*Immunology* 1996 **87** 372–380

# Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*: variables in the *in vivo* priming and *in vitro* cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0

A. BARNARD,* B. P. MAHON, J. WATKINS,† K. REDHEAD†‡ & K. H. G. MILLS *Infection and Immunity Laboratory, Department of Biology, St Patrick's College, Maynooth, Co. Kildare, Ireland and †The National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, UK*

## SUMMARY

In studies of the mechanism of immunity to *Bordetella pertussis* in a murine respiratory infection model, we have previously demonstrated that natural infection of immunization with a whole cell vaccine induces a potent protective immune response, which is mediated by T-helper type-1 (Th1) cells. In contrast an acellular vaccine generates Th2 cells and is associated with delayed bacterial clearance following respiratory challenge. In the present study we have investigated the apparent Th1/Th2 cell dichotomy in acquired immunity and have examined the factors that affect their induction or detection. The cytokine profiles of *B. pertussis*-specific T cells in immune animals were determined using antigen-stimulated *ex vivo* spleen cells or $CD4^+$ T-cell lines and clones established in the presence of interleukin-2 (IL-2) or IL-4. Antigen-specific T cells derived from mice immunized with the acellular vaccine were almost exclusively of the Th2 cell type. In contrast, T-cell lines and clones established following respiratory infection or immunization with the whole cell vaccine were predominantly of the Th1 type. However, a proportion of T cells from convalescent mice, especially when cultured in the presence of IL-4, secreted IL-4 and IL-5 with or without detectable IL-2 and interferon-$\gamma$ (IFN-$\gamma$), suggesting that Th0 or Th2 cells were also primed during natural infection *in vivo*. Furthermore, when mice were assessed 6 months after infection, spleen cells produced significant levels of IL-4 and IL-5, which were not evident at 6 weeks. The route of immunization and the genetic background of the mice were also found to influence the preferential priming of Th1 cells, and this was directly related to the level of protection against respiratory or intracerebral (i.c.) challenge. Our findings underline the critical role of $CD4^+$ Th1 cells in immunity to *B. pertussis*, but also demonstrate that a number of factors in the *in vivo* priming and *in vitro* restimulation can skew the apparent dominance of one Th cell type over another.

## INTRODUCTION

$CD4^+$ T cells mediate protective immunity against infectious pathogens by acting as helper cells for antibody responses and by mediating cellular immune responses against intracellular pathogens.[1] Studies with murine $CD4^+$ T-cell clones have established a clear assocation between cytokine secretion and function.[2] Type-1 $CD4^+$ T cells (Th1 cells) mediate delayed-type hypersensitivity reactions and stimulate macrophages to kill intracellular bacteria and parasites,[3] but can also provide help for IgG2a antibodies that are important in protection against viral infection.[4] Type-2 $CD4^+$ T cells (Th2 cells) are considered to be the main helper T cells, especially for IgA, IgE and IgG1 antibody responses, and in addition activate eosinophils against certain extracellular parasites.[3,5]

*Bordetella pertussis*, a Gram-negative bacterium that causes the respiratory disease whooping cough in humans, is a suitable pathogen to study the relative role of Th cell subpopulations in protective immunity in murine models. Intracerebral (i.c.) challenge of naive mice with *B. pertussis* has a lethal outcome and is used as a model for vaccine potency testing.[6] Resipiratory challenge results in a disease that has many characteristics of the infection in children and is considered to be the most suitable model for a study of immunity.[7] Pertussis appears to be a toxin-mediated disease that primarily affects infants and young children and can cause death.[8] A whole cell vaccine is effective at preventing severe disease.[6,9] However, because of the high frequency of local adverse reactions and

Received 31 October 1995; accepted 17 November 1995.

*Present address: Department of Molecular Medicine, King's College School of Medicine and Dentistry, The Rayne Institute, 123 Cold Harbour Lane, Denmark Hill, London SE5 9NU, UK.

‡Present address: Hoechst UK Ltd, Walton Manor, Milton Keynes, Bucks, UK.

Correspondence: Dr K. Mills, Infection and Immunity Laboratory, Biology Department, St Patrick's College, Maynooth, Co. Kildare, Ireland.

rare systemic manifestations of its toxicity, the uptake rate of this vaccine has often dropped below acceptable levels and in some countries its use has been discontinued.[10] This has motivated the development of less reactogenic acellular vaccines based on various purified *B. pertussis* components. However, until recently their protective efficacy has been disappointing.[11]

*Bordetella pertussis* has classically been considered to be an extracellular pathogen, hence it has been assumed that protection is mediated by humoral immunity, which would be associated with a Th2 response. It has been considered that local IgA or IgG transudating into the lungs prevents colonization by inhibiting bacterial adherence to ciliated epithelial cells in the lungs or prevents disease by neutralizing toxins. However, analysis of serum antibody responses in a clinical trial failed to show a correlation between their levels and protection.[11] There is now convincing evidence to suggest that *B. pertussis* can be taken up and survive within macrophages in the lungs.[12,13] Consistent with these observations, we have demonstrated protection in a murine respiratory model in the absence of a detectable antibody response.[14] Athymic mice failed to clear the infection after respiratory challenge. However, adoptive transfer of immune spleen cells or purified $CD4^+$ T cells from convalescent donors into nude or sublethally irradiated recipient mice conferred a high level of protection. Analysis of the protective T cells from immune donors revealed that they secreted interleukin-2 (IL-2) and interferon-$\gamma$ (IF-$\gamma$), but not IL-4 or IL-5, a cytokine profile characteristic of Th1 cells.[14,15] *B. pertussis* specific $CD4^+$ T cells from convalescent children (M. Ryan & K. H. G. Mills, unpublished observations) or adults that had suffered from whooping cough in childhood also secrete exclusively type 1 cytokines.[16] Furthermore, immunization of mice with a whole cell vaccine, which induced a high level of protection against respiratory challenge, appeared to favour the induction of Th1 cells.[15,17] In contrast, immunization with an acellular vaccine, comprising purified native filamentous haemagglutinin (FHA), pertactin and chemically detoxified pertussis toxin (PTd), adsorbed to alum, generates high antibody levels and predominantly Th2 cells. Mice immunized with this vacine show a delay in complete clearance of bacteria from the lungs following challenge.[15]

In the present investigation we have extended these findings using murine T-cell lines, T-cells clones and *ex vivo* T cells taken at extended intervals after immunization. Furthermore, we have examined the effect of immunization route and the genetic background of the mice on the induction of Th1/Th2 cells and protection against *B. pertussis* challenge. Although the data provide further evidence of the Th1/Th2 dichotomy in acquired immunity to *B. pertussis*, the findings also highlight a number of factors that influence the selective induction and detection of cytokine-producing T-cell subsets.

