Clin. exp. Immunol. (1985) **59**, 673–678.

Antibody to bacteriophage ϕX 174 synthesized by cultured human peripheral blood lymphocytes

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(Accepted for publication 28 September 1984)

SUMMARY

Following immunization of human subjects with the antigen bacteriophage ϕX 174, concomitant with the rise in serum antibody, cells appear in the circulation which *in vitro*, without antigen stimulation, synthesize antibody of the same class as serum antibody in most subjects studied. This function is inhibited by puromycin or irradiation, is independent of T cells and occurs within the first 36–72 h of culture. Such cells are found only in recently immunized subjects. Peripheral blood mononuclear cells (PBM) from all immunized subjects synthesize more antibody to ϕX 174 *in vitro* if antigen is present during cell culture; none was synthesized by antigen containing PBM cultures from unimmunized subjects. This antigen-induced antibody response is T cell and antigen dose-dependent and inhibited by puromycin or irradiation. Following primary immunization the antibody synthesized *in vivo* and *in vitro* is IgM. Following secondary immunization IgG antibody is immediately detected *in vivo* but *in vitro* antigen-induced antibody continues to be IgM for at least 3 months. IgG antibody then appears: once this class switch occurs, *in vitro* antigen-induced IgG antibody can be demonstrated in cultured PBM of subjects for many years, without further booster immunization.

Keywords antibody synthesis antigen-induced *in vitro* immune regulation

INTRODUCTION

The bacteriophage ϕX 174 is a T cell-dependent neoantigen used to measure *in vivo* antibody responses of man and other animals (Uhr, Finkelstein & Bauman, 1962; Ching, Davis & Wedgwood, 1966; Ochs, Davis & Wedgwood, 1971; Peacock, Jones & Gough, 1973; Wedgwood, Ochs & Davis, 1975; Jackson *et al.*, 1977). The primary response to i.v. injected bacteriophage ϕX 174 consists of antigen clearance within 3–4 days followed by appearance of IgM antibody in the serum with a peak level at 2 weeks. A second immunization with phage results in a brisk rise of antibody, with a class switch to IgG, reaching a peak at 1 week. We have studied *in vitro* antibody synthesis by cultured peripheral blood mononuclear cells (PBM) from immunized healthy humans with or without antigen in the cultures.

MATERIALS AND METHODS

Subjects. Eight healthy adults (four female and four male) were immunized with bacteriophage ϕX 174 after informed consent was obtained. Six additional subjects immunized 2–16 years earlier with ϕX 174 were also studied.

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Antigen. A single lot (8–79) of bacteriophage ϕX 174 prepared as previously described (Uhr *et al.*, 1962; Ochs *et al.*, 1971) was used for immunization of the eight principal subjects, and for induction of *in vitro* antibody synthesis. It was purified, tested for sterility and absence of pyrogens, and stored in 0·1 M ammonium acetate buffer (pH = 7·4) at −70°C, at a final concentration of 1×10^{11} plaque forming units/ml (PFU/ml). Antibody activity expressed as rate of inactivation (Kv) was determined by a phage neutralization assay (Ching *et al.*, 1966). To ascertain antigen-induced antibody formation, the activity of cultures without antigen was subtracted from the activity of cultures with antigen. To assess the relative contribution of IgM and IgG antibodies samples were pre-incubated with 2-mercaptoethanol (2-ME) (Grubb & Swahn, 1958; Ochs *et al.*, 1971). Residual activity after 2-ME treatment was designated IgG antibody; activity sensitive to 2-ME, IgM antibody. 2-ME did not affect phage viability in the concentration used.

Immunization. Subjects were given bacteriophage ϕX 174 i.v., 2×10^9 PFU/kg body weight. Blood was collected at 3 days, and at 1, 2 and 4 weeks. A second dose of phage was given 6 weeks after the primary immunization and blood collected at similar intervals.

