INFECTION AND IMMUNITY, Feb. 1991, p. 625–630
0019-9567/91/020625-06$02.00/0

Vol. 59, No. 2

Properties of Pertussis Toxin Mutant PT-9K/129G after Formaldehyde Treatment

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Received 12 July 1990/Accepted 26 November 1990

Formaldehyde treatment is a method routinely used to detoxify diphtheria, tetanus, and pertussis toxins as well as other molecules suitable for vaccine production. To investigate whether chemical detoxification alters the immunological properties of vaccine components, we have treated the pertussis toxin mutant PT-9K/129G with formaldehyde and tested the properties of the resulting molecules. Very low concentrations of formaldehyde stabilize the molecule without affecting the physicochemical and immunological parameters. Increasing doses of formaldehyde abolish the mitogenic and hemagglutinating activities of PT-9K/129G. At the same time, the molecule loses the ability to be recognized by a monoclonal antibody specific for a major protective epitope on the S1 subunit of pertussis toxin and its affinity for anti-pertussis toxin polyclonal antibodies is also reduced. In marked contrast, the ability of PT-9K/129G to be recognized by human T-cell clones is not affected by Formalin treatment. In vivo, the formaldehyde-treated molecules induce amounts of specific antibodies comparable with those of untreated molecules but significantly lower levels of toxin-neutralizing antibodies. Furthermore, the formaldehyde-treated molecules also show a reduced protective activity in the intracerebral challenge assay.

Formaldehyde treatment was introduced in 1923 (4, 14) to detoxify diphtheria toxin for vaccine use. Since then, this method has been successfully used for inactivation of tetanus, diphtheria, and pertussis toxins (PT) and preparation of many bacterial and viral vaccines. Such vaccines have proved to be very efficacious, and their use has been instrumental in the eradication of several diseases. One of the few drawbacks of chemical treatment is that chemically inactivated toxoids can revert to toxicity (18); therefore, each vaccine lot has to undergo extensive testing before use. With the advent of biotechnology, molecular genetics has provided new tools to inactivate toxins for vaccine use. In fact, it is now possible to modify one or more codons in their genes and obtain naturally nontoxic molecules which do not need chemical treatment and have no risk of reversion to toxicity (12). We have recently constructed genetically detoxified PT mutants and tested them in preclinical (10, 12) and phase I clinical (13) studies as potential vaccines against whooping cough. PT is a protein composed of five different subunits. Subunit S1 (A protomer), which is responsible for toxicity, is an enzyme with ADP-ribosyltransferase activity. Subunits S2, S3, S4, and S5 form the nontoxic B oligomer which binds to receptors on the surface of eucaryotic cells. The nontoxic mutant (PT-9K/129G) used in this study is enzymatically inactive and devoid of any toxic activity but maintains the ability to bind to receptors on eucaryotic cells.

Not only are genetically inactivated molecules safer theoretically in that there is no possibility of subsequent reversion to toxicity but results obtained so far have shown that they may also be superior immunogens compared with chemically detoxified molecules (6). In fact, even when using doses of the genetically engineered vaccine which are lower than those previously described for chemically detoxified molecules (16, 17, 20), we have obtained higher levels of toxin-neutralizing antibodies in adult volunteers (13). This observation raised the possibility that chemical treatment may modify the antigenic properties of these proteins and decrease their immunogenicity. To test this hypothesis, we have treated mutant PT-9K/129G with increasing doses of formaldehyde to test how the physicochemical and antigenic properties vary.

MATERIALS AND METHODS

Preparation of PT, PT-9K/129G, and B oligomer. PT, PT-9K/129G, and the B oligomer of PT were obtained and purified from the culture supernatants of wild-type strain *Bordetella pertussis* W28, recombinant strain *B. pertussis* W28-9K/129G expressing the double S1 mutation Arg-9→Lys and Glu-129→Gly (10, 12), and recombinant strain *B. pertussis* W28-8D/9G engineered to secrete only the B oligomer (11), respectively.

