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Immunogenic Correlation between Cross-Reacting Material (CRM197) Produced by a Mutant of *Corynebacterium diphtheriae* and Diphtheria Toxoid

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The in vivo immunizing potency of diphtheria toxoid and formalin-treated cross-reacting material (CRM197, a nontoxic mutant protein) was compared in guinea pigs. Major antigenic differences between the two untreated proteins were also tested in rats. The results showed that diphtheria toxoid and CRM197 were equally effective immunogens, but only if the latter was treated with formalin in the same concentration (0.7% vol/vol) as that of the toxoid. Formalin treatment rendered the antigens more resistant to enzymatic proteolysis by trypsin in vitro.

Uchida et al. [1] have reported the isolation of a series of corynephage β mutants that direct the synthesis of nontoxic proteins which are serologically related to diphtheria toxin. One of the cross-reacting materials, CRM197, was found to be serologically identical to and to be the same molecular size as diphtheria toxin (62,000 daltons). The absence of toxicity was due to the lack of adenosine diphosphoribosyl transferase activity of fragment A (22,000-dalton polypeptide) of CRM197 [2]. On the other hand, fragment B (40,000 daltons) appeared to be unaltered and has been shown to compete with toxin for surface receptors on sensitive eukaryotic cells [3].

In an immunologic study of these proteins, Pappenheimer et al. [3] showed that rabbit antisera to CRM197 contained antibodies to different diphtherial determinants and that these antibodies had poor avidity for toxin, in spite of the fact that CRM197 was serologically identical to diphtheria toxin. At least 90% of the antibodies precipitable in vitro by diphtheria toxin were also precipitable by fragment A. Thus, antisera to CRM197 contained antibodies primarily to antigenic determinants located on fragment A. Pappenheimer et al. [3] also showed that rabbit antisera obtained by immunization with purified fragment A precipitated poorly with the pure toxin.

On the other hand, antisera from rabbits immunized with CRM197 that had been treated with 0.2% formalin contained antibodies that were avidly recognized by diphtheria toxin, as shown by in vitro precipitation tests. None of these antibodies was to fragment A.

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One can reasonably deduce that formalin has a stabilizing effect by preventing proteolysis of CRM197, and this stabilization probably explains the greater immunizing effect of the diphtheria toxoid. In this communication we have shown that there is good correlation in the immune response obtained in guinea pigs immunized with formalin-treated CRM197 and diphtheria toxoid. We have also compared the immunogenic difference between CRM197 and diphtheria toxin when these antigens were injected into rats. The immune response of rats to these two antigens was identical to that of guinea pigs. In vitro proteolysis of untreated and formalin-treated antigens strongly suggested that the formalinization process produced diphtheria toxoid and CRM197 that were more resistant to enzymatic hydrolysis.

Materials and Methods

Strain. *Corynebacterium diphtheriae* strains C7(β197) and C7($-$)tox^- were supplied by Dr. A. M. Pappenheimer, Jr. (Biological Laboratories, Harvard University, Cambridge, Mass.).

CY growth medium. Ten grams of casamino acids (Difco Laboratories, Detroit, Mich.), 20 g of yeast extract (Difco), and 5 g of KH_2PO_4 were dissolved in 1 liter of distilled water. After the addition of 50% $CaCl_2 \cdot 2H_2O$, the pH was brought to 7.4, and the solution was boiled and filtered. Then 2 ml of solution II and 1 ml of Mueller and Miller's solution III [4] were added; 100-ml aliquots were placed in 1-liter Erlenmeyer flasks and autoclaved at 115 C for 20 min.

Production of CRM197. C7(β197) was seeded onto a plate of Loeffler's medium (75% [vol/vol] beef serum, 25% [vol/vol] nutrient broth, and 1.5% [wt/vol] glucose, pH 6.5; coagulation of the

medium was effected by heating for 3 hr at 85 C). After overnight incubation at 35 C, a single colony was used to inoculate Erlenmeyer flasks containing CY broth and 2% maltose without iron until a concentration of $\sim 5 \times 10^7$ organisms/ml was reached. The flasks were incubated at 35 C in a rotary shaker at 200 rpm for about 18 hr and then seeded in a 7-liter fermenter. The fermentation conditions were an air flow of 2 liters/min, agitation at 500 rpm with the pH automatically maintained at a constant value of 7.4, a Po_2 of 2.5%, and a growth period of 48 hr. The bacteria were then sedimented by centrifugation at 13,000 *g* for 20 min, and 0.01% thimerosal was added to the supernatant as preservative. The final yield was 15 Lf (limit flocculation units)/ml, with a protein concentration of 23.5 mg/ml.