Cell separation. PBM, obtained by Ficoll-hypaque gradient centrifugation (Ficoll-Paque, Pharmacia, Piscataway, New Jersey, USA) (Böyum, 1968), were washed three times with phosphate-buffered saline (PBS) and suspended in RPMI 1640 (Gibco, Grand Island, New York, USA), with added 10% fetal calf serum (Reheis Chemical Co, Phoenix, Arizona, USA), 1 mM L-glutamine, 100 units penicillin and 100 μg streptomycin/100 ml. More than 95% of cells were mononuclear. In some experiments cell preparations were separated into T and B cell enriched fractions. After 1 h exposure (37°C, 5% CO_2) of $4–10 \times 10^6$ PBM/ml in RPMI with 20% fetal calf serum in plastic culture flasks, non-adherent cells were removed by washing three times with media; 3–10% of non-adherent cells were myeloperoxidase positive, and >98% were viable. 2-amino ethylisothiouronium bromide treated sheep red blood cells (AET-SRBC) (Pellegrino *et al.*, 1975) were mixed with the non-adherent cells (ratio of 150:1), layered over cold (4°C) Ficoll-Paque and centrifuged at 500*g* for 20 min. The interface was removed, washed twice with PBS and suspended in media. Forty to sixty per cent of cells from the interface, designated 'B cells', had surface immunoglobulin detected by immunofluorescence; <2% formed sheep erythrocyte rosettes (E rosette positive), and 20–40% were myeloperoxidase positive. The pellet, containing >90% E rosette positive cells, was treated with 0·87% NH_4Cl to lyse the AET-SRBC. The resulting mononuclear cells were designated 'T cells'.

Culture conditions. PBM were cultured in round bottom microtitre plates (Costar, Cambridge, Massachusetts, USA (total volume 200 μl) or 5 ml polystyrene tubes (Falcon, Oxnard, California, USA) (total volume 1–2 ml), and incubated in 5% CO_2 at 37°C at a final concentration of 2×10^6 cells/ml. In some experiments lymphocyte subpopulations (B or T cells) were incubated in microtitre plates either alone or in co-culture; final cell concentrations were $0·25 \times 10^6$ B cells and/or $0·75 \times 10^6$ T cells. Except where noted antigen was added on day 0 at a final concentration of 1×10^6 PFU/ml. Cultures were incubated for 12 days, frozen and thawed once, and supernatants collected for antibody determination. Selected cultures were irradiated with 1,500 rad (^{137}Ce, 300 rad/min) or treated with puromycin (10 μg/ml).

RESULTS

Antibody responses in vivo

No subject had pre-existing serum antibody to ϕX 174. The primary and secondary antibody responses to bacteriophage ϕX 174 were within our previously defined normal range (Ochs *et al.*, 1982) in 7 subjects. One male subject (identified in figures by the symbol ●) had a low secondary response. Secondary antibody responses were greater in females than males.

Antibody synthesis in vitro

Spontaneous antibody synthesis. After 1° immunization, antibody was synthesized *in vitro*, in the absence of antigen, by PBM from three individuals at 1 week and six individuals 2 weeks after phage injection (Fig. 1a). By 4 weeks, less or no antibody was synthesized. Antibody was synthesized by only one subject 6 weeks after 1° immunization.

