Growth Responses of Coliform Bacteria to Purified Immunoglobulin G from Cows Immunized with Ferric Enterobactin Receptor FepA

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ABSTRACT

The ability of purified bovine immunoglobulin (Ig) G from cows immunized with ferric enterobactin receptor FepA to inhibit the growth of coliform bacteria derived from bovine intramammary infection was investigated in iron-restricted media. All isolates of *Escherichia coli* (n = 21) and *Klebsiella pneumoniae* (n = 21) were tested for growth in a chemically defined medium containing 0.5 mg/ml of apolactoferrin and in a pooled source of dry cow secretion. The addition of 4 mg/ml of purified bovine IgG directed against FepA in the synthetic medium resulted in significant growth inhibition for both *E. coli* and *K. pneumoniae* isolates. Growth reduction of *E. coli* was greater than that of *K. pneumoniae*. In dry cow secretions, the growth of each *E. coli* isolate but of less than half of *K. pneumoniae* isolates (43%) was inhibited by IgG from cows immunized with FepA. Purified bovine IgG from cows immunized with *E. coli* J5 had a minimal inhibitory effect on the growth of both *E. coli* and *K. pneumoniae* isolates in the synthetic medium. In dry cow secretions, IgG from cows immunized with *E. coli* J5 had no inhibitory effect on the growth of *E. coli* and *K. pneumoniae* isolates. Supplementation with 50 μM of ferric chloride to the medium completely reversed the inhibitory effects of the antibodies and lactoferrin. Bovine IgG directed against FepA apparently inhibited the growth of coliform bacteria by interfering with the binding of the ferric enterobactin complex to the cell surface receptor FepA.

(Key words: coliform bacteria, ferric enterobactin receptor, mastitis)

Abbreviation key: apoLf = apolactoferrin, BSB = Bacto Synthetic Broth® (Difco Laboratories, Detroit, MI), IROMP = iron-regulated outer membrane proteins, Lf = lactoferrin, LPS = lipopolysaccharide, MAb = monoclonal antibody, SDCS = standard dry cow secretion.

INTRODUCTION

Under the normal physiological environment with low iron availability, Gram-negative bacteria synthesize and secrete a group of low molecular mass compounds named siderophores that bind iron with high affinity (18). The siderophores are too large (600 to 1200 Da) to pass through the porin channels of the bacterial outer membrane (19, 20). Therefore, the siderophores require specific iron-regulated outer membrane proteins (IROMP) to enable their passage across the bacterial outer membrane and into the periplasm (6, 10). The ability to assimilate ferric siderophores through these receptors can be an important virulence factor (7, 9, 31, 35). Consequently, IROMP are often suggested as vaccine components to control bacterial infections (30, 32).

Enterobactin is the primary siderophore of *Escherichia coli* and many other Gram-negative bacteria (24). Ferric enterobactin receptor FepA, an IROMP, is crucial to the enterobactin-mediated system for iron uptake because FepA specifically binds ferric enterobactin (16). The molecular mass and antigenic properties of FepA were highly conserved among different genera of Gram-negative bacteria (24), including coliform isolates from naturally occurring bovine IMI (14). These characteristics make FepA a possible antigen choice for vaccine formulation to control coliform mastitis during the nonlactating period when most iron in bovine mammary gland secretions is bound to lactoferrin (Lf) (2). A previous study (13) indicated a murine monoclonal antibody (MAb) that was specific for blocking the FepA binding site inhibited the growth of coliform bacteria isolated from bovine IMI. A recent study (12) demonstrated that immunization with native FepA successfully elicited an immunological response in bovine serum and milk. However, the growth responses of coliform bacteria to
antisera from cows immunized with purified FepA are unknown. The objective of this study was to compare growth responses of coliform bacteria to highly purified bovine IgG from cows immunized with FepA in a chemically defined medium containing apolactoferrin (apoLf) and in a pooled source of dry cow secretions.

**MATERIALS AND METHODS**

**Bacterial Isolates**

The isolates tested were *E. coli* (n = 21) and *Klebsiella pneumoniae* (n = 21) from bovine IMI in five herds. A mutant strain of *E. coli*, AN193B3J3 (entA−), was kindly provided by Mark Coy (University of California, Berkeley). The mutant cannot produce enterobactin and lacks FepA outer membrane receptor activity. All coliform bacteria were stored on trypticase soy agar slants (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) at 25°C prior to use.

