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Lymphokine Secretion and Cytotoxic Activity of Human CD4$^+$ T-Cell Clones against *Bordetella pertussis*

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Human CD4$^+$ T-cell clones specific for pertussis toxin and other *Bordetella pertussis* antigens have been tested for their cytotoxic activity, lymphokine production, and capacity to induce immunoglobulin synthesis. Clones specific for the S1 subunit of pertussis toxin were cytotoxic for autologous Epstein-Barr virus-transformed B cells, which had been pulsed with the native antigen, the recombinant S1 subunit of pertussis toxin, or synthetic peptides derived from the S1 amino acid sequence. The killing of antigen-pulsed target cells was class II restricted. All of the T-cell clones produced mostly interleukin-2 and gamma interferon and assisted allogeneic B cells in the production of immunoglobulins M and G but not immunoglobulin E. The potential in vivo role of the cytotoxic activity of these clones is discussed.

Traditionally, immunity against bacterial infections has been investigated by studying the humoral response to whole bacteria or individual antigens. Recent studies have shown that cellular immunity may also have a relevant role, especially in instances where bacteria invade eukaryotic cells. For instance, L3T4$^+$ T lymphocytes specific for *Listeria monocytogenes* have been shown to confer adoptive protection against listeriosis (19), whereas in mycobacteria both class I- and class II-restricted T cells have been involved in antibacterial immunity (16). Usually, class I-restricted T lymphocytes kill the cells that present the antigen on their surface, whereas class II-restricted T cells provide help to B lymphocytes for immunoglobulin synthesis. However, a number of exceptions to this rule have been already reported, and CD4$^+$ T cells with cytotoxic properties have been described (16, 18, 23, 30).

Bordetella pertussis, the causative agent of whooping cough, adheres to the cilia of the upper respiratory tract and multiplies locally in the extracellular space, where it releases a number of toxins that cause the local and systemic damages that lead to the disease (25). In vitro, *B. pertussis* has also been shown to invade eukaryotic cells (12, 20, 33). However, the in vivo role of this intracellular stage is unknown. Immunity to *B. pertussis* can be acquired after disease or vaccination with whole inactivated bacterial cells. Recently, vaccines containing purified antigens have been tested in animal models and clinical studies (25). These vaccines contain a chemically detoxified form of pertussis toxin (PT) and other antigens such as filamentous hemagglutinin, the 69-kDa outer membrane protein, and/or the fimbriae. The cellular pertussis vaccine has been classically used as an adjuvant for immunoglobulin E (IgE) induction (24), and an acellular vaccine has been shown to induce PT-specific IgE in vaccinated children. This adjuvant effect can be obtained also with purified PT (26).

The nature of the immunity against *B. pertussis* is not yet known. In spite of the fact that in animal models passive immunization with monoclonal antibodies (MAbs) specific for PT protects against infection with virulent bacteria (37), no correlation between anti-PT antibodies and protection was found during a recent clinical trial (1). With the aim of investigating the role of the cellular immunity in *B. pertussis* infection and IgE induction, we generated a number of T-cell clones against PT, the 69-kDa outer membrane protein, and filamentous hemagglutinin. All clones were CD4$^+$ CD8$^-$ and proliferated in a specific manner in response to *Bordetella* antigens presented by autologous antigen-presenting cells (APC) (9, 10). We studied lymphokine production and the helper and cytotoxic properties of these T-cell clones. Furthermore, we found that they were cytotoxic for APC but unable to induce IgE synthesis.

MATERIALS AND METHODS

Antigens. A nontoxic PT mutant, PT-9K/129G, was used as the antigen in this study. This mutant is enzymatically inactive but maintains intact all of the B- and T-cell epitopes of PT (27, 34, 35). PT and PT-9K/129G were purified from the culture supernatants of the wild-type strain *B. pertussis* W28 and the recombinant strain *B. pertussis* W28 expressing the double mutant PT-9K/129G by Affi-Gel blue absorption and successive column affinity chromatography with Fetuin-Sepharose (34). In the experiments with denaturated PT-9K/129G, the antigen was resuspended in phosphate-buffered saline and heat inactivated at 100°C for 45 min.