Formaldehyde treatment of PT-9K/129G. Purified PT-9K/129G was dialyzed for 24 h at 4°C against phosphate-buffered saline (PBS) (pH 7.4) containing 0.025 M lysine (Ajinomoto, Tokyo, Japan) and 0.01% (wt/vol) thimerosal. After protein determination (8), various percentages (wt/vol) of formaldehyde (0.035 [F1], 0.042 [F2], 0.052 [F3], 0.070 [F4], 0.105 [F5], 0.140 [F6], 0.210 [F7], and 0.420 [F8]) were added to several aliquots of dialyzed PT-9K/129G (F0) preparations. The final protein/Formalin ratios (wt/wt) were 0.3, 0.25, 0.2, 0.15, 0.1, 0.075, 0.05, and 0.025, respectively. The mixtures were incubated at 37°C for 48 h and dialyzed exhaustively against saline (0.9% NaCl). The final preparations were again analyzed for protein content (8), and their patterns were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (7) and Western blot (immunoblot) analysis (11).

Hemagglutination. The hemagglutination assay was performed as previously described (15) by using glutaraldehyde-fixed chicken erythrocytes as target cells. Results were expressed as doses of hemagglutinin causing complete agglutination of erythrocytes.

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Radioimmunoassay. The affinity constant was determined by competitive radioimmunoassay in microtiter plates coated either with monoclonal antibody 1B7 or with goat immunoglobulins against PT (9, 10). The antibodies were used at a concentration of 10 μg/ml in a 5 mM glycine buffer (pH 9.2). After the plates were coated overnight at 4°C, they were saturated with 2.5% (wt/vol) bovine serum albumin in PBS (pH 8.0) and washed with PBS containing 0.125 ml of Tween 20 per liter. The plates, which contained twofold dilutions of PT and the Formalin-treated PT-9K/129G preparations to be tested, were then incubated with ^{125}I-labeled PT (10^5 counts per well).

After 3 h at room temperature, the plates were extensively washed, the wells were cut out, and the bound radioactivity was counted in a gamma counter. Each sample was analyzed in duplicate. PT was iodinated by the standard chloramine T procedure. The data were analyzed by a nonlinear regression analysis, and the affinity constant was calculated as previously described (9).

Proliferation assays. The mitogenic activities of PT-9K/129G and its Formalin-treated analog were assayed as the induction of proliferative response of human peripheral blood mononuclear cells from healthy adult donors. Briefly, Ficoll-Hypaque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) separated peripheral blood mononuclear cells were plated in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, Mass.) at 10^5 cells per well in 0.2 ml of RPMI 1640 (GIBCO Laboratories, Paisley, Scotland) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, and 10% human AB serum. Formalin-treated PT-9K/129G preparations were then added at final concentrations of 0.3 and 1.0 μg/ml. After 96 h of incubation, [^{3}H]thymidine (1 μCi; specific activity, 185 GBq/mmol; Amersham International, Amersham, United Kingdom) was added to the wells, which were harvested 16 to 18 h later on fiberglass filters with a cell harvester (Skatron, Lier, Norway). Mapping of T epitopes on PT-9K/129G treated with 0.035 (F1), 0.070 (F4), 0.140 (F6), and 0.42% (wt/vol) (F8) formaldehyde was performed by using S1-specific human CD4-positive T-cell clones (1, 2). The clones tested were T215, S106, S105, and S223, which recognize S1 peptides 27-39, 30-41, 180-194, and 212-235, respectively (1). The T-cell recognition assay was performed essentially as previously described (1, 2). Briefly, T-cell clones were incubated at 2×10^4 cells per well for 48 h in the presence of 2×10^4 mitomycin C-treated, Epstein-Barr virus-transformed autologous B lymphocytes and antigens in RPMI 1640 plus fetal calf serum in 96-well flat-bottomed plates. [^{3}H]thymidine (1 μCi) (Amersham) was then added to the wells, and after an additional 18 h, cultures were harvested on fiberglass filters with a cell harvester (Skatron). The incorporated radioactivity was determined by liquid scintillation. Results were expressed as the mean counts per minute of duplicate cultures.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as previously described (10). Briefly, wells of flat-bottomed polystyrene microtest plates (Costar) were coated with PBS (pH 7.4) containing 10 μg of purified PT or purified B oligomer and incubated for 2 h at 37°C and overnight at 4°C in a humidified chamber. After incubation of fivefold dilutions of guinea pig serum samples, specific alkaline phosphatase-conjugated immunoglobulin G antibodies (Miles, Yeda, Israel) were added to the wells. PBS (100 μl) (pH 7.4) was used in all steps, and incubations were performed at 37°C for 2 h. Three washings of the plates with PBS containing 0.05% Tween 20 and 0.02% NaN_3 were carried out between each incubation.