Diphtheria toxoid. A partially purified diphtheria toxoid containing 500 Lf/ml and 4.36 mg of protein/ml, originating from the normal production of vaccines for human use, was used (lot no. 1/S; ISVT Sclavo, Siena, Italy), produced from *C. diphtheriae* PW 8, strain CN 2000.

Formalin-treated CRM197. The crude CRM197 solution (15 Lf/ml) was diafiltered and concentrated to 850 Lf/ml by pressure ultrafiltration with an Amicon filter and a Diaflo XM-50 membrane (Amicon Corp., Lexington, Mass.). It was then dialyzed against phosphate-buffered saline (67 mM, pH 7.8) containing 25 mM L-lysine. Two aliquots were taken from the solution and treated with formalin; 0.2% formalin was added to one of these, in accordance with the procedure described by Pappenheimer et al. [3], and was stored for a week at room temperature (about 22 C). The solution contained 850 Lf/ml and 4.36 mg of protein/ml. The other aliquot was treated with 0.7% formalin and stored for a week at room temperature. This sample contained 695 Lf/ml and 3.6 mg of protein/ml. Other samples containing 0.2% formalin were stored for two weeks at room temperature or for one week at 37 C so that we could examine the effect of differences in temperature (i.e., reaction rate) on the formalinization process.

Diphtheria toxin. Reference toxin (lot no. HDM 49; ISVT Sclavo) with 3,000 minimal lethal doses (MLD)/ml, 75 Lf/ml, and 2.45 mg of protein/ml was used. The MLD was defined as the minimal amount of toxin that kills guinea pigs within four days.

Flocculation titration. The titration for Lf/ml was performed according to the method of Ramon [5], using a horse antiserum containing 100 units/ml and 15.3 mg of protein/ml (lot no. SDF 2; ISVT Sclavo) standardized against a reference diphtheria flocculating serum with 300 units/ml supplied by the Division of Biological Standards (National Institutes of Health, Bethesda, Md.).

Protection test in guinea pigs. A group of 320 healthy untreated guinea pigs was subdivided into 64 groups of five animals each. Twenty of these groups (four each) received sc 0.2% formalin-treated CRM197 in single doses of 12.5, 25, 50, 100, and 200 Lf. Twelve more groups that received 12.5, 25, and 50 Lf in single doses were inoculated, 40 days after the first injection, with a booster dose of 25, 50, and 100 Lf, respectively. Eight groups received 0.7% formalin-treated CRM197 in single doses of 12.5 and 25 Lf. The remaining 24 groups were subjected to a similar treatment, using diphtheria toxoid. The challenge dose was given 30 days after the single injection or 15 days after the booster, by inoculating sc 2, 4, 8, or 16 MLD, respectively, into each group. A control group of six animals received a challenge dose of 1 MLD. In addition, groups of four animals were vaccinated using the same doses described above and bled at the times reported for the challenge; the pool of the four sera of each group was titrated by the neutralization test, and the titer was compared with that in the sera of a group of control animals.

Immunization of rats. Two groups of 10 rats each were immunized sc with 12.5 Lf of CRM197 and standard diphtheria toxin, respectively. Blood samples were obtained from the animals 30 days after inoculation. Two groups of five rats each were used as controls.

Titration of antibodies to toxin. This test was performed according to the method reported in the *European Pharmacopoeia* [6] for guinea pigs or rabbits. A standard antiserum to diphtheria toxin was used with 6 units/ml (lot no. A-38; National Institutes of Health) and a standard diphtheria toxin with 75 Lf/ml (lot no. HDM 49; ISVT Sclavo).

Toxicity test in guinea pigs. Five guinea pigs weighing ~350 g each were inoculated sc with 100 Lf of CRM197. They were observed for 30 days after the injection.

Dermonecrotic test. Two rabbits were inoculated intradermally with 0.2 ml (25 Lf) of CRM197. They were observed for seven days after the injection.

Enzymatic proteolysis. Proteolysis was measured using trypsin (lot no. 20C–8030; Sigma Chemical Co., St. Louis, Mo.); the enzymatic activity was measured by the method of Fasold and Gundlach [7] using α-N-benzoyl-L-arginine ethyl ester (BAEE) (lot no. 81 C-0060; Sigma Chemical Co.) as substrate. The hydrolysis of BAEE to the corresponding product α-N-benzoyl-L-arginine was followed spectrophotometrically at 254 nm after incubation at 20 C at pH 8.0 in 0.02 M Tris-hydroxymethylaminomethane buffer. Calcium chloride was used in a final concentration of 0.02 M because of its stabilizing effect on trypsin. The enzyme:substrate ratio was 1:100 (wt/wt). One BAEE unit was defined as a change in OD at 254 nm of 0.001/min in a 3.2-ml volume under the conditions used. Trypsin had 6,500 BAEE units/mg.