## MATERIALS AND METHODS

*Bacteria and antigens*

Killed *B. pertussis* Wellcome 28 cells were prepared by heating a bacterial suspension in phosphate-buffered saline (PBS) at 80° for 30 min. *Bordetella pertussis* Wellcome 28 was grown at 36° under agitation conditions in Stainer-Scholte liquid medium.[14] Purified pertussis toxin (PT), glutaraldehyde and formalin-treated PT (PTd), a non-mitogenic genetically toxoided recombinant PT (rPT),[18] FHA and pertactin prepared from *B. pertussis* Tohama, were kindly provided by SmithKline Beecham (Rixensart, Belgium). The whole cell vaccine (WCV) used was The Third British Reference Preparation for pertussis vaccine (NIBSC 88/522).

*Mouse immunization*

BALB/c and CBA mice were bred and maintained under the guidelines of the Home Office or the Irish Department of Health. NIH and C57BL/6 mice were obtained from Harlan Olac Ltd (Blackthorn, UK). Except for mice used in the intracerebral (i.c.) challenge experiment, which were 4 weeks old, all other mice were 2–3 months old at the initiation of experiments. Unless stated otherwise, mice were immunized at 0 and 4 weeks with 2·0 IU of the whole cell vaccine (88/522) or an acellular vaccine comprising 12·5 $\mu$g each of FHA, pertactin and PTd adsorbed to alum (1·0 mg potassium aluminium sulphate) per dose. Immunogens were reconstituted at the appropriate concentration in PBS such that each mouse received 0·3 ml intraperitoneally (i.p.) or 0·2 ml subcutaneously (s.c.).

Bordetella pertussis *challenge*

Respiratory infection of mice was performed by aerosol challenge using a modification of the method described by Sato *et al.*[19] Bacteria from a 48-hr culture were resuspended in physiological saline containing 1% casein at a concentration of $2 \times 10^{10}$ colony-forming units (CFU)/ml. The challenge inoculum was administered to the mice for 12 min using a nebulizer in a sealed container within a class 3 exhaust protected cabinet. The course of respiratory infection was followed by performing CFU counts on individual lung homogenates using Bordet-Gengou agar plates, as described previously.[14] The limit of detection was approximately 3 CFU/lung. Results are mean viable *B. pertussis* counts for individual lungs from four mice per group per time-point. Challenge by the i.c. route was performed using *B. pertussis* strain 18323, as described by Kendrick *et al.*[20]

*Generation of B. pertussis specific T-cell lines and clones*

T-cell lines were established from spleens of mice 6 weeks after respiratory challenge or immunizations (at 0 and 4 weeks) with the whole cell or acellular vaccines. Spleens were removed and a single-cell suspension prepared in RPMI-1640 medium supplemented with 2% normal mouse serum. Cells were cultured at a concentration of $2 \times 10^6$/ml, and stimulated with killed whole bacteria ($10^6$/ml), rPT (0·2 $\mu$g/ml), FHA (1 $\mu$g/ml) or pertactin (1 $\mu$g/ml) at 37°in a $CO_2$ incubator. After 4 days fresh medium, antigen-presenting cells (APC; $2 \times 10^6$/ml irradiated syngeneic spleen cells) and either human recombinant (r)IL-2 (5 U/ml) or murine rIL-4 (0·5 ng/ml) were added. After a further 7 days, the surviving T cells were cultured at $1 \times 10^5$/ml with the specific *B. pertussis* antigen and APC ($2 \times 10^6$/ml irradiated syngeneic spleen cells), and maintained on an 11-day cycle of antigen and cytokine stimulation. Cloning of T-cell lines was performed by limiting dilution after two to three cycles of antigen stimulation *in vitro*. T-cell clones were maintained by restimulation with antigen as described for the T-cell lines. T-cell lines and clones were tested for antigen specificity and cytokine production at the end of the feed/starve cycle, 11 days after antigen stimulation.

*Cytokine assays*

*Ex vivo* spleen cells ($2 \times 10^6$/ml) or cultured T-cell lines or clones ($1 \times 10^5$/ml) and APC ($2 \times 10^6$/ml) were cultured with heat-killed *B. pertussis* cells ($10^6$/ml), FHA (5 μg/ml), pertactin (5 μg/ml) or rPT (0·2 μg/ml) in 200 μl wells of flat-bottomed 96-well microtitre plates at 37° in a $CO_2$ incubator. Supernatants were removed 24 hr after the initiation of culture to test for IL-2, and after 48 hr to test for IFN-γ, IL-4, IL-5 and IL-10. IL-2 was assayed by testing the ability of supernatants to support the proliferation of the IL-2- or IL-4-dependent CTLL-2 cell line in the presence of the anti-IL-4 antibody 11-B-11 (10 μg/ml). The results are expressed as international units (IU) per ml after reference to the international standard. IFN-γ, IL-4, IL-5 and IL-10 levels were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies (Pharmingen, San Diego, CA), as described elsewhere.[4,14]

*IgG subtype analysis*

The IgG subclass of serum antibodies was determined against *B. pertussis* sonicate or purified FHA, PT or pertactin using a modification of the ELISA previously described.[4,14]

## RESULTS

### Cytokine production by spleen cells from immune mice—effect of immunogen and route of immunization on Th1/Th2 cell induction

*In vitro* antigen stimulation of spleen cells from mice 6 weeks after respiratory infection or immunization with the whole cell vaccine (0 and 4 weeks) by the i.p. route produced high levels of IL-2 and IFN-γ, and undetectable levels of IL-4 and IL-5. In contrast, spleen cells from mice immunized with an acellular vaccine, comprising PTd, FHA and pertactin adsorbed to alum, produced moderate to high levels of IL-5, but undetectable IL-2 and IFN-γ (Fig. 1). IL-4 could not be detected in the supernatants from antigen-stimulated spleen cells from any of these mice.

Immunization with the accellular vaccine by the s.c. route confirmed the selective induction of Th2 cells, with detectable levels of IL-4 and moderate levels of IL-5, but undetectable IL-2 and IFN-γ. However, spleen cells from mice immunized with the whole cell vaccine by the s.c. route also produced low to moderate levels of IL-4 and IL-5, as well as IFN-γ and high levels of IL-2, suggesting the induction of Th2 or Th0 cells as well as Th1 cells using this route of immunization (Fig. 1).

