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Identification of Subregions of *Bordetella pertussis* Filamentous Hemagglutinin That Stimulate Human T-Cell Responses

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Filamentous hemagglutinin (FHA), a 220-kDa protein that mediates the adhesion of *Bordetella pertussis* to eukaryotic cells, is a component of acellular vaccines against whooping cough. To identify the subregions of FHA that are immunogenic for T cells, 16 human T-cell clones were raised against purified FHA and tested for the recognition of recombinant and proteolytic fragments. The clones were found to map either in the carboxy-terminal or the amino-terminal part of the FHA molecule, but none of them recognized the central region, which contains a sequence that is homologous to that of the eukaryotic protein fibronectin. These data suggest that subregions of FHA that do not contain sequences that are potentially cross-reactive with self proteins may be sufficient to induce an immune response against the whole protein.

Filamentous hemagglutinin (FHA) is a virulence factor expressed by *Bordetella pertussis*, the etiological agent of whooping cough (12). This protein has hemagglutinating activity and mediates adhesion of *B. pertussis* to eukaryotic cells in vitro and to ciliated cells of the upper respiratory tract during bacterial colonization (8, 9). Adhesion to macrophages in vitro is mediated by the Arg-Gly-Asp sequence, which is contained in a nonapeptide (sequences 1093 through 1101) that is homologous to the binding site of the eukaryotic adhesin fibronectin (8, 9). FHA is synthesized as a large precursor of 367 kDa which is cotranslationally or posttranslationally processed to give the mature polypeptide of 220 kDa, which is secreted into the culture medium (3). The purified 220-kDa fragment is subjected to further degradation upon storage: it is cleaved at a trypsinlike site (Arg-Arg-Ala-Arg-Arg) to give two fragments of 98 and 140 kDa that correspond to the amino-terminal and carboxy-terminal parts of the molecule, respectively (Fig. 1). The 140-kDa fragment can be further processed to a 125-kDa fragment or smaller fragments. Because of the possible role of FHA in pertussis pathogenesis and the protective activity of FHA shown in some animal models (4, 10), FHA has been included in new acellular pertussis vaccines together with detoxified pertussis toxin, the major virulence antigen of *B. pertussis* (6, 11). However, such a big molecule may not be needed in a vaccine, because fragments of it may be sufficient to induce an immune response against the whole protein. The identification of subregions of FHA containing B- and T-cell epitopes may be useful for the formulation of safer recombinant vaccines, which would avoid the risk of contaminating traces of active pertussis toxin in FHA preparations and solve problems of long storage. In addition, immunization with well-defined sequences of FHA would avoid the administration of a protein containing an epitope that is potentially cross-reactive with fibronectin, a host protein that may create problems of tolerance breakdown and autoimmunity.

We have previously demonstrated that T lymphocytes from adults with positive anamnesis of whooping cough respond to FHA and to other antigens when stimulated with inactivated *B. pertussis* phase I cells (2). Here we used purified FHA to stimulate peripheral blood mononuclear cells (PBMC) from two adults, one with positive anamnesis of whooping cough (donor 1) and another who received the cellular pertussis vaccine in childhood (donor 2). PBMC from these donors were cultured (10^5 PBMC per well in microtiter plates) for 6 days in the presence of purified FHA in RPMI medium containing 10% human serum. The proliferative response was measured by [^{3}H]thymidine incorporation during the last 18 h of culture. Figure 2 shows a strong proliferation with FHA at any antigen concentration, demonstrating that both donors have T lymphocytes that are specific for this antigen. We then cloned the T lymphocytes by limiting dilution and obtained 12 FHA-specific clones from donor 1 and 4 from donor 2. To identify the regions of FHA containing T-cell epitopes, we tested the clones in proliferation assays for recognition of three recombinant fragments (H1, H2, and H3 in Fig. 1) containing portions of FHA expressed in *Escherichia coli* as fusion proteins (3). In proliferation assays, T cells (2×10^4 per well in microtiter plates) were cultured for 3 days in the presence of the antigen and autologous antigen-presenting cells (2×10^4 mitomycin C-treated, Epstein Barr virus-transformed B lymphocytes per well) in RPMI medium containing 10% fetal calf serum. Cultures were pulsed with [^{3}H]thymidine for the last 18 h. The results of proliferation assays are reported in Table 1. Three different patterns of recognition of the fragments were found in the clones from donor 1. Four clones recognized both H2 and H3, and one clone recognized just H2. Seven clones recognized FHA but did not proliferate to any of the recombinant fragments; therefore they must be specific for epitopes outside of these regions. The clones from donor 2 showed two patterns of recognition: two clones were specific for H2 and two did not proliferate to any of the fragments. None of the 16 clones proliferated in the presence of H1, the fragment containing the Arg-Gly-Asp sequence.

