HOST RESPONSE TO INFECTION

Induction of a specific antibody response to *Bordetella pertussis* antigens in cultures of human peripheral blood mononuclear cells

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The role of specific antibodies in protective immunity to *Bordetella pertussis* has not yet been clearly defined. In the present work, the induction of a specific antibody response to *B. pertussis* in cultures of human peripheral blood mononuclear cells (PBMC) was investigated, on the assumption that the capacity of circulating lymphocytes to mount a specific response *in vitro* may provide a useful parameter for the evaluation of protective immunity. When PBMC from normal adult donors were cultured with a heat-inactivated *B. pertussis* whole-cell suspension, cells secreting antibodies to pertussis toxin, pertactin and filamentous haemagglutinin were generated consistently. The antibody response peaked between days 7 and 11 of culture and the antibodies produced were exclusively of the IgM class.

Introduction

The mechanisms of protective immunity against *Bordetella pertussis* infection, following natural exposure or vaccination, are still largely unknown [1–3]. Data from murine models indicate that natural immunity and both T- and B-cell compartments are involved [4–6]. Clinical trials have also suggested that different arms of the immune response may contribute to protection, by showing that cell-mediated and antibody responses to *B. pertussis* vaccine antigens occur early after primary immunisation [7–11]. While these immunogenicity studies failed to demonstrate a clear correlation between post-vaccination serum antibody levels and protection from pertussis [12–16], recent reports have shown that antibodies against some *B. pertussis* antigens are indeed associated with a lower likelihood of acquiring the disease [17, 18]. However, an early waning of humoral immunity was observed even in children who received highly efficacious acellular vaccines [8, 19].

Although serum antibody titres decay rapidly following antigen exposure, it is possible that memory B and T cells persist after vaccination or exposure and this may result in the capacity of circulating lymphocytes to mount a specific recall antibody response *in vitro* to *B. pertussis* antigens. To test this hypothesis, and based on previous experience in the induction and maintenance

of specific antibody responses in cultures of human peripheral blood mononuclear cells (PBMC) [20, 21], the antibody response to specific *B. pertussis* antigens was investigated in PBMC cultures of normal adult blood donors.

Materials and methods

Antigens

A heat-killed suspension of *B. pertussis* whole cells was an in-house preparation. Briefly, *B. pertussis* strain 18323 (ATCC 9797, serotype 1, 3) was grown on charcoal agar plates supplemented with cephalixin 20 µg/ml (Unipath, Milan, Italy) and incubated at 35°C in a moist atmosphere for 48 h. Colonies were harvested, suspended in phosphate-buffered saline (PBS) and adjusted to an optical density equivalent to 10⁹ cfu/ml. The preparation was heat-inactivated at 100°C for 1 h in capped tubes. The *B. pertussis* soluble antigens pertussis toxin (PT), pertactin (69-kDa antigen, 69K) and filamentous haemagglutinin (FHA) were kindly supplied by Chiron Biocin (Siena, Italy). Keyhole limpet haemocyanin (KLH) (Calbiochem, San Diego, CA, USA) and dextran B-512 (Sigma) were used as unrelated control antigens.

Cell preparation

Heparinised blood, obtained from seven healthy adult volunteer donors (age range 30–60 years), was first

passed through a synthetic wool column (Coop, Basel, Switzerland) to remove strongly adhering suppressor cells, as described previously [22], then layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged (400 g) at 20°C for 40 min. Cells at the interface were harvested, washed and suspended in tissue-culture medium.

Induction of antigen-specific antibody response

The method previously described in detail was followed [20, 21]. Briefly, PBMC were suspended at a concentration of 7×10^6 /ml in Iscove's Modified Dulbecco's Medium (IMDM; Sigma) supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin and streptomycin (50 U/ml each), polyethylene glycol (PEG, mol. wt 6000; Serva, Heidelberg, Germany) 4% and human serum 8%. The serum had been heat-inactivated and absorbed with 10^9 microbial cells/ml of serum, in the cold, with constant stirring by rotation, to remove anti-*B. pertussis* antibodies.