An examination of the specificity of the splenic T cells producing cytokines *in vitro* revealed that the type-1 cytokines, IL-2 and IFN-γ, were predominantly produced in response to *in vitro* restimulation with heat-killed bacteria (Fig. 1) or a formalin-treated bacterial sonicate (data not shown). Although moderate and low levels of these cytokines were also produced in response to FHA and PT, respectively, IL-2 and IFN-γ were undetectable following *in vitro* stimulation with pertactin. In contrast, spleen cells from mice immunized with the acellular vacine produced significant levels of type-2 cytokines, especially IL-5, in response to *in vitro* stimulation with FHA and PT.

### Cytokine production by *B. pertussis*-specific T-cell lines and clones established from convalescent or immunized mice

$CT4^+$ T-cell lines were established from mice that had been immunized with the pertussis whole cell or acellular vaccines or

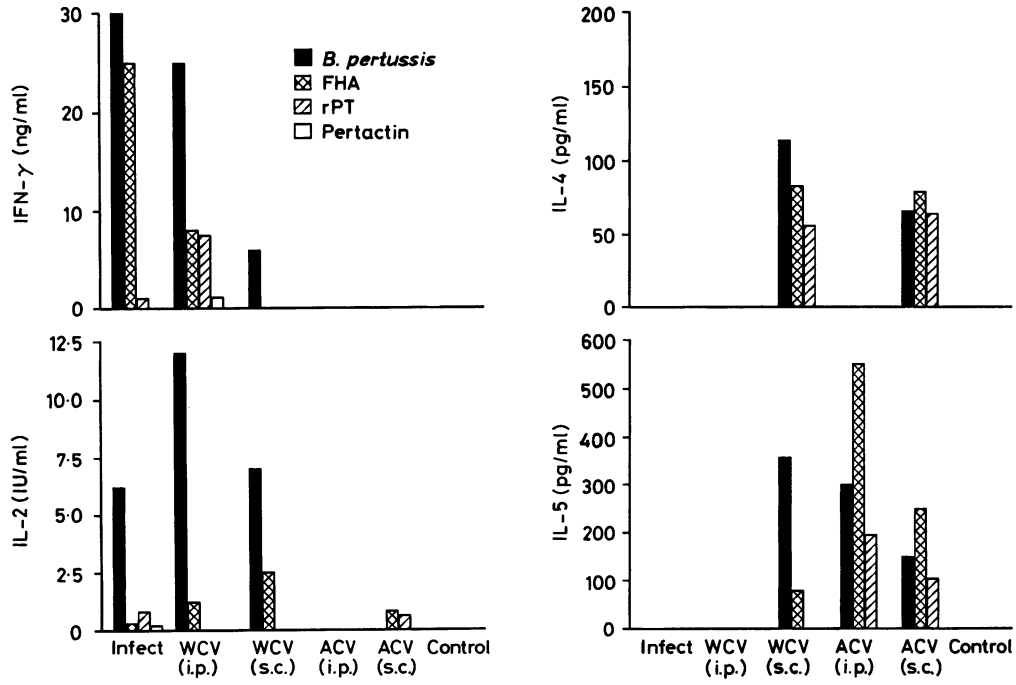


**Figure 1.** Effect of immunogen and route of immunization on the induction of *B. pertussis*-specific Th1 or Th2 cells. Cytokine production was assessed in the supernatants of spleen cells derived from BALB/c mice 6 weeks after respiratory infection or immunization (0 and 4 weeks) with the whole cell vaccine (WCV) or acellular vaccine (ACV) by the i.p. or s.c. routes. Spleen cells were cultured with heat-killed *B. pertussis*, FHA, rPT, or pertactin, and IL-2 estimated after 24 hr and IL-4, IL-5 and IFN-γ after 72 hr. Results are means of assays from four individual mice performed in triplicate.

had recovered from respiratory infection by stimulating spleen cells *in vitro* with either killed whole bacteria or purified FHA, rPT or pertactin. Because it has been suggested that the *in vitro* restimulation protocol, in particular the choice of exogenous cytokine, can select for Th1 or Th2 cell types, we used two experimental approaches in which either IL-2 or IL-4 was added to the cultured T cells. The T-cell lines were periodically tested for cytokine production by testing supernatants following stimulation with specific antigen and irradiated APC; the results are summarized in Table 1.

T-cell lines specific for FHA, PT, pertactin and other unidentified *B. pertussis* antigens were successfully established from convalescent mice. T-cell lines established in the presence of IL-2 all produced significant levels of IFN-$\gamma$, and a proportion also produced IL-4. In contrast, when IL-4 was added to the cultures, all except one produced IL-4, with only four out of the nine producing detectable IFN-$\gamma$.

It proved more difficult to establish T-cell lines from mice immunized with the whole cell vaccine. Nevertheless, T-cell lines were successfully generated by stimulation *in vitro* with killed bacteria and rPT, but not with pertactin or FHA. All of the lines established by stimulation with whole bacteria by either protocol secreted IFN-$\gamma$, but not IL-4 (Th1), whereas T-cell lines specific for PT secreted IL-4 and low or undetectable IFN-$\gamma$ (Th0/Th2).

T-cell lines specific for FHA, PT and pertactin were successfully generated from mice immunized with the acellular vaccine and these T-cell lines were more readily established when IL-4 was added to the cultures. In agreement with the predominant Th2 type responses detected using *ex vivo* spleen cells, all of the T-cell lines from mice immunized with the acellular vaccine produced significant levels of IL-4 (Table 1) and IL-5 (data not shown), but only one produced detectable IFN-$\gamma$.

A number of T-cell clones was generated from convalescent mice or mice immunized with the acellular vaccine. These were established from the T-cell lines described in Table 1 and from bulk cultures of spleen cells from other groups of immune mice stimulated *in vitro* with pertactin or rPT. Consistent with the observations from the *ex vivo* T cells, *B. pertussis*-specific $CD4^+$ T cells generated from convalescent mice were predominantly, but not exclusively, type 1, whereas T-cell clones derived from mice immunized with acellular vaccine were all of the Th2 cell type (Table 2). The clonal analysis also demonstrated that the heterogeneous cytokine profile observed in certain cases with polyclonal T-cells lines categorized as Th0

