Heat-Revealed Antigens in DNA-Rich Preparations from *Brucella abortus.** (28308)

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Antibodies against DNAase-sensitive antigens, and their production in rabbits following intravenous injection of DNA-rich material extracted from *Brucella abortus,* were described previously(1,2,3). It also has been ascertained(4) that not all of the sera from rabbits immunized with such DNA-rich material contained these antibodies and that concentration of DNAase-sensitive antigens varied considerably among different batches of DNA-rich preparations, even though they were obtained by the same procedure. DNA-rich preparations of *B. abortus* contained, therefore, not only antigens sensitive to DNAase treatment, but also other antigens in which DNA was apparently not involved in their reactions with antibody. A third class of antigens has now been detected in these DNA-rich preparations, namely antigens that become reactive only after the preparations have been heated at a relatively high temperature. The purpose of this communication is to describe these heat-revealed antigens.

Materials and methods. The methods for preparation of the DNA-rich materials, and the procedure for the complement-fixation test have been described(4).

Antisera were prepared by immunizing white New Zealand rabbits with DNA preparations from smooth *Brucella abortus.* Injections were given intravenously 4 times a week for 4 weeks, and each animal received a total quantity of material containing 100 mg of DNA.

In the studies on temperature effects, DNA-rich preparations from *Brucella abortus* were heated in a water bath either at 100°C for 10 minutes(5) or at 85°C for 3 hours. Samples were then chilled either rapidly, by transferring the heated samples to chilled tubes immersed in crushed ice, or slowly, by placing the heated samples first in a water bath at 60°C for 3 hours and next in an ice-water bath.

Heated and native DNA preparations were both fractionated by centrifugation in a cesium chloride density gradient by the method of Meselson and coworkers(6), and the fractions were collected by a procedure described by Szybalski(7). The fractions were dialyzed against physiological saline at 4°C before they were tested for reactive antigens by the method of complement-fixation.

Results. Effect of heating on a DNA-rich preparation of Brucella abortus. In Table I are shown the results of an analysis of heated and non-heated DNA preparations for complement-fixing antigens reacting with a rabbit antiserum prepared against a DNA-rich preparation from smooth *Brucella abortus.* A marked increase in complement-fixing antigens was found in the heated sample when compared with the non-heated control. Al-

TABLE I. Effect of Heating on a DNA-rich Preparation from Smooth *Brucella abortus* Tested by Complement-Fixation

Antigen	μg DNA*	Antiserum S-4 diluted 1 → 500	Buffer
Native DNA	2.0	.005†	.335
	.5	.073	.330
	.12	.290	.335
	.02	.333	.330
	.006	.328	.335
	.001	.333	.335
Heated at 100°C 10 min., chilled rapidly	2.0	.003	.321
	.5	.005	.330
	.12	.002	.328
	.02	.015	.336
	.006	.246	.330
	.001	.329	.320
Buffer	none	.335	.338

* Based on U.V. absorption at 260 mμ, using purified calf thymus DNA as the standard.

† Values are optical densities at 541 mμ and are proportional to % of cells lysed. Extent of complement-fixation is inversely proportional to optical density.

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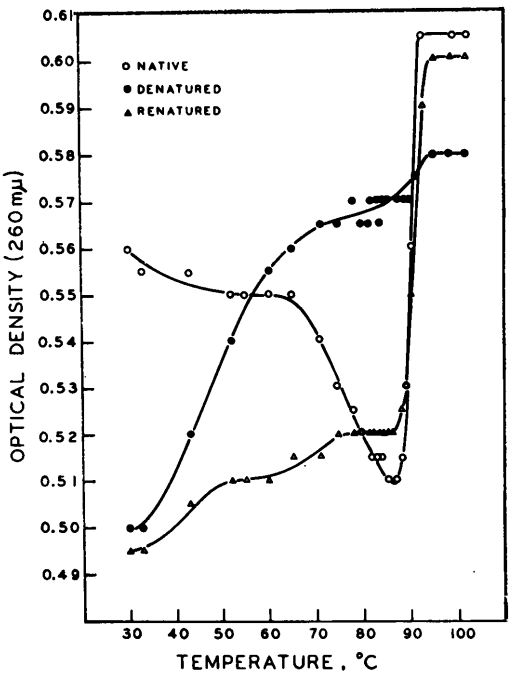