After the subregions of FHA containing T-cell epitopes were identified, the problem was whether the polypeptides derived from the spontaneous proteolytic cleavage of FHA contained these epitopes. We therefore electroeluted from an 8% sodium dodecyl sulfate–polyacrylamide gel the fragments of 140, 125, and 98 kDa (7) and used them as antigens

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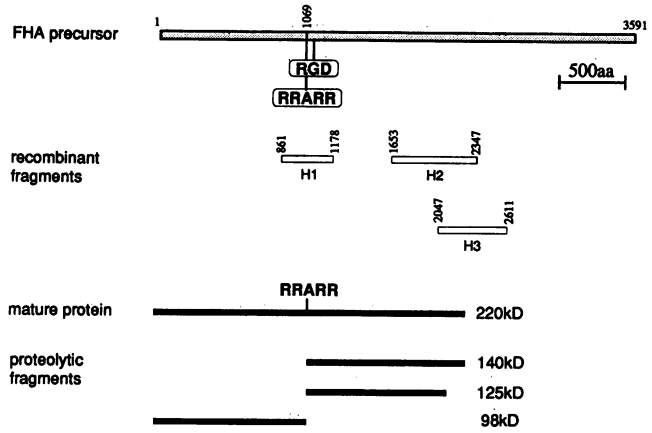

FIG. 1. Schematic representation of the FHA molecule. From top to bottom: FHA precursor, mature FHA, recombinant H1, H2, and H3 fragments, and proteolytic fragments of 140, 125, and 98 kDa generated from the mature FHA. Boxes contain the amino acid sequences of proteolytic site Arg-Arg-Ala-Arg-Arg and of the Arg-Gly-Asp tripeptide, indicated by the single-letter amino acid code.

in proliferation assays with the clones. Clones RR-F19, RR-F45, and DB-F10, which had recognized either H2 or both of the H2 and H3 recombinant fragments, preferentially recognized the 140- and 125-kDa fragments, as expected, whereas RR-F3, RR-F37, and DB-F11, which had not been previously mapped, recognized mainly the amino-terminal 98-kDa fragment (Table 2). However, for each clone, a lower but significant proliferation was observed also with the other electroeluted bands, because each band had a low contamination with proteolytic fragments deriving from other regions of the FHA molecule. The presence of these fragments was observed with Western blotting and could not be avoided even by careful electroelution, given the well-known susceptibility to degradation of the FHA molecule (3). To investigate the MHC class II restriction of the clones, monoclonal anti-DR, -DP, and -DQ antibodies were added in proliferation assays (1). Only the anti-DR monoclonal antibodies inhibited the response of clones RR-F2, DB-F10, and DB-F11 to FHA (Fig. 3), indicating that the clones recognize the antigen in the context of DR molecules.

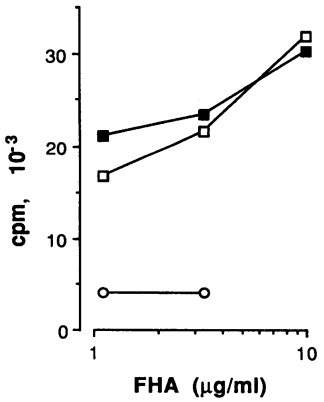

FIG. 2. Proliferative response of PBMC from donor 1 (■), donor 2 (□), and a nonimmune person (○) to the purified 220-kDa form of FHA. Background proliferation values in the absence of antigen were 1.0×10^{-3} and 0.2×10^{-3} for donors 1 and 2, respectively.

TABLE 1. Recognition of FHA recombinant fragments by T-cell clones

Donor and clone	Recognition of antigen (mean cpm [10^{-3}])[a]				
	None[b]	FHA	H1	H2	H3
Donor 1					
RR-F2	0.2	**4.5**	0.2	**11.7**	**6.8**
RR-F3	0.2	**9.1**	0.2	0.2	0.2
RR-F12	0.2	**13.6**	0.2	0.2	0.2
RR-F19	0.5	**14.1**	0.3	**6.5**	**7.1**
RR-F21	0.5	**11.2**	0.5	0.5	0.4
RR-F33	0.5	**20.1**	0.4	0.4	0.4
RR-F37	0.4	**8.9**	0.4	0.4	0.3
RR-F43	0.5	**12.4**	0.5	**8.8**	0.6
RR-F44	0.2	**4.8**	0.2	**7.1**	**8.9**
RR-F45	0.6	**11.8**	0.4	**13.8**	**16.5**
RR-F46	0.4	**8.5**	0.4	0.5	0.4
RR-F47	0.5	**4.0**	0.4	0.5	0.5
Donor 2					
DB-F6	0.7	**6.4**	0.8	1.0	0.7
DB-F9	0.9	**6.1**	0.9	**12.8**	0.9
DB-F10	0.8	**11.0**	0.7	**7.8**	0.7
DB-F11	1.0	**13.9**	0.9	0.8	0.8