Solutions of diphtheria toxin, CRM197, 0.2%–0.7% formalin-treated CRM197, and diphtheria toxoid at concentrations of 1 mg/ml for each antigen were equilibrated by dialysis against 0.02 M Tris-hydroxymethylaminomethane buffer, pH 8.0. Aliquots of 2.5 ml of each antigen were removed, and 10 μl of 2% (wt/vol) dithiothreitol solution (lot no. 610206; Calbiochem, San Diego, Calif.) and 10 μl of 0.002 M calcium chloride solution were added to each.

Each sample was incubated at 37 C for 90 min with 10 μl of 1% (wt/vol) trypsin solution in 10^{-3} N HCl (enzyme:antigen ratio, 1:10). After this period, 7.5-μl aliquots of each sample solution were analyzed by tandem-crossed immunoelectrophoresis under the conditions described below. Five-tenths milliliter of a 2% (wt/vol) BAEE solution (enzyme:substrate ratio, 1:100) was then added to each remaining sample. Hydrolysis of the substrate was followed by spectrophotometric analysis at 254 nm to measure enzymatic activity after incubation with the antigens.

Slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed according to the technique of Weber and Osborn [8], using a dual vertical slab apparatus (Bio-Rad Laboratories, Richmond, Calif.). The acrylamide concentration was 10% in the running gel and 5% in the stacking gel. The length of the former was 9 cm, and a current of 80 mA was applied to each gel for 8 hr. The electrophoresis was performed at a constant temperature of about 15 C, cooling the core cell of the apparatus with running tapwater.

Immunoelectrophoresis and immunodiffusion. Immunoelectrophoresis according to the method of Grabar and Burtin [9] was employed, using a horse antiserum containing 100 units/ml and 15.3 mg of protein/ml (lot no. SDF 2; ISVT Sclavo) as the standard. A constant current of 8 mA was applied to the agarose gel using 0.02 M Tris-barbiturate buffer, pH 8.6, for 50 min. Tandem-crossed immunoelectrophoresis was performed as described by Kroll [10], with a diffusion time of 30 min. The first-dimensional electrophoresis was done at 10 V/cm for 1.5 hr in agarose gel using 0.02 M Tris-barbiturate buffer, pH 8.6. The second-dimensional electrophoresis was done at 2 V/cm for 18 hr in agarose gel containing 0.14 units of horse antiserum/cm^2. Immunodiffusion was performed by the method of Ouchterlony [11], using the above horse antiserum.

Protein determination. Protein concentra-

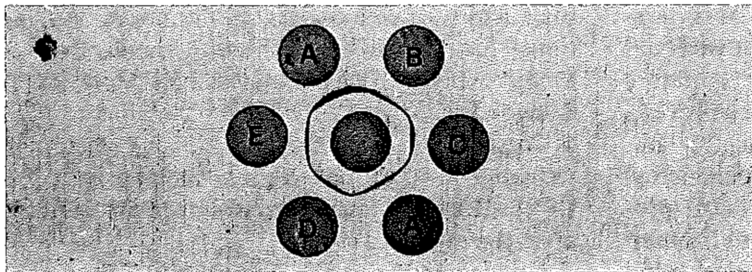

Figure 1. Agar immunodiffusion showing serologic identity among diphtheria toxin, diphtheria toxoid, and untreated and formalin-treated cross-reacting material (CRM197). The central well contained 10 μl of a standard horse antiserum (100 units/ml). The surrounding wells contained, respectively, (*A*) 10 μl of reference diphtheria toxin, 75 Lf (limit flocculation units)/ml diluted to 15 Lf/ml; (*B*) diphtheria toxoid, 500 Lf/ml diluted to 15 Lf/ml; (*C*) crude CRM197, 15 Lf/ml; (*D*) 0.2% formalin-treated CRM197, 850 Lf/ml diluted to 15 Lf/ml; and (*E*) 0.7% formalin-treated CRM197, 695 Lf/ml diluted to 15 Lf/ml.

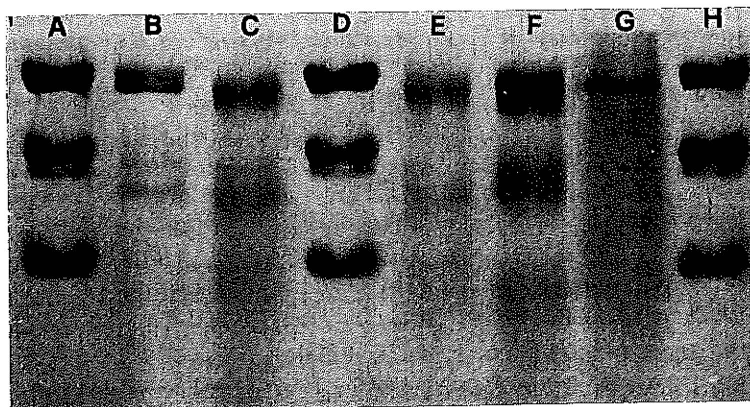

Figure 2. Electrophoretic mobility in slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (*A*) 10 μg of bovine serum albumin (67,000 daltons), (*B*) 25 μg of untreated cross-reacting material (CRM197), (*C*) 25 μg of 0.2% formalin-treated CRM197, (*D*) 10 μg of ovalbumin (43,000 daltons), (*E*) 25 μg of 0.7% formalin-treated CRM197, (*F*) 25 μg of diphtheria toxoid, (*G*) 25 μg of reference diphtheria toxin, and (*H*) 10 μg of chymotrypsinogen A (25,000 daltons).

