Effect of Heat on Antigenicity and Immunogenicity of the Antigenic Determinant Shared by *Haemophilus influenzae* Type b and *Escherichia coli* K100

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*Escherichia coli* K100 produces an antigenic determinant similar to, or identical with, the capsular antigen of *Haemophilus influenzae* type b. Studies of the effects of heat on the immunogenicity, erythrocyte-modifying capacity, and antigenicity of this cross-reacting antigen (CRA) revealed the following findings. Immunization of rabbits with viable or formaldehyde-killed suspensions of *E. coli* K100, producing CRA, engendered CRA antibodies in significant titers, as demonstrated by hemagglutination of erythrocytes modified by *H. influenzae* type b antigen. Heating of the suspensions for 1 h at 56 or 100°C destroyed the immunogenicity of CRA, and the heated suspensions did not prime for a secondary antibody response. Supernatants of heated suspensions also were non-immunogenic. Repeated freezing and thawing of heated suspensions of *E. coli* K100 or their supernatants did not restore immunogenicity. Heat also abolished the immunogenicity of *H. influenzae* type b. The loss of immunogenicity of *E. coli* K100 by heat was not due to alteration of the antigenic determinant, since heated suspensions and supernatants thereof modified erythrocytes for agglutination by *H. influenzae* type b antiserum. The latter supernatants also inhibited hemagglutination by *H. influenzae* type b antibodies and absorbed the latter. We conclude that striking differences exist in the effects of heat on CRA on the one hand and of enterobacterial common antigen and lipopolysaccharide O antigen of enteric bacteria on the other. Heating of the latter two antigens does not abolish their priming effect, and repeated freezing and thawing restores the immunogenicity of heated antigens.

*Haemophilus influenzae* type b is by far the most important pathogen of the species and is responsible for meningitis, epiglotitis, otitis, and other infections, notably of infants and young children. The capsular antigen, endowing the microorganism with its virulence, is a polyribophosphate (18). Rodriguez et al. (12), studying its biological and serological characteristics, showed that the antigens produced by various strains are identical. The antigen has a molecular weight of about 150,000 (12) and is immunogenic in adults (1) but not in rabbits or infants (10). It is well known that microorganisms may share antigenic determinants with unrelated species of bacteria, plants, and/or animals. The studies of Schneerson et al. (13) established the fact that certain strains of *Escherichia coli*, namely those with K100 antigen, produce a heat-stable, cross-reactive antigen (CRA) with properties similar to those of the capsular antigen of *H. influenzae* type b. Antibodies produced in rabbits by immunization with the appropriate *E. coli* strains precipitate the *H. influenzae* type b antigen. Because the crude O antigens of *Enterobacteriaceae* and the purified lipopolysaccharide preparations obtained therefrom as well as the ethanol-soluble enterobacterial common antigen lose their immunogenicity, although not their antigenicity, by heating (8, 16), it was deemed of interest to determine the effect of heat on the antigenic and immunogenic characteristics of CRA produced by *H. influenzae* type b and *E. coli* K100. The results of this investigation are presented.

**MATERIALS AND METHODS**

**Bacterial strains.** The strain of *E. coli* O75:K100:H5 NIH (Easter) was obtained through the courtesy of John B. Robbins, Food and Drug Administration, Bethesda, Md. The strain of *E. coli* O75:K100 (Baker) was isolated in this laboratory from the cerebrospinal fluid of a patient with meningitis due to two serotypes of *E. coli* (4), and the strain of *E. coli* O75:K100 negative (Thompson) was isolated from

† Deceased 24 June 1976.
the sputum of a patient with cystic fibrosis. Two strains of *H. influenzae* type b (8549 and 5517) were recovered from the cerebrospinal fluid of patients with meningitis. The strains were maintained by repeated transfers on chocolate agar and by preservation in skim milk at −70°C.

For the preparation of antigens, the *E. coli* strains were grown on brain veal agar in Kolle flasks for 18 h at 37°C. The resulting growth was suspended in 25 ml of phosphate hemagglutination buffer (Difco; pH 7.3) per flask. The *H. influenzae* strains were grown for 18 h at 37°C in a CO2 incubator in petri dishes of 14-cm diameter on chocolate agar made with Mueller-Hinton base. The resulting growth was suspended in 4.5 ml of saline solution containing 0.5% formaldehyde per dish.