The enzyme substrate reaction which developed at room temperature after the addition of 1 mg of *p*-nitrophenylphosphate per ml in 1 M diethanolamine (pH 9.8) containing 0.5 mM $MgCl_2$ was measured at 405 mm in a Titertek Multiskan (Flow Laboratories, McLean, Va.). Antitoxin antibody levels were expressed as geometric mean absorbance values of undiluted sera (ABS-MAX) from guinea pigs tested individually.

Toxin neutralization. The ability of the antibodies raised in guinea pigs to neutralize the clustering effect of PT on Chinese hamster ovary (CHO) cells was evaluated essentially as previously described (3, 5, 10). Briefly, sera from guinea pigs obtained after vaccination with one or two doses of Formalin-treated preparations of PT-9K/129G were directly diluted in the wells of flat-bottomed microplates (Costar) in 25 μl of Dulbecco modified Eagle medium (DMEM; Flow Laboratories). Purified wild-type PT (120 pg) in 25 μl of DMEM was added to each well, and the plates were incubated for 3 h at 37°C. After the incubation period, 0.2 ml of DMEM containing 10^4 CHO cells, previously treated with 1 mg of trypsin per ml, was added to each well and incubated for 48 h at 37°C in 5% CO_2 atmosphere. As a positive control, the clustering effect of PT alone was determined in each plate. Neutralizing titers were expressed as the reciprocal of the highest serum dilution causing complete inhibition of the clustering activity induced by the wild-type toxin.

Immunogenicity. Groups of six white guinea pigs were inoculated intradermally with 3 or 25 μg of the Formalin-treated PT-9K/129G preparations adsorbed onto aluminium hydroxide (0.5 mg/dose). Four weeks later, the animals were bled and given booster doses identical to the doses given at the first immunization. Two weeks after the booster doses, sera were collected and tested by ELISA for antitoxin titers and toxin-neutralizing activity in the CHO cell assay.

Vaccine potency. Groups of 16 male CD1 mice were vaccinated intraperitoneally according to the World Health Organization recommendations (19) with 0.5 ml of diphtheria-pertussis-tetanus vaccine containing PT-9K/129G preparations treated with 0.035 (F1), 0.070 (F4), and 0.420% (F8) formaldehyde as the pertussis component. Fourteen days later, mice were injected intracerebrally with 300 median lethal doses deriving from a suspension of virulent *B. pertussis* 18323 (Sclavo, Siena, Italy). Mice were observed for 14 days and deaths were recorded.

RESULTS

Formaldehyde treatment of PT-9K/129G. Eight samples, each containing 5 mg of PT-9K/129G, were treated with doses of formaldehyde ranging from 0.035 to 0.420% (wt/vol) for 48 h at 37°C as described in Materials and Methods and summarized in Table 1. Five micrograms of each sample was then loaded onto a polyacrylamide gel to test the effect of chemical treatment on the SDS-PAGE profile of PT-9K/129G. Treatment with increasing doses of formaldehyde proportionally changed the natural electrophoretic pattern (Fig. 1a). The overall pattern, composed of subunits S1 (A protomer) and S2, S3, S4, and S5 (B oligomer), was not changed by low doses of formaldehyde. However, bands with molecular weight higher than that of S1, which by Western blot analysis were shown to be composed mainly of multimers of the B-oligomer subunits, were already visible at the lower Formalin dose and increased in intensity at higher