Subunit S1 from *B. pertussis* was expressed in *Escherichia coli* and fused to the NH_2-terminal 98 amino acids of the MS2 polymerase (28). The fusion protein was partially purified by lysing the bacteria and collecting the inclusion bodies and further purified by electroelution as described previously (4). Synthetic peptides 27 through 40 and 180 through 194 of subunit S1 were purchased from Novabiochem (Laufelfingen, Switzerland).

Media for cell stimulation and growth. The culture medium was RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with L-glutamine (2 mM), 1% nonessential amino acids, 1% sodium pyruvate, 50 μg of gentamicin per ml, 50 μM 2-mercaptoethanol, and 10% heat-inactivated pooled human type 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 of

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human recombinant interleukin-2 (IL-2; Hoffman-La Roche, Inc., Nutley, N.J.) per ml.

T-cell clones. T-cell clones specific for *B. pertussis* were obtained from peripheral blood of an immune donor with the HLA class II phenotype DR 1, 2, DR w16, DQ w1, DQ w5. The isolation and antigen specificity of the clones were described previously (9, 10). Clones of T lymphocytes were analyzed for cell surface phenotype by direct immunofluorescence on a fluorescence-activated cell sorter (FACStar; Becton Dickinson & Co., Erembodegem, Belgium). Phycoerythrin- and fluorescein-conjugated OKT3, OKT4, and OKT8 MAbs (Ortho Diagnostic Systems, Inc., Raritan, N.J.) were used.

Immortalization of B lymphocytes with EBV. Peripheral blood mononuclear cells from the immune donor were transformed with Epstein-Barr virus (EBV) as described previously (36). Briefly, 10^7 peripheral blood mononuclear cells were suspended in 10 ml of RPMI-FCS containing 30% of the supernatant of the EBV-producing marmoset cell line B95.8 and 600 ng of cyclosporin A (Sandoz, Basel, Switzerland) per ml; the peripheral blood mononuclear cells were then distributed in a flat-bottom 96-well plate at 5×10^4 cells per well. The EBV lines DOS, EDR, PE117, EBV1, EBV8 were kind gifts from Antonio Lanzavecchia (Basel Institute for Immunology, Basel, Switzerland).

Cytotoxicity assay. After overnight pulsing with antigen (5 μg/ml, unless otherwise specified), EBV-transformed target cells were pelleted, resuspended, and labeled with 100 μCi of sodium [^{51}Cr]chromate (Amersham International, Amersham, United Kingdom) for 1 h at 37°C. After three washes, target cells were added to 96-well round-bottom plates (Costar, Cambridge, Mass.) in 100 μl of medium at 10^4 cells per well in triplicate wells. Effector cells were then added in 100 μl at the indicated effector/target (E/T) cell ratios. The plates were incubated for 4 h and centrifuged, and cell supernatants were collected from each well. ^{51}Cr release was counted in a gamma counter. The percent specific lysis was calculated by using the following formula: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. For statistical analysis of the data, the Student *t* test was used. Differences were considered significant when *P* values were <0.05.

Inhibition of cytolytic activity with MAbs against major histocompatibility complex (MHC) molecules. The effector clones and B-cell targets (E/T ratio, 10:1) were incubated during the 10-h cytotoxicity assay with increasing concentrations (given as percentages [volume/volume]) of hybridoma supernatants containing MAb L243 (anti-HLA-DR), recognizing a nonpolymorphic HLA epitope present on all DR molecules, or W6/32 (anti-class I, A, B, C), kindly provided by Antonio Lanzavecchia.

Quantitation of IL-4, gamma interferon (IFN-γ), and IL-2. Viable T blasts of antigen-specific T-cell clones were extensively washed and incubated at 10^6 cells per ml in RPMI-FCS in the presence of phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO) (0.5 ng/ml) and anti-CD3 MAb (OKT3; Ortho Pharmaceutical Corp., Raritan, N.J.) (50 ng/ml) for 24 h or in the presence of mitomycin C (Sigma)-treated autologous EBV-transformed B cells and denatured PT for 12, 24, 36, 48, and 72 h at 37°C in 5% CO_2 humidified atmosphere. The cells were then centrifuged at 400 × *g* for 10 min; the culture supernatants were collected, filtered through a 0.22-μm-pore-size filter, and then stored in aliquots at −70°C until used. Since no significant changes in the levels of cytokines after different stimulation periods were found, in this report only the results at 72 h are presented.