**Bacterial antigens.** For the study of antigenicity and immunogenicity, the following preparations were used. Bacteria were suspended in hemagglutination buffer; portions were kept unheated or were heated for 1 h at either 56 or 100°C. Suspensions were also prepared in 0.5% formaldehyde and were used after being sterilized. Supernatants were prepared from some of these suspensions by centrifugation at 23,500 × *g* for 10 min. Portions from supernatants of unheated suspensions were heated for 1 h at 100°C. These supernatants were stored at −20°C until used.

**Immunization.** Albino rabbits, weighing 2 to 3 kg, were each injected intravenously with 1 ml of antigen on days 0, 3, 5, 7, 10, 12, 14, and 17. The animals were bled before immunization and on days 14 and 21. In certain experiments, rabbits were challenged after primary immunization, as described below, and sera were obtained 4 and 7 days after the booster injection. Three rabbits were used for each titration.

**Antisera.** The following antisera were used: *E. coli* CRA antisera were prepared by intravenous immunization of rabbits with unheated suspensions of *E. coli* O75K100 (Easter) or (Baker). *H. influenzae* type b antisera were prepared by intravenous immunization of rabbits with formaldehyde-killed suspensions of *H. influenzae* type b (strains 8549 and 5517). *E. coli* O75 antiserum was purchased from Difco Laboratories, Detroit, Mich.; *H. influenzae* type b rabbit antiserum was made available by the Department of Public Health, Division of Biological Laboratories, Boston, Mass., and the Division of Laboratories and Research, New York State Department of Health, Albany.

**Titration of antibodies.** Antibodies against CRA were titrated by using the hemagglutination test according to the previously described procedure (17). Briefly, washed rabbit erythrocytes (2.5% suspension) were mixed with antigen (supernatants of appropriate cultures); the mixtures were incubated at 37°C for 30 min and washed to remove excess antigen. Serum in serial twofold dilutions (0.2 ml) was mixed with equal amounts of antigenically modified erythrocytes. The mixtures were incubated for 30 min at 37°C, and the resulting hemagglutination was read grossly after centrifugation at 1,300 × *g* for 2 min.

**Erythrocyte-modifying capacity of CRA.** CRA from *E. coli* was mixed with erythrocytes, as described above, and the suspension was tested with *H. influenzae* type b antiserum in appropriate dilutions. Hemagglutination indicated the presence of CRA. In all experiments, antigen from *E. coli* lacking CRA was used for control purposes.

**Antibody-combining capacity of CRA.** To determine the specificity and quantity of CRA, the material in serial twofold dilutions (0.2 ml) was mixed with equal amounts of 2 hemagglutinating units of the corresponding antiserum. The mixtures were incubated at 37°C for 30 min. Erythrocytes modified with CRA from either *E. coli* or *H. influenzae* type b (0.2 ml) were then added to the mixtures, and the hemagglutination test was completed as described above. Inhibition of hemagglutination was considered as evidence of the specificity of CRA, and the highest dilution of the antigen in inhibition hemagglutination was considered indicative of its quantity.

In additional experiments, CRA antisera were mixed with equal amounts of supernatants of bacterial suspensions. The mixtures were incubated at 37°C for 1 h and then centrifuged. The supernatants, together with appropriate controls, were titrated as described above. Reduction of the antibody titer by at least 75% was considered as evidence of the specificity of the CRA antibodies. The bacterial suspensions instead of supernatants were used for neutralizing CRA antibodies in the absorption tests, described below.

**Absorption tests.** Antisera in appropriate dilution were absorbed with equal amounts of bacterial suspensions. The mixtures were incubated at 37°C for 1 h, centrifuged, and titrated as described above.

**Gel diffusion precipitation test.** The gel diffusion precipitation test was carried out as described previously (15). Briefly, agarose at a concentration of 0.5% was used as the medium in plastic petri dishes. The circular wells were 4 to 6 mm in diameter. The distance between the edges of antibody and antigen wells was 2 to 3 mm. The plates were kept at 4°C, and precipitation was recorded during a 7-day period.

**RESULTS**

**Immunogenicity.** Preliminary experiments have revealed that, as expected, *E. coli* O75 (Easter) produces antigen that cross-reacts with *H. influenzae* type b and is detected by the hemagglutination test utilizing *H. influenzae* antiserum. CRA also is produced by the *E. coli* O75 (Baker) strain, in contrast to the *E. coli* O75 (Thompson) strain. Supernatants of heat-killed (100°C) suspensions of Easter and Baker strains modified erythrocytes for hemagglutination of *H. influenzae* type b antiserum in titers similar to those obtained with *H. influenzae* type b modified erythrocytes.

