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HUMAN PERIPHERAL BLOOD LYMPHOCYTES FROM RECENTLY VACCINATED INDIVIDUALS PRODUCE BOTH TYPE-SPECIFIC AND INTERTYPIC CROSS-REACTING NEUTRALIZING ANTIBODY ON IN VITRO STIMULATION WITH ONE TYPE OF POLIOVIRUS[1]

FONS G. C. M. UYTDEHAAG,* HENK G. LOGGEN,* TON LOGTENBERG,† ROBERT A. LICHTVELD,‡ BERT VAN STEENIS,* JACK A. A. M. VAN ASTEN* AND ALBERT D. M. E. OSTERHAUS*

*From the *National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands; the †Department of Clinical Immunology, University Hospital, Utrecht, The Netherlands; and ‡The Netherlands Army, Utrecht, The Netherlands*

An in vitro system of poliovirus-specific antibody production by peripheral blood B cells on stimulation by the virus has been developed. Virus-neutralizing antibodies in culture supernatant fluids, or virus-specific antibody-secreting cells (ASC) were detected by microneutralization assay and ELISA-SPOT test, respectively.

After booster immunization with polio vaccine, anti-poliovirus-neutralizing ASC were present in circulation. This response was measurable between 5 and 12 days after booster vaccination. At between 12 and 90 days, another subset of B cells was found in peripheral blood that only produced poliovirus-specific neutralizing antibody after in vitro antigenic stimulation. The in vitro virus-induced response required B cells, monocytes, and T4⁺ (T helper) cells, and was shown to result from de novo protein synthesis.

The anti-poliovirus-neutralizing response in vitro could be dissected in a type-specific and intertypic cross-reactive response by using various antigen concentrations for in vitro stimulation. Evidence was obtained by absorption studies for the existence of intertypic cross-reactive neutralization-inducing epitopes.

Human polioviruses, among which three serotypes can be defined that show a restricted antigenic homology, are members of the *Picornaviridae*, a family of viruses causing a variety of diseases in man and animals (for review, see Reference 1). Infection with poliovirus or immunization with live attenuated or inactivated vaccine evokes an immune response in which the formation of virus-specific neutralizing antibodies is considered to be the major component (1). Studies on this humoral immune reactivity to poliovirus have involved mainly the measurement of antibodies in serum and have revealed conflicting results regarding the fine specificity of the antibody response (2–5) (A. Osterhaus, unpublished observations). Analysis of the humoral immune response to poliovirus at the cellular level would likely be an important prerequisite for a better understanding of mechanisms involved in the induction, the regulation, and the fine specificity of the response. The development of satisfactory human in vitro models to allow such studies is especially important, because polioviruses and other members of the *Picornaviridae* family recently have been subject to new approaches which may lead to the production of synthetic and anti-idiotype vaccines (6–9).

Methods have been established to assess the specific antibody response in cultures of human B cells after stimulation with influenza virus (10, 11), varicella zoster virus (12), and rabies virus (13). In the present paper, we report the in vitro induction of a secondary antigen-specific and $T4^+$ cell-dependent neutralizing antibody response of human peripheral blood lymphocytes (PBL)[2] by the purified inactivated polioviruses type 1 (strain Mahoney), type 2 (strain MEF_1), and type 3 (strain Saukett). By using the three different types of poliovirus, the host repertoire of antibody production could be examined in terms of type-specific as well as cross-reactive antibodies. Kinetic studies revealed evidence for the existence of at least two functionally different B cell subsets involved in the immune response against poliovirus.

MATERIALS AND METHODS

Immunizations. Vaccination against diphtheria (D), pertussis (P), tetanus (T), and poliomyelitis was introduced in the Netherlands in 1957 with the use of a combined vaccine (14). All children born after 1960 have been immunized at the ages of 3, 4, 5, and 12 mo with this vaccine. Booster immunizations were given at the ages of 4 and 9 yr with a D, T, poliovaccine. The vaccine contains per milliliter: 2.5 Lf D toxoid (DT), 4 IU killed *Bordetella pertussis*, 5 Limes floculationes (Lf) tetanus toxoid (TT), and killed poliovirus type 1 (Mahoney) 40 D antigen units (DU), type 2 (MEF_1) 4 DU, and type 3 (Saukett) 7.5 DU, 1.5 mg $AlPO_4$, 2 mg 2-phenoxyethanol, and 25 μg formaldehyde. Healthy male subjects, 18 to 25 yr old, were given an intramuscular booster immunization with D, T, poliovaccine. Blood samples were collected before and at various times after immunization as indicated.

Lymphocyte preparation. Mononuclear cells were isolated from heparinized blood by density gradient centrifugations on Ficoll-Isopaque. Mononuclear cells were depleted from T helper/inducer cells or T suppressor cells by treatment with monoclonal antibody RIV6 and rabbit complement or monoclonal antibody FK18 and rabbit complement, respectively.

RIV6 is a mouse monoclonal antibody against a T cell differentia-

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[1] This paper was presented in part at the 16th International Leucocyte Culture Conference, Cambridge, England, August 19–25, 1984.

[2] Abbreviations used in this paper: ASC, antibody-secreting cells; NT, microneutralization test; DT, diphtheria toxoid; D, diphtheria; TT, tetanus toxoid; T, tetanus; KPV, killed poliovirus vaccine; AP, alkaline phosphatase; PBL, peripheral blood lymphocytes; VN, virus neutralization; DU, D antigen units.

tion antigen present on approximately 60% of circulating peripheral T lymphocytes. This population functionally defines the human helper/inducer T cell subset for T-B cooperation (see results, Table II). The production and further characteristics of RIV6 will be described elsewhere (Kreeftenberg et al., manuscript in preparation). The FK18 mouse monoclonal antibody recognizes 20 to 40% of peripheral blood T lymphocytes. This population functionally defines the same population as the OKT8 monoclonal antibody (15). FK18 was the generous gift of F. Koning (Department of Immunohematology and Bloodbank, University Hospital Leiden, The Netherlands).

Ten to 50 million PBL in 1 ml of culture medium were incubated with RIV6 antibody at a final dilution of 1/100 or FK18 antibody at a final dilution of 1/50 for 1 hr at 4°C. After incubation, the cells were spun down, the supernatant fluid was removed, and fresh rabbit complement was added to the cells at a final dilution of 1/2 in 1 ml medium. After incubation for 2 hr at 37°C, viable cells were isolated on Ficoll-Isopaque, were washed twice, and were used in the experiments. Percentages of B cells and T cell subsets were determined by immunofluorescence analysis, using monoclonal antibodies Leu-1, Leu-2b, Leu-3a, Leu-12 (Becton Dickinson, Mountain View, CA), RIV6, and FK18. Cells (1×10^6) were incubated for 30 min at room temperature with 100 μl unconjugated monoclonal antibody. The cells were washed twice, were resuspended in 25 μl (1/10 diluted) FITC-conjugated goat anti-mouse IgG (H+L) (Becton Dickinson), and were incubated for 30 min at 0°C. The cells were then washed twice and were resuspended in 0.5 ml PBS containing 1% BSA, 0.05% NaN_3. Percentages of fluorescent cells were determined by FACS analysis (Becton Dickinson).