TABLE 1. Antigen specificity of T-cell clones reactive to *B. pertussis*[a]

Clone	Antigen specificity
S223	aa 212–235 of S1 subunit of PT
S208	aa 30–41 of S1 subunit of PT
S106	aa 30–41 of S1 subunit of PT
S105	aa 180–194 of S1 subunit of PT
T215	aa 27–39 of S1 subunit of PT
R2F	Filamentous hemagglutinin
RR7	69-kDa outer membrane protein

[a] T-cell clones were obtained by in vitro stimulation of peripheral blood mononuclear cells from an immune donor with either purified PT or whole *B. pertussis*. aa, amino acids.

For the measurement of IFN-γ and IL-4 in the supernatants, the IMRX-γ IFN radioimmunoassay (Centocor Inc., Malvern, Pa.) and the Quantikine immunoassay (RD Systems, Minneapolis, Minn.), respectively, were used according to the manufacturers' instructions. IL-2 was determined by testing its effect on the proliferation of the IL-2-dependent T-cell line CTLL, as described elsewhere (22). Briefly, 4×10^3 CTLL cells were incubated in duplicate in a total volume of 200 μl with serial dilutions of T-cell clone supernatants in RPMI-FCS. Proliferation was assessed by the incorporation of 0.5 μCi of ^{3}H-labeled thymidine (Amersham) per well during the last 4 h of a 24-h incubation period. Values with 5 standard deviations over those of control supernatants were regarded as positive.

Measurement of immunoglobulin synthesis. Enriched B-cell suspensions from the peripheral blood of normal donors were prepared as described elsewhere (8, 22). They usually contained 50 to 70% B cells, 10 to 15% macrophages, and less than 1% T cells, as judged by cytofluorographic analysis with anti-CD20 (B1; Kontron, Zurich, Switzerland), anti-CD14 MAb (OKT14; Ortho), and anti-CD3 MAb (OKT3; Ortho), respectively. They will be referred as B lymphocytes for simplicity.

The culture system used for the induction of immunoglobulin synthesis was performed in duplicate tubes containing 4×10^5 B cells and 2×10^5 clonal T blasts that had been preactivated by PMA plus anti-CD3 alone or in the presence of 100 U of recombinant IL-4 (2×10^8 U/mg of protein; the kind gift of Glaxo, Verona, Italy) and 5 μg of anti-IFN-γ polyclonal antibody (Genzyme, Boston, Mass.) in 1 ml of RPMI-FCS. After 10 days of culture at 37°C in a 5% CO_2 humidified atmosphere, supernatants were collected and assayed for their immunoglobulin content. The radioimmunoassay procedure for the measurement of IgG, IgM, and IgE content has been reported in detail elsewhere (8, 22).

RESULTS

PT-dependent lysis of autologous EBV B-cell targets by T-cell clones. We have previously described T-cell clones specific for PT generated from peripheral blood of a donor who had suffered from whooping cough in his childhood (9). Most of these clones were specific for S1, one of the five subunits of PT that has been shown to be highly immunogenic in animal models (37). The fine specificity of such clones and of clones reactive to other *B. pertussis* antigens is shown in Table 1.

The cytotoxic activity of the clones specific for PT was

TABLE 2. Cytotoxic activity of human T-cell clones specific for PT[a]

Effector	Target pulsed with:	% Specific cytotoxicity at the following E/T cell ratios:		
		7/1	15/1	30/1
S106	No antigen	0.1	0.4	0.7
	PT-9K/129G (2.5 μg/ml)	21.6	37.3	58.6[b]
	PT-9K/129G (5 μg/ml)	27.0	47.4	67.9[b]
S223	No antigen	0.3	0.6	0.7
	PT-9K/129G (2.5 μg/ml)	36.9	51.4	72.6[b]
	PT-9K/129G (5 μg/ml)	47.7	60.9	70.1[b]
T215	No antigen	0.9	1.0	1.3
	PT-9K/129G (2.5 μg/ml)	26.2	39.7	52.3[b]
	PT-9K/129G (5 μg/ml)	38.8	54.7	71.5[b]
RR7	No antigen	0.1	0.8	0.9
	PT-9K/129G (2.5 μg/ml)	0.7	2.0	2.1
	PT-9K/129G (5 μg/ml)	1.6	0.6	1.4

