Clin. exp. Immunol. (1987) **68,** 275–281

The primary and secondary cellular immune responses to whole cell *Bordetella pertussis* vaccine and its components

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(Accepted for publication 5 December 1987)

SUMMARY

The cellular immune responses of Balb/c mice and Wistar rats immunized in hind footpads with intact killed *Bordetella pertussis* were found to differ from those of similar animals immunized with other bacteria including *Bordetella bronchiseptica*, *Salmonella typhimurium* and *Escherichia coli*. All the bacteria stimulated increases in cell number, proliferation and interleukin 2 (IL-2) production in popliteal lymph nodes which peaked 3–5 days after injection and decreased to resting levels by day 7. However, *B. pertussis* also caused a second peak in all three parameters at 11 days after immunization. This peak was not seen following injection with any of the other bacteria. *Bordetella pertussis* also caused systemic effects, increased cellular proliferation in bone marrow and thymus, with similar biphasic kinetics. It possesses a potent toxin, distinguishing it from the closely related *B. bronchiseptica*. The use of purified materials confirmed that the presence of this pertussis toxin (PT) was responsible for the later peak in stimulation, whereas lipopolysaccharide (LPS) in combination with PT and also the filamentous haemagglutinin (FHA) could mimic the early peak of stimulation. Primary immunization with *B. pertussis* was also shown to generate lymph node cells which responded *in vitro* to secondary challenge with *B. pertussis* cells, FHA or PT. Both proliferation and IL-2 production were enhanced, except with FHA which only increased IL-2 production. Lymph node cells from mice immunized with *E. coli* showed no such responses.

Keywords *Bordetella pertussis* vaccine mouse cellular immunity interleukin 2

INTRODUCTION

Concern over the reactogenicity of whole-cell *Bordetella pertussis* vaccines has generated considerable interest in the development of acellular vaccines containing isolated and purified antigens. The potential of individual antigenic components to induce protective immune responses has been assessed by their protective effects in tests including intracerebral (Kendrick *et al.*, 1947; Sato *et al.*, 1979; Munoz, Arai & Cole, 1981) and aerosol challenge (Sato *et al.*, 1980; 1981; 1982) in mice, and by their abilities to elicit antibody responses (Sato *et al.*, 1982; Ashworth *et al.*, 1983). Knowledge of the immunobiology of pertussis is, however, incomplete. The relative importance of the humoral and cellular responses in protection against infection of the respiratory tract, and of the effects of different antigenic components of the bacterium on the immune response of human infants is unclear. We have investigated the cellular immune responses in mice and rats, following a primary injection of whole-cell pertussis vaccine, or isolated pertussis antigens and also following secondary

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in vitro challenge with these same purified components. The lymphocyte content and cellular proliferation in lymph nodes draining the injection site, and proliferation in thymus and bone marrow were studied together with interleukin 2 (IL-2) production by lymph node cells as a specific measure of T cell activation (Robb, 1984).

MATERIALS AND METHODS

Animals. The mice used were 10–12 week old Balb/c supplied by Olac (Bicester, Oxfordshire). Rats were 2 months old male Wistars (Olac).

Bacterial vaccine and purified components. The second British Reference Preparation for pertussis vaccine (NIBSC 66/84) reconstituted in phosphate buffered saline (PBS) was used as the source of killed whole *B. pertussis* cells. The material was washed once in PBS, before immunization.

Three other bacterial species were chosen to compare with *B. pertussis*: *Bordetella bronchiseptica* (NCTC 8344), although very similar to *B. pertussis*, does not produce pertussis toxin (Pittman, 1979); *Escherichia coli* 0111 strain J5 which is like *B. pertussis* in having an endotoxin lacking a polysaccharide component; *Salmonella typhimurium* (LT2) which is a mouse pathogen.

Late exponential cultures of *B. bronchiseptica* NCTC8344 grown in Stainer and Scholte medium (as described in Stainer & Scholte, 1971), *E. coli* 0111 strain J5 and *S. typhimurium*. LT2 grown in brain-heart infusion broth (Difco) at 37°C under agitated conditions were harvested by centrifugation, resuspended in PBS and killed by heating to 56°C for 30 min in a water bath. The cells were washed once in PBS before their use for immunization.