The effect of heat on the immunogenicity of CRA produced by *E. coli* and *H. influenzae* was studied next (Table 1). Heating for 1 h at either 56 or 100°C abolished the immunogenicity of CRA present in bacterial suspensions. The specificity is documented by the facts that CRA produced by *E. coli* K100 stimulated antibody titers against *H. influenzae* type b similar to those stimulated by the latter microorganism and that the CRA-negative Thompson strain of

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E. coli failed to engender such antibodies. In accord with previous findings (13), formaldehyde-killed suspensions caused the production of CRA antibodies in titers similar to those stimulated by viable suspensions (Table 1). As expected from these results, supernatants of heated suspensions of E. coli K100 failed to stimulate CRA antibody production. The loss of immunogenicity of CRA by heat was not due to the destruction of the antigen, as documented below.

Previous studies with the common antigen of Enterobacteriaceae and with lipopolysaccharides of gram-negative bacteria revealed that repeated freezing and thawing restores to a significant degree the immunogenicity lost due to heating (8, 16). Accordingly, heated suspensions of E. coli K100 and H. influenzae, as well as supernatants thereof, were treated by repeated freezing and thawing. These antigens did not engender CRA antibodies.

Since heated lipopolysaccharides in amounts that fail to engender O antibodies in rabbits nonetheless prime these animals to a booster injection of minimally immunogenic unheated antigen (8), it was deemed of interest to learn whether rabbits immunized with heated suspensions of the Easter and Baker strains of E. coli were primed for an accelerated antibody response to CRA. Animals so immunized were challenged with an unheated suspension of the Easter strain and, for control purposes, with H. influenzae type b as well. Nonimmunized rabbits were used in parallel. Antibodies were not produced after the booster immunization. Thus, important differences in the immunogenicity of lipopolysaccharide O antigens and of the CRA of E. coli have come to light. The immunogenicity of CRA is destroyed by heating at 56 and 100°C and is not restored by repeated freezing and thawing; further, the animals immunized with these antigens are not primed for a secondary antibody response.

Erythrocyte-modifying capacity. In view of the fact that heated suspensions of the E. coli strains producing CRA failed to induce CRA antibodies in the rabbits, it was of interest to determine the possible effect of heat on the antigenic determinant. In the first series of experiments, therefore, erythrocytes were modified with supernatants from two CRA-positive strains. In parallel, similar preparations were obtained from a strain of H. influenzae type b (5517).

Modified erythrocytes were tested with H. influenzae type b antiserum and with CRA antiserum obtained by immunization with unheated suspensions of E. coli (Easter). For control purposes, E. coli O75 antiserum without CRA antibodies was used. CRA was heat stable, became attached to erythrocytes in either the unheated or heated state, and reacted with H. influenzae type b antibodies (Table 2). Thus, heat does not abolish the erythrocyte-modifying capacity of CRA and its reactivity with CRA antibody. The higher titers obtained with E. coli (Easter) serum of erythrocytes modified with heated rather than with unheated supernatant was due to the presence of O antibodies, as is evident from the fact that E. coli O antiserum without CRA antibodies caused hemagglutination of erythrocytes treated
with *E. coli* antigen. It is known that heat enhances the erythrocyte affinity of lipopolysaccharide O antigens (7). These experiments, then, show that the loss of immunogenicity by heat of CRA produced by *E. coli* K100 is not due to its loss of reactivity with CRA antibodies.

It is of interest to note that repeated (8 to 10) passages of freshly isolated strains of *H. influenzae* type b on chocolate agar resulted in significant loss of erythrocyte-modifying CRA present in the supernatants of these suspensions, although the bacterial cells themselves were still strongly agglutinated by homologous antiserum.

The antigenicity of CRA was studied further by hemagglutination inhibition and absorption tests. It was shown that CRA produced by *E. coli* K100 neutralized *H. influenzae* type b antibodies, irrespective of whether unheated or heated supernatants of bacterial suspensions were used. Thus, it can be concluded that heat does not destroy the antigenic determinant K100. Since it was shown by Robbins and co-workers (11) that the Easter strain of *E. coli* produces several antigens that cross-react with *H. influenzae* type b, it is not surprising that complete inhibition by *H. influenzae* antigens was not obtained when *E. coli* (Easter) antiserum was used in hemagglutination inhibition tests.