[a] $CD4^+$ effector T cells were added to target cells at the indicated E/T ratios. Target cells were autologous EBV-B (10^4 cells per well) that had been pulsed with mutant PT-9K/129G for 18 h before the 4-h ^{51}Cr release assay.
[b] $P < 0.01$ versus the unstimulated control.

tested against autologous EBV-B cells pulsed overnight with various doses of the nontoxic PT mutant PT-9K/129G. All of the PT-reactive clones lysed target cells preincubated with PT-9K/129G, whereas no killing was observed with clone RR7, which is specific for the 69-kDa protein (Table 2). Moreover, there was a dose-dependent effect of the antigen on B-cell killing.

Killing of EBV-B cells preincubated with denatured PT-9K/129G or the S1 subunit. PT is a complex bacterial protein toxin composed of five subunits, designated S1, S2, S3, S4, and S5 (21, 29). The subunit S1 is an enzyme with ADP-ribosyltransferase activity on GTP-binding proteins located on the membranes of eukaryotic cells. The other subunits form the B oligomer, which binds to the receptors on the cell surface and translocates the S1 subunit across the membrane. Both PT and the B oligomer are mitogenic for T lymphocytes in vitro (27). The mutant PT-9K/129G is devoid of enzymatic activity but retains the mitogenicity of wild-type PT. Therefore, to exclude the possibility that the killing observed was a result of a nonspecific activation of effector T cells, we next performed a series of experiments in which denatured PT-9K/129G or the sole S1 subunit was used as the antigen for sensitization of target cells. The results of a representative experiment (Fig. 1) indicate that the T-cell clones were able to recognize and lyse targets preincubated with native PT-9K/129G and, to a lesser extent, those pulsed with the denatured antigen or S1 subunit. These findings indicate that the killing observed is not due to the mitogenic effect of native PT-9K/129G on T cells and is specifically directed to the S1 subunit.

Fine antigenic specificity of PT-reactive T cells. T lymphocytes recognize an antigen only after some type of metabolic processing by APC that presents denatured proteins or cleaved peptides in association with the relevant MHC molecules (15, 40). However, short peptides capable of associating directly with the MHC molecule on the surface of the APC may substitute these processed fragments in stimulating an antigen-specific immune response (6). Using this approach, we preincubated EBV-B target cells with two

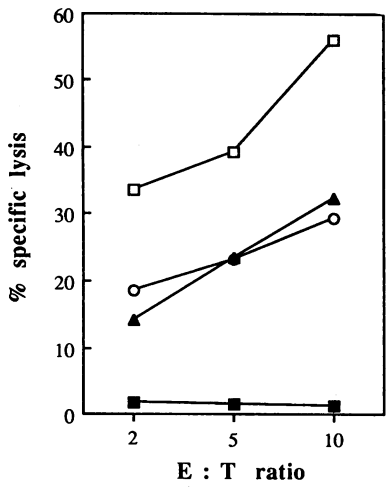

FIG. 1. Specific lysis of EBV-B target cells pulsed with no antigen (■), native (□) or denatured (○) PT-9K/129G, or the PT-9K/129G S1 subunit (▲) by the $CD4^+$ cytotoxic clone T215.

synthetic peptides homologous to the S1 subunit and then tested their susceptibility to killing by the clones T215 and S223. Clone T215 showed strong and specific cytotoxic activity when autologous EBV-B cells were preincubated with either PT-9K/129G or synthetic S1 peptide 27-40, whereas no killing was demonstrated with the unrelated peptide 180-194 (Table 3). In contrast, clone S223, which is specific for an epitope not present on the peptides tested, did not cause any lysis of target cells pulsed with the peptides. These results indicate that the killing of S1-sensitized target cells is highly specific, depending on the T-cell epitope recognized.