Purified active pertussis toxin (PT) was isolated from 3-day suspension cultures of *B. pertussis* strain Wellcome 28 (W28) by affinity chromatography as described by Irons & MacLennan (1979). Purified PT was treated with glutaraldehyde as described by Munoz *et al.* (1981) to yield a PT toxoid (PTd). Purity was confirmed by SDS polyacrylamide gel electrophoresis using silver staining.

Purified filamentous haemagglutinin (FHA) was kindly supplied by Dr Y. Sato (National Institute of Health, Japan). Purify was confirmed as for PT.

Lipopolysaccharide (LPS) was purified from *B. pertussis* W28 by the method of Staub (1965). An endotoxin reference preparation (Mallinckrodt Inc, St Louis, MO, USA) was used as a source of *E. coli* LPS.

When required the protein content of the preparations was determined by the method of Schacterle & Pollock (1973) using BSA as a standard.

Immunizations. Balb/c mice were immunized with 5×10^7 killed bacteria (NIBSC 66/84) per mouse contained in 50 μl of saline injected into hind footpads. Active pertussis toxin (10 ng), PTd (10 ng), FHA (30 ng) and LPS (300 ng) were injected into the hind footpad of each mouse. These amounts are believed to be equivalent to those present in 5×10^7 *B. pertussis* bacteria (Ashworth *et al.*, 1983). Wistar rats each received 5×10^8 bacteria in 50 μl of saline in each hind footpad.

Cell suspensions. Popliteal lymph nodes were removed and single cell suspensions prepared as previously described by Spitz *et al.* (1985). After three washes in RPMI 1640 culture medium (Flow) the cells were resuspended in RPMI 1640 supplemented with 3% (v/v) fetal calf serum. Viable cells were counted by trypan blue exclusion and the cell concentration adjusted to 5×10^6 cell/ml for IL-2 production and 1×10^6 cell/ml for the determination of proliferative activity.

Interleukin 2 production. The capacity of lymphoid cells to produce IL-2 *in vitro* was studied by culturing 5×10^6 cells/ml at 37°C in the presence of 5% (v/v) CO_2 (Spitz *et al.*, 1985). After 36 h the cells were centrifuged and the cell-free supernatant frozen at −20°C before the assay of IL-2.

Interleukin 2-dependent cell line assay. Interleukin 2 activity in culture supernatants was determined by its effect on the proliferation of an IL-2 dependent T cell line, CTLL (Gearing, Johnstone & Thorpe, 1985). CTLL cells (5×10^3) were incubated in a total volume of 100 μl of RPMI 1640 containing 5% v/v fetal calf serum and dilutions of test supernatants. Proliferation was measured by the incorporation of [^{3}H]thymidine (0·5 μCi, specific activity 20 μCi/mM Amersham) during the last 4 h of a 24 h incubation period as described by Gearing *et al.* (1985).

Cell proliferation assay. Lymph node cells were distributed into 96-well microtitre plates (Flow)

at 5×10^4 cells/well in RPMI 1640 containing 3% v/v fetal calf serum (each animal's cells were assayed in duplicate). After 18 h in culture, 0·5 μCi of [^{3}H]thymidine were added and the cells were harvested 4 h later on to fibreglass filters (Whatman); incorporated radioactivity was determined by scintillation counting as described by Spitz *et al.* (1985).

Determination of proliferation in thymus and bone marrow. At different times after immunization with *B. pertussis*, rats were injected intraperitoneally with 0·2 mg of colchicine per 100 g body weight in 0·5 ml pyrogen-free saline. After 3 h a second injection of colchicine was given and 3 h later thymuses were removed and cell suspensions prepared. The cells were smeared on slides, fixed, and stained with haematoxylin to allow the identification of metaphase figures. The numbers of cells which had accumulated in colchicine metaphase were recorded by two independent observers, each counting a total of 2000 cells per slide. This method has been previously described in detail by Edwards *et al.* (1981).

