INFECTION AND IMMUNITY, May 1994, p. 1830–1834
0019-9567/94/$04.00+0
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Vol. 62, No. 5

Formaldehyde Treatment of Proteins Can Constrain Presentation to T Cells by Limiting Antigen Processing

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Received 29 November 1993/Returned for modification 1 January 1994/Accepted 16 February 1994

Proteins to be used as vaccines are frequently treated with formaldehyde, although little is known about the effects of this treatment on protein antigenicity. To investigate the effect of formaldehyde treatment on antigen recognition by T cells, we compared the in vitro T-cell response to proteins that have been formaldehyde treated with the response to untreated proteins. We found that peripheral blood mononuclear cells from individuals vaccinated with three formaldehyde-treated proteins (pertussis toxin, filamentous hemagglutinin, pertactin) of *Bordetella pertussis* showed little or no response to the formaldehyde-treated proteins but proliferated very well in response to the corresponding untreated protein. These findings were further confirmed with CD4$^+$ T-cell clones specific for defined epitopes of the bacterial proteins. We found that some epitopes are presented poorly or not at all when formaldehyde-treated proteins are used, whereas other epitopes are equally presented to T-cell clones when either formaldehyde-treated or untreated antigens are used. However, T-cell recognition could be restored by either antigen degradation before formaldehyde treatment or heat denaturation after such treatment. Parallel digestion with trypsin of both formaldehyde-treated and untreated proteins showed that fragments generated from the two forms of the same antigen were different in size. These results demonstrate that formaldehyde treatment can constrain antigen presentation to T cells and that this may be due to an altered proteolytic processing of formaldehyde-treated proteins.

Formaldehyde treatment is a process widely used in vaccine preparation to stabilize protein components or to inactivate toxin molecules such as diphtheria, pertussis, or tetanus (14, 15). Formaldehyde reacts with ε-amino groups of lysines to give an unstable product that can then react with a second amino group to form a stable methylene bridge. These reactions can occur either between amino acids of the same molecule, resulting in internal cross-linking of the protein, or between two molecules, resulting in dimerization (14).

Generally, formaldehyde-treated vaccines have been proven very effective in inducing protective antibody responses, but there is little information about the effects of this treatment on antigen recognition by T cells. Since T cells recognize antigens as peptides bound to major hystocompatibility complex (MHC) molecules (17), in principle, formaldehyde treatment could affect antigen presentation by interfering with either (i) proteolytic degradation into peptides, (ii) peptide binding to MHC, or (ii) T-cell receptor recognition of the peptide-MHC complex.

In this study, we investigated the effect of formaldehyde treatment on presentation of three proteins of *Bordetella pertussis* (pertussis toxin [PT], filamentous hemagglutinin [FHA], and pertactin [69K]) to T cells. We studied antigen-specific responses of peripheral blood mononuclear cells (PBMC) from both naturally infected individuals whose T cells have been primed by "native" bacterial proteins (5) and healthy individuals whose T cells have been primed by a whooping cough vaccine (13). Furthermore, we used CD4$^+$ T-cell clones raised against non-formaldehyde-treated FHA, 69K, or PT which are specific for defined epitopes of the three proteins (4–6).

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Here we report that formaldehyde treatment constrains presentation of some epitopes to T cells in vitro and show evidence that this may depend on a different proteolytic processing of the formaldehyde-treated proteins compared with native proteins.

MATERIALS AND METHODS

Antigens. The genetically detoxified PT mutant PT-9K/129G and the FHA protein were purified from the culture supernatant fluid and the 69K protein was purified from the cell paste of recombinant strain *B. pertussis* W28-9K/129G (11, 13). Before formaldehyde treatment, the three proteins were dialyzed for 24 h at 4°C against phosphate-buffered saline (PBS; pH 7.4) containing 0.025 M lysine and 0.01% thimerosal. Formaldehyde was added to FHA (0.4 mg/ml) at a 0.14% final concentration and to 69K (0.7 mg/ml) at a 0.24% final concentration. PT-9K/129G was treated with various concentrations of formaldehyde (0.02 to 7%) in order to obtain a molecule devoid of mitogenicity for T lymphocytes, a feature of the mutant PT-9K/129G as well as of the wild-type PT (10). The sample treated with 3.5% formaldehyde was then chosen for this study because it was nonmitogenic for PBMC from a nonimmune donor. After formaldehyde treatment, the three proteins were incubated at 37°C for 48 h and dialyzed exhaustively against PBS. In some cases, formaldehyde-treated proteins were heat denatured at 95°C for 20 min. In order to measure the antigen-specific T-cell response to the non-formaldehyde-treated PT-9K/129G, this molecule was heat denatured (95°C for 45 min) to get rid of its mitogenic effect.