FIG. 1. Melting point curves of native DNA, O—O; heated and rapidly chilled DNA, ●—●; and heated and slowly cooled DNA, △—△.

though the unheated preparation contained DNAase-sensitive antigens, the heat-revealed antigens were insensitive to the action of DNAase. Further attempts were therefore made to determine the nature of the antigens which appeared after heating.

Fractionation by differential centrifugation. A sample of the DNA-rich material from smooth *Brucella abortus* was separated into a light and a heavy fraction by differential ultracentrifugation, as described by Plescia, *et al.*(8). Centrifugation was carried out in a Spinco preparative ultracentrifuge at 10,000 r.p.m. for 1.5 hours using the #40 rotor. The supernatant (hereafter referred to as 10 Sup) was decanted and the sediment (hereafter referred to as 10 Sed) was resolubilized in saline to its initial volume. A portion of each fraction was heated at 100°C for 10 minutes and chilled rapidly. Both the heated and non-heated samples were analyzed for complement-fixing antigens. The results are shown in Table II. The heat-revealed antigens were found predominantly in the 10 Sed fraction, whereas the DNAase-sensitive antigens were found in the 10 Sup fraction(8).

Influence of rate of cooling on appearance of heat-revealed antigens. When a double-stranded DNA is heated at temperatures above its melting point, it generally becomes single-stranded(5). Recombination of the strands, resulting in a renatured DNA, occurs if the DNA preparation is cooled slowly, but not if it is chilled rapidly. The melting points of double and single-stranded DNA differ greatly and are therefore used to distinguish them. The melting point curves for our heated DNA preparations from smooth *Brucella abortus*, cooled either slowly or rapidly, and for a non-heated control are given in Fig. 1. The melting point (Tm) of the non-heated control was 91.5°C. The curve of the slowly cooled sample resembled that of the non-heated control indicating that renaturation had occurred; in contrast, the rapidly chilled sample yielded a curve typical of denatured DNA.

The rapidly chilled and the slowly cooled heat-treated samples, as well as the non-heated control, were tested for complement-fixing antigens (Table III). In comparison to the non-heated control, an equal increase in complement-fixing activity occurred in both heated samples. The rate of cooling

TABLE II. Effect of Heating on "10 Sed" and "10 Sup" Fractions of a DNA-rich Preparation from Smooth *Brucella abortus*.

Antigen	μg DNA*	Antiserum S-4 diluted 1 → 500	Buffer
10 Sed (non-heated)	1.5	.020†	.360
	.3	.217	.360
	.1	.350	.359
	.02	.350	.360
10 Sed (heated at 100°C 10 min., chilled rapidly)	1.5	.000	.330
	.3	.000	.320
	.1	.003	.324
	.02	.003	.320
10 Sup (non-heated)	2.0	.038	.349
	.5	.256	.358
	.12	.306	.351
	.03	.355	.359
10 Sup (heated at 100°C 10 min., chilled rapidly)	2.0	.010	.360
	.5	.015	.350
	.12	.119	.360
	.03	.322	.350
Buffer	none	.355	.355

* See footnote Table I.
† " " " "

TABLE III. Complement-Fixing Antigens in Native and Heated DNA Preparations from Smooth *Brucella abortus*.

Antigen	μg DNA*	Antiserum (S-pool) diluted 1 → 500	Buffer
Native DNA	2.0	.003†	.332
	.5	.237	.336
	.02	.335	.335
Heated at 100°C 10 min., chilled rapidly	2.0	.006	.335
	.5	.003	.338
	.02	.021	.340
Heated at 100°C 10 min., held at 60°C 3 hr	2.0	.006	.330
	.5	.002	.333
	.02	.011	.340
Buffer	none	.334	.339

* See footnote Table I.
† " " " "

therefore was not a critical factor, an indication that single-stranded DNA was not likely to be part of the heat-revealed antigens.