MHC restriction of antigen-specific T-cell clones. MAbs directed against the nonpolymorphic framework determinants of class I or class II-DR molecules were added to the wells during the assay. The anti-DR MAb significantly inhibited lysis of antigen-pulsed EBV-B cells (Table 4). Conversely, treatment with the anti-class I MAb did not cause inhibition of cytolysis but sometimes resulted in an increased level of cytotoxicity. These data indicate that the killing is class II DR restricted. It was therefore interesting to investigate whether the restriction element was DR1, as

TABLE 3. Peptide specificity of cytotoxic $CD4^+$ clones against the S1 subunit of PT[a]

T-cell clone	Antigen	% Specific lysis at the following E/T cell ratios:		
		2/1	5/1	10/1
T215	None	0.1	0.1	1.6
	PT-9K/129G	27.1	35.9	45.7[b]
	S1 peptide 27-40	18.2	36.7	44.1[b]
	S1 peptide 180-194	0.6	1.3	1.5
S223	None	1.1	1.3	1.6
	PT-9K/129G	13.1	16.1	22.1[b]
	S1 peptide 27-40	0.5	0.8	1.0
	S1 peptide 180-194	0.4	0.6	0.5

[a] Autologous EBV-B cells preincubated for 18 h with the mutant PT-9K/129G or synthetic S1 peptide were used as target cells in a 4-h ^{51}Cr release cytotoxicity assay.
[b] $P < 0.01$ versus the unstimulated control.

TABLE 4. Effect of anti-class I and anti-class II MAbs on lysis of PT-9K/129G-treated EBV-B cells by clone T215

% Specific lysis in the presence of MAbs[a]		
None	Anti-class I	Anti-class II-DR
62.0	55.3 (10)	44.0 (10)[b]
	61.4 (5)	40.3 (5)[b]
	63.3 (3)	45.0 (3)[b]

[a] The results presented indicate the percent release of ^{51}Cr above the background of spontaneous release in medium alone. The E/T cell ratio was 10:1. The numbers within parenthesis indicate the concentration (expressed as percent, volume/volume) of hybridoma supernatants containing MAbs.
[b] $P < 0.05$ versus the control.

previously shown in proliferation assay for the same clones (9, 10). Thus, allogeneic EBV-B cell lines with different DR specificities were then used as targets to define the DR allele involved in the cytotoxic recognition. The results of a representative experiment (Fig. 2) indicate that a marked antigen-dependent increase in cytotoxicity was seen only when target cells shared the HLA-DR allele with the effector. In fact, only the EBV-B lines DOS and EDR, which are homozygous for DR1, were recognized and lysed.

Cytokine production and helper activity by *B. pertussis*-specific T-cell clones. It has been recently shown that, as in the murine system (13, 38), human helper T (Th) lymphocytes can be divided into at least three different subsets (22). The first (Th1) is capable of producing IFN-γ, IL-2, and tumor necrosis factor; the second (Th2) produces IL-4, IL-5, and IL-6; and the third (Th0) produces IFN-γ, IL-4, and IL-2. Moreover, it has been found, in both human and murine systems, that IL-4 is essential for the induction of IgE synthesis, whereas IFN-γ exerts an inhibitory effect (7, 8, 32). Since PT has been reported to increase the levels of IgE in serum during *B. pertussis* infection (26) and PT vaccination induces PT-specific IgE (17), we investigated the pattern of lymphokine synthesis and immunoglobulin induction by T-cell clones specific for PT and other *B. pertussis* antigens. Interestingly, when a potent aspecific stimulus such as PMA plus anti-CD3 MAb was used, all of the clones were able to produce high amounts of IFN-γ and IL-2. Two clones (S105 and R2F) also produced significant levels of IL-4 (Table 5). However, when PT was used as a specific stimulus, IFN-γ, but neither IL-2 nor IL-4, was detected in the supernatant. Whether this is due to the lack of production of these cytokines after antigen stimulation or to consumption by T cells during proliferation remains to be established. In other experiments we have found that T-cell clones unable to secrete detectable amounts of IL-4 usually do not express IL-4 mRNA (31).