Secondary challenge of lymph node cells in vitro. Sixteen days after primary injection of mice with either *B. pertussis* or *E. coli*, the popliteal lymph nodes were removed and cell suspensions were prepared as described above. Cells from five animals were pooled to enable challenge with pertussis antigens at concentrations of 5 μg/ml PT, PTd and FHA, 50 μg/ml LPS and 5×10^8 cells/ml for the whole bacteria. One ml of culture containing 5×10^6 cells in RPMI 1640 with 3% v/v fetal calf serum was incubated for 36 h in the presence of the different antigens after which the supernatants were removed and assayed for IL-2 activity using the CTLL line. Aliquots of cells were also pulsed with [^{3}H]thymidine between 32 and 36 h in culture and the proliferative activity determined as described above.

RESULTS

Localized primary immune response in mouse popliteal lymph nodes. Immunization with either the J5 strain of *E. coli*, or *S. typhimurium* or *B. bronchiseptica* resulted in a stimulation of the cells in the popliteal lymph node draining the site of injection. There was an increase in cell number, rate of proliferation and IL-2 production which peaked at day 3–5 (Fig. 1). When *B. pertussis* was used, a peak of stimulation between day 2 and 3 was followed by a marked decline in cell number and rate of proliferation between day 4 and 5 (IL-2 production did not decline). A second phase of activity was then seen with maximum proliferation and IL-2 production at 11 days followed by a decline between 11 and 17 days (see Fig. 1). Cell numbers continued to increase at 7–16 days. The absence of PT in the closely related bacterium *B. bronchiseptica* would suggest that it is the PT component of *B. pertussis* which causes this second peak of activity. Purified components of *B. pertussis* were also injected individually or in combination and cell number and IL-2 production measured (Fig. 2). The magnitude of each response was much lower than when whole bacteria were used. Nonetheless it was possible to confirm that LPS in combination with PT and FHA could cause an early peak in cell accumulation and IL-2 production. The presence of PT could stimulate a second peak of IL-2 production and maintain cell numbers.

Primary cellular immune response in rat thymus and bone marrow. The proliferative activity in thymus and bone marrow of rats immunized in the hind foot pads with *B. pertussis* was measured (Fig. 3). As with the popliteal lymphocytes, a biphasic response was observed with an initial peak in mitotic figures at 1–2 days followed by a trough at 7 days, a second peak at 11 days and a return to background levels by day 15. The results are expressed as an increase in the percentage of cells which can be arrested in metaphase by colchicine over a 6 h period compared to the percentage seen from matched control animals. Normal basal levels of proliferation are 4% for thymus and 13% for bone marrow.

Secondary challenge of popliteal lymph node cells in vitro. The proliferation and production of IL-2 by popliteal lymph node cells from mice immunized with *E. coli* or *B. pertussis* and challenged in culture with various antigens 16 days after injection is shown in Fig. 4. The highest titre of IL-2 was obtained following challenge with *B. pertussis* of leucocytes from mice immunized with whole *B. pertussis* cells. However, significant levels were also produced following challenge with FHA and to a lesser degree, PT. This effect was not seen when cells from animals immunized with *E. coli* were challenged with the same panel of antigens.