Mononuclear cells were depleted of monocytes by adherence to plastic Falcon flasks at 37°C in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS; Rehatuin Armour Pharmaceutical Co., Kankakee, IL). After 60 min, supernatants containing lymphocytes were harvested and were washed twice in serum-free RPMI 1640 medium. After the adherence procedure, the cell suspension usually contained between 5 and 10% monocytes, as determined by nonspecific esterase staining and fluorescence analysis with anti-human Leu-M3 monoclonal antibody (Becton Dickinson).

Culture conditions. PBL containing 5 to 10% monocytes were suspended in RPMI 1640 medium containing 25 mM HEPES, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), 10^{-5} M 2-mercaptoethanol, and 10% FCS (Rehatuin Armour Pharmaceutical Co.). Cells were cultured in 24-well, flat-bottomed plates (Costar Data Packaging, Cambridge, MA) in the presence of various concentrations of killed poliovirus type 1 Mahoney, type 2 MEF_1, or type 3 Saukett (a gift from Dr. A. L. van Wezel, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands) at a cell density of 2×10^6 PBL/ml in a final volume of 1 ml/well.

After 10 days of culture at 37°C in a humidified atmosphere of 5% CO_2 in air, supernatant fluids were harvested and were assayed for anti-poliovirus type-specific neutralizing antibodies, for antibodies against TT, DT, and total Ig. In some experiments, cultures were terminated at day 7 after initiation, and poliovirus-specific antibody-secreting cells (ASC) were enumerated by ELISA-SPOT, whereas antibody in supernatant fluids was determined as described above.

Determination of poliovirus type-specific neutralizing antibodies. The content of poliovirus type-specific neutralizing antibodies in culture supernatant fluids was determined by routinely used microneutralization test (NT) as described (16). Titers of neutralizing antibody were expressed as reciprocals of dilutions of supernatant fluids neutralizing 100 $TICD_{50}$ in NT.

Absorption of poliovirus type-specific neutralizing antibody from culture supernatant fluids. Supernatant fluids from cultures stimulated with type 1, type 2, or type 3 of poliovirus, exhibiting titers between 2 and 64 of neutralizing antibodies against all three types, were divided into four equal samples. These samples were incubated with poliovirus type 1 (45 μg/ml), type 2 (25 μg/ml), or type 3 (15 μg/ml), or without virus, at 4°C for 16 hr. To remove free virus particles and virus complexed to antibody, the samples were layered on top of a linear 20 to 50% w/w sucrose gradient. The gradients were centrifuged for 6 hr at 35,000 rpm in a Beckman SW41 rotor. Fractions were collected by bottom unloading and were assayed for poliovirus type-specific neutralizing antibody activity.

Determination of anti-DT neutralizing antibody. The neutralizing activity against DT in supernatant fluid was determined by in vitro NT as described recently (17). In brief, culture supernatant fluids and a reference anti-DT serum were diluted in Medium 199 (RIVM) containing 20 mg/L phenol red, 100 IU/ml penicillin, 100 μl/ml streptomycin, and 10% FCS (complete M199). Fifty microliters of diluted culture supernatant fluid were mixed in flat-bottomed microtiter tissue culture plates (Greiner Labor Technik, Nürtingen, FRG) with 50 μl of the Lr/10,000 test dose in M199. This dose corresponds to the lowest amount of DT (2.5×10^{-3} Lf) able to induce metabolic inhibition in the presence of 10^{-4} IU anti-DT of the reference.

After incubation for 1 hr at 20°C, 50 μl of a suspension containing 2.5×10^5 Vero cells/ml (African green monkey kidney cell line) in complete M199 were added. The plates were incubated for 6 days at 37°C and were screened for complete metabolic inhibition as indicated by a red color of the culture supernatant fluids. Neutralizing titers were calculated by multiplication of 10^{-4} IU with the dilution factor of the sample showing complete metabolic inhibition in the test, and were expressed as IU anti-DT/ml.

Enzyme-linked immunosorbent assays (ELISA). Anti-TT-specific IgG was assayed by ELISA as described (18). The concentration of anti-TT-specific antibody in ng/ml was calculated by determining the ratio of the slopes of the best-fitting line of both reference and sample (13). The method used for standardization of the reference serum has been described (13). Total Ig in supernatant fluids was assayed as described (13).

ELISA-SPOT test. B cells secreting poliovirus type-specific IgG or IgM antibody were enumerated by a modification of the ELISA-SPOT test, as described by Sedgwick and Holt (19). Flat-bottomed microtiter plates (Titertek Type III; Flow Laboratories Inc., McLean, VA) were coated with 150 μl bovine anti-poliovirus types 1, 2, and 3, IgG antibody (20 μg/ml) in carbonate buffer, pH 9.6, by incubation at 20°C for 16 hr. After washing with PBS, wells were filled with 100 μl of poliovirus type 1 (45 ng/ml), type 2 (250 ng/ml), or type 3 (250 ng/ml) diluted in PBS containing 1% BSA (Boseral, Organon Teknika, Turnhout, Belgium). After 2 hr incubation at 37°C, plates were washed with PBS and were incubated for 45 min at 37°C with PBS, 1% BSA, to block remaining binding sites. PBL stimulated in vitro with poliovirus or nonstimulated control PBL were added to wells in 100 μl RPMI 1640, 1% BSA, at various cell densities (10^6, 0.5×10^6, 0.25×10^6/ml) and were incubated in a vibration-free incubator in a humidified atmosphere of 5% CO_2 in air for 3 hr. The wells were washed with PBS, 1% BSA, 0.05% Tween-20, and were incubated with 100 μl goat anti-human IgM-alkaline phosphatase conjugate or goat anti-human IgG-alkaline phosphatase (AP) conjugate (Tago Inc., Burlingame, CA) for 16 hr at 20°C. After washing with PBS, 1% BSA, 0.05% Tween-20, the AP substrate 5-bromo-4-chloro-3-indolylphosphate (5-BCIP) was added in gelling agarose. The AP substrate was a 2.3 mM 5-BCIP (Sigma Chemical Co., St. Louis, MO) solution in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma Chemical Co.) containing 5 mM $MgCl_2 \cdot 6H_2O$ 0.01% Triton X-405 (Sigma Chemical Co.), 0.01% NaN_3, and 0.6% 36°C gelling agarose (Sigma Chemical Co.).

To visualize IgM and IgG ASC, plates were incubated at 37°C in a humidified atmosphere for 16 hr and 2 hr, respectively. SPOTS were counted microscopically under 320× magnification.