Cell cultures. The culture medium was RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% human serum (RPMI-HS). For the growth of T-cell lines

and clones, RPMI-HS was supplemented with 50 U of recombinant interleukin-2 (Cetus, Emeryville, Calif.) per ml.

PBMC proliferation assay. Blood from two healthy adult volunteers was collected 30 days after they had received the first injection of the acellular pertussis vaccine Acelluvax (Biocine-Sclavo, Siena, Italy), containing formaldehyde-treated PT-9K/129G, FHA, and 69K (13). PBMCs were separated by Ficoll-Hypaque density gradient centrifugation and frozen. For the proliferation assays, thawed PBMC (10^5) in 0.2 ml of RPMI-HS were cultured in 96-well flat-bottom microplates in the presence or absence of bacterial proteins in duplicate wells. After 5 days, 0.5 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom) was added in each well, and DNA-incorporated radioactivity was measured after an additional 16 h by liquid scintillation counting.

Establishment of T-cell clones. MHC class II-restricted $CD4^+$ T-cell clones specific for PT, FHA, and 69K were previously obtained from one individual naturally infected with *B. pertussis* (4–6); other $CD4^+$ T-cell clones specific for PT were obtained from three vaccinees receiving an acellular pertussis vaccine containing PT-9K/129G (3, 12). Briefly, PBMC (10^5) were cultured in 0.2 ml of RPMI-HS in 96-well flat-bottom microplates in the presence of 9 μg of bacterial proteins per ml. After 7 days, recombinant interleukin-2 (30 U/ml) was added, and after an additional 15 days, T-cell lines were cloned by limiting dilution (0.3 cell per well) in the presence of irradiated (3,000 rads) allogenic PBMC (5×10^5 per ml), phytohemagglutinin (1 μg/ml; Wellcome, Dartford, United Kingdom), and recombinant interleukin-2 (100 U/ml) in 20-μl cultures in Terasaki trays. The T-cell clones obtained were screened for the capacity to proliferate in response to the bacterial protein used as a stimulator. For this, T cells (2×10^4) were cultured with mytomicin-treated Epstein-Barr virus-transformed B cells (2×10^4) in 0.2 ml of RPMI-fetal calf serum in 96-well flat-bottom microplates in triplicate wells in the presence of various concentrations of the antigen. After 2 days, 0.5 μCi of [^{3}H]thymidine was added, and the radioactivity incorporated was measured after an additional 16 h by liquid scintillation counting. Epstein-Barr virus-transformed B-cell lines were obtained as described previously (5).

Proteolytic digestion of 69K. The three different forms of 69K (untreated, formaldehyde treated, and formaldehyde treated and boiled) were first dialyzed against a buffer containing 50 mM Tris (pH 7.4), 0.1 M urea, and 2 mM $CaCl_2$. The protein samples were incubated with trypsin (Boehringer, Mannheim, Germany) at protein/trypsin ratios (wt/wt) of 1:0.3 (untreated), 1:1 (formaldehyde treated), and 1:0.005 (formaldehyde treated and boiled) at 37°C for 30 min. Optimal protein/trypsin ratios were determined in preliminary experiments aimed at finding comparable degrees of digestion of the different antigen forms. A denaturing solution containing 2-mercaptoethanol and sodium dodecyl sulfate (SDS) was then added to stop the reaction, and the samples were electrophoresed on an SDS–10% polyacrylamide gel (PAGE) according to the method of Laemmli (8). Western blot (immunoblot) was performed as described before (16), using polyclonal anti-69K mouse antibodies.

RESULTS

T cells specific for *B. pertussis* proteins fail to recognize the corresponding formaldehyde-treated antigens. While assessing the T-cell response of an individual who had been infected by *B. pertussis*, we found that his PBMC proliferated in response to the purified or recombinant proteins PT, FHA, and 69K but showed little or no response to the corresponding formaldehyde-treated proteins (data not shown). This observation prompted us to reinvestigate the T-cell response in healthy individuals 1 month after they had been immunized with the first dose of a whooping cough vaccine composed of formaldehyde-treated PT 9K/129G, FHA, and 69K proteins in combination with aluminum hydroxide as adjuvant (13).