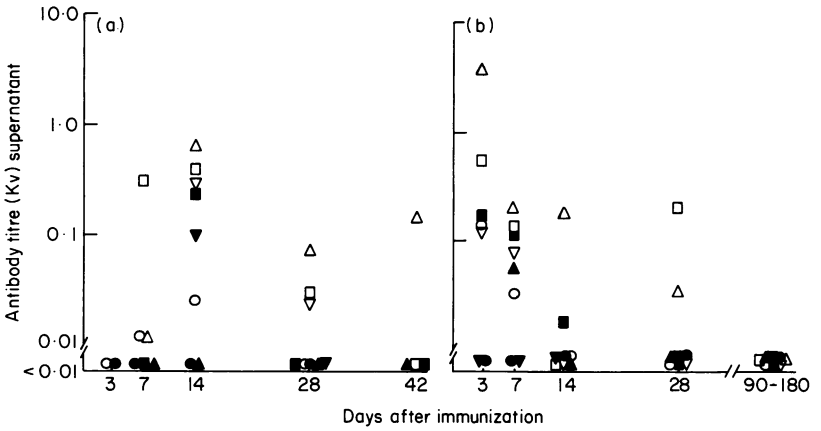

Fig. 1. Spontaneous *in vitro* antibody synthesis by PBM in 12 day cultures without added antigen. Values are either of pools from triplicate wells, or means of values from three wells. (a) presents the response after 1° immunization, (b) after 2° immunization.

Following 2° immunization, cells spontaneously secreting antibody *in vitro* appeared in the circulation by 3 days in five subjects (Fig. 1b). Spontaneous *in vitro* antibody synthesis was less at 1 week and detectable in only two subjects at 2 and 4 weeks. None was found thereafter.

Addition of puromycin to cultures or cell irradiation abrogated spontaneous antibody production, indicating synthesis rather than release of preformed antibody. Spontaneously synthesized antibody was detected only in cultures containing B cells and was neither augmented nor suppressed by addition of autologous T cells (T:B ratio of 3:1). Spontaneous antibody synthesis was demonstrable at 36 h of culture and maximal at 72 h, but not thereafter.

Following both 1° and 2° immunization, antibody was synthesized spontaneously by all subjects before and during (but not after) the peak serum antibody response. Peak values for spontaneous *in vitro* antibody synthesis correlated with peak serum antibody values ($r = +0{\cdot}75$, $P \leqslant 0{\cdot}02$ for the 1°; $r = +0{\cdot}64$, $P < 0{\cdot}05$ for the 2° response). Only IgM was synthesized during the 1° response and serum antibody was only IgM (Fig 2, column 1). During the 2° response, IgG antibody was synthesized too, and IgG antibody appeared in the serum. Spontaneous antibody synthesis had

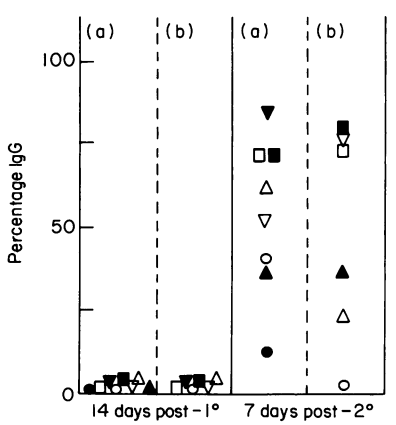

Fig. 2. Anti-phage antibody resistant to 2-ME presented as percentage of total antibody activity (percentage IgG), comparing serum antibody (a, *in vivo*) and antibody spontaneously produced by PBM in culture (b, *in vitro*) after 1° and 2° immunization. No spontaneously produced antibody was detected on day 14 post-1° in two males (● ▲) or on day 7 post-2° (● ▼).

generally disappeared 4 weeks after immunization even though serum antibody levels remained high. PBM of unimmunized donors did not spontaneously synthesize antibody to bacteriophage.

Antigen-induced antibody synthesis. Antibody response to *in vitro* antigen stimulation, measured by a net increase of antibody activity in antigen stimulated compared to non-stimulated cultures, was demonstrated in one individual at day 7, in seven individuals at day 14, and in all individuals at days 28 and 42 following primary immunization (Fig. 3a). The antibody produced was mainly IgM.

