with virus of a different serotype. Although the neutralizing antibody response to type 2 virus has not been thoroughly investigated, the isolation of cross-reactive monoclonal antibodies which neutralize both type 1 and type 2 viruses (M. Ferguson, personal communication; Uhlig *et al.*, 1990) demonstrates the existence of type-common epitopes shared by these two serotypes. Our results on the cell-mediated immune response to poliovirus suggest that Th cells directed against sites conserved between type 2 and type 1 and between type 2 and type 3 viruses may also play a role in heterotypic immunity to poliovirus infections.

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(Received 5 December 1990; Accepted 23 January 1991)