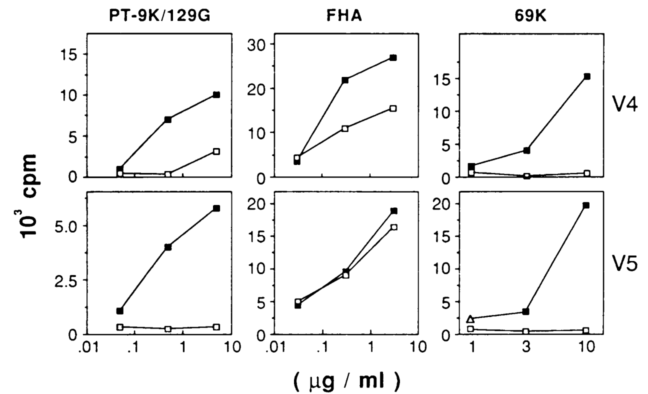

FIG. 1. Formaldehyde-treated proteins are poorly antigenic for PBMC that recognize the corresponding untreated proteins. Shown is the proliferation of PBMC from two individuals (V4 and V5), vaccinated with formaldehyde-treated PT 9K/129G, FHA, and 69K, in response to various concentrations of untreated (closed squares) or formaldehyde-treated (open squares) proteins.

Figure 1 shows that PMBC from two vaccinees also respond very poorly to the formaldehyde-treated proteins, while they proliferate very well in response to the untreated proteins. This finding was rather intriguing, since, as already reported (13), T cells from vaccinees were primed in vivo by formaldehyde-treated proteins. It must be noted that in all experiments shown here the FHA and 69K antigens were treated with a concentration of formaldehyde identical to that used for vaccine preparation, whereas for the PT antigen we used a higher concentration of formaldehyde to avoid a PT mitogenic effect.

Formaldehyde treatment limits presentation of some epitopes to human T-cell clones. Since the PBMC proliferative response to a given antigen is usually polyclonal, we tried to elucidate further the inhibitory effect of formaldehyde treatment by using $CD4^+$ T-cell clones established with non-formaldehyde-treated protein as antigens and specific for defined epitopes of FHA, 69K, and PT. T-cell clones specific for PT have been obtained from PBMC of three vaccinated (3) and one naturally infected (4) individual. Clones specific for FHA and 69K were obtained from PBMC of one naturally infected individual (5, 6).

Figure 2 shows proliferation experiments with a panel of representative T-cell clones specific for different epitopes of the three proteins and with different abilities to recognize formaldehyde-treated antigens presented by autologous Epstein-Barr virus-transformed B cells. Indeed, three patterns can be identified: (i) T-cell clones that recognize formaldehyde-treated and untreated antigens equally well; (ii) T-cell clones showing a proliferation that is significantly lower with the formaldehyde-treated than with the untreated antigen; and (iii) T-cell clones that proliferate in response to the untreated form of the protein but that do not respond at all to the formaldehyde-treated antigen. Interestingly, in the case of diminished T-cell response to formaldehyde-treated antigens,

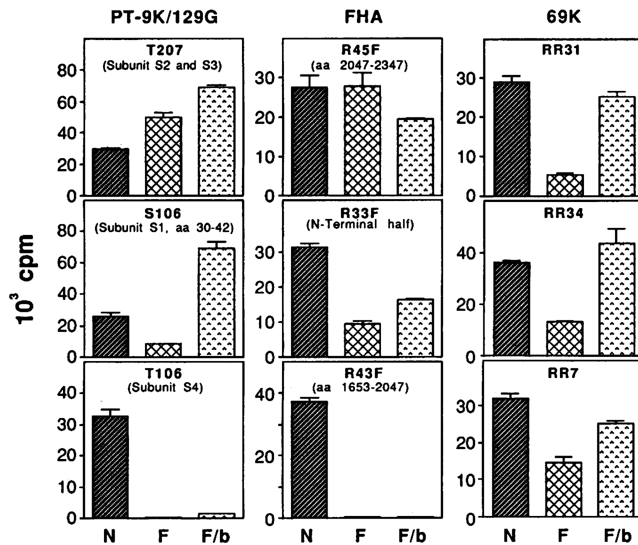

FIG. 2. The inhibitory effect of formaldehyde depends on the epitope presented to T cells. Shown is the proliferation of $CD4^+$ T-cell clones specific for different epitopes of the same protein in response to 3 μg of untreated (N), formaldehyde-treated (F), or formaldehyde-treated and boiled (F/b) proteins per ml. Numbers in the panels indicate clone number and the epitope recognized. aa, amino acids.

treatment of the proteins with increasing concentrations of formaldehyde causes a parallel decrease in T-cell proliferation, demonstrating that the constraint imposed by the formaldehyde is dose dependent (data not shown).