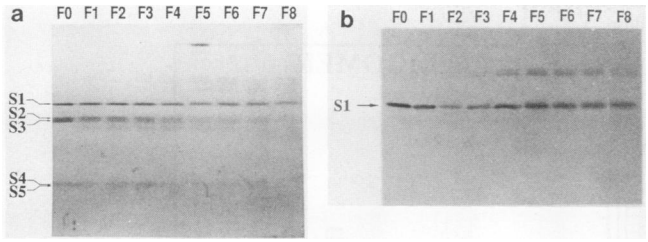

FIG. 1. (a) Electrophoretic pattern of PT-9K/129G not treated (lane F0) and treated with increasing doses (0.035 to 0.420%) of formaldehyde (lanes F1 through F8). The positions of bands corresponding to the five subunits are indicated to the left of the gel. (b) Western blot of the SDS gel shown in Fig. 1a, using monoclonal antibody 1B7 against the S1 subunit.

doses (results not shown). At Formalin concentrations above 0.105%, most of the B oligomer is present in high-molecular-weight form, and the individual bands are no longer detectable. Interestingly, the band corresponding to the S1 subunit, although decreasing in intensity, never disappears, indicating that this subunit is not cross-linked by formaldehyde as efficiently as the subunits of the B oligomer.

In vitro properties of Formalin-treated molecules. Table 1 summarizes some of the in vitro properties of the samples treated with different doses of formaldehyde. Two typical properties of PT-9K/129G, such as mitogenicity for T cells and hemagglutinating activity, are not affected by low doses of Formalin; however, they are progressively decreased by intermediate doses and totally abolished by doses above 0.105%. Similarly, the affinity of PT-9K/129G for toxin-neutralizing polyclonal antibodies was decreased up to 2 orders of magnitude by chemical treatment. Interestingly, the protective epitope recognized by the monoclonal antibody 1B7 in radioimmunoassay was not available for antibody binding even after 0.052% Formalin treatment (Table 1), while in a Western blot analysis, the S1 subunit was recognized by monoclonal antibody 1B7 in all Formalin-treated samples, even if to a lower extent (Table 1 and Fig. 1b). The alteration of the B-cell epitopes caused by the Formalin treatment raised the question of whether the T-cell epitopes of PT-9K/129G are also affected. The samples were therefore tested for recognition by four human T-cell clones

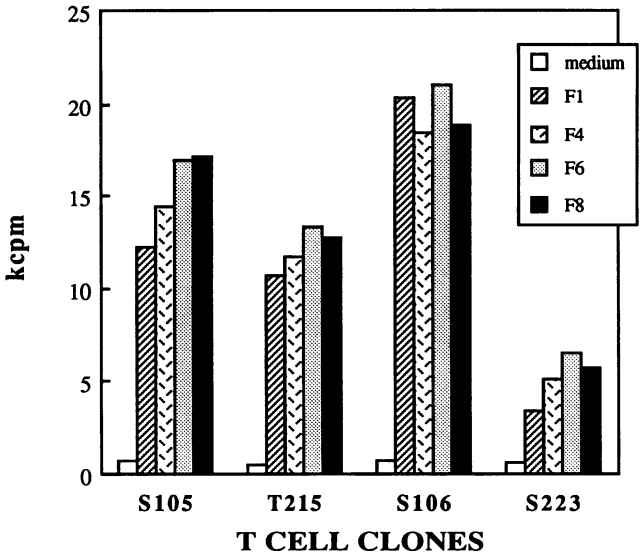

FIG. 2. Response of human T-cell clones specific for amino- and carboxy-terminal epitopes of the wild-type S1 subunit to various Formalin-treated preparations of PT-9K/129G.

specific for the S1 subunit. The results (Fig. 2) demonstrate that the T-cell epitopes were not affected by chemical treatment.