Temperature requirements for appearance of heat-revealed antigens. When the same DNA-rich preparation from smooth *Brucella abortus* was heated for 3 hours at 85°C, a temperature short of the melting point, and analyzed for complement-fixing antigens, it was found (Table IV) that essentially the same increase in complement-fixing antigens had occurred as on heating at 100°C for 10 minutes. This finding also indicates that single-stranding was incidental and not required for the appearance of the heat-revealed antigens.

Fractionation of heat-revealed antigens by centrifugation in a cesium chloride density gradient. To determine the extent of association, if any, of the heat-revealed antigens with DNA, native as well as heated DNA preparations (100°C) were centrifuged in a cesium chloride density gradient. In addition, a portion of each fraction obtained by centrifugation of unheated material was heated following fractionation. The results of complement-fixation tests with these materials are shown in Tables V and VI. Complement-fixing antigens were recovered in a pool of the light fractions of heated material but not in the 260 mμ absorbing fraction. When the fractions of the native DNA preparation, obtained by density gradient centrifugation, were heated at 100°C for 10 minutes, complement-fixing antigens could not be detected in any of the fractions. A pool of all the fractions was also heated at 100°C for 10 minutes, but no increase in complement-fixing activity was detected.

Source of antigens. Preparations of DNA-rich material from mucoid *Brucella abortus* cells were also tested for their ability to yield heat-revealed antigens. They were always found in preparations from smooth cells, whether or not the preparations contained DNAase-sensitive antigens, but they were never found in presumably similar preparations from mucoid cells. Antisera containing antibodies against heat-revealed antigens also contained agglutinins against smooth *Brucella abortus* cells; Olitzki was the first to report the presence of such agglutinins in antisera obtained from rabbits immunized with DNA preparations from smooth *Brucellae*(9). Antibodies against the heat-revealed antigens were absorbed by smooth *Brucella abortus* cells but not by non-smooth cells. All of this suggests that heat-revealed antigens are similar to, or possibly identical with, smooth cell-surface antigens.

Chemical nature of the heat-revealed antigens. The antigens were tested for their sensitivity to the action of a variety of en-

TABLE IV. Effect of Heating at Different Temperatures on Appearance of Heat-Revealed Antigens in a DNA Preparation from Smooth *Brucella abortus*.

Antigen	μg DNA*	Antiserum (S-pool) diluted 1 → 500	Buffer
Native prep.	2.0	.020†	.345
	1.0	.195	.350
	.1	.344	.350
	.02	.350	.360
Prep. heated at 100°C for 10 min., followed by rapid chilling	2.0	.005	.340
	1.0	.010	.340
	.1	.000	.350
	.02	.008	.345
Prep. heated at 85°C for 3 hr, followed by rapid chilling	2.0	.008	.348
	1.0	.004	.350
	.1	.000	.345
	.02	.057	.348
Buffer		.340	.350

* See footnote Table I.
† " " " "

TABLE V. Complement-Fixing Antigens in Fractions of Heated DNA Preparations Obtained by Centrifugation in a CsCl Gradient.

Antigen	Dilutions based on stock solution containing 40 γ DNA per ml	Antiserum S-pool 1:500	Buffer
260 mμ fractions (11-13) of heated (100°C, 10 min., rapid chilling) DNA	1→ 8	.320†	.340
	1→ 16	.344	.350
	1→ 80	.348	.350
	1→ 160	.350	.350
Light fractions (18-21) of heated (100°C, 10 min., rapid chilling) DNA	1→ 8	.005	.340
	1→ 16	.003	.350
	1→ 80	.008	.340
	1→ 160	.045	.345
Buffer		.340	.352

† See footnote Table I.

TABLE VI. Complement-Fixing Antigens in Heated and Unheated Fractions of Native DNA Preparations Obtained by Centrifugation in a CsCl Gradient.

