

HUMAN T CELL IMMUNITY AGAINST *BORDETELLA PERTUSSIS* ANALYZED AT CLONAL LEVEL

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Summary. - *Cloned peripheral blood T lymphocytes from an immune donor were grown in interleukin 2 and tested for proliferation in response to inactivated Bordetella species (B. pertussis, B. parapertussis and B. bronchiseptica) and mutants deficient for the expression of virulence-associated antigens. All the T cell clones obtained were CD4⁺8⁻ and recognized specifically the Bordetella antigens when presented by autologous B cells. On the basis of the responsiveness to the whole inactivated bacteria, it was possible to cluster the twelve clones obtained into four groups with the following specificity: 1) filamentous hemagglutinin (FHA); 2) B. pertussis specific antigens; 3) virulence-associated Bordetella specific antigens; 4) non-virulence-associated Bordetella specific antigens. Employing two new B. pertussis deletion mutants, clone 6 (representative of cluster 1), was found to recognize the C-terminus of FHA. Furthermore, 3 out of 4 clones of cluster 3 were specifically stimulated by the soluble 69,000 M.W. protein from the outer membrane of B. pertussis. Surprisingly, none of the twelve clones obtained by stimulation in vitro with whole inactivated bacteria recognized PT. Thus, PT does not seem to be the most representative antigen on the whole inactivated bacteria. However, when a new generation of clones was obtained using soluble PT as the in vitro stimulus, it was observed that 11 clones of this group recognized this antigen. Furthermore, the majority of them was against the subunit S1 of PT. Therefore, we can conclude that a T cell memory against PT exists in a donor who has had pertussis several years before. In conclusion, these results provide useful information in the attempt to obtain a simplified acellular vaccine for whooping cough.*

KEY WORDS: T cell immunity, *Bordetella pertussis*, PT specific T cell clones.

Riassunto (Immunità delle cellule T umane contro la *Bordetella pertussis* analizzata a livello clonale). - *Cloni linfocitari T ottenuti da un donatore immune sono stati mantenuti in coltura con IL-2. Diverse specie di Bordetella inattivata (B. pertussis, B. parapertussis e B. bronchiseptica) ed alcuni mutanti che non esprimono gli antigeni della virulenza sono stati utilizzati per saggiare la risposta proliferativa dei cloni ottenuti. Tali cloni erano tutti CD4⁺CD8⁻ e riconoscevano specificamente gli antigeni di Bordetella quando presentati da cellule B autologhe. Sulla base della risposta al batterio intero inattivato, è stato possibile suddividere i dodici cloni ottenuti in quattro gruppi con le seguenti specificità: 1) emagglutinina filamentosa (FHA); 2) antigeni specifici di B. pertussis; 3) antigeni specifici di Bordetella associati alla virulenza; 4) antigeni specifici di Bordetella non associati alla virulenza. Utilizzando due mutanti di B. pertussis si è visto che il clone 6 (rappresentativo del gruppo 1) riconosce la parte C terminale di FHA. Inoltre 3 dei 4 cloni appartenenti al gruppo 3) erano stimolati specificamente da una proteina solubile della membrana esterna di B. pertussis, di peso molecolare 60.000. Contrariamente alle aspettative, nessuno dei cloni ottenuti in seguito a stimolazione con l'intero batterio inattivato era in grado di riconoscere la tossina della pertosse (PT). Quindi non sembra che PT sia tra gli antigeni più rappresentativi del batterio inattivato. Tuttavia sono stati prodotti nuovi cloni linfocitari T utilizzando PT come antigene per la stimolazione in vitro, ed è stato osservato che 11 cloni di questo gruppo erano in grado di riconoscere specificamente PT. Questi risultati forniscono utili informazioni per poter ottenere un vaccino acellulare contro la pertosse.*

PAROLE CHIAVE: immunità dei linfociti T umani, *Bordetella pertussis*, cloni T specifici per PT.