Immunogenicity. To evaluate the effect of formaldehyde on the immunogenicity of PT-9K/129G, groups of six guinea pigs were immunized with 3 or 25 μg of the samples treated with 0.035 (F1), 0.042 (F2), 0.070 (F4), and 0.42% (F8) formaldehyde and adsorbed onto 0.5 mg of aluminum hydroxide. Four weeks later, the guinea pigs received a booster dose. Blood samples were taken 2 weeks later. Sera were tested by ELISA for the antitoxin levels against wild-type PT and B oligomer. The total amounts of anti-PT antibodies induced by F1, F2, F4, and F8 preparations were not affected by Formalin treatment (Fig. 3). In fact, after the first immunization, all sera showed the same level of anti-PT antibodies as F0, which increased after the second injection. However, while antibodies obtained from samples treated with low doses of formaldehyde recognized PT and to a

TABLE 1. Effect of formaldehyde treatment on in vitro properties of the genetically detoxified pertussis toxin mutant PT-9K/129G

Vaccine	% Formaldehyde (wt/vol)	PT/F[a]	Mitogenicity[b] of PT-9K/129G		HA titer[c] (μg/well)	Affinity constant[d]		Binding[e] to 1B7
			1 μg	0.3 μg		Gamma globulins	1B7	
F0			23.0	12.0	0.5–1	1.15×10^9	5.54×10^7	+++
F1	0.035	0.300	43.6	15.4	4	1.61×10^9	7.4×10^7	+++
F2	0.042	0.250	30.1	10.1	4	1.67×10^9	5.04×10^7	+++
F3	0.052	0.200	29.6	10.7	4	8.25×10^9	—[g]	++
F4	0.070	0.150	11.8	2.1	4	ND[f]	—	++
F5	0.105	0.100	4.7	0.9	9	1.85×10^8	—	++
F6	0.140	0.075	2.6	0.6	>10	1.03×10^8	—	++
F7	0.210	0.050	2.0	0.3	>10	5.60×10^7	—	+
F8	0.420	0.025	0.5	0.6	>10	6.75×10^7	—	+

[a] Protein-to-formaldehyde ratio (wt/wt).
[b] Results are expressed as the mean counts per minute (10^3) of duplicate cultures.
[c] Hemagglutination titer, expressed as the dose of hemagglutinin causing complete agglutination of glutaraldehyde-fixed chicken erythrocytes.
[d] The affinity constant was determined by competitive radioimmunoassay of microtiter plates coated either with monoclonal antibody 1B7 or goat gamma globulins against PT. The affinity constant was evaluated by a nonlinear regression analysis of experimental data and expressed as liters per mole.
[e] The binding to monoclonal antibody 1B7 was detected by Western blot analysis. +++, Strong binding; ++, medium binding; +, weak binding.
[f] ND, Not determined.
[g] —, Values were below detectable levels.