tions were determined by the technique of Lowry et al. [12], using bovine serum albumin as the standard (lot no. F-33206; Armour Pharmaceutical Co., Chicago, Ill.).

Statistical methods. The statistical analyses were performed according to Hartley's method [13] using a Hewlett Packard model no. 9845 computer (Hewlett Packard, Palo Alto, Calif.).

Results

After 48 hr of incubation under the conditions described above, the culture filtrate of C7(β197) contained 15 Lf of CRM197/ml. Partially purified CRM197 treated with formalin in the ways described in Materials and Methods was compared with diphtheria toxin and toxoid by double diffusion in agar and appeared to be immunologically identical (figure 1). SDS-PAGE indicated that 0.2% and 0.7% formalin-treated CRM197 had the same molecular size (62,000 daltons) as diphtheria toxin and toxoid when compared with standard proteins (figure 2). Immunoelectrophoresis (figure 3) showed different degrees of mobility between untreated and formalinized antigens.

All of the antigens gave rise to a single main arc, but 0.2% formalin-treated CRM197 was less mobile than 0.7% formalin-treated CRM197, and the latter was even less mobile than diphtheria toxoid.

This degree of mobility was also confirmed by tandem-crossed immunoelectrophoresis (figure 4), which showed a more compact tandem between diphtheria toxoid and 0.7% formalin-treated CRM197 than between diphtheria toxoid and 0.2% formalin-treated CRM197. The greater amount of formalin in 0.7% formalin-treated CRM197 would explain its faster mobility with respect to 0.2% formalin-treated CRM197 on the basis of a greater capacity to bind added L-lysine, which increases the superficial charges of proteins, while slight differences in superficial charge between 0.7% formalin-treated CRM197 and diphtheria toxoid would be suspected.

The toxicity test showed that at necropsy the guinea pigs treated with CRM197 were in perfect health, without loss of weight, edema, necrosis, inflammation of adrenal glands or lungs, or pleural exudate. The dermonecrotic test showed no reaction at the site of the injection.

The immunoresponse of guinea pigs treated with 0.2% formalin-treated CRM197 and toxoid was compared after one or two sc injections. The results after challenge with 2, 4, 8, and 16 MLD are shown in table 1, and the corresponding analysis of the variance is shown in table 2. The range of the challenge dose was chosen on the basis of prelim-

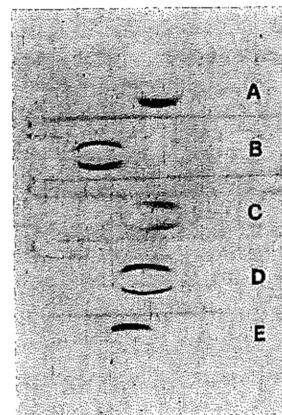

Figure 3. Immunoelectrophoresis of 2 μg each of (*A*) diphtheria toxin, (*B*) diphtheria toxoid, (*C*) cross-reacting material (CRM197), (*D*) 0.2% formalin-treated CRM197, and (*E*) 0.7% formalin-treated CRM197. Each central well contained 50 μl of a standard horse antiserum (100 units/ml). The anode is to the left.

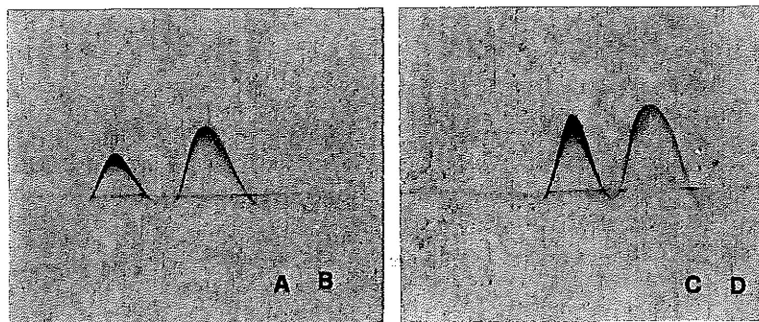

Figure 4. Tandem-crossed immunoelectrophoresis of (*A*) 2 μg of diphtheria toxoid, (*B*) 4 μg of 0.2% formalin-treated cross-reacting material (CRM197), (*C*) 4 μg of diphtheria toxoid, and (*D*) 4 μg of 0.7% formalin-treated CRM197. The agarose gel contained 0.14 units of a standard horse antiserum (100 units/ml)/cm². The anode is to the left.