Introduction

It is widely recognized that there is a need for a new acellular vaccine for the prevention of whooping cough. This may be composed by proteins purified from *B. pertussis* or rather by recombinant and/or synthetic peptides which are immunodominant epitopes of *B. pertussis* antigens. The identification of these peptides will require the analysis of T cell memory from donors who are immune against *B. pertussis* since they have had whooping cough during their life. In this study, several T cell clones from a donor who had suffered from pertussis in childhood, were generated and analyzed for their fine specificity.

Materials and methods

Source of T cell

Peripheral blood mononuclear cells (PBMC) were collected from a healthy donor (R.R.) with positive anamnesis for whooping cough in childhood and never vaccinated against *B. pertussis*.

Antigens

Virulent (phase I) and non-virulent (phase III) *B. pertussis* (BP), *B. parapertussis* (BPP) and *B. bronchiseptica* (BB) strains were used together with transposon-induced *B. pertussis* mutants lacking one or more virulence-associated antigens (see Table 1 for summary).

Bordetella strains were grown on Bordet-Gengou agar at 35 °C for 72 h and *Salmonella typhi* on LB agar at 37 °C overnight. For inactivation, bacteria were harvested from plates, washed in PBS and resuspended in a 0.5% formalin solution in PBS and left overnight on a Coulter mixer (Coulter Electronics Ltd., Luton, UK). After extensive washing, bacteria were resuspended in PBS and stored at 4 °C. The OD₅₂₅ of the suspensions was determined and the number of cells per milliliter was calculated on previously established standard curves. Production of hemolysis on BG agar and/or toxic effect on CHO cells [2] were adopted as parameters for the control of phase I *Bordetella* species. Soluble PT was purified from *B. pertussis* phase I culture supernatant as described by Sekura [8], was precipitated in ammonium sulfate and kept at 4 °C. Before use it was resuspended in PBS and heat-inactivated (at 100 °C for 45 min). Subunits S1, S2, S3, S4, S5 were expressed in *E. coli*, each fused to the aminoterminal 98 aminoacids of the MS2 DNA polymerase [3]. The fusion proteins were partially purified by lysing the bacteria and collecting the inclusion bodies as described [3]. The protein concentration obtained was generally between 0.5-1 mg/ml with the 70% represented by the fusion protein. Inclusion bodies were resuspended in PBS and stored at -20 °C. For PBMC stimulation, S1 was further purified by electroelution, as described [3].

Media for cell stimulation and growth

The culture medium was RPMI 1640 (GIBCO Laboratories, Paisley, Scotland) supplemented with L-glutamine (2 mM), 1% non essential aminoacids, 1% sodium pyruvate, 50 µg/ml gentamycin, 5 × 10⁻⁵ M 2-mercaptoethanol, and 10% heat-inactivated pooled human AB serum (RPMI-HS) or fetal calf serum (RPMI-FCS). To support the antigen-independent growth of T-cell clones, RPMI-HS was supplemented with 50 U/ml human recombinant interleukin 2 (rIL-2; Hoffmann-La Roche, Nutley, NJ).

Cloning of *B. pertussis*-specific T lymphocytes

PBMC isolated from heparinized blood of donor R.R. by Ficoll-Hypaque were cultured in flat-bottomed Cluster 96 plates at the concentration of 10⁵/well in 0.2 ml RPMI-HS in the presence of 1 × 10⁶ *B. pertussis* cells. This antigen concentration was found to induce a strong proliferative response by PBMC from this donor. After 7 days, the activated T cells were expanded in medium supplemented with rIL-2 and after another 7 days, lymphoblasts were cloned by limiting dilution [1, 9]. Cells were seeded at 0.3 per well in Terasaki trays in the presence of 10⁵ allogeneic mitomycin C-treated PBMC, in RPMI-HS containing phytohemagglutinin (PHA) (1 µg/ml) and IL-2. About 50% of the clones obtained proved antigen-specific when tested in proliferation assays against *B. pertussis*. These clones were maintained in culture by periodic restimulation in the presence of allogeneic mitomycin C-treated PBMC, PHA and rIL-2. T lymphocyte clones were analyzed for cell surface phenotype by direct immunofluorescence on a fluorescence-activated cell sorter (FACstar, Becton Dickinson, Erembodegem, Belgium). Both phycoerythrin- and fluorescein-conjugated OKT3, OKT4, OKT8 monoclonal antibodies (Ortho Diagnostic Systems, Raritan, NJ) were used.