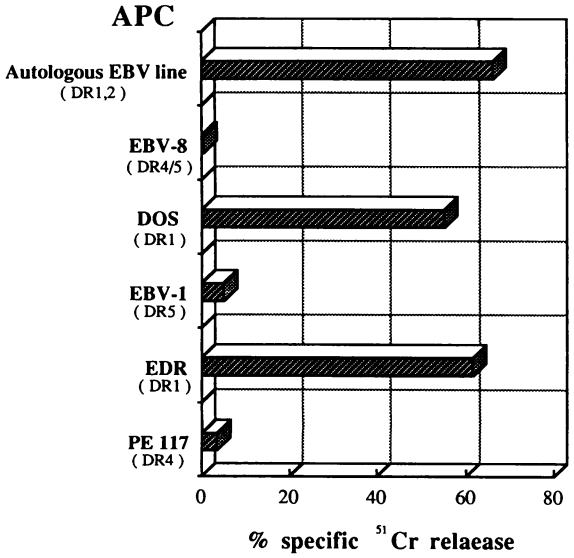

FIG. 2. DR1-restricted cytotoxic activity of the clone T215 to different EBV-B cells preincubated with 5 μg of PT-9K/129G per ml. The results are given as the percent specific lysis with an E/T ratio of 10:1. The values of cytotoxicity obtained with untreated target cells were less than 3%.

TABLE 5. Lymphokine production by T-cell clones

Stimulant and clone	IFN-γ (IU/ml)	IL-2 (U/ml)	IL-4 (ng/ml)
PMA + anti-CD3[a]			
S223	10	85	<0.02
S208	128	53	0.27
S106	46	70	0.23
S105	96	128	1.5
R2F	114	136	1.2
RR7	149	129	0.6
Antigen[b]			
S223	9	0.3	<0.02
S208	77	0.1	<0.02
S106	9	0.3	<0.02
S105	10	0.1	<0.02
R2F	ND[c]	ND	ND
RR7	ND	ND	ND

[a] Cells were stimulated with 50 ng of soluble anti-CD3 per ml and 0.5 ng of PMA per ml for 24 h.
[b] Clones specific for S1 subunit were stimulated with 3 μg of purified holotoxin per ml for 72 h. (Proliferation of the clones was also measured as a control of antigen stimulation.)
[c] ND, not determined.

Induction of immunoglobulin synthesis was investigated by culturing preactivated T cells with allogeneic B lymphocytes purified from the peripheral blood of two donors. As shown in Table 6, all clones provided help for IgM and IgG but were not able to induce IgE synthesis. Since the addition of exogenous IL-4 in the same experiment induced the synthesis of IgE (Table 6), we conclude that our clones are unable to provide help for IgE production. In the same experiment, a clone known to provide help for IgE synthesis, DP18 (31), induced IgE production even in the absence of exogenous IL-4 (Table 6). Taken together, these data indicate that human PT-specific T-cell clones behave like murine Th1 cells.

DISCUSSION

T lymphocytes are usually classified as cytotoxic or helper cells according to their function. In this report, we have shown that human $CD4^+$ T-cell clones specific for PT have both cytotoxic and helper properties in vitro. The finding that a single clone has both activities is not surprising, because the selective induction of cytotoxic or helper activity in T cells may be due to distinct activating signals (11). For instance, different APC and/or different environmental conditions may activate one of the two functions, depending on the type of immune response required (2, 11).

The ability to lyse APC is a property typical of class

TABLE 6. Induction of IgM, IgG, and IgE synthesis in allogeneic B cells by PMA plus anti-CD3-activated T-cell clones[a]

Clone	Immunoglobulin synthesis (ng/ml)					
	IgM		IgG		IgE	
	Expt 1 (20)[b]	Expt 2 (220)	Expt 1 (360)	Expt 2 (100)	Expt 1 (0.5)	Expt 2 (0.2)
S223	4,820	15,740	2,200	3,960	0.6 (16.1)[c]	0.2 (1.4)
S208	13,380	500	3,420	1,200	0.5 (4.5)	0.2 (0.5)
S106	2,400	21,000	320	4,680	0.4 (5.1)	0.2 (1.7)
S105	13,160	15,580	3,700	2,840	0.4 (5.8)	0.2 (5.6)
R2F	9,360	ND[d]	2,040	ND	0.6 (17.6)	ND
RR7	13,300	ND	3,260	ND	0.5 (5.6)	ND
DP18	6,970	2,180	2,150	2,100	5.9 (7.0)	0.9 (2.3)

[a] Allogeneic B cells (4×10^5) from two different donors were cultured in the presence of 2×10^5 T cells preactivated with PMA plus anti-CD3 MAb as described in Materials and Methods. T cell-induced immunoglobulin synthesis was calculated by subtracting the values obtained in parallel cultures containing cycloheximide (100 μg/ml). DP18 was a T-cell clone specific for *Dermatophagoides pteronyssinus* that showed a Th2 phenotype when stimulated with PMA plus anti-CD3 MAb (31).