**Table 1.** Cytokine production by *B. pertussis*-specific T-cell lines from infected or immunized mice established in the presence of IL-2 or IL-4

| | | IL-2 restimulation | | | | IL-4 restimulation | | | |
|---|---|---|---|---|---|---|---|---|---|
| *In vivo* priming | *In vitro* antigen | Line no. | IFN-$\gamma$ (ng/ml) | IL-4 (pg/ml) | Th type | Line no. | IFN-$\gamma$ (ng/ml) | IL-4 (pg/ml) | Th type |
| Inf | BP | 1A | 78 | < 50 | 1 | 1B | 38 | < 50 | 1 |
| | BP | 2A | 38 | < 50 | 1 | 2B | 16 | 623 | 0 |
| | BP | 3A | 51 | < 50 | 1 | 3B | 45 | 294 | 0 |
| | Petactin | 4A | 7 | < 50 | 1 | 4B | < 0·5 | 607 | 2 |
| | Pertactin | 5A | 10 | < 50 | 1 | 5B | < 0·5 | 1000 | 2 |
| | PT | 6A | 3 | 202 | 0 | 6B | 2 | 501 | 0 |
| | PT | 7A | 17 | 290 | 0 | 7B | < 0·5 | 450 | 2 |
| | FHA | 8A | 3 | 640 | 0 | 8B | < 0·5 | 1083 | 2 |
| | FHA | 9A | 2 | 92 | 0 | 9B | < 0·5 | 1466 | 2 |
| WCV | BP | 10A | 28 | < 0·5 | 1 | 10B | 49 | < 50 | 1 |
| | BP | 11A | 70 | < 0·5 | 1 | 11B | 8 | < 50 | 1 |
| | Pertactin | 12A | < 0·5 | < 50 | * | 12B | < 0·5 | < 50 | * |
| | Pertactin | 13A | < 0·5 | < 50 | * | 13B | < 0·5 | < 50 | * |
| | PT | 14A | 5 | 1000 | 0 | 14B | < 0·5 | < 50 | * |
| | PT | 15A | < 0·5 | 264 | 2 | 15B | < 0·5 | < 50 | * |
| | FHA | 16A | < 0·5 | < 50 | * | 16B | < 0·5 | < 50 | * |
| | FHA | 17A | < 0·5 | < 50 | * | 17B | < 0·5 | < 50 | * |
| ACV | BP | 18A | < 0·5 | 221 | 2 | 18B | 4 | 275 | 0 |
| | BP | 19A | < 0·5 | < 50 | * | 19B | < 0·5 | < 50 | * |
| | Pertactin | 20A | < 0·5 | > 2000 | 2 | 20B | < 0·5 | > 2000 | 2 |
| | Pertactin | 21A | < 0·5 | < 50 | * | 21B | < 0·5 | 587 | 2 |
| | PT | 22A | < 0·5 | < 50 | * | 22B | < 0·5 | 450 | 2 |
| | PT | 23A | < 0·5 | < 50 | * | 23B | < 0·5 | < 50 | * |
| | FHA | 24A | < 0·5 | 446 | 2 | 24B | < 0·5 | 403 | 2 |
| | FHA | 25A | < 0·5 | 505 | 2 | 25B | < 0·5 | > 2000 | 2 |

T-cell lines were established from mice following respiratory infection (Inf) or immunization with the whole cell vaccine (WCV) or the acellular vaccine (ACV) by stimulating spleen cells with heat-killed *B. pertussis* (BP) or soluble *B. pertussis* antigens in the presence of IL-2 or IL-4. Results are mean cytokine levels from triplicate assays performed at least twice. T-cell lines were arbitrarily classified into cell types as follows: Th1 (IFN-$\gamma$ > 1·0 ng/ml and IL-4 < 100 pg/ml), Th2 (IFN-$\gamma$ < 1·0 ng/ml and IL-4 > 100 pg/ml) or Th0 (IFN-$\gamma$ > 1·0 ng/ml and IL-4 > 100 pg/ml).

* T-cell lines which produced very low or undetectable IFN-$\gamma$ or IL-4 or did not survive beyond one to two rounds of antigen stimulation.

 

**Table 2.** Cytokine production by antigen-specific CD4$^+$ T-cell clones established from mice infected with *B. pertussis* or immunized with a pertussis acellular vaccine

| *In vivo* priming | Clone | Antigen specificity | IFN-γ (ng/ml) | IL-2 (IU/ml) | IL-4 (pg/ml) | IL-5 (pg/ml) | IL-10 (pg/ml) | Th cell type |
|---|---|---|---|---|---|---|---|---|
| Infection | 1A.3 | BP | 61 | 5·4 | < 50 | < 50 | < 50 | 1 |
| | 1A.12 | BP | > 100 | 2·2 | < 50 | < 50 | < 50 | 1 |
| | 1B.2 | BP | > 100 | 0·9 | < 50 | < 50 | < 50 | 1 |
| | 2A.2 | BP | > 100 | 12·7 | < 50 | < 50 | < 50 | 1 |
| | 2A.3 | BP | 26 | 5·4 | 80 | 52 | < 50 | 1 |
| | 3A.1 | BP | 46 | < 0·1 | < 50 | 57 | < 50 | 1 |
| | 3A.6 | BP | > 100 | < 0·1 | < 50 | < 50 | < 50 | 1 |
| | 3B.1 | BP | > 100 | 17 | 52 | < 50 | < 50 | 1 |
| | 3B.4 | BP | > 100 | 3·1 | < 50 | 75 | < 50 | 1 |
| | 3B.5 | BP | 0·5 | < 0·1 | 817 | 65 | < 50 | 2 |
| | 31.2 | Pertactin | 33 | 0·6 | 167 | 1287 | < 50 | 0 |
| | 31.6 | Pertactin | 43 | 0·3 | 60 | < 50 | < 50 | 1 |
| Acellular vaccine | 20B.2 | Pertactin | < 0·5 | < 0·1 | > 2000 | > 2000 | < 50 | 2 |
| | SP.6 | PT | < 0·5 | < 0·1 | 539 | 682 | 200 | 2 |
| | SP.7 | PT | < 0·5 | < 0·1 | > 2000 | 178 | < 50 | 2 |
| | SP.11 | PT | < 0·5 | < 0·1 | > 2000 | 921 | 578 | 2 |
| | EP.1 | PT | < 0·5 | 0·1 | > 2000 | 137 | < 50 | 2 |
| | NP.2 | PT | < 0·5 | 0·1 | > 2000 | 221 | 125 | 2 |

Cytokine levels were assayed in supernatants of T-cell clones stimulated with killed *B. pertussis* or purified antigens in the presence of irradiated syngeneic APC. Results are the means of triplicate assays performed at least twice.

in Table 1, may reflect a mixture of Th1 and Th2 clones. However, clone 31.2, established from a bulk culture, did produce IL-2, IL-4, IL-5 and IFN-γ, which is more representative of a Th0 profile (Table 2).