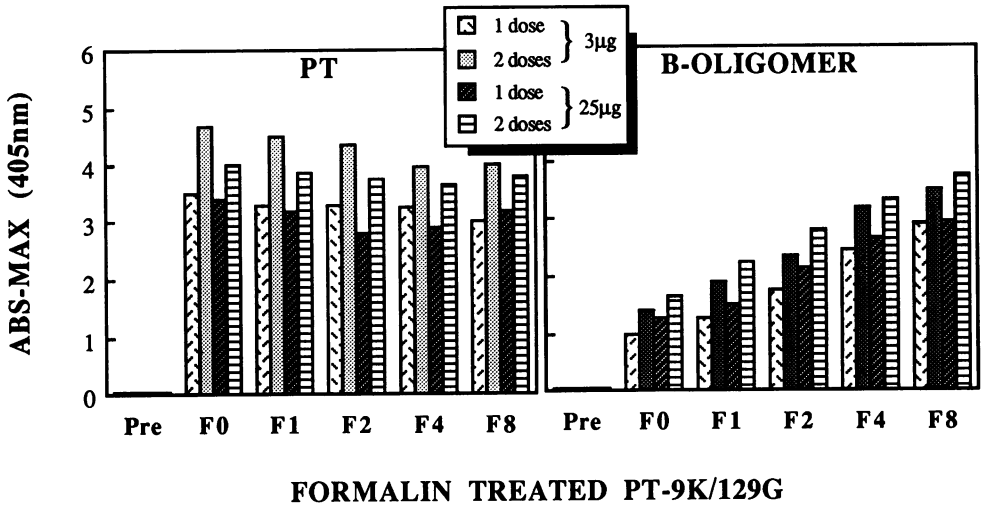

FIG. 3. ELISA levels of anti-PT and anti-B oligomer antibodies raised in guinea pigs injected with of 3 or 25 µg of PT-9K/129G treated with different doses of formaldehyde. Antitoxin antibody levels are expressed as geometric mean absorbance values (ABS-MAX) of undiluted sera.

lower extent the B oligomer (Fig. 3), the antibodies obtained from samples treated with high doses of formaldehyde recognized the B oligomer and PT at the same level. Interestingly, the change in specificity of the antibodies correlated with a decrease in the toxin-neutralizing titers of the antibodies obtained; samples treated with low doses of formaldehyde had neutralizing titers above 1/1,280, whereas samples treated with high doses of formaldehyde were unable to neutralize the toxin activity at dilutions above 1/160 (Fig. 4). We conclude that while the total amount of antibodies is not affected by Formalin treatment, the fine specificity and the properties of the antibodies obtained are altered.

Vaccine potency. We next studied the ability of some Formalin-treated preparations of PT-9K/129G to protect mice from a lethal intracerebral infection with virulent *B. pertussis*. The vaccine formulations were composed of PT-9K/129G not treated (F0) or treated with 0.035 (F1), 0.070 (F4), or 0.420% (F8) (wt/vol) formaldehyde, combined with diphtheria and tetanus toxoids and adsorbed onto 2 mg of aluminum hydroxide. Mice were immunized intraperitoneally with various dilutions of vaccine, and 2 weeks later, immunizations were followed by intracerebral challenge with the virulent strain *B. pertussis* 18323. The results reported in Table 2 show that low doses of formaldehyde (F1) did not affect the protective activity of PT-9K/129G, while intermediate (F4) and high (F8) doses of formaldehyde reduced it significantly.

DISCUSSION

Formaldehyde is usually used to detoxify toxins and inactivate viruses for vaccine purposes. Since these native molecules are too toxic or dangerous to be used without Formalin treatment, so far studies on the effect of Formalin treatment on the immunological properties of antigens have not been possible. The availability of mutants of PT made nontoxic by genetic manipulation (12) gave us the unique

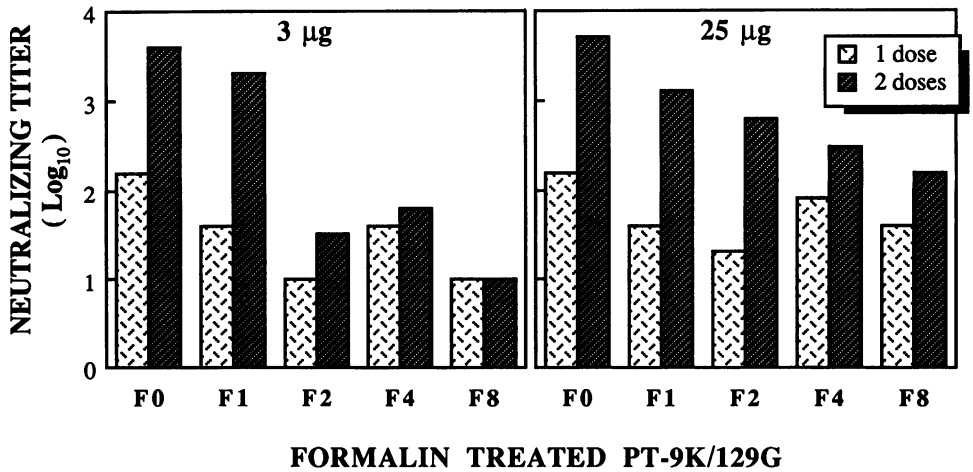

FIG. 4. Neutralizing titers of antibodies raised in guinea pigs by one or two injections of 3 and 25 µg of PT-9K/129G treated with increasing doses of formaldehyde. Neutralizing titers are expressed as reciprocals of the highest serum dilutions resulting in 100% inhibition.