[a] Results are from duplicate cultures. Values in boldface type are significantly ($P < 0.05$; Student t test) above background levels.
[b] Background proliferation without antigen.

We conclude that 7 out of 16 T-cell clones recognize the H2 recombinant fragment. Of these, four recognize also the overlapping H3 fragment and therefore map in the region of positions 2047 through 2347, whereas three map in the amino-terminal part of H2 at positions 1653 through 2046. We analyzed the sequence of H2 with the algorithm by Margalit et al., which predicts the presence of T-cell epitopes on the basis of the amphipathic helix model (5). We found that there are several segments with good probabilities of forming amphipathic alpha helixes and therefore to become T-cell antigenic sites, but there are two with probabilities as high as 72 and 80% in sequences 1798 through 1835 and 2033 through 2058, respectively (data not shown). The latter segment is located partly in the region where H2 and H3 overlap. The clones that do not recognize any of the

TABLE 2. Recognition of FHA proteolytic fragments by T-cell clones

Donor and clone	Recognition of proteolytic fragments (mean cpm [10^{-3}])[a]			
	None[b]	140 kDa	125 kDa	98 kDa
Donor 1				
RR-F3	0.5	3.2	6.7	**14.5**
RR-F19	0.5	**6.5**	**8.5**	3.1
RR-F37	0.4	4.2	4.9	**12.1**
RR-F45	0.3	**12.8**	**12.1**	5.1
Donor 2				
DB-F10	1.8	**15.2**	**17.4**	5.2
DB-F11	0.6	3.5	3.8	**8.1**

[a] Results are from duplicate cultures. All clones showed a significant ($P < 0.05$; Student t test) response to the three fragments, but each showed a preferential proliferation to some of them. Values in boldface type indicate for each clone responses that were at least twofold higher than the lowest proliferation value observed. None of the clones showed a proliferation when an unrelevant protein (PT9K/129G) was added in the control wells.
[b] Background proliferation without antigen.

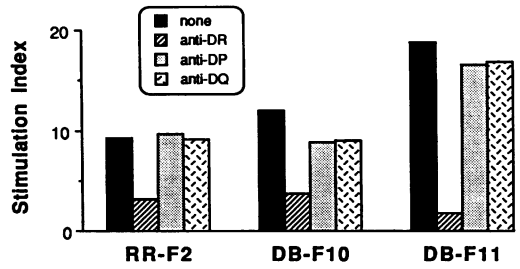

FIG. 3. Inhibition of proliferative response to FHA by anti-class II monoclonal antibodies. The ability of anti-class II antibodies to inhibit the proliferation of T-cell clones was tested by incubating the clones with homologous antigen-presenting cells, the antigen, and anti-DR, anti-DP, and anti-DQ antibodies. The figure shows that the clones are DR restricted, since stimulation is inhibited only by anti-DR monoclonal antibodies.

recombinant fragments may map either within positions 1 through 860 or within positions 1179 through 1652, which were not covered by the recombinant fragments. However, when three of these clones were tested with the proteolytic fragments, they were found to proliferate in the presence of the 98-kDa fragment. This indicates that they map within positions 1 through 860, which is the region covered by the 98-kDa fragment but not overlapping H1. Finally, none of the T-cell clones from either donor recognized the recombinant fragment H1 (positions 861 through 1178), which contains the Arg-Gly-Asp tripeptide. Although the number of clones analyzed is not high, it could be hypothesized that these two donors do not have in their repertoire T cells with specificity for the sequence containing Arg-Gly-Asp.

The regions of FHA that we have identified as containing T-cell epitopes could be better defined by the synthesis of peptides homologous to amphipathic segments. Furthermore, the construction of several recombinant fragments covering sequences 1 through 860 and 1179 through 1652 may help to identify the other regions of FHA recognized by T lymphocytes. All of the relevant fragments of FHA should be then analyzed for the presence of B-cell epitopes, which, together with T-cell epitopes, may induce an immune response against the whole FHA when included in a vaccine.

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