### Persistence of Th1/Th2 responses

In order to examine the development of the CD4$^+$ T-cell response over time and to establish if the dichotomy of CD4$^+$

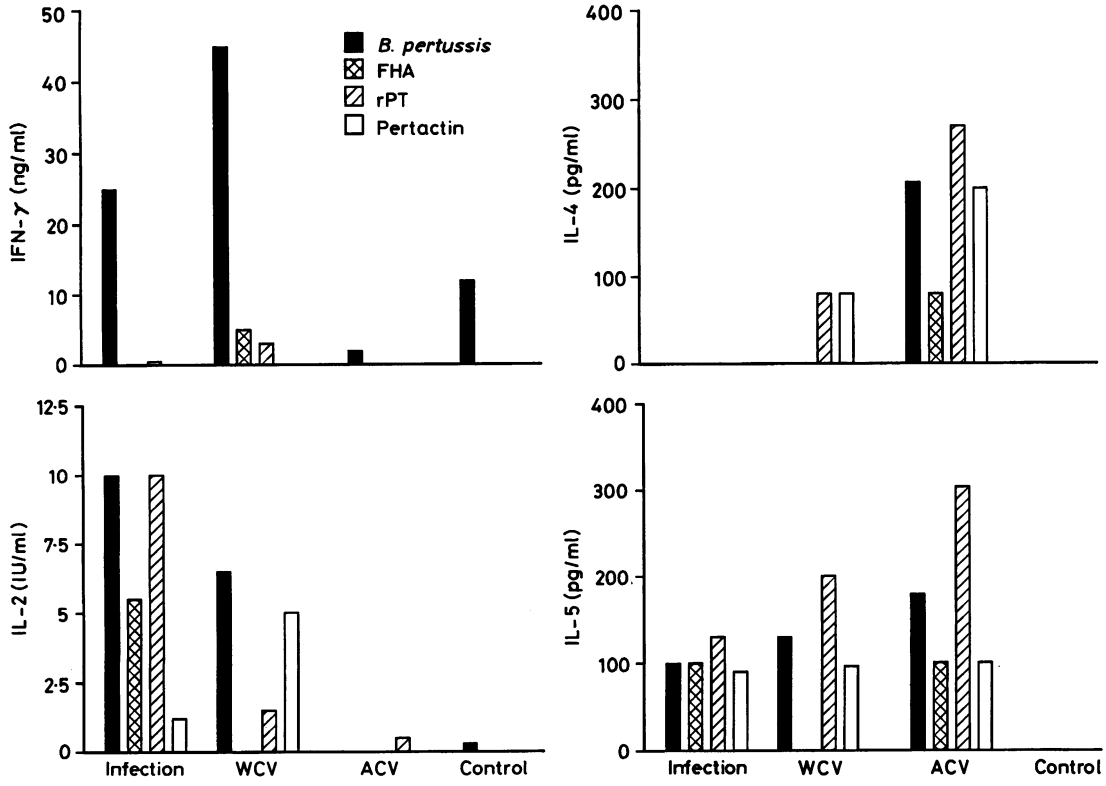


**Figure 2.** Persistence of Th1 and Th2 responses 6 months after immunization or infection. Cytokine production was assessed in spleen cells from mice 6 months after respiratory infection or i.p. immunization (0 and 4 weeks) with the WCV or ACV.

Th1 and Th2 responses detected at 6 weeks (Fig. 1) persisted over a more prolonged period after *in vivo* priming, cytokine production was assessed at intervals after immunization or challenge using antigen-stimulated spleen cells. The findings in Fig. 2 demonstrated that *B. pertussis*-specific $CD4^+$ T-cell responses were still strong 6 months after respiratory infection or immunization (0 and 4 weeks) with the whole cell vaccine. Although the responses had waned somewhat, they were still detectable when tested at 11 and 24 months post-immunization (data not shown). A persistent type-2 response, with significant levels of IL-4 and IL-5 (compared with IL-5 only at 6 weeks; Fig. 1) was also detected 6 months after two immunizations with acellular vaccine (Fig. 2).

Interestingly, the predominantly Th1 response observed after immunization with the whole cell vaccine appeared to have switched to a Th0 or Th1/Th2 type at 6 months. Significant levels of IL-4 and IL-5 were detected in supernatants from spleen cells stimulated with whole killed bacteria, and to a lesser extent in response to PT, FHA or pertactin. Low levels of IL-4 and IL-5 were also detected in supernatants of antigen-stimulated spleen cells taken from mice 6, 11 or 24 months after respiratory infection (Fig. 2 and data not shown). Nevertheless, the predominant cytokines produced by T cells from convalescent mice at prolonged intervals after infection were still IL-2 and IFN-$\gamma$.

### IgG subclass analysis of *B. pertussis*-specific serum antibodies correlates with T-cell cytokine profile from immune mice

As T-cell derived cytokine have distinct roles in immunoglobulin class switching,[3] with Th1 cytokines associated with IgG2a and Th2 with IgG1, we investigated the IgG subclasses of the anti-*B. pertussis* antibody responses in immune mice. Following immunization with the acellular vaccine, mice developed strong antibody responses against PT, pertactin and FHA and the dominant subclasses were IgG1 and IgG3 (Table 3 and data not shown). Immunization with the whole cell vaccine induced moderate titres of IgG specific for FHA, PT and pertactin. However, in these mice the ratio of IgG1 to IgG2a was reversed. When examined 6 weeks after infection, mice had very low levels of specific antibodies; significant levels were only detected in convalescent mice 8 weeks after challenge, and the ratios of IgG1 : IgG2a specific for FHA and pertactin were also low. However, IgG1 was the dominant subclass of the anti-PT antibody response in these mice.

**Table 3.** IgG1 and IgG2a serum antibodies to *B. pertussis* antigens in mice following immunization or respiratory infection

| | IgG1 : IgG2a specific for | | |
|---|---|---|---|
| *In vivo* priming | FHA | Pertactin | PT |
| Respiratory infection | 0·41 | 0·42 | 3·3 |
| Whole cell vaccine | 0·33 | 0·74 | 1·0 |
| Acellular vaccine | 1·2 | 1·57 | 4·4 |

Serum antibody specific for the *B. pertussis* antigens FHA, pertactin and PT were tested by ELISA using antibodies specific for mouse IgG1 and IgG2a. Antibodies levels were measured 6 weeks after immunization with the whole cell or acellular vaccines, and in convalescent mice 8 weeks after respiratory challenge. The results are expressed as a ratio of the mean IgG1 : IgG2a levels for eight mice per group from two separate experiments.