Antigen	Dilutions based on stock solution containing 40 γ DNA per ml	Antiserum S-pool 1:500	Buffer
260 mμ fractions (14-16) of native DNA	1→ 8	.343†	.350
	1→ 160	.343	.350
260 mμ fractions (14-16) of native DNA following heating at 100°C for 10 min., and rapid chilling	1→ 8	.347	.350
	1→ 160	.348	.355
Light fractions (20-23) of native DNA	1→ 8	.340	.345
	1→ 160	.343	.345
Light fractions (20-23) following heating at 100°C for 10 min., and rapid chilling	1→ 8	.345	.350
	1→ 160	.345	.350
Buffer		.340	.352

† See footnote Table I.

zymes and specific inactivating agents. They proved to be insensitive to DNAase, RNAase, alkaline phosphatase, panprotease, and lipase, but sensitive to the action of sodium periodate. Also, the DNA preparation gave a positive test with phenol-sulfuric acid. These results suggest the possibility that the heat-revealed antigens may be polysaccharide in nature.

Discussion. The foregoing data show that heating of DNA-rich material from smooth *Brucella abortus* cells results in the appearance of additional complement-fixing antigens. These were insensitive to DNAase and could be produced by heating the preparations at temperatures short of melting the DNA. It is unlikely, therefore, that the heat-revealed antigens involve DNA or that single-stranding of DNA is necessary. However, these heat-revealed antigens are apparently associated with nucleic acid in the DNA-rich preparation prior to heating, because heating was effective only when applied to the DNA-rich preparation *prior* to its fractionation into a nucleic acid fraction and a less dense fraction.

The simplest way of interpreting these findings at the moment is to assume the existence of complexes between a dense material, presumably nucleic acid, and a lighter material, most likely carbohydrate in nature. It can be assumed further that the antigenic portion of such complexes is not accessible to the antibody, and that heating causes a dissociation of the complex to liberate the antigenic moiety, which in fact proved to be less dense than the DNA. If such assumptions are correct, it would be expected, because of the demonstrated differences in density between the heat-revealed antigens and

nucleic acid, that the assumed complexes also might become dissociated by centrifugal force in a density-gradient. No evidence for such a dissociation was obtained, in terms of an increase in complement-fixing antigens in the light fractions, when a DNA preparation from smooth cells was centrifuged in a density gradient. Therefore, if dissociation did occur, it would have to have been different from that caused by heating. The finding that there was no increase in complement-fixing antigens when either the light fractions or a pool of all the fractions obtained by density-gradient centrifugation were heated at 100°C for 10 minutes can be interpreted to mean that some sort of dissociation did occur, and that it was different from that caused by heating. Of possible relevance are the recent observations by Greer *et al.*(10) that heating of DNA at elevated temperatures short of that required for melting causes depurination. In contrast, density-gradient centrifugation presumably does not cause depurination. Attempts are in progress to determine whether or not heat-revealed antigens contain purines.

The fact that the heat-revealed antigens were found only in DNA-rich preparations from smooth and not in preparations from non-smooth *Brucella abortus* cells, coupled with the finding that antibodies against the heat-revealed antigens were absorbed by smooth *Brucella abortus* cells, strongly suggests that the heat-revealed antigens are similar to, or identical with, certain cell-surface antigens. The observation by Olitzki *et al.*(11) that diaminopimelic acid, a constituent of cell walls, is also present in the DNA-rich preparations is of interest in this regard. However, it remains to be determined whether the cell-surface antigens, which appear to involve carbohydrates on the basis of chemical tests, are naturally complexed with nucleic acids or are artifacts of extraction. It is hoped that current studies with cell walls and protoplasms, kindly furnished by Drs. E. Ribi and J. Foster, will resolve this problem. Preliminary observations indicate that the bulk of the heat-revealed antigens is not derived from the protoplasm. Additional results from preliminary studies, in which pneumococcal type-specific polysaccharide was added to *Brucella abortus* suspensions at the time of extraction with 0.5% phenol, suggest that the nucleic acid complex with heat-revealed antigens might be more than an artifact of extraction.