inary tests using 0.2% formalin-treated CRM197. Our results showed a marked difference in protection level afforded by 0.2% formalin-treated CRM197 in comparison to diphtheria toxoid. Since these differences in immunogenicity might have been due to differences in the rate of the formalin reaction with CRM197, we also tested the immunogenicity of CRM197 incubated with 0.2% formalin at room temperature for two weeks or at 37 C for one week; both antigens were injected sc into guinea pigs using single doses of 12.5 and 25 Lf for each animal.

The level of the antibody response to toxin 30 days later and the protection afforded against the challenge of 2, 4, 8, and 16 MLD of diphtheria toxin were practically the same as those obtained by vaccinating the animals with the previously described 0.2% formalin-treated CRM197. The reaction rate between 0.2% formalin and CRM197 was monitored using the SDS-PAGE procedure, since CRM197 when subjected to this procedure showed as well as the principal band of 62,000 daltons two additional bands: one of 40,000 daltons and the other of 22,000 daltons. After formalin treatment under the conditions described, the principal band was more marked, while the other two were noticeably weaker (figure 5). (This comparison was referred to in the profile on SDS-PAGE of diphtheria toxoid and 0.7% formalin-treated CRM197 [figure 2].) After seven days at room temperature, the reaction between 0.2% formalin and CRM197 was like that at 37 C for the

Table 1. Results of protection and neutralization tests for antibodies to diphtheria toxin in guinea pigs immunized with cross-reacting material (CRM197) and diphtheria toxoid.

	Lf		No. of animals per group surviving challenge with an MLD of				
Antigen	Injection 1	Injection 2	2	4	8	16	Antibody to toxin (IU/ml)
CRM197, 0.2% formalin-treated	12.5	. . .	3	2	0	0	0.01
	25	. . .	4	2	2	2	0.1
	50	. . .	4	5	3	2	0.1
	100	. . .	5	5	4	4	>0.1 to <1
	200	. . .	5	5	5	5	>0.1 to <1
	12.5	25	5	5	5	4	>1 to <10
	25	50	5	5	5	5	10
	50	100	5	5	5	5	>10 to <25
Diphtheria toxoid	12.5	. . .	5	5	5	5	1
	25	. . .	5	5	5	5	1
	50	. . .	5	5	5	5	1
	12.5	25	5	5	5	5	>1 to <10
	25	50	5	5	5	5	10
	50	100	5	5	5	5	>10 to <25
CRM197, 0.7% formalin-treated	12.5	. . .	5	5	5	0	>0.01 to <0.1
	25	. . .	5	5	5	5	>0.1 to <1

NOTE. There were five animals per group and four groups per injection 1. Injection 2 was given 40 days after injection 1. Lf = limit flocculation units; MLD = minimal lethal dose; IU = international units.

Table 2. Analysis of variance in the protection test of guinea pigs immunized with cross-reacting material (CRM197) and diphtheria toxoid.

Comparison*	Line no.	Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*	*P* value
Formalin-treated CRM197, injection 1	1	Between challenge doses	8.950	3	2.983	5.264	<0.05
	2	Between single doses	36.800	4	9.200	16.235	<0.01
	3	Error	6.800	12	0.566	...	...
		Total	52.500	19	...	...	...
Formalin-treated CRM197 and diphtheria toxoid, injection 1	1	Between challenge doses	4.475	3	1.491	5.264	<0.01
	2	Between immunizing doses	18.400	4	4.600	16.235	<0.01
	3	Between vaccines	27.225	1	27.225	96.088	<0.01
	4	Interaction 1 and 3	4.475	3	1.491	5.264	<0.05
	5	Interaction 2 and 3	18.400	4	4.600	16.235	<0.01
	6	Error	6.800	24	...	...	...
		Total	79.775	39	...	...	...
Formalin-treated CRM197, injections 1 and 2	1	Between challenge doses	6.666	3	2.222	2.943	...
	2	Between immunizing doses	37.500	1	37.500	46.675	<0.01
	3	Between vaccine doses	6.333	2	3.167	4.195	<0.05
	4	Error	12.833	17	0.755	...	...
		Total	63.333	23	...	...	...
Formalin-treated CRM197 and diphtheria toxoid, injections 1 and 2	1	Between challenge doses	0.125	3	0.041	1.000	...
	2	Between immunizing doses	0.083	2	0.041	1.000	...
	3	Between vaccine doses	0.041	1	0.041	1.000	...
	4	Error	0.708	17	...	...	...
		Total	0.958	23	...	...	...