TABLE 2. Mouse protective activity (intracerebral challenge)

Vaccine	Dose (μg)	No. of survivors/ total no. tested
F0	15	13/16
	5	11/16
	1.7	6/16
F1	15	12/16
	5	13/16
	1.7	5/16
F4	15	8/16
	5	8/16
	1.7	3/16
F8	15	2/16
	5	1/16
	1.7	1/16

opportunity to immunize animals with the native antigens and therefore ascertain whether there is a difference in the immune responses to native and Formalin-treated antigens. We have previously shown that a mild formaldehyde treatment (0.035% [wt/vol]) stabilizes the nontoxic PT mutant PT-9K/129G but does not change its biological and immunological properties (10). In this study, we have treated the same molecule with increasing concentrations of formaldehyde (up to 0.420% [wt/vol]) and we have studied how the biological, physicochemical, and immunological properties of the antigen are modified. It should be noted that the most severe treatment used in this study (0.420% formaldehyde for 48 h at 37°C) is still below the conventional treatment used, for instance, for diphtheria toxin, which is stored for several weeks at 37°C in the presence of 0.750% formaldehyde.

We have shown that Formalin treatment alters in a dose-dependent fashion the electrophoretic pattern, biological properties such as the mitogenic and the hemagglutinating activities, and also the affinity of the molecule for antibodies raised against the native toxin (10). An interesting finding has been the observation that after Formalin treatment of the holotoxin, the immunodominant protective epitope located in the S1 subunit and recognized by many monoclonal antibodies becomes unavailable for antibody binding. However, this epitope is likely to be masked by the subunits of the B oligomer, since it can be detected in a Western blot, when the S1 subunit is no longer complexed with the other subunits (Fig. 1b). In marked contrast, the T-cell epitopes also located in the S1 subunit are not affected by Formalin treatment. These observations correlate well with the immunogenic properties of the molecules. In fact, the immune system was equally stimulated by native or Formalin-treated antigens, and the total amount of antibodies produced and presumably cellular immunity were not affected by Formalin treatment. However, the specificity and quality of the antibodies produced changed; samples treated with higher Formalin concentrations produced predominantly anti-B oligomer antibodies which were unable to neutralize the native toxin in vitro. The protective activity of the antigen in the intracerebral challenge assay was also decreased in a dose-dependent fashion by treating the molecule with formaldehyde. These observations show that Formalin treatment does not affect the immunogenicity of antigens but changes the quality of the immune response which is induced. In particular, the natural B-cell epitopes present in the molecules are affected by Formalin treatment, and therefore high doses of antigens may be required in order to induce protective immunity with Formalin-treated antigens. As a consequence, the immune system produces many antibodies directed against nonprotective epitopes (or with lower affinity for the protective epitopes) and the cellular immunity is overstimulated. Both conditions may favor the appearance of untoward reactions of the Arthus type or delayed-type hypersensitivity. We conclude that classical Formalin detoxification, although very useful, changes the immunogenicity of antigens and therefore, when possible, genetic manipulations should be used to produce safe immunogens with unchanged antigenic conformation compared with the native protein. On the other hand, treatment of antigens with low doses of formaldehyde (such as sample F1) stabilizes the molecule without changing its physical or immunological parameters (10). Such treatment can be very useful for vaccine preparations.

ACKNOWLEDGMENTS

We thank Franco Giovannoni for animal studies, Sclavo Quality Control Center for some of the in vitro tests, Catherine Mallia and Antonella Mori for secretarial assistance, and Giorgio Corsi and Fabio Gambassi for graphic work.

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