RESULTS

In vitro induction of the anti-poliovirus response. Unless indicated otherwise, human PBL were collected 30 days after booster vaccination with a vaccine containing DT, TT, and the three types of poliovirus. As shown in Figure 1, the in vitro induction of poliovirus-specific neutralizing antibody responses clearly depends on the antigen concentration in vitro. In the absence of virus, antibody was not produced. Irrespective of the type of poliovirus used to stimulate an immune response in vitro, optimal virus-specific neutralizing antibody responses were seen at concentrations of 1 to 70 ng virus/culture (Fig. 1). This has been found in all 20 individuals studied (Fig. 2). At a higher concentration of virus in the culture, i.e., 45 μg for type 1, 25 μg for type 2, and 15 μg for type 3, abrogation of the response was noticed (Fig. 1). To rule out the possibility that residual viral antigen in culture supernatant fluids accounted for this decreased virus-neutralizing antibody response, various doses of inactivated poliovirus, type 1, type 2, or type 3, were tested for their ability to block poliovirus-neutralizing activity of a WHO reference serum (CVV 901025 A7) in virus neutralization (VN). Samples of the reference serum and of an anti-poliovirus antibody-negative control serum were incubated with or without poliovirus antigens under conditions described in *Materials and Methods* for stimu-

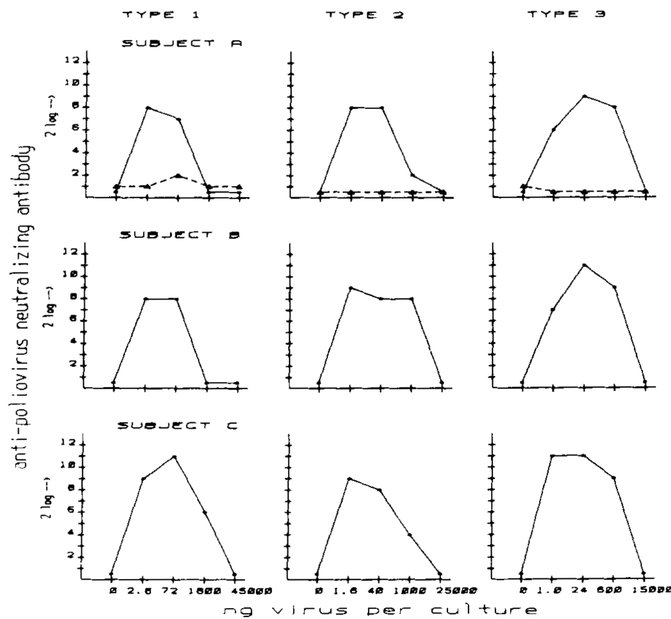

Figure 1. Antigenic requirements for the in vitro poliovirus-induced virus-specific homotypic neutralizing antibody response without (●——●) or in the presence (Δ-----Δ) of cycloheximide in culture. Three representative experiments are shown.

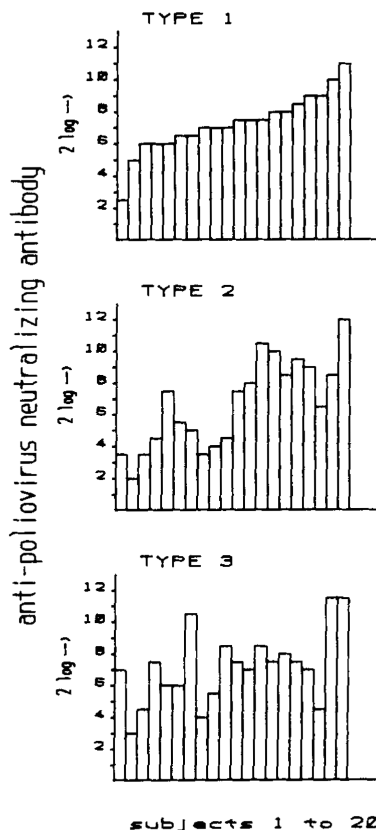

Figure 2. In vitro induced anti-poliovirus homotypic neutralizing antibody responses of PBL of 20 individuals taken 30 days after booster vaccination with DT poliovirus vaccine. Cultures were stimulated with type 1 (2.8 ng), type 2 (1.6 ng), or type 3 (1 ng) of poliovirus.

lation of PBL with poliovirus. After 10 days at 37°C, supernatant fluids were harvested and were assayed for virus-neutralizing antibody activity. Although virus-neutralizing antibody activity in VN was inhibited when high doses of virus were added to the samples (45 μg/ml for type 1, 25 μg/ml for type 2, and 15 μg/ml for type 3), a complete reduction of neutralizing antibody titers, as seen in cultures of PBL stimulated with identical doses of virus in vitro, has never been observed (Fig. 3). In addition, the negative control serum remained negative after the addition of high concentrations of antigen, arguing against receptor blocking as a mechanism to explain decreased responses (Fig. 3). Together, these results indicate that antigen present in the culture supernatant fluids does not affect the detection of specific antibody in VN. The precise mechanism(s) of the decreased response is under study. In the presence of cycloheximide, PBL did not respond to an antigenic stimulus, indicating the requirement for de novo protein synthesis in this synthesis in this system (Fig. 1).

Specificity of the anti-poliovirus antibody response. In all cultures of PBL, each type of poliovirus exclusively elicited neutralizing antibodies against the stimulating type of virus when used at relatively low concentrations. In none of these cultures was a significant response against either the heterologous type of poliovirus or against TT or DT detected (Fig. 4). Furthermore, no substantial raise in total Ig was observed in cultures at any antigen concentration tested (Fig. 5). These results demonstrate clearly the antigen specificity of the in vitro induced response.

Apart from the stimulation of type-specific antibody responses, each type of virus could also induce a heterotypic neutralizing antibody response at supra-optimal concentrations (Fig. 4). Antibodies directed to the other recall antigens DT and TT were again not detectable, arguing against polyclonal activation of PBL at these antigen concentrations. In supernatant fluids of unstim-

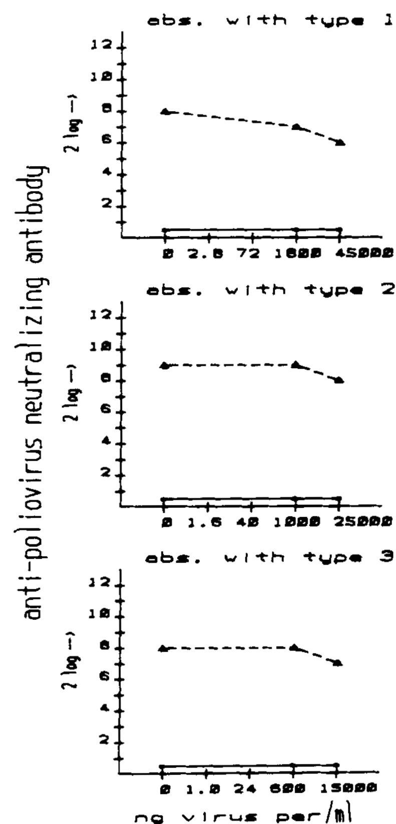

Figure 3. Absorption of a polyclonal reference serum (▲-----▲) with neutralizing antibody titers against poliovirus type 1, type 2, and type 3 and of negative control serum (●——●) with different concentrations of poliovirus type 1, type 2, or type 3.

