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

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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and have revealed conflicting results regarding the fine specificity of the antibody response (2–5) (A. Osterhaus, unpublished observations).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), 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 floculantes (Lf) tetanus toxoid (TT), and killed poliovirus type 1 (Mahoney) 40 D antigen units (DU), type 2 (MEF), 4 DU, and type 3 (Saukett) 7.5 DU, 1.5 mg AlPO4, 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 differentiation antigen.

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.
from culture supernatant fluids. The production and further characteristics of RIV6 will be defined as the same population as the OKT8 monoclonal antibody (15). T lymphocytes. This population functionally defines the human rabbit complement was added to the cells at a final dilution of 1/2 bodies Leu-1, Leu-2b, Leu-3a, Leu-12 (Becton Dickinson, Mountain Co., Kankakee, IL). After 60 min, supernatants containing lymphocytes were harvested and were washed twice in serum-free RPMI containing human Leu-M3 monoclonal antibody (Becton Dickinson). After 60 min at room temperature with 100 μl of uncoated 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 37°C, viable cells were isolated on Ficoll-Isoaque, 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), FITC, and PKH1. Cells (1 × 10⁶) were incubated for 30 min at 37°C in RPMI 1640 medium containing 25 mM HEPES, 2 mM L-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 10% FCS containing 1% BSA. 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% monocyties, as determined by non-specific esterase staining and fluorescence analysis with anti-human Leu-M3 monoclonal antibody (Becton Dickinson). Mononuclear cells containing virus or nonstimulated control cells were plated in Falcon flasks at 37°C in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS; Reheult Armour Pharmaceutical Co., Kankakee, IL). After 60 min, supernatants containing lymphocytes were harvested and were washed twice in serum-free RPMI 1640 medium. The plates were incubated for 30 min at 37°C in RPMI 1640 medium containing 25 mM HEPES, 2 mM L-glutamine, 100 μM penicillin, and 10% FCS containing 1% BSA (Becton Dickinson). After 60 min, supernatants were resuspended in RPMI 1640 medium containing 25 mM HEPES, 2 mM L-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 10% FCS containing 1% BSA. The plates were incubated at 35°C and were screened for complete metabolic inhibition as indicated by the ratio of the slopes of the best-fitting line of both reference and sample. 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 I; Flow Laboratories Inc., McLean, VA) were coated with 100 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 1 hr. After washing with PBS, the 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 supernatants containing poliovirus (Roche, Germany). 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 control PBL were added to wells containing 100 μl poliovirus type-neutralizing antibody-negative control serum were incubated at several concentrations (10³, 0.5 × 10⁸, and 0.25 × 10⁸) and were incubated in a vibration-free incubator in a humidified atmosphere of 5% CO₂ in air for 3 hr. The wells were washed with PBS, 1% BSA, 0.05% Tween-20, and were incubated with 10 μl goat anti-human IgM-alkaline phosphatase conjugate or goat anti-human IgG-alkaline phosphatase conjugate (Tago Inc., Burlingame, CA) for 1 hr at 20°C. After washing with PBS, 1% BSA, 0.05% Tween-20, the AP substrate was 2-5-aminophenyl 4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO) solution in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma Chemical Co.) containing 5 mM MgCl₂, 0.01% NaN₃, 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 320X 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, a reduction 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-
IN VITRO POLIOVIRUS-SPECIFIC ANTIBODY PRODUCTION

**Figure 1.** Antigenic requirements for the in vitro poliovirus-induced virus-specific homotypic neutralizing antibody response without (○—○) or in the presence (△—△) 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.

**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.

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 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 rise 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 unstimulated 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...
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 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. 7A). This type of response had sharply de-
After revaccination, in vitro antigen-inducible antipoliovirus type-specific responses were observed on day 12 and on day 30 (Fig. 7B 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 antipoliovirus 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. 7A). 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. 7B 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

**Figure 6.** Evidence for intertypic cross-reactive neutralization-inducible 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.
### TABLE 1

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

<table>
<thead>
<tr>
<th>Stimulating Virus</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASC/10^6 NT</td>
<td>ASC/10^6 NT</td>
<td>ASC/10^6 NT</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>Donor A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8 ng/culture</td>
<td>506</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>72 ng/culture</td>
<td>218</td>
<td>1457</td>
<td>2048</td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 ng/culture</td>
<td>0</td>
<td>6</td>
<td>&lt;2</td>
</tr>
<tr>
<td>40 ng/culture</td>
<td>87</td>
<td>406</td>
<td>256</td>
</tr>
<tr>
<td>Type 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.19 ng/culture</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>24 ng/culture</td>
<td>0</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Unstimulated PBL</td>
<td>0</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Donor B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8 ng/culture</td>
<td>127</td>
<td>11</td>
<td>256</td>
</tr>
<tr>
<td>72 ng/culture</td>
<td>135</td>
<td>88</td>
<td>128</td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 ng/culture</td>
<td>47</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>40 ng/culture</td>
<td>82</td>
<td>56</td>
<td>8</td>
</tr>
<tr>
<td>Type 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.19 ng/culture</td>
<td>0</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>24 ng/culture</td>
<td>11</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Unstimulated PBL</td>
<td>0</td>
<td>0</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

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

* NT: Neutralizing antibody titers expressed as reciprocals of supernatant fluid dilution neutralizing 100 TCID50 virus in NT.

* 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 boosted 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.
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 Steershoven, 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 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).

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 antiviral antibodies modulate antiviral antibody responses in man.

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