Culture conditions

PBMC suspensions were mixed with the *B. pertussis* whole cell (1×10^7 /ml) antigen preparation. Polymyxin B (Sigma) 10 µg/ml was added in some experiments to eliminate effects due to lipopolysaccharide (LPS) present in the *B. pertussis* antigen; this did not affect the magnitude of the antibody response. The mixtures were distributed in 0.1-ml volumes in the wells of micro-test plates (Falcon Plastics, Lincoln Park, NJ, USA) and incubated at 37°C in a humidified CO₂ 5% incubator. Where indicated, recombinant human interleukin-2 (IL-2) (Genzyme, Cambridge, MA, USA) 75 U/ml was added after culture for 4 days. At different time intervals, the contents of five wells were pooled. The cells were washed, resuspended in IMDM and assayed for the presence of specific antibody-forming cells. From each sample, two dilutions were tested in triplicate.

Assay for antibody-secreting cells (ASC)

Cultured PBMC secreting specific anti-*B. pertussis* antibodies were counted by a modification of the ELISPOT test [23]. Briefly, flat-bottomed, 96-well microtitration plates (Greiner, Frickenhausen, Germany) were coated (/well) with 0.1 ml of PT 3 µg/ml, FHA 1 µg/ml or 69K 3 µg/ml in PBS, pH 7.3, for 2 h at 37°C and overnight at 4°C in a humidified chamber. Plates were washed with PBS, Tween 20 (Sigma) 0.05% and NaN₃ 0.01% and incubated for 2 h at 37°C with PBS containing albumin (Sigma) 1% as blocking agent. The plates were rinsed, the cells were added and then incubated at 37°C in a CO₂ incubator for 3 h. After washing, the plates were incubated overnight at 4°C with 100 µl/well of an optimal dilution of alkaline-phosphatase (AP)-conjugated goat anti-human immunoglobulins (Sigma). In

some experiments AP-conjugated anti-human IgM and anti-human IgG (Sigma) were also used. After extensive washing, 0.1 ml of the AP substrate 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) 1 mg/ml in 1 M 2-amino-2-methyl-1-propanol buffer (Sigma) was added. After incubation for 1 h at room temperature, the plates were rinsed with de-ionised water and allowed to dry. The blue spots were counted with a stereomicroscope at $\times 40$ magnification. Negative controls (i.e., without antibody-secreting cells) were included in all experiments and did not result in spot development. The requirement for *de novo* protein synthesis for spot development was assessed by adding cycloheximide (Sigma) 175 µg/ml to the cells and incubating at 37°C for 2 h, before seeding them in the antigen-coated wells. This treatment resulted in >90% inhibition of spot number. In some control experiments, cultured PBMC were assayed for the presence of sheep red cell (SRC)-specific plaque-forming cells with a modification of the haemolysis in gel method [22].

Statistical analysis

Values are presented as mean and SEM. Statistical significance of differences was calculated by a two-tailed Student's *t* test for paired data. A *p* value <0.05 was considered significant.

Results

Induction of antibody response in vitro

The first experiments established that culture conditions previously described as optimal for the induction of a specific antibody response to SRC and *C. albicans* antigens in cultures of normal human PBMC [20, 21] were also suitable for the induction of a specific anti-*B. pertussis* response. Cells were suspended in medium containing *B. pertussis*-absorbed human serum. The cultures received heat-inactivated *B. pertussis* whole cells as antigen and the antibody response was evaluated by visualising and counting cells making antibody to the *B. pertussis* antigens PT, 69K and FHA. The results of three experiments with PBMC from three different blood donors are shown in Fig. 1. The data demonstrate that, in the presence of *B. pertussis*, antibody-forming cells specific for the three *B. pertussis* antigens were induced. The response was completely antigen-dependent (*p* < 0.05) and was of approximately the same magnitude for the three antigens.

The next experiments were aimed at establishing the antigen specificity of the induced response. *B. pertussis*-stimulated cells were seeded in plastic wells coated with FHA, in the absence or in the presence of different amounts of soluble FHA (29–286 µg/ml) or with the unrelated antigens KLH or dextran B-512 143 µg/ml. The results are shown in Fig. 2. As expected, soluble FHA inhibited spot formation in a

Fig. 1. Antigen dependence of the response. PBMC were cultured without (■) or with (□) *B. pertussis* whole-cell antigen preparation. At day 4, IL-2 75 U/ml was added. At day 7, the cells were assayed for anti-FHA, 69K and PT antibody-secreting cells (ASC). The results (ASC/ 10^6 cells seeded in culture) are expressed as mean and SEM of three experiments with PBMC from three different blood donors ($p < 0.05$).