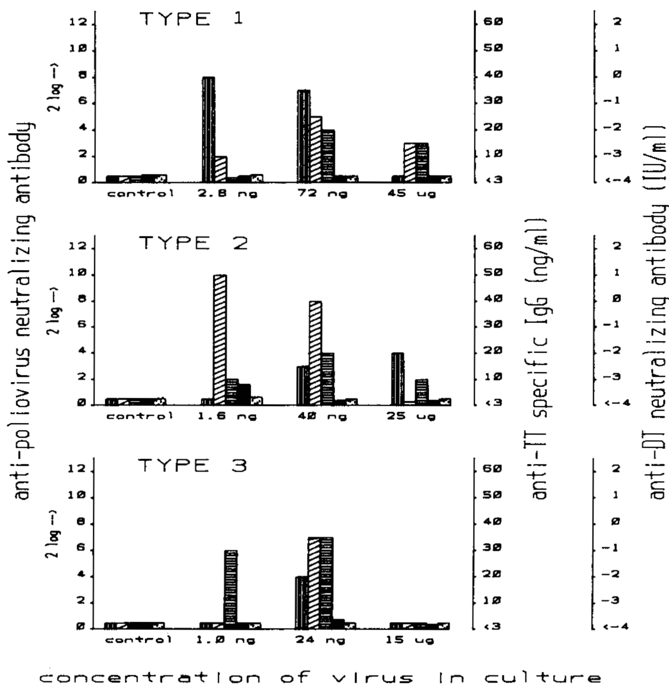

Figure 4. Fine specificity of the in vitro poliovirus-induced poliovirus-specific antibody response. Neutralizing antibodies against poliovirus type 1 (▥), type 2 (▨), and type 3 (▤) were measured in virus NT; values of anti-poliovirus-neutralizing antibody responses in four donors ranged from 64 to 1024 for type 1, 16 to 1024 for type 2, and 32 to 2048 for type 3. Antibody against DT (■) and TT (□) were measured in NT and ELISA, respectively.

ulated cultures, no antibody responses to DT and TT were found, which excludes spontaneous antibody synthesis (Fig. 4 and *vide infra*).

Because the production of oligoclonal antibodies stimulated by viruses is a well-described phenomenon (20–25), we addressed the queston of whether the heterotypic antibody responses could have resulted from oligoclonal activation of PBL by supra-optimal concentrations of virus in vitro. In that case, heterotypic antibodies (e.g., anti-types 2 and 3) resulting from stimulation of PBL by one type of virus (e.g., type 1) in vitro should be able to react only with their respective types of virus (e.g., types 2 and 3), but not with the stimulating type of virus (e.g., type 1). To examine this possibility, culture supernatant fluids from PBL stimulated in vitro with supra-optimal concentrations of type 1, type 2, or type 3 of poliovirus, which exhibited low titers of neutralizing antibodies against all three types of poliovirus, were incubated each with type 1, type 2, or type 3 of poliovirus. After the immune complexes had been removed (see *Materials and Methods*), type-specific virus-neutralizing antibodies in absorbed and in "sham"-absorbed culture supernatant fluids were determined. It was found—as shown in Figure 6—that, regardless of the type of virus used for stimulation of PBL in vitro, poliovirus type-specific neutralizing antibody in culture supernatant fluids could not only bind to a corresponding type of virus, but also to each of the other two types of viruses. These results indicate strongly that true intertypic cross-reacting neutralizing antibodies are produced by PBL on stimulation with each of the types of polioviruses in vitro. In addition, these results illustrate again that the induction of type-specific responses is an antigen-specific event.

To find out whether the intertypic cross-reacting virus-

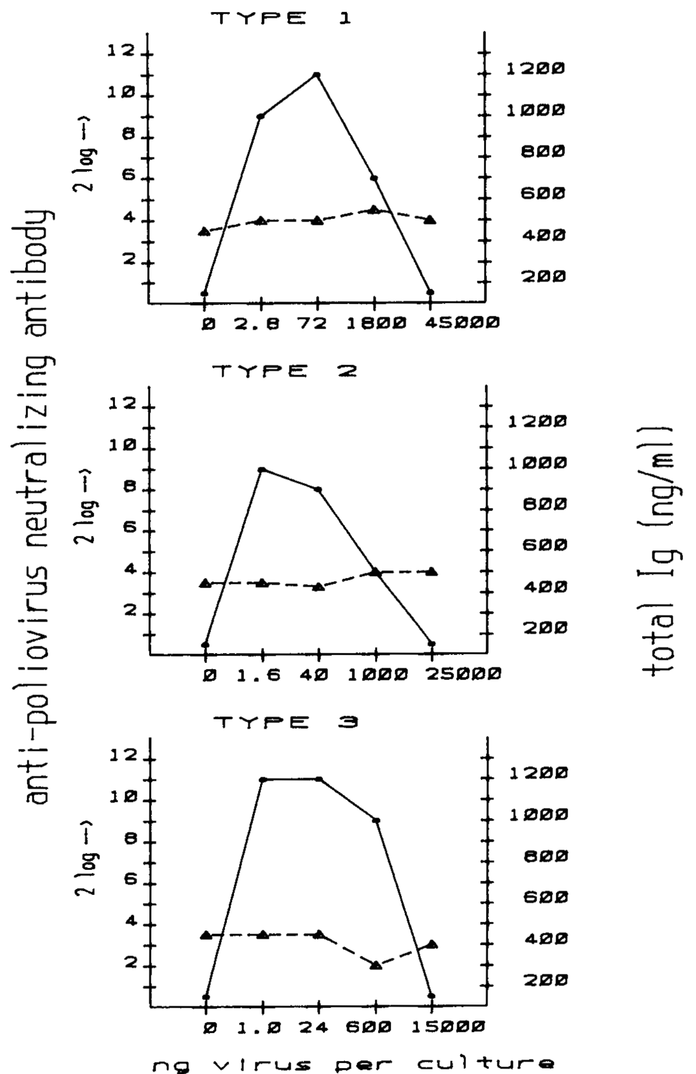

Figure 5. The in vitro poliovirus-induced poliovirus-specific (●——●) and total Ig (▲-----▲) response of PBL taken 30 days after vaccination with DT, TT, and poliovirus vaccine.

neutralizing activity present in culture supernatant fluids indeed represented anti-poliovirus antibody, a poliovirus type-specific ELISA-SPOT test was developed. This test allowed us to study the production of both homotypic and heterotypic antibody at the cellular level. As shown in Table I, cells secreted poliovirus type-specific IgM and IgG antibody in cultures stimulated with relatively low amounts of virus, whereas at supra-optimal concentrations, intertypic cross-reacting IgM and IgG antibody was also secreted.