Following 2° immunization antigen-induced antibody synthesis was found in three of five individuals on day 3, and in all tested by day 7 and thereafter (Fig. 3b). *In vitro* antigen-induced antibody synthesis during the 28 days after 2° immunization was mainly IgM although serum antibody was mostly IgG (Fig. 4, column 1). In the 3–9 months thereafter an increasing proportion of *in vitro* antigen-induced antibody was IgG (Fig. 4, column 2). PBM of unimmunized donors did not synthesize antibody to bacteriophage in the presence of antigen. Addition of puromycin to cultures or cell irradiation abrogated antigen-induced antibody production, again indicating synthesis, not release. Antigen-induced antibody synthesis was demonstrable 48 h after introduction of antigen and continued thereafter throughout the 12 days of culture.

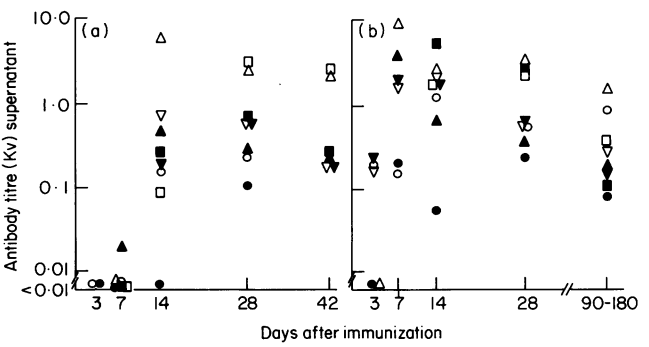

Fig. 3. Antigen-induced *in vitro* antibody synthesis by PBM in 12 day cultures with added antigen. Values (from pooled triplicate wells, or means or values from three wells) were corrected for spontaneously synthesized antibody by subtracting spontaneous antibody activity (without added antigen) from the activity found with antigen present. (a) presents the response to 1° immunization, (b) 2° immunization.

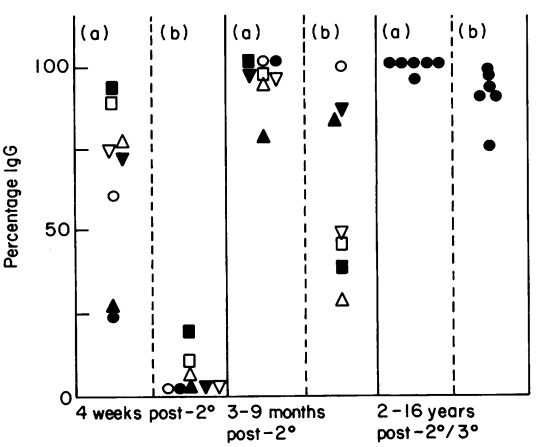

Fig. 4. Anti-phage activity resistant to 2-ME presented as percentage of total activity (percentage IgG), in serum (a, *in vivo*) and in antigen stimulated PBM in culture (b, *in vitro*), from samples taken at times indicated after 2° immunization.

Characteristics of antigen-induced response

Persistence of antigen reactive cells. Six subjects repeatedly immunized 2–16 years previously all had high serum antibody activity (geometric mean Kv = 335, range 142–590) of the IgG class, none had PBM that spontaneously synthesized antibody. When cultured with antigen, PBM of all produced antibody which was also mainly IgG (Fig. 4, column 3). PBM of these subjects produced less antigen-induced antibody than recently immunized subjects. Also, PBM from recently immunized subjects produced antibody in every well of quadruplicate cultures, with little variation in antibody activity in each well. In contrast cultured PBM from subjects immunized many years previously showed marked variation in antibody activity between quadruplicate wells and in some instances antibody was detectable in only one of four wells. These findings presumably reflect the limiting number of antigen reactive PBM in the cultures (total cells in each well approx. $0{\cdot}5 \times 10^5$ B cells, $3{\cdot}5 \times 10^5$ T cells).

Antigen concentration dependence. PBM from previously immunized subjects were cultured with various concentrations of phage. A concentration dependent increase in antibody synthesis was found, with minimal antibody activity (Kv < 0·02) at 10^3 PFU/ml increasing to a maximum (Kv ~ 10) at concentrations of 10^6–10^8 PFU/ml. At 1×10^9 PFU/ml antibody synthesis was diminished or undetected (Kv 1·2 to < 0·01).