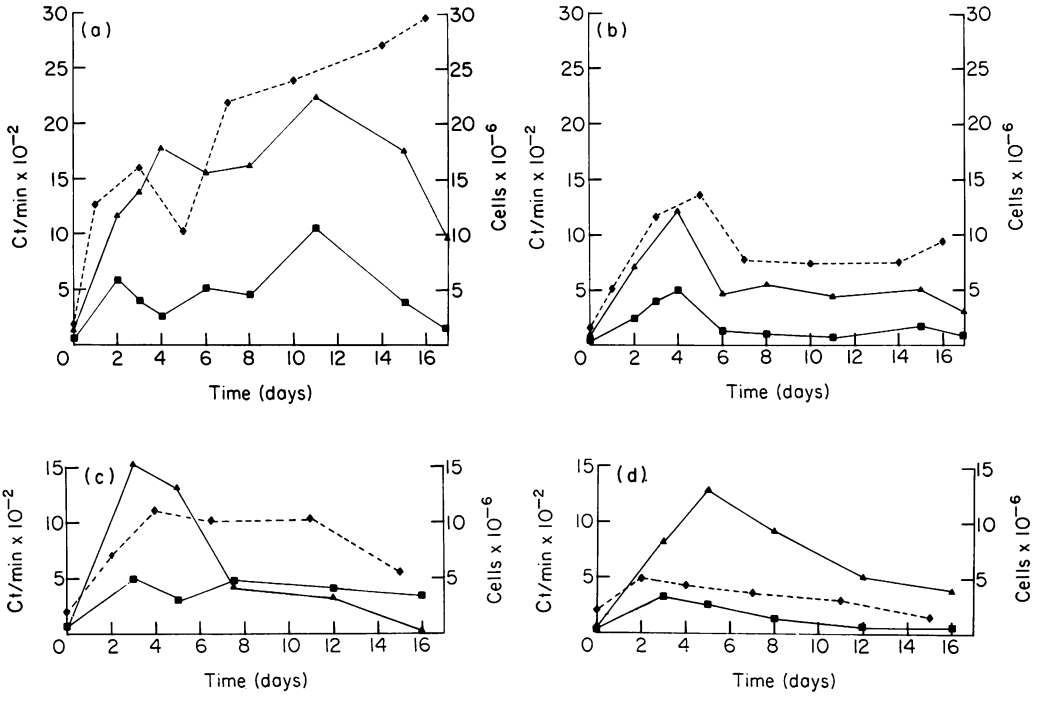

Fig. 1. Kinetics of responses of popliteal lymph node cells from mice immunized in the hind footpads with either (a) *B. pertussis* (b) *E. coli*, (c) *Salmonella typhimurium* or (d) *Bordetella bronchiseptica*. (♦) Total number of leucocytes accumulated (cells $\times 10^{-6}$ in two lymph nodes). (■) Proliferation (ct/min $\times 10^{-2}$ of tritiated thymidine incorporated by 2×10^5 leucocytes). (▲) IL-2 production (ct/min $\times 10^{-2}$ of tritiated thymidine incorporated by an IL-2 dependent cell line (CTLL) in the presence of 40% supernatant from lymph node leucocytes cultured for 18 h at a density of 5×10^6 cells/ml). Each point is the mean of three determinations.

Antigen-specific increases in cell proliferation were observed in cells from mice immunized with *B. pertussis* and challenged *in vitro* with *B. pertussis* bacteria, PT and also LPS derived from *B. pertussis*. Despite the production of IL-2 no cell proliferation was seen in response to challenge with FHA.

DISCUSSION

Infection of the respiratory tract by *B. pertussis* is facilitated by a number of virulence factors including adhesins such as FHA and toxins such as PT. The effects of these molecules, particularly PT, on cells of the murine immune system can be profound; less than 10 ng of PT can affect lymphocyte recirculation resulting in lymphocytosis (Weiss & Hewlett, 1986) and it can potentiate IgG and IgE antibody responses (Munoz, 1985). This report shows that PT is also responsible for stimulating local and systemic increases in cell-mediated immunity which appear after an initial response primarily to LPS has subsided. PT has also been reported to cause similarly delayed effects in the serum glucose response of mice (Kreeftenberg *et al.*, 1984) and in the mouse weight gain test (Redhead & Seagroatt, 1986).

In our studies, we found that injection of 5×10^7 intact killed bacteria was a much more effective stimulus of immunity than injection of purified components in amounts equivalent to those contained in the same number of bacteria. This could be due to the bacteria remaining as a localized depot at the site of injection so avoiding dilution of the components. In support of this hypothesis, Alonso *et al.* (1986) have shown that using very high doses of PT (up to 2·5 μg per mouse) can cause substantial localized delayed type hypersensitivity responses.