Kinetics of the anti-poliovirus antibody response. Type-specific neutralizing antibody responses in vitro of PBL of four donors were studied at various intervals after booster immunization. PBL were cultured in the absence of antigen or in the presence of concentrations of antigen that favored the induction of type-specific responses against only the homologous type of virus. Before booster vaccination, PBL of all individuals consistently failed to respond to in vitro antigenic stimulus (data not shown). When cultured on day 5 after revaccination, PBL produced poliovirus-specific neutralizing antibodies without in vitro antigenic stimulation. Antibody production against the other recall antigen TT and DT was also noticed (Fig. 7*A*). This type of response had sharply de-

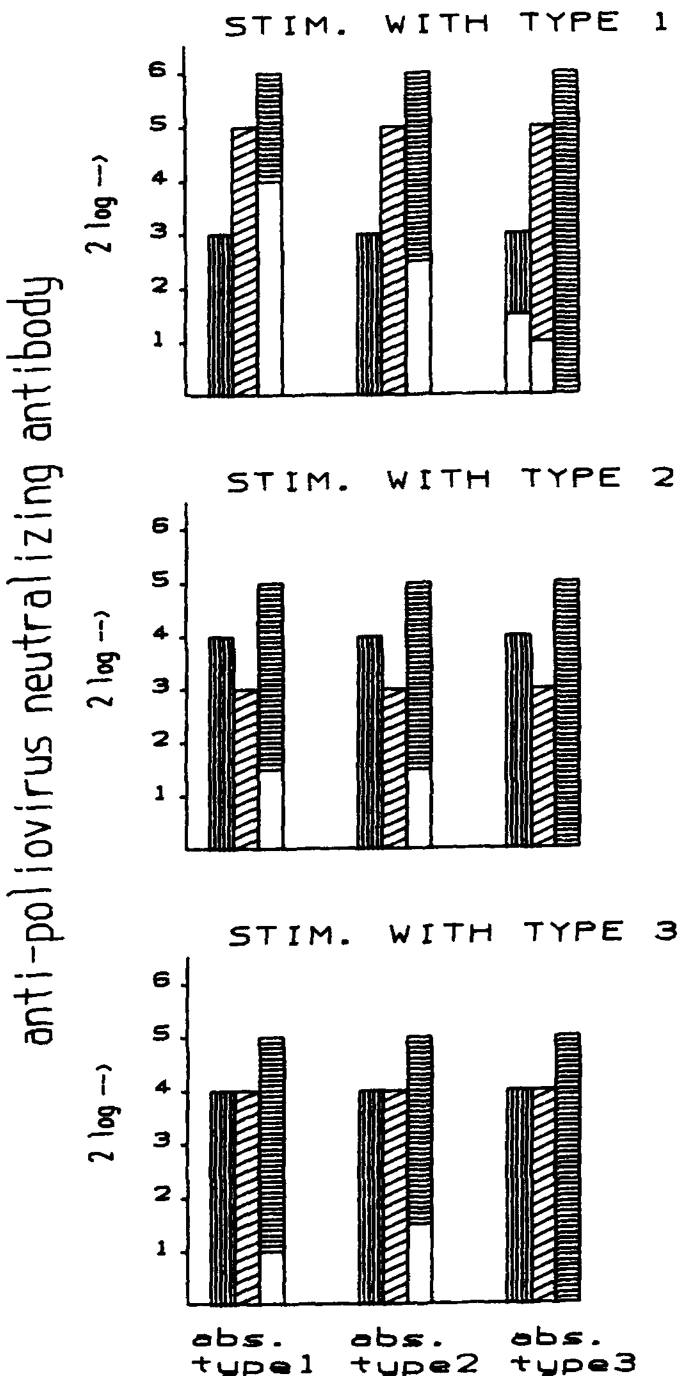

Figure 6. Evidence for intertypic cross-reactive neutralization-inducing epitopes on polioviruses. Culture supernatant fluids of cultures stimulated with supra-optimal concentrations of type 1, type 2, or type 3 of poliovirus, exhibiting neutralizing antibody titers against all three types, were absorbed with type 1, type 2, or type 3 of poliovirus (see *Materials and Methods*). Neutralizing antibodies before (shaded columns) and after absorption (open columns) were determined against type 1 (▥), type 2 (▨), and type 3 (▤) of poliovirus in VN.

creased on day 12 (Fig. 7*B*) and was absent on day 30 after booster immunization (Fig. 7*C*). The same phenomena were observed when B cells were tested in an ELISA-SPOT test directly after isolation from peripheral blood, i.e., without culturing (unpublished observation). These data indicate that temporarily spontaneous antigen-specific antibody production by PBL in vitro occurs against each of the antigens to which the donors have been recently immunized.

After revaccination, in vitro antigen-inducible anti-poliovirus type-specific responses were observed on day 12 and on day 30 (Fig. 7*B* and *C*). Although this pattern of the response was similar in all four donors, the absolute values of individual responses were variable: responses measured on day 30 after booster varied from 64 to 1024 for type 1, 16 to 1024 for type 2, and 32 to 2048 for type 3. The persistence of the in vitro response was also variable. PBL of some subjects could be stimulated by virus up to 1 yr after booster immunization, whereas PBL of other subjects became unresponsive 8 wk after revaccination (data not shown).

T helper cell requirement for in vitro antigen-induced anti-poliovirus antibody production. The induction of a type-specific neutralizing anti-poliovirus response in vitro required the presence of T cell help in the system. PBL depleted of T cell-help cells before culture by treatment with the OKT4-like monoclonal antibody RIV6 and rabbit complement failed to respond to an antigenic stimulus in vitro (Table II). Treatment with another monoclonal antibody, FK18, which functionally resembles OKT8, did not significantly influence the induction by antigen of a type-specific neutralizing anti-poliovirus antibody response.

DISCUSSION

Until now, the human immune response to poliovirus has been exclusively evaluated by serologic studies. The development of in vitro poliovirus-induced virus-specific culture and of assay systems described in the present study has allowed the investigation of the anti-poliovirus response at the cellular level.

In this study, we show evidence for the transient appearance of circulating B cells spontaneously secreting poliovirus-specific and DT-specific neutralizing antibody after in vivo booster immunization with these antigens (Fig. 7*A*). The transient appearance in circulation of this B cell subset seems to be a general phenomenon of the human immune response, because these cells have been demonstrated shortly after immunization with a wide variety of bacterial (26–29) and viral (11, 13, 30) antigens. The kinetics of the anti-poliovirus-specific spontaneously ASC was similar to the pattern seen with other antigens (26–30). It remains to be determined whether these B cells are in a terminal stage of differentiation or have the potency to give rise to other B cell subsets, as suggested by Brieva and Stevens (31).