### Respiratory challenge-protection correlates with a strong Th1 response

In order to establish the correlation between the method of *in vivo* priming and the cytokine profile of systemic T cells with the ability to resist infection, groups of 16–20 immune or control mice were challenged by aerosol inoculation with viable *B. pertussis*, and colony counts were performed on the lungs at intervals after challenge. Unimmunized control mice showed an increase in the numbers of bacteria in the lungs in the first 5 days and significant levels were still detectable 14 days after challenge (Fig. 3). Mice challenged 6 weeks after previous infection or immunization with the whole cell vaccine by the i.p. route showed a substantial drop in the numbers of bacteria in the lungs within 2 days and had undetectable levels 5 days after challenge. In contrast, clearance was delayed until 14 days after challenge in mice immunized with the acellular vaccine by the same route and schedule. Although numbers of bacteria dropped rapidly after challenge in these mice, low numbers of bacteria persisted in the lungs 9 days later.

When compared with the i.p. route, immunization with the whole cell or acellular vaccine by the s.c. route, which was associated with higher IL-5/IL-4 and lower IFN-$\gamma$ levels (Fig. 1), did not confer as high a level of resistance against respiratory challenge (Fig. 3). Mice immunized with the whole cell vaccine by the s.c. route still had detectable bacteria in the lungs 9 days after challenge, while mice immunized with the acellular vaccine by this route had not completely cleared the infection by day 14.

### Th1/Th2 responses and protection against i.c. challenge in different strains of mice

We and others have routinely used BALB/c strain of mouse for

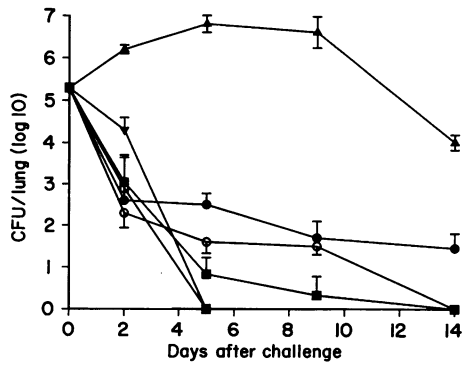


**Figure 3.** Effect of immunogen and route of immunization on protection against *B. pertussis* respiratory challenge. BALB/c mice were aerosol challenged 6 weeks after previous infection (▼) or immunization (0 and 4 weeks) with the whole cell vaccine i.p. (□) or s.c. (■) or the acellular vaccines i.p. (○) or s.c. (●). A group of naive mice (▲) was challenged at the same time. Clearance of *B. pertussis* was followed by perfoming viable counts on individual lungs at intervals after challenge. Results are mean (± SD). CFU estimated for individual lungs from four mice per group at each time-point.

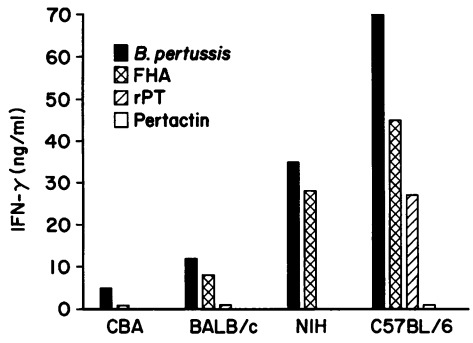


**Figure 4.** IFN-γ production by spleen cells from different strains of mice following immunization with the whole cell vaccine.

the respiratory infection model and for studies on CD4$^+$ T-cell responses to *B. pertussis*.[14,16,17] However, NIH mice are normally used in the Kendrick test, where mice are challenged i.c. with *B. pertussis* to determine the potency of pertussis vaccines.[6,20] Here we tested the correlation between Th cell responses and protection against i.c. challenge induced by the whole cell vaccine in four mouse strains with different genetic backgrounds.

Spleen cells from BALB/c (H-2$^d$), CBA (H-2$^k$), NIH (H-2$^q$) and C57BL/6 (H-2$^b$) mice that had been immunized with the whole cell vaccine were tested for cytokine production stimulation with *B. pertussis* antigens *in vitro*. Spleen cells from all four strains of mice produced moderate to high levels of IFN-γ and IL-2 in response to killed bacteria and lower levels upon stimulation with PT and FHA, but low or undetectable levels of IL-4 and IL-5 to any of the antigens tested (Fig. 4 and data not shown). Significantly higher concentrations of IFN-γ were secreted by spleen cells from NIH and C57BL/6 compared with CBA and BALB/c mice (Fig. 4). This was also observed for spleen cells from convalescent mice (data not shown).

Groups of 4-week-old BALB/c, CBA, NIH and C57BL/6 mice were immunized once by the i.p. route with graded doses of the whole cell vaccine, and challenged i.c. with live *B. pertussis* 2 weeks later. The numbers of mice surviving from the total challenged are shown in Table 4. There were no survivors among the groups of 20 unimmunized control mice from all four strains. The level of protection in the immunized CBA and BALB/c mice was poor; 3/20 CBA and 9/20 BALB/c (compared with 18/20 NIH or C57BL/6) mice survived challenge following immunization with the highest dose (1·0 IU) of the whole cell vaccine. Furthermore, 9/20 NIH and 10/18 C57BL/6 mice immunized with 0·2 IU of the whole cell vaccine survived the i.c. challenge.

**Table 4.** Influence of genetic background on protection against *B. pertussis* i.c. challenge following immunization with the whole cell vaccine

| Mouse strain | Whole cell vaccine dose (IU) | Survivors |
|---|---|---|
| CBA (H-2$^k$) | 1 | 3/20 |
| | 0·2 | 1/19 |
| | 0·04 | 0/20 |
| | 0 | 0/20 |
| BALB/c (H-2$^d$) | 1 | 9/20 |
| | 0·2 | 2/20 |
| | 0·04 | 0/20 |
| | 0 | 0/20 |
| NIH (H-2$^q$) | 1 | 18/20 |
| | 0·2 | 9/20 |
| | 0·04 | 2/20 |
| | 0 | 0/20 |
| C57BL/6 (H-2$^b$) | 1 | 18/20 |
| | 0·2 | 10/18 |
| | 0·04 | 3/20 |
| | 0 | 0/20 |

Groups of mice were immunized with graded doses of the pertussis whole cell vaccine and challenged i.c. with *B. pertussis* 2 weeks later. Results are expressed as the number of animals surviving 14 days after challenge out of the total number challenged in that group.

## DISCUSSION

It has now been firmly established that Th1 cells play an important role in protection against intracellular pathogens. Natural infection with viruses and intracellular bacteria appears to preferentially activate Th1 cells.[2,4] However, attempts to induce Th1 cells by immunization, especially with subunit or purified recombinant antigens, have proved more difficult.[21] In this study we demonstrate a dichotomy in the CD4$^+$ T-cell response following immunization or infection with *B. pertussis*, which is directly relevant to protection against the pathogen *in vivo*. Protection against respiratory or i.c. challenge with *B. pertussis* was found to correlate with the induction of antigen-specific Th1 cells, which in turn was influenced by a number of factors including the nature of immunogen, the route of immunization and strain of mice. Furthermore, the classification of T-cell subsets into Th1, Th2 or Th0 on the basis of cytokine secretion was also affected by the timing of the test post-immunization and the technique employed to detect the Th cell subpopulations *in vitro*.