* See table 1.

same incubation period, and the failure of the antigen to provide complete protection in guinea pigs was probably due to a lower stabilizing effect of 0.2% formalin on CRM197 in comparison to that of 0.7% formalin on diphtheria toxin.

To evaluate this hypothesis we prepared 0.7% formalin-treated CRM197 as indicated in Materials and Methods. The antigen was inoculated into guinea pigs in single doses of 12.5 and 25 Lf. The antibody response to toxin after 30 days and the protection against the challenge using diphtheria toxin are shown in table 1. The protection that was provided by this antigen in a 25-Lf dose in a single injection is in good agreement with the results given by diphtheria toxoid (table 1). It should be noted, however, that the level of antibodies to toxin was slightly lower than that formed in the animals that received the same dose of diphtheria toxoid.

We wondered whether, in addition to the probable stabilizing effect of the formalin, this slight difference in antibody titer could be due to a basic antigenic difference in the CRM197 molecule in comparison to that of the diphtheria toxin. To answer this question, we required an animal model that could be inoculated with diphtheria toxin and CRM197 without formalin treatment. For this reason we used rats, whose resistance to the toxicity of diphtheria toxin is well known. Groups of rats were inoculated with either CRM197 or diphtheria toxin, as described in Materials and Methods, and bled 30 days after treatment. Antibodies to the toxin were titrated in the serum pool as described previously. The results clearly suggest that both CRM197 and diphtheria toxin are equally immunogenic without formalin treatment (table 3).

Further evidence that formalin-treated antigens were more resistant to enzymatic proteolysis than untreated antigens was provided by in vitro experiments using trypsin. As is already known [3, 14, 15], CRM197 and diphtheria toxin are sensitive to the proteolytic action of trypsin, and reducing chemicals split nicked diphtheria toxin or CRM197 into A and B fragments (22,000 daltons and 40,000 daltons, respectively). Under the conditions described in Materials and Methods, the stabilities of CRM197, diphtheria toxin, 0.2%–0.7% formalin-treated CRM197, and diphtheria toxoid toward trypsin were compared by tandem-crossed immunoelectrophoresis (figure 6). After incubation for 90 min, CRM197 and diphtheria toxin lost their antigenic identity, while 0.2%–0.7% formalin-treated CRM197 and diphtheria toxoid still reacted to homologous antibodies.

At the end of the incubation period with the single antigens, the enzymatic activity of trypsin was tested by the addition of the specific synthetic substrate BAEE; hydrolysis was followed by

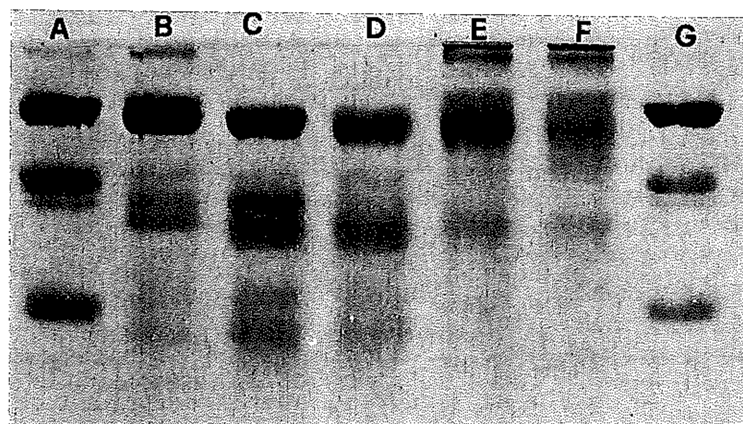

Figure 5. Electrophoretic patterns in sodium dodecyl sulfate–polyacrylamide gel electrophoresis showing the time dependence of treatment with 0.2% formalin of 25 μg of cross-reacting material (CRM197): (*A* and *G*) 10 μg each of bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), and chymotrypsinogen A (25,000 daltons); (*B*) untreated CRM197; (*C*) CRM197 plus formalin, 1 hr at 22 C; (*D*) CRM197 plus formalin, 24 hr at 22 C; (*E*) CRM197 plus formalin, seven days at 22 C; and (*F*) CRM197 plus formalin, seven days at 37 C.

measuring the OD at 254 nm. Trypsin showed enzymatic activity in each sample (figure 7), excluding any occasional enzymatic inhibition.

Discussion

Pappenheimer et al. [3] studied the antigenicity of proteins serologically related to diphtheria toxin such as CRM197 by evaluating the quantitative response in rabbits of serum antibodies which could be precipitated in vitro by diphtheria toxin. They showed that 0.2% formalin-treated CRM197 was able to stimulate the production of antibodies in rabbits and that these antibodies were almost completely precipitated by diphtheria toxin. They attributed the high antigenicity of formalin-treated CRM197 in comparison to untreated CRM197 to the stability of formalin-treated protein to the proteolytic degradation in vivo. On the basis of these observations, we tested the immunogenicity of formalinized CRM197 and compared it with diphtheria toxoid in guinea pigs treated with the antigens in different amounts and with various immunization techniques. The immunoresponse was assayed by the protection test and by titration of antibodies to the toxin.