A second, functionally distinct B cell subset was identified which produced poliovirus-specific neutralizing antibody only after an in vitro antigenic stimulus and in the presence of T cell help (Fig. 7*B* and *C*, and Table II). In contrast to observations in influenza and herpesvirus systems for in vitro antigen-induced antibody response (32), the memory B cell response against polioviruses was detectable in all donors tested. In the studies on the in vitro antibody responses to influenza virus, the selection of donors was based on serum antibody levels against the virus. The authors showed that nonresponsiveness of PBL was due to the absence of recirculating memory B cells (32). Our observations again emphasize that a *recent* booster immunization is required to reproducibly study B cell activation by antigen in human PBL. Similar observations were made by Lane et al. (33) and Cupps et al. (30). These authors demonstrated that 2 to 3 mo after

TABLE I

Intertypic cross-reactive antibody response of human PBL to poliovirus in vitro

Stimulating Virus	Anti-poliovirus Type-specific Response								
	Type 1			Type 2			Type 3		
	ASC/10^6ly.[a]		NT[b]	ASC/10^6ly.		NT	ASC/10^6ly.		NT
	IgG	IgM		IgG	IgM		IgG	IgM	
Donor A									
Type 1									
2.8 ng/culture	*31*[c]	*500*	*64*	12	6	4	0	0	<2
72 ng/culture	*218*	*1437*	*2048*	*312*	*37*	*64*	*0*	*0*	*8*
Type 2									
1.6 ng/culture	0	6	<2	*781*	*687*	*512*	0	0	4
40 ng/culture	*87*	*406*	*256*	*1687*	*1812*	*1024*	*50*	*81*	*128*
Type 3									
0.19 ng/culture	0	0	2	31	6	4	*43*	*106*	*64*
24 ng/culture	*0*	*5*	*<2*	*168*	*168*	*64*	*352*	*125*	*256*
Unstimulated PBL	0	6	<2	0	0	<2	6	0	<2
Donor B									
Type 1									
2.8 ng/culture	*127*	*11*	*256*	33	0	4	0	0	<2
72 ng/culture	*135*	*88*	*128*	*178*	*55*	*32*	*11*	*0*	*16*
Type 2									
1.6 ng/culture	47	0	<2	*460*	*260*	*1024*	0	0	4
40 ng/culture	*82*	*56*	*8*	*470*	*35*	*256*	*11*	*26*	*16*
Type 3									
0.19 ng/culture	0	0	<2	8	0	<2	*100*	*35*	*64*
24 ng/culture	*11*	*0*	*16*	*41*	*11*	*128*	*122*	*78*	*512*
Unstimulated PBL	0	0	<2	0	0	4	0	0	<2

[a] ASC: Measured in ELISA-SPOT test (see *Materials and Methods*). Expressed/10^6 lymphocytes.

[b] NT: Neutralizing antibody titers expressed as reciprocals of supernatant fluid dilution neutralizing 100 $TCID_{50}$ virus in NT.

[c] Homotypic responses at optimal antigen concentrations as well as heterotypic responses at optimal and supra-optimal concentrations of antigen are italicized.

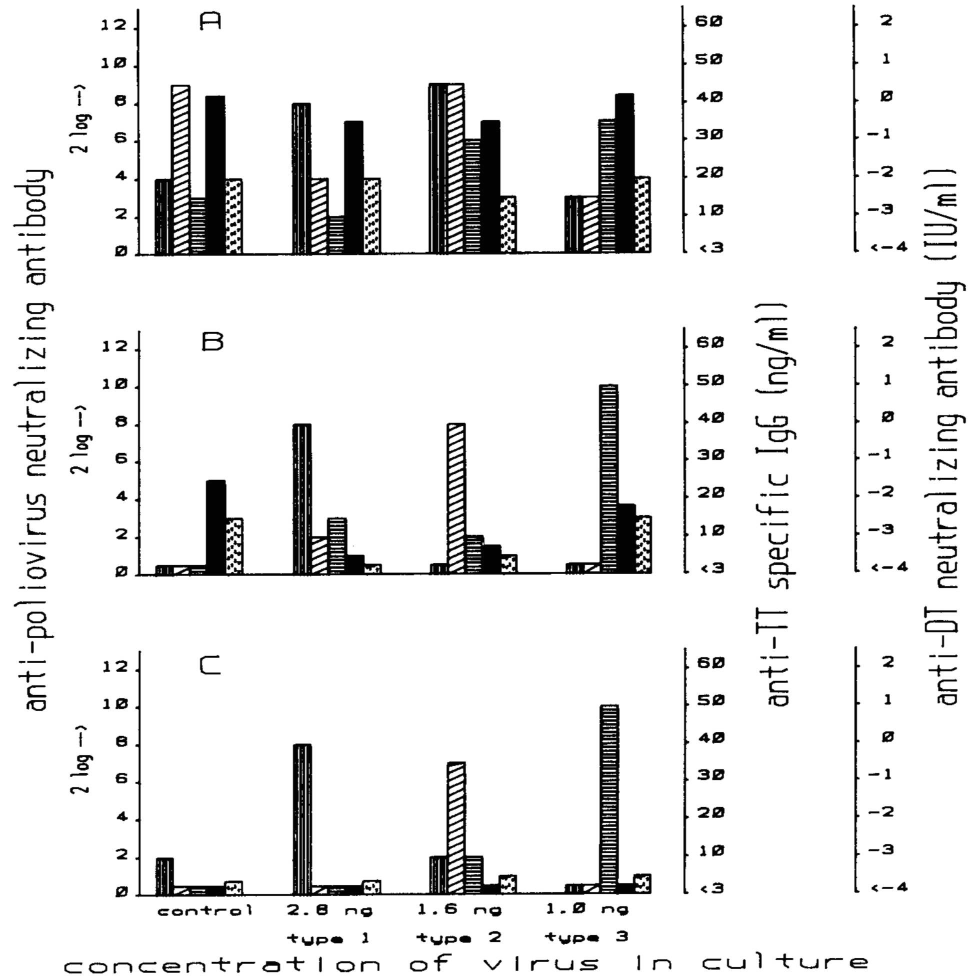

Figure 7. In vivo kinetics of the in vitro antibody response against poliovirus. PBL taken before immunization with poliovirus showed no in vitro response. PBL were isolated from blood of boostered donors on day 5 (*A*), day 12 (*B*), and day 30 (*C*). After stimulation with polioviruses in vitro, antibody responses against polioviruses type 1 (▥), type 2 (▨), type 3 (▤), and against DT (■), and TT (□) were measured. Ranges for anti-poliovirus responses in four different donors are given in the legend to Figure 4.

TABLE II
T cell dependency of the in vitro neutralizing antibody response of human PBL against poliovirus

Cell Population	% Positive Cells in FACS Analysis with Monoclonal Antibodies[a]				Homotypic Neutralizing Antibody Response in Vitro[b]		
	Leu-1	Leu-2a/FK18	Leu-3a/RIV6	Leu-12	Type 1	Type 2	Type 3
UF-PBM[c]							
Donor 1	66.6	25.8/23.9	36.9/34.4	10.9	32	64	1024
Donor 2	43.0	16.8/12.2	37.4/38.5	15.0	32	512	ND[f]
RIV6[d]							
Donor 1	54.9	56.5/46.3	0.8/0.5	9.0	<2	<2	<2
Donor 2	28.1	39.6/34.4	0.5/0.6	16.1	<2	<2	ND
FK18[e]							
Donor 1	74.0	2.9/0.9	67.9/67.6	7.5	8	32	512
Donor 2	76.3	0.9/0.4	74.0/74.5	10.8	32	64	ND

[a] Cells were stained as described in *Materials and Methods* and were analyzed on a Becton Dickinson FACS analyzer.
[b] Neutralizing antibody titers expressed as reciprocals of supernatant fluid dilution neutralizing 100 $TCID_{50}$ virus in VN.
[c] Unfractionated peripheral blood non-nuclear cells.
[d] Peripheral blood mononuclear cells after treatment with RIV6 (T4-like) and rabbit complement (see *Materials and Methods*).
[e] Peripheral blood mononuclear cells after treatment with FK18 (T8-like) and rabbit complement (see *Materials and Methods*).
[f] ND, not determined.

immunization with KLH and Hepatitis B surface antigen, respectively, human PBL of some subjects became refractory to stimulation by these antigens in vitro, whereas PBL of other subjects manifested an antigen-induced antibody response in vitro 6 mo or more after the final immunization.