[b] Numbers within parentheses in subheadings indicate the spontaneous immunoglobulin synthesis in allogeneic B-cell cultures in the absence of preactivated T-cell clones.

[c] Values within parentheses express IgE concentrations measured in parallel cultures carried out in the presence of IL-4 (100 U/ml) and anti IFN-γ polyclonal antibody (5 μg/ml).

[d] ND, not determined.

I-restricted $CD8^+$ T cells, and the relevance of class II-restricted CD4-mediated cytotoxicity is still a subject of discussion (5). A major argument concerns the question whether $CD4^+$ cytotoxic T lymphocytes are generated in vivo or acquire cytotoxic properties after long in vitro culture. Studies by Fleischer have shown that a large fraction of $CD4^+$ lymphocyte clones can acquire specific cytotoxicity during in vitro cultivation (14), whereas other evidence has demonstrated that in vivo-primed, freshly isolated $CD4^+$ T cells kill APC in an antigen-specific and MHC-restricted way (39, 41). Thus, CD4-mediated killing seems to not be restricted to in vitro long-term cultured cell lines but may be relevant in vivo as well.

Until recently, cell-mediated immunity against intracellular organisms was thought to depend on $CD4^+$ antigen-specific helper T lymphocytes, which, after activation, release lymphokines, such as IFN-γ, that are able to activate antimicrobial effector functions in macrophages (18). However, evidence obtained in several experimental systems has challenged this relatively simple scenario (18, 23, 30). In the human immune response to mycobacteria, it was found, for example, that mycobacterial antigens not only stimulate Th cells to produce macrophage-activating factors but also stimulate $CD4^+$ cytotoxic T lymphocytes, which lyse the human macrophages that harbor the pathogen (16). Furthermore, $CD4^+$ T-cell clones generated from lymphocytes infiltrating the liver of a patient with chronic hepatitis B showed strong and specific cytotoxicity against autologous B cells preincubated with hepatitis B surface antigen (3). These new findings have suggested that the classical concept of Th cell-mediated immunity needs to be reconsidered.

In the case of *B. pertussis*, class II-restricted cytotoxic T cells may play several roles. (i) They may cause downregulation of the specific immunity by killing the antigen-presenting B cells, which capture the antigen by membrane-associated immunoglobulins. This function has been suggested to justify in general the existence of class II-restricted cytotoxic T cells (5). (ii) They may kill human monocytes or other cells that bear intracellular *B. pertussis*. This mechanism may be important for the termination of latent *Bordetella* infections and the control of bacterial carriage. In fact, it has been suggested that *B. pertussis* survives intracellularly in a form that is resistant to normal immune defenses and antibiotics and that this form is responsible for intraepidemic carriage of *B. pertussis* (12, 20). (iii) Class II-restricted cytotoxic T cells may eliminate eukaryotic cells that have been intoxicated by PT and are no longer able to function properly.

Whether these clones are also involved in protection against disease remains an open question. In other systems, however, similar mechanisms have been shown to be relevant. For instance, a *Plasmodium bergei*-specific murine $CD4^+$ T-cell clone that displays cytotoxic activity and produces IFN-γ and IL-2 in vitro is able to confer protection in naive mice against sporozoite challenge (23). Although we can only speculate on the possible functions of the cytotoxic activity of the T-cell clones described herein, their helper phenotype provides important clues to the immune response against *B. pertussis*. They secrete IL-2 and IFN-γ and assist in the production of IgM and IgG, which are known to be important in immunity against whooping cough. Furthermore, we have shown that our PT-specific T-cell clones are unable to induce IgE synthesis in vitro. These data, together with our previous observation that enzymatically inactive mutants of PT are unable to induce an IgE-mediated anaphylaxis in mice (27, 34), suggest that the adjuvant effect of PT for IgE production is not due to PT-specific Th cells and therefore should be attributed to intrinsic properties of PT.

In conclusion all of these findings suggest that Th cell-mediated immunity may play a crucial role in in vivo infection with *B. pertussis*.

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