Figure 2 also shows that, in the case of diminished T-cell response to formaldehyde-treated antigens, proliferation was somewhat reestablished when the formaldehyde-treated antigens were boiled. In contrast, when the T-cell response to formaldehyde-treated proteins was absent, the boiled proteins also were not recognized at all by the T-cell clones. Altogether, these data demonstrate that the inhibitory effect of formaldehyde can be both qualitative and quantitative depending on the epitope recognized by T cells, and it is not related to the uptake of the antigen. Similar results were obtained with several MHC class II-matched Epstein-Barr virus-transformed B-cell lines as antigen-presenting cells (data not shown).

Furthermore, we have found that the T-cell response to formaldehyde-treated 69K is not restored in the presence of various concentrations (1 to 5%) of immune human serum (from a vaccinee) containing high titers of anti-69K antibodies (data not shown). This demonstrates that immune complexes do not play a critical role in T-cell recognition of epitopes that are poorly presented in the presence of a formaldehyde-treated antigen.

Formaldehyde treatment constrains antigen processing. We then asked whether the constraint on protein recognition by T cells imposed by formaldehyde treatment was at the level of antigen processing. We took advantage of the partial degradation that FHA spontaneously undergoes in vitro (7) to treat with formaldehyde a protein that is partially preprocessed (Fig. 3) and asked whether it would be recognized better by T cells. Figure 4 shows that T cells, which poorly recognize formaldehyde-treated FHA, proliferate equally well in response to untreated FHA and to a spontaneously degraded FHA that is treated with formaldehyde. This demonstrates that T-cell recognition of formaldehyde-treated proteins is improved by partial degradation before the treatment and suggests that the constraint imposed by formaldehyde is probably at the level of antigen processing.

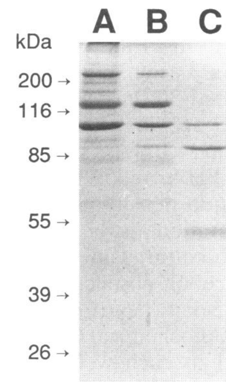

FIG. 3. Formaldehyde treatment of native and preprocessed FHA. Shown are the results of SDS-PAGE of formaldehyde-treated native FHA (lane A); formaldehyde-treated, preprocessed FHA (lane B); and untreated, preprocessed FHA (lane C).

To prove an effect on antigen processing formally, we investigated the protease sensitivity of untreated and formaldehyde-treated proteins. Thus, different forms of the 69K protein were digested with trypsin. The Western blot in Fig. 5 shows that trypsin treatment of the 69K protein generates fragments of different sizes from untreated, formaldehyde-treated, or formaldehyde-treated and boiled proteins. Furthermore, to obtain comparable degrees of protein digestion, we had to use 3-fold more trypsin with the formaldehyde-treated 69K than with the untreated protein, but for the formaldehyde-treated 69K that had been boiled, we used 1,000-fold less trypsin. This experiment demonstrates that formaldehyde treatment alters protease sensitivity and suggests that different epitopes might be generated by intracellular processing of different forms of the same protein.

DISCUSSION

In the present study, we have found that some $CD4^+$ T cells specific for *B. pertussis* proteins (PT, FHA, and 69K) fail to recognize the same proteins treated with formaldehyde. The inhibitory effect of formaldehyde can be qualitative or quantitative, depending on the epitopes recognized by T cells.

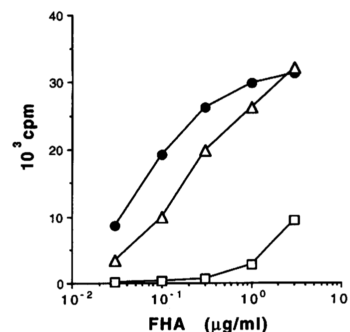

FIG. 4. Processing before formaldehyde treatment restores presentation to T cells. Shown is the proliferation of FHA-specific T-cell clone R33F in response to various concentrations of untreated (circles), formaldehyde-treated (squares), or degraded and formaldehyde-treated (triangles) FHA.