The poliovirus-specific response could be dissected in a virus type-specific and a heterotypic response, depending on the dose of virus used for in vitro stimulation. A number of possibilities was considered to explain the in vitro induced heterotypic responses of recently in vivo activated B cells.

a) Spontaneous in vitro production of antibody by in vivo activated human B cells has been described in several systems (11, 13, 26–29, 33) and also in this study. This possibility is excluded by the data from kinetic studies as shown in Figure 7.

b) Polyclonal and oligoclonal activation of B cells by nonspecific T cell help induced by supra-optimal concentrations of virus in vitro have been considered. The data presented in Figures 4 to 7 argue strongly against these possibilities.

c) Antigen-specific B cells could have been activated by nonspecific help in the absence of antigen (34). This alternative is highly unlikely, because in supernatant fluids of nonstimulated human PBL taken 30 days after in vivo immunization, no anti-poliovirus activity was detectable (Fig. 4).

d) Intertypic cross-reactive neutralization-inducing epitopes could exist. Evidence for intertypic cross-reactive epitopes of polioviruses has emerged from studies in several laboratories (4, 5, 35, 36). However, cross-reactivity was never detected by virus neutralization in those studies. In other investigations that did reveal data in support of intertypic neutralization determinants, heterotypic neutralizing antibody titers in sera of laboratory animals (2, 3) or of human beings (14, 37–39) were always low, or were only obtained after hyperimmunization of animals over a prolonged period of time (Van Steenis, personal communication). Together, these studies may indicate that intertypic neutralization-inducing epitopes in comparison with epitopes involved in type-specific neutralization are less immunogenic, less accessible to the immune system, or less frequently expressed, perhaps by the heterogenicity of polioviruses. In any case, increasing the concentration of a poliovirus type in in vivo or in vitro systems should be expected to enhance the heterotypic antibody response. This was indeed observed in the present study. Heterotypic antibody responses are most pronounced when poliovirus is used in cultures in 12- to 25-fold higher concentrations than optimal for generating type-specific responses (Fig. 4). Furthermore, suppression of heterotypic responses seems more difficult to achieve by high concentrations of antigen than suppression of type-specific responses (Fig. 4, and UytdeHaag et al., unpublished observations). However, conclusive evidence for the existence of intertypic cross-reactive neutralization-inducing epitopes emerged from the studies in which absorption of heterotypic antibody from culture supernatant fluids by both the corresponding nonstimulating types of poliovirus as well as the stimulating types of virus was demonstrated. This was found for cultures stimulated with type 1, type 2, or type 3 of poliovirus (Fig. 6). Consequently, we suggest that apart from a major antigenic site involved in neutralization (7, 8), other intertypic cross-reactive neutralization-inducing epitopes are also present which probably play a minor role in the induction of neutralizing immune response. Evidence for multiple *intratypic* neutralization-inducing epitopes has been reported (40–44), and we also recently found neutralizing monoclonal antibodies which express heterotypic activity (Osterhaus et al., unpublished observation).

In conclusion, we have described a reproducible system for the antigen-specific triggering of human PBL and the assessment of in vitro secreted antibody to poliovirus. We demonstrated the utility of this system to study the kinetics and the fine specificity of the anti-poliovirus response. Understanding the sequential events of the human anti-poliovirus response after booster immunization will allow the study of mechanisms by which anti-idiotypic antibodies modulate antiviral antibody responses in man.

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REFERENCES

1. **Minor, P. D., O. Kew, and G. C. Schild.** 1982. Poliomyelitis—epidemiology, molecular biology and immunology. *Nature 299:109.*
2. **Wenner, H. A., C. A. Miller, P. Kamitsuka, and J. C. Wilson.** 1954. Preparation and standardization of antiserums prepared with the three known types of poliomyelitis viruses. *Am. J. Hyg. 59:221.*
3. **Furesz, J.** 1959. The antibody response of roosters to poliomyelitis virus. *J. Immunol. 84:251.*
4. **Ferguson, M., P. D. Minor, D. I. Magrath, Q. Yi-Hua, M. Spitz, and G. C. Schild.** 1984. Neutralization epitopes on poliovirus type 3 particles: an analysis using monoclonal antibodies. *J. Gen. Virol. 65:197.*
5. **Mertens, T., U. Pika, and H. J. Eggers.** 1983. Cross antigenicity among enteroviruses as revealed by immunoblot technique. *Virology 129:431.*
6. **Pfaff, E., M. Mussgay, H. G. Bohm, G. E. Schulz, and H. Schaller.** 1982. Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. *EMBO. J. 1:869.*
7. **Minor, P. D., G. C. Schild, J. Bootman, P. M. A. Evans, M. Fergusson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, L. D. Clarke, R. C. Mountford, and J. W. Almond.** 1983. Location and

primary structure of a major antigenic site for poliovirus neutralization. *Nature 301:674.*