Fig. 2. Antigen specificity of the response. Cells were cultured with *B. pertussis* antigen. At day 4, IL-2 75 U/ml was added. At day 7, the cells were assayed for anti-FHA secreting cells in the presence of increasing amounts of soluble FHA (■: 2a = 29, 2b = 54, 2c = 143, 2d = 286 µg/ml) or of dextran B-512 (▨) or KLH (▨) 143 µg/ml. Results are shown for one of three experiments performed with similar results. Data are expressed as percentage of ASC in control wells (□).

dose-dependent manner, while KLH and dextran did not. Moreover, *B. pertussis*-stimulated cells did not develop spots when plated on dextran-coated plastic wells, nor plaques of lysis on SRC (data not shown). Thus, the *B. pertussis*-induced response was specific for *B. pertussis* antigens.

Kinetics of the response and effect of IL-2 addition

The time course of appearance of specific anti-*B. pertussis* antibody-forming cells was studied. PBMC were cultured with or without *B. pertussis* whole-cell antigen. At different time intervals, the ASC assay was performed on PT-coated plates. The results are shown

in Fig. 3. The antigen-dependent response appeared after a lag of several days, reached a peak on day 7, then started to decline. Again, no sizeable response was induced in the absence of antigen. These results were confirmed in three experiments with PBMC from three different donors.

Because of the previously reported enhancing effect of IL-2 on the induction of a specific antibody response *in vitro* [20], all the above experiments were performed in the presence of IL-2. However, in some experiments, the requirement for IL-2 for the induction of anti-*B. pertussis* response was investigated. The results are shown in Fig. 4. At days 7–9, the antibody response was significantly higher in cultures that received IL-2 ($p < 0.05$). However, by day 11, cultures not treated with IL-2 were also able to mount a significant antibody response ($p < 0.05$). In all instances, a statistically significant response depended on the presence of antigen. Thus, IL-2 appeared to accelerate the induction of the anti-*B. pertussis* antibody response.

Fig. 3. Kinetics of the anti-*B. pertussis* antibody response. Cells were cultured without (●) or with (■) *B. pertussis* antigen. At day 4, IL-2 75 U/ml was added and the ASC assay was performed at different time intervals. The results are expressed as anti-PT ASC/ 10^6 cells seeded in culture. Results are shown for one of three experiments performed with similar results.

Fig. 4. Effect of IL-2 addition on the anti-PT response. Cells were cultured with or without *B. pertussis* antigen (BP) and with or without IL-2 75 U/ml, added at day 4. Cells were assayed for anti-PT ASC at day 7–9 (a) and at day 11 (b). The results (ASC/ 10^6 cells seeded in culture) are expressed as mean and SEM of eight experiments.

Class of anti-B. pertussis antibody produced

In some experiments the numbers of anti-*B. pertussis* ASC of IgM and IgG class were also evaluated, at different times, from day 4 to day 14 of culture. The results are shown in Fig. 5. At all time points only IgM antibody-secreting cells were detected. The same result was obtained in cultures performed without

added IL-2 (data not shown). As the number of anti-PT ASC detected with anti-IgM antibody equalled those detected with anti-total Ig serum, the above results were not a consequence of an inadequacy of the anti-IgG antibody used. Thus, *B. pertussis* induced an exclusively IgM antibody response in PBMC cultures.

Fig. 5. Class of anti-*B. pertussis* antibody produced. Cells were cultured in the presence of *B. pertussis* antigen and IL-2 75 U/ml was added at day 4. At different time points, the cultures were assayed for total immunoglobulin (■), IgM (□) and IgG (▤) anti-PT ASC. The results (ASC/ 10^6 cells seeded in culture) are expressed as mean and SEM of three experiments.