Another observation was made recently which may be pertinent to the problem of unique associations between nucleic acid and cell-surface antigens. Immunization of rabbits with a DNA preparation isolated from Ehrlich-Lettré mouse tumor cells resulted in antisera that were cytotoxic for the mouse tumor cells; these cytotoxic serum factors could not be neutralized by an excess of the immunizing DNA preparation but were adsorbed by intact tumor cells. One explanation for this observation could be that cell-surface antigens, comparable to the heat-revealed antigens in the *Brucella* preparation, are present as complexes in the DNA-rich immunizing preparation, and are not, therefore, in a reactive form. If this explanation proves to be correct, it would suggest a more general occurrence of a unique association of cell-surface antigens with nucleic acids.

Summary. Heating of DNA-rich preparations from smooth, but not from mucoid, *Brucella abortus* cells resulted in the appearance of complement-fixing antigens that were insensitive to DNAase but sensitive to sodium periodate. The effect of heating was not due to its single-stranding of DNA, and DNA itself apparently was not required for antigenic activity of the heat-revealed antigens. Prior to heating, however, the heat-revealed antigens appear to be complexed with DNA. This is supported by the finding that following density-gradient centrifugation, which separates a DNA preparation into a nucleic acid fraction and less dense fractions, heating neither the individual fraction nor the pooled fractions resulted in the appearance of heat-revealed antigens. Evidence to date suggests that the heat-revealed antigens are similar to, or identical with, smooth cell-surface antigens, and that, at least in cellular extracts, the unique association of such cell-surface antigens with nu-

cleic acids might be a fairly general phenomenon.

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Synergistic Renal Effects of DCI and Cortisone in Mice Poisoned with Bacterial Endotoxin. (28309)

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The adrenergic blocking agent DCI or 3,4-dichloroisoproterenol provided a measure of protection against bacterial endotoxin when given subcutaneously to mice by McLean and Berry(1). Cortisone had been found to protect mice from these lipopolysaccharides by Berry *et al.*(2,3) and the handling of nitrogenous waste products was implicated(4). In the present work the influence of DCI on nitrogen excretion and its interaction with cortisone has been investigated.

Methods. Male CF1 mice were used in this study. Saline blanks were given subcutaneously as were the doses of DCI 10 mg/kg and cortisone acetate 5 mg/mouse. Difco crystalline lipopolysaccharide from *E. coli* (Code 0.27:B8, Control 118023) was the endotoxin used. An approximate LD_{50} of 0.3 mg/mouse was injected intraperitoneally. The DCI dose was given twice; 5 hours before and 1 hour before the endotoxin. The cortisone doses were given in the same way. The average weight of the mice used in these experiments was about 23 g. The micro-Kjeldahl procedure was used for determination of urinary nitrogen and blood non-protein nitrogen (NPN). Urine was collected under toluene in conical centrifuge tubes. Pairs of mice were restrained by small stainless steel screens over glass funnels. The animals were without food and water throughout the collection period, which was 17 hours (5 P.M.-10 A.M.). For determination of NPN one volume of blood (about 1 cc) was mixed with 2 volumes of 10% trichloroacetic acid, centrifuged at 3000 RPM for 3 minutes and then filtered. One cc of the filtrate was combined with 0.5 cc 36N H_2SO_4.

Results. Three separate experiments were conducted, each with 6 values per group. Two sets of values were combined to give the data presented in Fig. 1 and the final experiment, carried out some months later than the others, confirmed the earlier findings. The results are presented graphically in the Figure. DCI did not increase urinary nitrogen or urinary volume outside normal limits. Cortisone did augment these parameters and the differences were significant. In combination DCI and cortisone acetate doubled urine volume and markedly increased urinary nitrogen excretion during the 17 hours of the test. Endotoxin alone depressed urinary nitrogen elimination and blood non-protein nitrogen levels reached very high values. If the mice were pretreated