8. **Evans, D. M. A., P. D. Minor, G. S. Schild, and J. W. Almond.** 1983. Critical role of an eighth-amino acid sequence of VP1 in neutralization of poliovirus type 3. *Nature 304:459.*
9. **UytdeHaag, F. G. C. M., and A. D. M. E. Osterhaus.** 1985. Induction of neutralizing antibody in mice against poliovirus type II with monoclonal anti-idiotypic antibody. *J. Immunol. 134:1225.*
10. **Callard, R. E.** 1979. Specific in vitro antibody response to influenza virus by human blood lymphocytes. *Nature 282:734.*
11. **Yarchoan, R., B. R. Murphy, W. Strober, H. S. Schneider, and D. L. Nelson.** 1981. Specific anti-influenza virus antibody production in vitro by human peripheral blood mononuclear cells. *J. Immunol. 127:2588.*
12. **Souhami, R. L., J. Babbage, and R. E. Callard.** 1983. Specific in vitro antibody responses to varicella-zoster. *Clin. Exp. Immunol. 46:98.*
13. **UytdeHaag, F. G. C. M., A. D. M. E. Osterhaus, H. G. Loggen, R. H. J. Bakker, J. A. A. M. van Asten, J. G. Kreeftenberg, P. van der Marel, and B. van Steenis.** 1983. Induction of antigen-specific antibody response in human peripheral blood lymphocytes in vitro by a dog kidney cell vaccine against rabies virus (DKCV). *J. Immunol. 131:1234.*
14. **Van Wezel, A. L., G. van Steenis, P. van der Marel, and A. D. M. E. Osterhaus.** 1984. Inactivated poliovirus vaccine: current production methods and new developments. *Rev. Infect. Dis. 6 (Suppl.) 2:5335.*
15. **Koning, F.** 1982. An OKT8-like mouse monoclonal antibody. *Abstr. Book. 5th European Immunology Meeting, Istanbul.*
16. **Dömök, I., and D. I. Magrath.** 1979. Guide to poliovirus isolation and serological techniques for poliomyelitis surveillance. *WHO Offset Publication 46.*
17. **Kreeftenberg, J. G., J. van der Gun, F. R. Marsman, S. K. Bhandari, and S. C. Maheshwari.** 1985. Investigations on a mouse model to estimate the potency of the diphtheria component in vaccines. *J. Biol. Stand. 13:1.*
18. **Hagenaars, A. M., R. W. van Delft, and J. Nagel.** 1984. Comparison of ELISA and toxin neutralization for the determination of tetanus antibodies. *J. Immunoassay 5:1.*
19. **Sedgwick, J. D., and P. G. Holt.** 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody secreting cells. *J. Immunol. Methods 57:301.*
20. **Johnson, K. P., B. Vandvik, and E. Norrby.** 1982. Immunological responses to viruses in multiple sclerosis: humoral immunity. *Clin. Immunol. Allergy 2:333.*
21. **Dalakas, M. C., J. L. Sever, D. L. Madden, N. M. Papadopoulos, I. C. Shekarchi, P. Albrecht, and A. Krezlewicz.** 1984. Late postpoliomyelitis muscular atrophy: clinical, virologic and immunologic studies. *Rev. Infect. Dis. 6 (Suppl.) 2:562.*
22. **Mehta, P. D., B. A. Patrick, H. Thormar, and H. M. Wisniewski.** 1982. Oligoclonal IgG bands with and without measles antibody activity in sera of patients with subacute sclerosing panencephalitis (SSPE). *J. Immunol. 129:1983.*
23. **Vartdal, F., B. Vandvik, and E. Norrby.** 1982. Intrathecal synthesis of virus-specific oligoclonal IgG, IgA and IgM antibodies in a case of varicella-zoster meningoencephalitis. *J. Neurol. Sci. 57:121.*
24. **Link, H., M. A. Laurenzi, and A. Fryden.** 1981. Viral antibodies in oligoclonal and polyclonal IgG synthesized within the central nervous system over the course of mumps meningitis. *J. Neuroimmunol. 1:287.*
25. **Wolinsky, J. S., M. N. Wazham, J. L. Hess, J. J. Townsend, and J. R. Baringer.** 1982. Immunochemical features of a case of progressive rubella panencephalitis. *Clin. Exp. Immunol. 48:359.*
26. **Stevens, R. H., E. Macy, C. Morrow, and A. Saxon.** 1979. Characterization of a circulating subpopulation of spontaneous antitetanus toxoid antibody producing B cells following in vivo booster immunization. *J. Immunol. 122:2498.*
27. **Geha, R. S.** 1981. Dynamics of human circulating antigen reactive cells following secondary immunization with tetanus toxoid. *Clin. Immunol. Immunopathol. 19:196.*
28. **Thomson, P. D., and W. S. Harris.** 1977. Detection of plaque-forming cells in peripheral blood lymphocytes *in vitro. Clin. Exp. Immunol. 3:393.*
29. **Kehrl, J. H., and A. S. Fauci.** 1983. Activation of human B lymphocytes after immunization with pneumococcal polysaccharides. *J. Clin. Invest. 71:1032.*
30. **Cupps, T. R., P. K. Goldsmith, D. J. Volkman, J. L. Gerin, R. H. Purcell, and A. S. Fauci.** 1984. Activation of human peripheral blood B cells following immunization with hepatitis B surface antigen vaccine. *Cell. Immunol. 86:145.*
31. **Brieva, J. A., and R. H. Stevens.** 1984. Human in vivo antigen-induced lymphoblastoid B cells are capable of cyclical antibody production in vitro. *J. Immunol. 133:147.*
32. **Callard, R. E., G. W. McCaughan, J. Babbage, and R. L. Souhami.** 1982. Specific in vitro antibody responses by human blood lymphocytes: apparent non-responsiveness of PBL is due to a lack of recirculating memory B cells. *J. Immunol. 129:153.*
33. **Lane, H. C., L. C. Edgar, G. Whalen, and A. S. Fauci.** 1983. Kinetics of the sequential presence in human peripheral blood of antigen-specific activated, inducible and memory B lymphocytes. *Fed. Proc. 42:411.*
34. **Peters, M., and A. S. Fauci.** 1983. Selective activation of antigen-specific human B cells in recently immunized individuals by nonspecific factors in the absence of antigen. *J. Immunol. 130:678.*
35. **Blondel, B., R. Crainic, O. Akacem, P. Bruneau, M. Girard, and F. Horodniceanu.** 1982. Evidence for common, intertypic antigenic determinants on poliovirus capsid polypeptides. *Virology 123:461.*
36. **Romanova, L. I., E. A. Tolskaya, and V. I. Agol.** 1981. Antigenic and structural relatedness among non-capsid and capsid polypeptides of polioviruses belonging to different serotypes. *J. Gen. Virol. 52:279.*
37. **Sabin, A. B.** 1952. Transitory appearance of type 2 neutralizing antibody in patients infected with type 1 poliomyelitis virus. *J. Exp. Med. 96:99.*
38. **Bodian, B., and R. S. Paffenberger.** 1954. Poliomyelitis infection in households. Frequency of viremia and specific antibody response. *Am. J. Hyg. 60:83.*
39. **Salk, J. E.** 1957. Poliomyelitis vaccination in the Fall of 1956. *Am. J. Public Health 47:1.*
40. **Uhlig, H., G. Rutter, and R. Dermick.** 1983. Evidence for several unrelated neutralization epitopes of poliovirus type 1, strain Mahoney, provided by neutralization tests and quantitative enzyme-linked immunosorbent assay (ELISA). *J. Gen. Virol. 64:2809.*
41. **Blondel, B., R. Crainic, and F. Horodniceanu.** 1982. Le polypeptide structural VP1 du poliovirus type 1 induit du anticorps neutralisants. *C. R. Acad. Sci. Paris 249:91.*
42. **Emini, E. A., S. Y. Kao, A. J. Lewis, R. Crainic, and E. Wimmer.** 1983. Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. *J. Virol. 46:466.*
43. **Emini, E. A., A. J. Dorner, L. F. Dorner, B. A. Jameson, and E. Wimmer.** 1983. Identification of a poliovirus neutralization epitope through use of neutralizing antiserum raised against purified viral structure proteins. *Virology 124:144.*
44. **Osterhaus, A. D. M. E., A. L. van Wezel, G. van Steenis, A. G. Hazendonk, and G. Drost.** 1982. Production and potential use of monoclonal antibodies against polio viruses. *Dev. Biol. Stand. 50:221.*