Discussion

The results presented in this study demonstrate that *B. pertussis* whole cells can induce an antigen-specific antibody response in cultures of PBMC from normal human blood donors. Seven different adult donors (age range 30–60 years) were tested repeatedly: they all responded, without appreciable differences in the magnitude of the response. Previous exposure of the donors to *B. pertussis* *in vivo*, due to infection or to vaccination, was not ascertained. However, it has been shown that most healthy Italian adults have been exposed to *B. pertussis* with acquisition of antigen-specific cell-mediated immunity and a type 1 cytokine pattern [24]. PBMC from all seven donors were indeed able to proliferate in response to *B. pertussis* antigens (data not shown). Thus, it is likely that the antibody response induced *in vitro* was the outcome of the specific stimulation of memory cells generated *in vivo*. The finding that *B. pertussis* induced an exclusively IgM antibody response in PBMC cultures may be due to the fact that the IgM to IgG switch in tissue culture is difficult to obtain and has been described only when subculture procedures were followed and in the presence of adequate co-stimuli [25]. On the other hand, memory cells which have already undergone switch recombination *in vivo* can be induced *in vitro* by antigen to mount an IgG response. Thus, an earlier study showed that, in *C. albicans*-stimulated cultures, both IgM and IgG ASC were generated consistently [21]. However, *C. albicans* is a normal component of the human microbial flora and, as a consequence, the human immune system is constantly exposed to its antigens, whereas contact with *B. pertussis* antigens is sporadic and, in the case of the donors, may have occurred several years previously. Therefore, antigen-specific memory B cells may not have been circulating at the time of blood collection. Moreover, memory cells may not be long-lived, but may require intermittent stimulation by antigen. In particular, it has been proposed that, in the absence of further stimulation, a memory B cell may revert to its naive phenotype [26]. In agreement with this hypothesis, PT and FHA were shown to induce specific IgG synthesis *in vitro* in lymphocytes obtained from convalescent pertussis patients, but not in lymphocytes obtained from healthy donors [27] – although, in this report, antigen-specific IgM production was not investigated.

The data from the present study showed that the presence of antigen in the PBMC culture was a necessary and mostly sufficient requirement to elicit an anti-*B. pertussis* antibody response *in vitro*. Addition of IL-2 caused a shift in the kinetics of the response, without significantly affecting its magnitude. On the other hand, in the previous study it was reported that the in-vitro antibody response to SRC was strictly dependent on the addition of IL-2 or IL-12 to the antigen-stimulated cultures [20, 28]. This apparent difference in the requirements for the efficient induc-

tion of a specific antibody response may simply be the consequence of the described effects of *B. pertussis* antigens on cytokine production. *B. pertussis* antigen stimulation resulted in the preferential induction of a type 1 cytokine profile in cultures of PBMC or T cells from healthy adults [24]. Moreover, it was shown that pertussis vaccines induced a predominantly type 1 cytokine profile in infants [7] and a type 1 cytokine profile was demonstrated in both acutely infected or convalescent children [29]. Finally, IL-12 was shown to be produced by macrophages in response to live or killed *B. pertussis* [30]. Thus, it is likely that, in cultures in the present study, the *B. pertussis* antigen (and not SRC) was able to induce a sufficient level of the appropriate cytokines to allow the efficient induction of a specific antibody response.

The ability to induce and evaluate a specific antibody response to *B. pertussis* antigens in human PBMC cultures may provide a better insight into the mechanism and role of such a response following exposure to the micro-organism by natural infection or vaccination. In a similar way, antibody production to rHBsAg *in vitro* has been proposed as a useful mean of exploration of the immune response to hepatitis B vaccine [31]. Further work is necessary to fully define and to exploit the possibilities provided by this approach and, in particular, to estimate whether it can help to define the state of memory induced by natural exposure or by primary vaccination in infants. In this regard, we are presently undertaking a study to evaluate the capacity to mount a specific anti-*B. pertussis* antibody response in cultures of PBMC from unexposed infants and from children receiving anti-pertussis acellular or whole-cell vaccines.

We are grateful to the blood donors and the staff of the AVIS Association of Bergamo. Thanks are also due to Dr P. Mastrantonio and Professor A. Cassone for reading the manuscript and providing constructive criticisms. This work was supported in part by grants from NIH-NIAID (contract no. N01-A1-25138) and from Istituto Superiore di Sanità ('Proper' project, contract no. 97A/P).

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