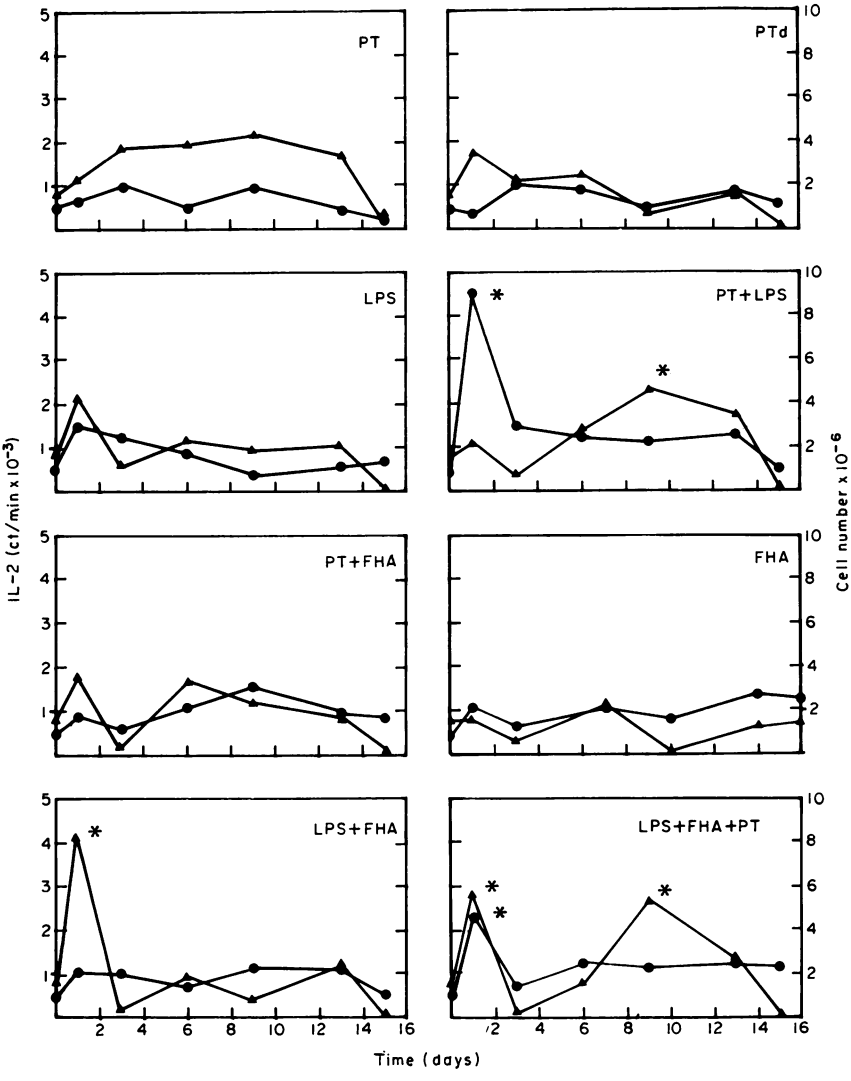

Fig. 2. Kinetics of responses of popliteal lymph node cells from mice immunized in hind footpads with component antigens derived from *B. pertussis*. (●) Total leucocyte accumulation (number of leucocytes in two lymph nodes). (▲) IL-2 production (ct/min of tritiated thymidine incorporated by CTLL cells in the presence of 40% supernatant from lymph node leucocytes cultured for 18 h at a density of 5×10^6 cells/ml). *Significant increases above control level ($P < 0{\cdot}05$). PT, pertussis toxin; PTd, toxoided pertussis toxin; LPS, lipopolysaccharide; FHA, filamentous haemagglutinin.