T cell dependence. T cell depleted PBM from immunized subjects obtained when cells spontaneously secreting antibody were no longer detectable synthesized only minute amounts of antibody or none at all when cultured with antigen. Addition of autologous T cells (T:B ratio of 3:1) in every instance increased the amount of antibody secreted from often undetectable amounts (mean Kv ~ 0·02, range < 0·01–0·09) to easily measurable activity (mean Kv 0·3, range 0·03–10). T cells alone did not synthesize demonstrable antibody.

DISCUSSION

Studies of *in vitro* antibody synthesis by human PBM initially employed polyclonal activators, such as pokeweed mitogen (Waldmann *et al.*, 1974; Ginsburg, Finkelman & Lipsky, 1978; Stevens & Saxon, 1978; Möller, 1979). More recently, antigen-induced antibody synthesis has been studied using antigens to which subjects have been exposed through natural infection, such as varicella zoster (Souhami, Babbage & Callard, 1981), influenza (Callard, 1979) and Herpes simplex type I (Froelich & Lum, 1982); or by active immunization with antigens such as Keyhole limpet haemocyanin (KLH) (Volkman, Lane & Fauci, 1981) and tetanus toxoid (Mudawwar, Yunis & Geha, 1978; Brenner & Munro, 1981). In the general secondary or recall responses were examined rather than the full sequence of the humoral immune response from primary induction through recall and class switch. We have shown that following 1° and 2° immunization with bacteriophage ϕX 174 two different *in vitro* antibody responses occur: a spontaneous T cell-independent and an antigen-induced T cell-dependent response.

Spontaneous *in vitro* synthesis of IgG antibody from PBM of recently immunized subjects has also been reported for tetanus toxoid and salmonella antigens (Stevens *et al.*, 1979; Thomson & Harris, 1974). These were clearly secondary or recall responses. In our studies and theirs the cells only appeared transiently in the peripheral blood. The synthesis of antibody in culture was short lived (36–72 h), and of the same class as the serum antibody. The presence of these cells suggests dispersion of B cells into the circulation from the rapidly proliferating B cell pool that is actively synthesizing antibody throughout the lymphoid network. The appearance corresponds in time with peak serum antibody levels (7–14 days post-1° and 3–7 days post-2° immunization). The disappearance of PBM spontaneously synthesizing antibody, even though serum antibody levels remain high, suggests feedback control and diminished proliferation of activated B cells, or 'homing' of the B cells that once circulated.

The antigen-induced response is clearly different. It involves cell–cell interaction (T cell dependence at least), is antigen dose-dependent and persists for years after immunization, exhibiting 'memory'. The discrepancy between the class of antibody synthesized in culture and that found in serum after 2° immunization has been noted also with the antigen KLH (Lane *et al.*, 1981).

Why circulating antigen reactive units should continue to produce only IgM antibody long after the class switch to IgG antibody in the serum has occurred is not clear. It is possible that during the recall response 'switched' cells (committed to IgG antibody) are driven immediately to active antibody synthesis and 'home' in the lymphoid organs; thus only 'unswitched' antigen reactive cells are found in the circulation. The detection months (and years) after immunization, of antigen reactive PBM that produce IgG antibody suggests that sufficient memory units are eventually committed to IgG antibody to be released into the circulation. Our data indicate that in the resting state the numbers of these reactive units in the PBM are very few.

We wish to acknowledge Joan H. Palmer for excellent secretarial assistance, and Catherine G. Lindgren for technical assistance. This work was supported by grants from the National Institutes of Health (AI-07073), and from the March of Dimes Birth Defects Foundation (6-273). A portion of this work was conducted through the Clinical Research Center facility at the University of Washington supported by NIH Grant RR-37. JB supported by training grant 5-T32-HDO7171-02.

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