The original description of Th1 and Th2 subpopulations of CD4$^+$ was based on cytokine secretion by murine T-cell clones.[2] Studies in mice on a number of infectious pathogens, including *Leishmania*[3] and poliovirus,[4] have successfully employed T-cell cloning techniques to define distinct Th1 and Th2 responses and their association with protection against or susceptibility to the infection. A number of other techniques, including quantitative polymerase chain reaction (PCR) for cytokine mRNA, intracellular staining with anti-cytokine antibodies and cytokine quantification in supernatants from antigen or polyclonally activated spleen, lymph node or peripheral blood lymphocytes have also been used to define Th1, Th2 or Th0 responses. However, these approaches have often indicated heterogeneous mixtures of Th1 and Th2 cell types. Although the generation of antigen-specific T-cell clones may overcome this ambiguity, as demonstrated in this and other studies,[22] culture of T cells, especially in the presence of exogenous cytokines, can alter the pattern of cytokines secreted and may select for specific cell types.

Regardless of the read-out system employed (*ex vivo* spleen cells or T-cell lines and clones), immunization with *B. pertussis* components in alum resulted in a clear Th2-type response. In contrast, analysis of *B. pertussis*-specific spleen cells 6 weeks

after respiratory infection or immunization with the whole cell vaccine suggested a clear type 1 response. Furthermore, anti-FHA and anti-pertactin serum IgG subclass analysis revealed that the dominant Th2 and Th1 responses correlated closely with high or low ratios of IgG1 to IgG2a, respectively. However, cytokine production by T-cell lines and clones established from convalescent mice allowed the detection of Th2 and Th0 cell types in addition to the dominant Th1 population. While the majority of T-cell lines and clones produced IFN-$\gamma$ and IL-2, without detectable IL-4 or IL-5, a minority produced IL-4 and IL-5 or all four cytokines tested. T-cell lines with Th0 or Th2 cytokine profiles were particularly evident following the addition of exogenous IL-4 to the cultures. It has recently been demonstrated that regulatory cytokines can selectively promote the development of Th1 or Th2 cells.[22] In our study, the addition of exogenous IL-4 *in vitro* may have inhibited IFN-$\gamma$-producing cells and stimulated IL-4-producing cells, thus allowing the expansion of the Th2 subpopulation. In contrast, the production of high levels of IL-2 and IFN-$\gamma$, either in bulk spleen cell cultures or the T-cell lines stimulated in the presence of IL-2, may inhibit proliferation and cytokine secretion by Th2 cells that may be present, thus allowing the selective expansion of the Th1 subpopulation. Therefore an apparent dominance of Th1 cells defined on the basis of cytokines secreted by antigen-stimulated spleen cells may not permit the detection of a minority of Th2 or Th0 cells within these polyclonal populations *ex vivo*. This is consistent with the recent suggestion that the classification of T-cell subsets into distinct Th1 or Th2 cell types based on arbitrary parameters may be an oversimplification of the situation *in vivo*.[23] Indeed the study at intervals after *in vivo* priming suggested that Th2 or Th0 cells could be detected by testing cytokine production by *ex vivo* spleen cells. In contrast to the dominant Th1 response at 6 weeks, low levels of IL-4 and IL-5, as well as moderate to high levels of IL-2 and IFN-$\gamma$, were detected from antigen-stimulated spleen cells at 6, 11 and 24 months after respiratory infection or immunization with the whole cell vaccine. The failure to detect type 2 cytokines at 6 weeks may reflect an over-dominance of type 1 cytokine production early in the immune response and a possible greater persistence of memory Th2 population.

It has been reported previously[24] that IL-4 production is difficult to detect following primary stimulation *in vitro*, but can be readily detected following restimulation or in long-term cultured T cells. Our findings are partly in agreement with this observation; IL-4 was undetectable in supernatants of antigen-stimulated spleen cells taken from mice 6 weeks after i.p. immunization with the acellular vaccine, despite the demonstration of high levels of IL-5. In contrast, established T-cell lines and clones from these mice all secreted high levels of IL-4 and IL-5. However, when mice were examined 6 months after immunization significant levels of IL-4 were detected in antigen-stimulated spleen cell supernatants. Taken together with the demonstration of IL-4 production 6 weeks after s.c. immunization and 6, 11 and 24 months after i.p. immunization with the whole cell vaccine, these findings suggest that the failure to detect IL-4 following a single *in vitro* stimulation with antigen may reflect insensitivity of the assays to detect low levels of the cytokine, or inhibition of its production in the presence of high levels of IFN-$\gamma$.

The functional significance of a dominant Th1 or Th2 type response in relation to protective immunity against a pathogenic organism *in vivo* is clearly demonstrated in this study. The rate of *B. pertussis* clearance following respiratory challenge closely reflects the profile of T-cell responses detected. Previous infection or immunization with the whole cell vaccine by the i.p. route induced strong Th1 responses and conferred a high level of protection against respiratory challenge. In contrast, immunization approaches, such as the use of purified antigens with alum or injection by the s.c. route, which enhanced type 2 cytokine production, were associated with delayed clearance after challenge.

One explanation for the selective induction of Th2 cells by the acellular vaccine may be provided by the suggestion that the putative protective antigens FHA, pertactin and PT are preferential targets for Th2 cells and do not stimulate Th1 cells. Although our findings indicate that these may not be the immunodominant antigens of *B. pertussis* for Th1 cells, the results both from T-cell lines and clones did demonstrate the priming of pertactin-specific Th1 cells. Analysis of cytokine production using spleen cells suggested that PT and FHA may also be recognized by a minority of Th1 cells from convalescent mice. Furthermore, we have recently demonstrated that further manipulation of the *in vivo* priming or the *in vitro* restimulation techniques can permit the generation of Th1 cells using purified *B. pertussis* components (B. P. Mahon, M. Ryan & K. H. G. Mills, unpublished observations).[25]

It has been suggested that *in vivo* priming of Th1 or Th2 cells may be influenced by a number of other factors, including the APC or the cytokine milieu in the lymphoid tissue at the site of inoculation.[22,26] The switch to higher levels of IFN-$\gamma$ and low or undetectable IL-4 and IL-5 following i.p., compared with s.c., immunization provides evidence for the involvement of APC, because macrophages, which are considered to be the main APC for Th1 cells,[26] are present in high numbers in the peritoneal cavity. Furthermore, the targeting of particulate and soluble antigens to phagocytic and non-phagocytic APC populations provides an alternative explanation for the selective induction of Th1 and Th2 cells by the whole cell and acellular vaccines, respectively.