The combination of lymphocytosis and stimulation of lymphocyte proliferation caused by PT is a major derangement of normal lymphoid function which probably contributes to the maintenance of *B. pertussis* infections. Toxoiding can remove these toxic effects of PT but leaves many antigenic determinants unaffected. Vaccines comprising toxoid PT, either alone or in combination with FHA, have been produced, and are currently in clinical trials. Specific antibodies are produced following injection with these preparations, and in mice protection against challenge with live bacteria (Munoz *et al.*, 1981) can be demonstrated. Our results indicate that T cells reactive to FHA and PT are generated following immunization with whole *B. pertussis* cells. The relative importance of these T cells in immunity to *B. pertussis* is as yet unknown. If a T cell response is important, then

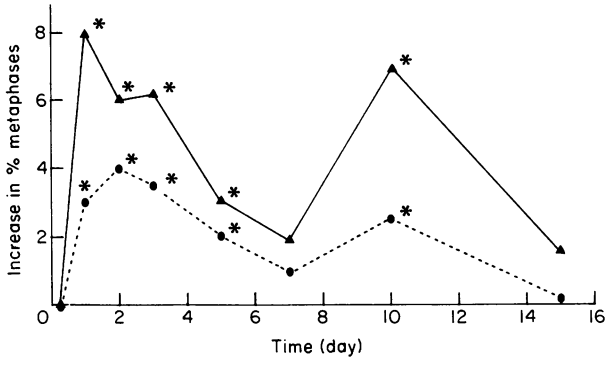

Fig. 3. Kinetics of proliferation in (●) rat thymus and (▲) bone marrow, following footpad injection of WCPV. Proliferation is expressed as the increase in % of cells which could be arrested in metaphase by colchicine, in a 6 h period, over the level in saline injected animals. s.e.m. were less than 0·2%. Asterisks mark significant increases above control level ($P < 0.05$).

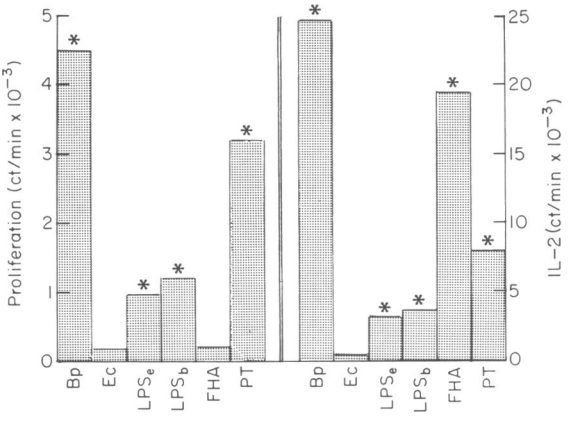

Fig. 4. Proliferation and production of IL-2 by lymph node leucocytes from mice immunized 16 days earlier with WCPV following secondary challenge *in vitro* with component antigens derived from *B. pertussis*. Proliferation is expressed as ct/min $\times 10^{-3}$ of tritiated thymidine incorporated by 2×10^5 leucocytes; IL-2 levels are expressed as ct/min $\times 10^{-3}$ of tritiated thymidine incorporation by CTLL cells in the presence of 40% supernatant from lymph node leucocytes cultured for 36 h at a density of 5×10^6 cells/ml. *Significant increases above control level ($P < 0.05$). M, medium only control; Bp, *Bordetella pertussis*; Ec, *Escherichia coli*; LPSe, lipopolysaccharide from *E. coli*; LPSb, lipopolysaccharide from *B. pertussis*; FHA, filamentous haemagglutin in; PT, pertussis toxin.

component vaccine preparations biased towards antibody production may not be completely effective. A note of caution should however be introduced as excessive T cell stimulation can lead to the production of many different lymphokine activities which can have serious consequences. These include inflammation, delayed type hypersensitivity and even autoimmune disease (Gearing *et al.*, 1986). We are currently examining the T cell responses of human peripheral blood leucocytes taken from normal healthy donors, clinical personnel exposed to patients with whooping cough and also patients with whooping cough, following *in vitro* challenge with component antigens of *B. pertussis*.

We would like to thank Drs A. Perris, E. Griffiths and R. Thorpe for discussion and Ms V. Watson for typing the manuscript.

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