When assayed by SDS-PAGE, the formalin-treated CRM197 had a molecular size of 62,000 daltons, identical to that of the toxin and toxoid, which suggested that the methylenic bonds, typical of formalinized proteins, are intramolecular, as Blass et al. [16] reported in the case of diphtheria toxoid. The antigenicity of formalin-treated CRM197 could not therefore be attributed to the formation of polymers of high molecular weight under the conditions used.

On the other hand, formalin treatment does not lead to the formation of new antigenic determinants, as occurs for other proteins such as albumin treated with glutaraldehyde [17], because the immunodiffusion precipitin test, immunoelectrophoresis, and tandem-crossed immunoelectrophoresis showed serologic identity between 0.2%–0.7% formalin-treated CRM197 and diphtheria toxin and toxoid. If the animals are treated with a single inoculation of 0.2% formalin-treated CRM197, total protection against a challenge dose of 16 MLD is only obtained with an amount of antigen equal to 200 Lf, while 12.5 Lf of the toxoid is sufficient; thus, the latter is 16 times more effective. The titer of antibodies to the toxin, expressed in international units/ml, is also clearly higher in the latter case. The analysis of the variance indicates a high significance at the level of $P < 0.01$ between the single immunizing doses of 0.2% formalin-treated CRM197 and the comparison between these and the single doses of toxoid (table 2). On the other hand, if a booster dose of 0.2% formalin-treated CRM197 is given 40 days after the first injection, complete protection is found at the 12.5-Lf dose level with a booster dose of 25 Lf. In this case, the titration of antibodies to the toxin gives a comparable value to that found with the toxoid using the same method of vaccination.

These observations led to the same hypotheses

Table 3. Results of neutralization tests for antibodies to diphtheria toxin in rats immunized with cross-reacting material (CRM197) or reference diphtheria toxin.

Antigen	No. of rats	Lf	Antibodies to toxin (IU/ml)
CRM197	10	12.5	>0.1 to <1
Diphtheria toxin	10	12.5	>0.1 to <1
Control	10	. . .	<0.01

NOTE. Lf = limit flocculation units; IU = international units.

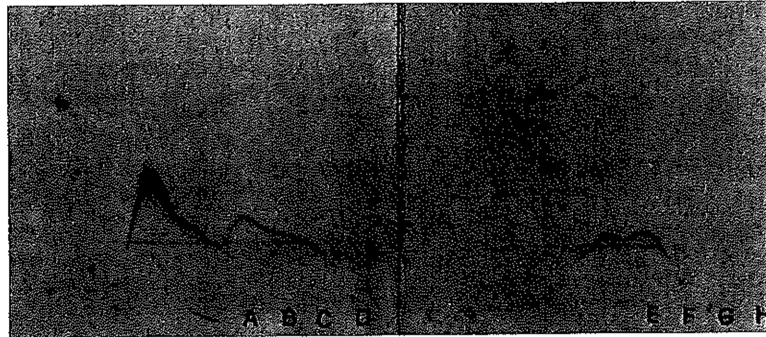

Figure 6. Tandem-crossed immunoelectrophoresis of untreated and formalin-treated antigens of *Corynebacterium diphtheriae* after exposure to trypsin: (*A*) standard diphtheria toxoid; (*B*) proteolysis of diphtheria toxoid; (*C*) proteolysis of 0.7% formalin-treated cross-reacting material (CRM197); (*D*) proteolysis of 0.2% formalin-treated CRM197; (*E*) standard diphtheria toxin; (*F*) standard CRM197; (*G*) proteolysis of diphtheria toxin; and (*H*) proteolysis of CRM197. The agarose gel contained 0.14 units of a standard horse antiserum (100 units/ml)/cm². All antigens were used at 3 μg. The anode is to the left.

as those proposed by Pappenheimer et al. [3] regarding the stabilizing effect of formalin on diphtheria toxin and CRM197. Thus, the immunogenic differences between single doses of 0.2% formalin-treated CRM197 and toxoid could be attributed to the greater stability of the latter against proteolysis. However, when a booster dose of both antigens is given, the same degree of protection and the same serum level of antibodies to toxin are found, a result indicating that a booster dose compensates for the lower degree of stability of the 0.2% formalin-treated CRM197. The validity of this hypothesis was confirmed by the immunologic responses of the animals treated with 0.7% formalin-treated CRM197. A single dose of 25 Lf of this antigen gave the same protection against a challenge of diphtheria toxin as the toxoid. The level of antibodies to the toxin produced in these animals was slightly lower, however, than that found in the animals that received the same dose of toxoid, but higher than that in animals that received 0.2% formalin-treated CRM197.