In addition to the immunogen, and site of inoculation, this study also demonstrated that the strain of mice influenced the cytokine profile of the responding T-cell population and subsequent protection against bacterial challenge. An examination of the T-cell responses following immunization with the whole cell vaccine in four different strains of mice revealed that NIH and C57BL/6 mice, compared BALB/c or CBA strains, secreted significantly higher levels of IFN-$\gamma$ and showed significantly better survival following i.c. challenge. The i.c. challenge test developed by Kendrick *et al.*[20] is used routinely to measure pertussis vaccine potency, and the results of Medical Research Council trials in the 1950s suggested that potency in this test correlated with vaccine efficacy in humans.[6] When taken together with our recent demonstration that respiratory infection in children results in the preferential activation of Th1 cells and the failure to find a correlation between protection and serum antibody levels in clinical trials,[11] these findings suggest that Th1 cells may also play a key role in immunity against whooping cough in humans. However, it remains to be determined if enhanced survival following i.c. challenge or relatively earlier complete bacterial clearance following respiratory challenge of mice with a strong

Th1 response will correlate with the prevention of *B. pertussis* infection in children. Nevertheless, future strategies for the development of improved potency acellular vaccines must consider the induction of Th1 cells.

## ACKNOWLEDGMENTS

We are grateful to Carine Capiau and Yves Lobet, SmithKline Beecham, Rixensart, Belgium for providing purified pertussis antigens, and to the Wellcome Trust for financial support.

## REFERENCES

1. Mills K.H.G. (1989) Recognition of foreign antigen by T cells and their role in immune protection. *Curr Opin Infect Dis* **2,** 804.
2. Mossmann T.R. & Coffman R.L. (1989) Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol* **46,** 111.
3. Scott P., Pearce E., Cheever A.W., Coffman R.L. & Sher A. (1989) Role of cytokines and $CD4^+$ T-cell subsets in the regulation of parasite immunity and disease. *Immunol Rev* **112,** 161.
4. Mahon B.P., Katrak K., Nomoto A., Macadam A., Minor P.D. & Mills K.H.G. (1995) Poliovirus-specific $CD4^+$ Th1 clones with both cytotoxic activity and helper activity mediate protective humoral immunity against a lethal poliovirus infection in transgenic mice expression the human poliovirus receptor. *J Exp Med* **181,** 1285.
5. Coffman R.L., Seymour B.W., Lebman D.A. *et al.* (1988) The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol Rev* **102,** 5.
6. The Whooping Cough Immunisation Committee of the Medical Research Council (1956) Vaccination against whooping-cough: relation between protection in children and results of laboratory tests. *Br Med J* **2,** 454.
7. Pittman M., Furman B.L. & Wardlaw A.C. (1980) *Bordetella pertussis* respiratory tract infection in the mouse: pathophysiological responses. *J Infect Dis* **142,** 56.
8. Weiss A.A. & Hewlett E.L. (1986) Virulence factors of *Bordetella pertussis*. *Annu Rev Microbiol* **40,** 661.
9. Robinson A., Irons L.I. & Ashworth L.A.E. (1985) Pertussis vaccine: present status and future prospects. *Vaccine* **3,** 11.
10. Miller D.L., Ross E.M., Alderslade R., Bellman M.H. & Rawson N.S.B. (1981) Pertussis immunization and serious acute neurological illness in children. *Br Med J* **282,** 1595.
11. Ad Hoc Group for the Study of Pertussis Vaccines (1988) Placebo-controlled trial of two acellular pertussis vaccines in Sweden—protective efficacy and adverse events. *Lancet* **i,** 955.
12. Bromberg K., Tannis G. & Steiner P. (1991) Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. *Infect Immun* **59,** 4715.
13. Tuomanen E.I., Zapiain L.A., Galvan P. & Hewlett E.L. (1984) Characterization of antibody inhibiting adherence of *Bordetella pertussis* to human respiratory epithelial cells. *J Clin Microbiol* **20,** 167.
14. Mills K.H.G., Barnard A.L., Watkins J. & Redhead K. (1993) Cell mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. *Infect Immun* **61,** 399.
15. Redhead K., Barnard A., Watkins J. & Mills K.H.G. (1993) Effective immunization against *Bordetella pertussis* is dependent on induction of cell-mediated immunity. *Infect Immun* **61,** 3190.
16. Peppoloni S., Nencioni L., Di Tommaso A. *et al.* (1991) Lymphokine secretion and cytotoxic activity of human $CD4^+$ T-cell clones against *Bordetella pertussis*. *Infect Immun* **59,** 3768.
17. Peterson J.P., Ibsen P.H., Haslov K. & Heron I. (1992) Proliferative responses and gamma interferon and tumor necrosis factor production by lymphocytes isolated from tracheobronchal lymph nodes and spleens of mice aerosol infected with *Bordetella pertussis*. *Infect Immun* **60,** 4563.
18. Lobet Y., Feron C., Dequesne D., Simoen E., Hauser P. & Locht C. (1993) Site-specific alterations in the B oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *J Exp Med* **177,** 79.
19. Sato Y., Izumiya K., Sato H., Cowell J.L. & Manclark C.R. (1980) Aerosol infection of mice with *Bordetella pertussis*. *Infect Immun* **29,** 261.
20. Kendrick P.L., Eldering G., Dixon M.K. & Misner J. (1947) Mouse protection tests in the study of pertussis vaccine. *Am J Public Health* **37,** 803.
21. Moore A., McGuirk P., Adams, S. *et al.* (1995) Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ Th1 cells. *Vaccine* **13,** 1741.
22. O'Gara A. & Murphy K. (1994) Role of cytokines in determining T-lymphocyte function. *Curr Opin Immunol* **6,** 458.
23. Kelso A. (1995) Th1 and Th2 subsets: paradigms lost? *Immunol Today* **16,** 374.
24. Weinberg A.D., English M. & Swain S.L. (1990) Distinct regulation of lymphokine production is found in fresh versus in vitro primed helper T cells. *J Immunol* **144,** 1800.
25. Cahill E.S., O'Hagan D.T., Illum L., Barnard A., Mills K.H.G. & Redhead K. (1995) Immune responses and protection against *Bordetella pertussis* infection after intranasal immunization of mice with filamentous haemagglutinin in solution or incorporated in biodegradable microparticles. *Vaccine* **13,** 455.
26. Gajewski T.F., Pinnas M., Wong T. & Fitch F.W. (1991) Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen presenting cell populations. *J Immunol* **146,** 1750.