Gel diffusion studies revealed that the *E. coli* strains producing CRA yielded an antigen that was precipitated by *H. influenzae* type b serum and that the precipitation line was identical with that elicited by *H. influenzae* type b antigen. A second additional line was present with the CRA-positive *E. coli* strains and absent from *H. influenzae*. This line was seen only when supernatants from unheated suspensions were used and not with heated supernatants or supernatants obtained from heated suspensions. The presence of more than one line agrees with observations reported by Robbins et al. (11).

**DISCUSSION**

It is well known that immunogenicity depends not only on the nature of the antigen but also on the species of animals used for immunization. Thus, haptens may be non-immunogenic in rabbits but immunogenic in mice and humans. Recently, it was shown that lipopolysaccharides of gram-negative bacteria and the entrobacterial common antigen lose their immunogenicity by heating, although the ability of the heated preparations to neutralize antibodies remained unaffected (8, 16). It is of particular interest that immunogenicity could be restored, to a significant degree, by repeated freezing and thawing of the heated antigens. In the present investigation, the effect of heat on immunogenicity, erythrocyte affinity, and antigenicity of the cross-reacting antigen (CRA) of *H. influenzae* type b and *E. coli* was studied. As shown by Robbins et al. (11), it is the K100 antigen that is similar to or identical with the capsular antigen of *H. influenzae* type b. The latter antigen is a polyribophosphate. The present experiments have revealed that the immunogenicity of CRA, obtained from two strains of *E. coli* (Easter and Baker), was abolished by heating for 1 h at 56 or 100°C. The question thus arose of whether immunogenicity depends upon the presence of viable bacterial cells. That colonization with CRA-positive *E. coli* results in the formation of cross-reactive antibodies in newborn rabbits and adult rhesus monkeys was shown by Handzel et al. (5) and Myerowitz et al. (6). Further, formalin-killed suspensions of *E. coli* engender cross-reacting antibodies (13), an observation confirmed in the present study.

**Table 2. Erythrocyte-modifying capacity of CRA in different culture supernatants**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th><em>E. coli</em> (Easter)</th>
<th><em>E. coli</em> (Baker)</th>
<th><em>H. influenzae</em> type b 5517</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean hemagglutinin titer (reciprocal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UH*</td>
<td>UH-H</td>
<td>H</td>
</tr>
<tr>
<td><em>E. coli</em> (Easter)</td>
<td>480</td>
<td>3,340</td>
<td>&gt;10,240</td>
</tr>
<tr>
<td><em>E. coli</em> O75</td>
<td>&lt;10</td>
<td>1,280</td>
<td>3,340</td>
</tr>
<tr>
<td><em>H. influenzae</em> type b (6843)</td>
<td>480</td>
<td>240</td>
<td>480</td>
</tr>
<tr>
<td><em>H. influenzae</em> type b (Boston)</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Erythrocyte-modifying antigen. UH, Supernatant from unheated suspension; UH-H, supernatant from unheated suspension, heated for 1 h at 100°C; H, supernatant from heated suspension (1 h, 100°C).
* ND, Not done.
tion agrees with the observation that purified CRA, too, is nonimmunogenic in rabbits (10). If, as suggested by these observations, CRA in solution acts as a hapten in the rabbit, this characteristic differs strikingly from that of soluble lipopolysaccharides (O antigens) and the enterobacterial common antigen (8, 16).

Loss of immunogenicity by heating could be readily explained if heat altered the antigenic determinant. However, hemagglutination, hemagglutination inhibition, and absorption tests carried out with heated preparations of CRA clearly indicate that its antigenicity was not destroyed by heat.

In further studies on the differences between immunogenic and non-immunogenic CRA, it will be important to keep in mind that release from the H. influenzae cells of the antigen varies between different strains and that under certain cultural conditions larger and smaller units are found (1, 2).

Striking differences between CRA of E. coli K100 on the one hand and lipopolysaccharide O antigens as well as the enterobacterial common antigen on the other have come to light in the present investigation. Heating reduces the immunogenicity of all antigens. However, only heated O antigen and the enterobacterial common antigen become immunogenic upon repeated freezing and thawing. Further, only the K100 antigen, in contrast to the others, fails to prime the rabbit for a secondary response. With all three antigens the antigenicity is preserved. It remains for future investigations to determine whether the effect of heat on other K antigens of E. coli, as described by Órskov et al. (9) and Semjen et al. (14), resembles that on CRA. It will also be of interest to learn whether heated CRA attached to erythrocytes or other particles becomes immunogenic for rabbits.

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LITERATURE CITED