To test whether this difference could be due to basic antigenic differences in the CRM197 molecule in comparison to that of the diphtheria toxin, we immunized rats with either CRM197 or diphtheria toxin. Chang and Neville [18] have suggested that the resistance of rats to diphtheria toxin may be attributed to the lack of a mechanism by which fragment A is transported across the cytoplasmic membrane and not to the absence of receptors for toxin on the cell membranes. The serum level of antibodies to the toxin 30 days after immunization was the same toward the two antigens, and we can therefore conclude that in rats CRM197 and diphtheria toxin have the same immunogenicity. The slight difference found in the levels of antitoxin produced by guinea pigs against 0.7% formalin-treated CRM197 and diphtheria toxoid may be due either to the variability of the biologic test or to the different degree of stability caused by 0.7% formalin in CRM197 and diphtheria toxin.

Hypotheses for the increased stability of formalinized antigens to enzymatic proteolysis would re-

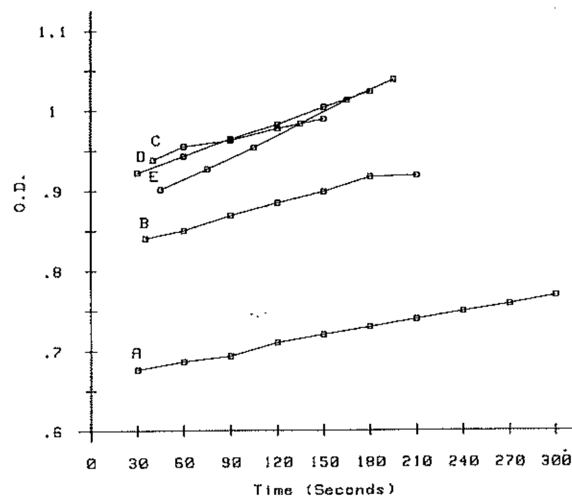

Figure 7. Enzymatic activity control of trypsin after incubation for 90 min with untreated and formalin-treated antigens of *Corynebacterium diphtheriae*: (*A*) cross-reacting material (CRM197); (*B*) diphtheria toxin; (*C*) diphtheria toxoid; (*D*) 0.7% formalin-treated CRM197; and (*E*) 0.2% formalin-treated CRM197. The method of Fasold and Gundlach [7] was used, with α-N-benzoyl-L-arginine ethyl ester as the substrate.

quire a more direct demonstration of the fate that these antigens undergo in vivo. Baseman et al. [19] showed that ^{125}I- or ^{131}I-labeled diphtheria toxin and toxoid were eliminated in different ways and at different times when inoculated iv into rabbits or guinea pigs; the toxoid was eliminated more slowly than toxin. Furthermore, the toxoid did not bind to the sensitive cell membranes [14]. Pappenheimer et al. [3] also reported that the complex of diphtheria toxin–antitoxin was not sensitive to the action of trypsin at a neutral pH.

These observations and the in vivo results that we observed led us to investigate the sensitivity of formalinized antigens to trypsin proteolysis in vitro in comparison to untreated CRM197 and diphtheria toxin. As shown in Results, after incubation with trypsin for 90 min under reducing conditions, CRM197 and diphtheria toxin lost their antigenic identity as compared with the reference antigens by tandem-crossed immunoelectrophoresis. In contrast, 0.2%–0.7% formalin-treated CRM197 and diphtheria toxoid again showed serologic identity, although at lower levels than reference diphtheria toxoid. Significantly, 0.2% formalin-treated CRM197 had a smaller area of immunoprecipitation than that of 0.7% formalin-treated CRM197 and diphtheria toxoid.

Our findings show that formalin-treated antigens (CRM197 and diphtheria toxoid) are more resistant to proteolytic degradation by trypsin than antigens without formalin treatment, and they suggest that 0.7% formalin-treated CRM197 may be considered a more suitable antigen than 0.2% formalin-treated CRM197 but similar to diphtheria toxoid as also shown by the in vivo results.

Stabilization by formalin of these antigens may be explained by the transformation of amino groups of lysine and arginine residues [16], and in particular, the three arginine residues present in the 14-amino acid loop that is sensitive to nicking by trypsin [14]. These results significantly strengthen the hypothesis suggested for the improvement of proteolytic resistance of formalinized antigens, and a further explanation could be given for their own immunogenic characteristics on the basis of a slower enzymatic degradation(s).

It therefore seems evident that the natural lack of toxicity of CRM197 does not influence the antigenicity of this protein and that its properties as an immunogenic agent are most likely related to its stability.

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