Immortalization of B lymphocyte with Epstein Barr Virus (EBV)

PBMC from donor R.R. were transformed with EBV as described [6].

Briefly, 10⁷ PBMC were resuspended in 10 ml RPMI-FCS containing 30% supernatant of the EBV-producing marmoset cell line B95.8 and 600 ng/ml cyclosporin A (Sandoz, Basel, Switzerland) and were distributed in a flat-bottomed 96-well plate at 5 × 10⁴/well.

Proliferation assays

T lymphocyte clones were incubated at 2 × 10⁴ cells/well for 3 days in the presence of 2 × 10⁴ mitomycin C-treated Epstein Barr virus-transformed autologous B lymphocytes (EBV-B cells) and of different antigen concentrations in 96-well flat-bottomed Cluster 96 plates (Costar, Cambridge, MA). All assay cultures were pulsed

for the last 16-18 h with 1 μ Ci of [3H]thymidine (sp. act. 185 GBq/mmol; Amersham Int., Amersham, UK). Cells were then harvested on glass-fiber filters with a cell harvester (Skatron, Lier, Norway) and incorporated radioactivity was determined by liquid scintillation counting.

Results and discussion

Although no specific serological or cellular tests have been established for the direct measurement of host immunity to pertussis infection, donors with positive and negative anamnesis for whooping cough in childhood were screened in a previous study [1] for antibody titers against PT by ELISA, and for their proliferative response to *B. pertussis* [1]. The aim was to identify donors suitable for the generation of T-cell clones specific for *B. pertussis* antigens. Indeed, in this way we were able to identify

donor R.R. who possessed high humoral and cellular responses against *B. pertussis*. This donor had had pertussis during his childhood.

Therefore, peripheral blood from donor R.R. was employed to obtain a first generation of T cell clones directed against *Bordetella* antigens [1]. Specific clones were identified by means of proliferation in response to killed *B. pertussis* as shown in Fig. 1.

To analyze further the fine specificity of the anti-*B. pertussis* clones, a panel of *Bordetella* wildtype strains as well as mutants was employed as antigen in proliferation tests together with a negative control such as *Salmonella typhi* (Table 1). This approach resulted in clustering the 12 clones obtained in four different groups which recognized: 1) FHA; 2) *B. pertussis*-specific antigen(s); 3) virulence-associated *Bordetella* antigen(s); 4) non-virulence-associated *Bordetella* antigens [1]. All the clones tested are specific for *Bordetella* species since they did not recognize inactivated *S. typhi*. The prediction that cluster 1 recognizes FHA was further confirmed by employing two new *B. pertussis* mutants (D. Relman, unpublished data), which allowed the clone's specificity to be defined for the C terminal portion of the FHA [1]. Interestingly, the antigen recognized by three clones of cluster 3 was also positively identified as the 69,000 MW outer membrane protein described by Novotny *et al.* [5], while the specificity of the fourth clone seems to be directed to a virulence factor different from the 69,000 MW protein [1]. In addition, the possibility that cluster 2 recognizes PT, suggested by the experiments with whole inactivated bacteria, was disproved by employing soluble PT as antigen [1]. Since cluster 4 recognizes non-virulence-associated *Bordetella* antigens, one could conclude that PT is not the most immunogenic structure of inactivated whole *B. pertussis* recognized *in vitro* by memory T cells from a donor who had whooping cough during childhood.

A new series of T cell clones was therefore generated from PBMC of the same R. R. donor employing PT as the antigen for *in vitro* stimulation. As shown in Table 2, T cell clones against PT can be obtained from an immune donor.