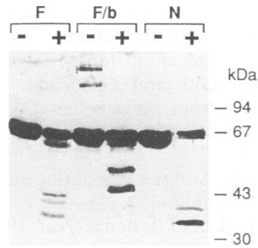

FIG. 5. Formaldehyde treatment affects proteolytic digestion. Shown are the results of a Western blot of undigested (−) or trypsin-digested (+), formaldehyde-treated (F), formaldehyde-treated and boiled (F/b), and untreated (N) 69K protein.

Indeed, some epitopes are not recognized at all, some epitopes are equally recognized, and other epitopes are partially recognized. With regard to this point, it has to be stressed that our previous data on T-cell clone recognition of formaldehyde-treated PT (10) had been obtained by using a formaldehyde-treated protein that had been boiled to avoid possible mitogenic effects. Indeed, also in the present work we found that boiling could restore a T-cell response.

The fact that methylene bridges induced by formaldehyde treatment (14) influence T-cell responses to proteins parallels the data obtained with other amino acid modifications, such as N-glycosylation (1), alkylation (18), and iodination (2), which have been shown to influence antigen presentation to T cells. In principle, the presence of methylene bridges in the proteins treated with formaldehyde could alter either (i) antigen processing, (ii) peptide-MHC association, or (iii) T-cell receptor recognition of the peptide-MHC complex. In an attempt to distinguish among these possibilities, we treated with formaldehyde a partially degraded (i.e., preprocessed) protein (FHA) and found that T-cell clones that are otherwise unable to proliferate in response to formaldehyde-treated FHA recognize the degraded and then formaldehyde-treated protein. Most important, we have demonstrated that the formaldehyde treatment changes protein sensitivity to protease activity. Indeed, trypsin digestion of the purified and the formaldehyde-treated 69K protein generates fragments of different sizes. Therefore, we favor the possibility that the main constraint imposed by formaldehyde treatment is at the level of antigen processing, although we cannot rule out the other two hypotheses, i.e., interference with either peptide-MHC association or T-cell receptor recognition of the peptide-MHC complex.

In the light of these findings, it is not surprising that some T cells from naturally infected individuals respond to purified proteins but do not recognize the formaldehyde-treated forms, since those T cells have been primed in vivo by antigen-presenting cells that have processed "native" bacterial proteins. Conversely, it was intriguing to observe also that some T cells from individuals immunized with a whooping cough vaccine respond to purified proteins but do not recognize formaldehyde-treated proteins, since those T cells have been primed in vivo by formaldehyde-treated antigens. Remarkably, T-cell responses were not restored even when experiments were performed in the presence of sera (from vaccinated individuals) with high titers of specific antibodies induced by the formaldehyde-treated proteins. This allows us to rule out a role for immune complexes in modifying antigen processing of formaldehyde-treated antigens. Yet, to reconcile these apparently contradictory results, it is tempting to speculate that, at least for the generation of some epitopes, there must be a difference in the processing of the formaldehyde-treated antigens in vivo versus that in vitro. This could be due to the activity of extracellular proteases induced by the inflammation (9) occurring at the immunization site or to a partial denaturation induced by the absorption of the protein on the aluminum hydroxide used as adjuvant. Alternatively, "professional" antigen-presenting cells such as dendritic cells could be responsible for a more effective processing of formaldehyde-treated proteins in vivo, which allows generation of T-cell epitopes similar to those from the untreated proteins.

Finally, our data provide evidence that one of the most common treatments, i.e., formaldehyde detoxification, used in vaccine preparation can limit the generation of some T-cell epitopes by constraining antigen processing. Therefore, formaldehyde-treated antigens should not be used to test in vitro the efficacy of T-cell responses primed by formaldehyde-treated vaccines. Future experiments in animal models will aim at both clarifying the processing of formaldehyde-treated antigens in vivo and investigating whether this could influence the T-cell repertoire primed and expanded in vivo.

ACKNOWLEDGMENTS

We thank C. Pennatini for performing trypsin digestion of the 69K protein and Giorgio Corsi for artwork.

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