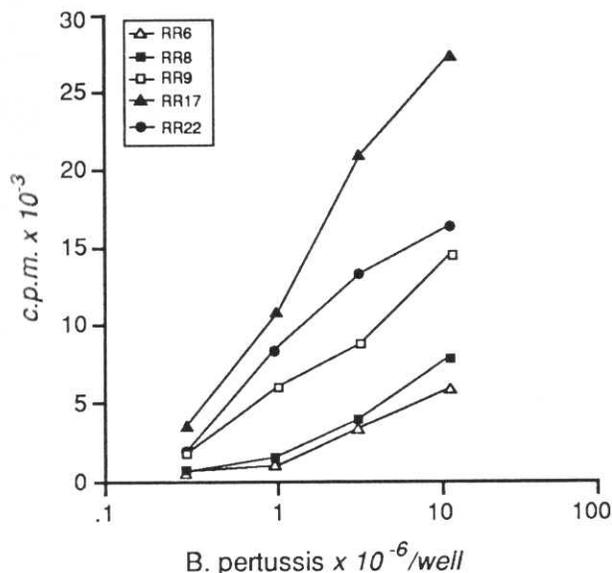


Fig. 1. - Specific proliferative response of T cell clones to killed *B. pertussis*.

Table 1. - Proliferation of human T cell clones against *B. pertussis* in response to *Bordetella* species and mutants

Cluster	Clone No.	Bacterial target									Prediction of the antigen recognized
		BP	BPP	BB phase I III	BP 353 (FHA')	BP 356 (S ₂)	BP 348 (AC, HLY)	BP 347 (vir)	<i>S. typhi</i>		
1	6	+	+	+	-	-	+	+	-	-	FHA
2	23, 32	+	-	-	-	+	+	+	-	-	<i>B. pertussis</i> -specific
3	7, 8 31, 34	+	+	+	-	+	+	+	-	-	Vir-regulated <i>Bordetella</i> specific
4	9, 17, 26, 28, 30	+	+	+	+	+	+	+	+	-	No-vir- regulated <i>Bordetella</i> specific

Table 2. - Response of human T cell clones to purified pertussis toxin (PT) and its recombinant subunits

Clone	Antigen					
	PT	S1	S2	S3	S4	S5
RR-T106	+	-	-	-	+	-
RR-T207	+	-	+	+	-	-
RR-T209	+	+	-	-	-	-
RR-T215	+	+	-	-	-	-
RR-T216	+	+	-	-	-	-
RR-T218	+	+	-	-	-	-
RR-T219	+	+	-	-	-	-
RR-T220	+	+	-	-	-	-
RR-T226	+	+	-	-	-	-
RR-T227	+	+	-	-	-	-
RR-T229	+	+	-	-	-	-

To identify the PT subunit recognized by each clone, *E. coli*-derived inclusion bodies, containing the five subunits as fusion proteins [3], were employed as antigens in proliferation assays. Results are shown in Table 2. Nine of the eleven clones (82%) were specific for S1, revealing an immunodominance of this subunit on the other. Of the other two clones one (T106) was directed to S4 and the other (T207) recognizes both S2 and S3 which have 67% omology [4]. None of the clones proliferated to S5. Therefore, we can conclude that indeed a T cell memory against PT exists in a donor who had suffered of pertussis. Furthermore, these data suggest that among the 5 subunits of PT,

the immunodominant one is S1, which is the enzymatically active moiety usually defined as oligomer A. This renders PT quite unique among bacterial toxins. In fact, for diphtheria and cholera toxins, the oligomer B is the most immunogenic.

On the basis of these results it can be suggested that the protective immunity generated by the inactivated whole-cell *B. pertussis* vaccine may be directed against bacterial antigens different from PT. Therefore, antigens such as FHA and 69,000 MW may be sufficient to induce protection against the disease if used in an acellular vaccine. However, this conclusion does not imply that a vaccine composed of only PT would not be efficient. Indeed, this has been the case when tested in an animal model [7], which is considered rather artificial but also predictive for vaccine effectiveness. A vaccine composed of PT or by its subunits, or even more simply by peptides derived from PT, may be considered to be completely innovative in comparison with the cellular vaccine, even in terms of the immune responses capable of evoking. In this case, the approach of analyzing T cell immunity at the clonal level may turn out to be a useful instrument for predicting vaccine effectiveness in volunteers receiving *B. pertussis* antigens. This issue is particularly relevant in view of the controversial interpretation raised by the field trial performed in Sweden with the Japanese acellular vaccine composed of soluble PT and FHA [10].

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