**Immunization**

To obtain desired serum, 15 multiparous cows, ranging from 160 to 230 DIM, were assigned to three treatment groups of 5 cows blocked by breed and DIM. The treatment groups were immunized with 100 or 500 µg of FepA or *E. coli* J5 (5 × 10⁹ boiled cells/ml). The cows were injected subcutaneously posterior to the scapula on approximately 200 DIM and received booster immunizations 14 and 28 d later. Serum (25 ml of blood) was collected from all cows on d 35 after the primary immunization. Sera from cows immunized with FepA were pooled for IgG purification because IgG responses did not differ between immunization doses (12).

**Purification of Bovine IgG**

Bovine IgG was purified from serum by ammonium sulfate precipitation and protein G affinity chromatography as described previously (12). The IgG fraction of pooled serum samples was first precipitated by using ammonium sulfate. Then, precipitated IgG was further purified by protein G affinity chromatography (Protein G Sepharose®4 Fast Flow; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The affinity column (1.5 × 5 cm) equilibrated with the binding buffer (20 mM sodium phosphate, pH 7.0) was prepared as described by the manufacturer. The IgG sample was dialyzed against binding buffer and was applied to the column. The ratio of total applied protein to bed volumes was 20 mg/ml. The flow rate was 0.8 ml/min. The column was washed with 10 bed volumes of binding buffer. The bound bovine IgG was eluted by 10 bed volumes of elution buffer (0.1 M glycine-HCl, pH 2.7). One hundred microliters per milliliter of eluate of 1 M Tris-HCl (pH 9.0) was placed in the fraction collector tubes prior to use. The purification profile of bovine IgG was monitored at 280 nm. The protein-containing fractions were pooled and dialyzed against PBS at 4°C. The purity of the IgG was determined by 12% SDS-PAGE utilizing the discontinuous buffer system of Laemmli (11). The purified bovine IgG was stored at −20°C prior to use. The IgG concentration was measured by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

All glassware was washed in 0.1N concentrated nitric acid for 4 h and rinsed three times in distilled, deionized water. Water for buffer and media was distilled and subjected to the NANOpure system (Barnstead Thermolyne, Dubuque, IA); conductivity was tested prior to use. Dialysis tubing was treated with sodium carbonate and EDTA (2).

**Standard Dry Cow Secretion**

Standard dry cow secretion (SDCS) was prepared from mammary secretions obtained from 20 cows at d 21 of the dry period. The secretion from all cows was pooled and centrifuged at 48,000 × g at 4°C for 60 min. The fat layer was removed, and the supernatant was decanted. The cell-free, fat-free supernatant was sterilized by filtration through a series of membrane filters (prefilter and 12.0, 5.0, 1.2, 0.8, and 0.45 µm; Schleicher & Schuell, Keene, NH). The filtered secretion was stored at −20°C prior to use.

**In Vitro Growth of Coliform Bacteria**

In vitro growth of coliform bacteria was as described by Toddhunter et al. (28) with the following exceptions. Prior to in vitro growth assays, coliform bacteria were grown for 6 h at 37°C in 5 ml of trypticase soy broth containing 300 µM α-α′-dipyridyl. The cultures were washed twice in PBS and serially diluted in PBS to achieve approximately 10⁶ cfu/ml. All in vitro growth assays were performed in 96-well microtiter plates (Sarstedt, Newton, NC); the total volume was 260 µl per well consisting of 250 µl of synthetic medium inoculated with 10 µl of diluted coliform bacteria. Growth assay in SDCS consisted of 240 µl of SDCS inoculated with 20 µl of a mixture of diluted bacteria plus IgG. An equal volume (10 µl:10 µl) of diluted coliform bacteria and concentrated IgG were mixed and incubated at 37°C for 30 min prior to
use. The cultures were aerobically incubated in a humidified atmosphere at 37°C for 10 h for growth assay in the synthetic medium or for 18 h for growth assay in the SDCS. All in vitro microcultures were completed in duplicate. Following incubation, the cultures were serially diluted in PBS, and four 10-μl spots of diluted and undiluted cultures were delivered onto the surface of a MacConkey agar plate (Difco Laboratories, Detroit, MI). MacConkey agar plates were incubated for 12 h at 37°C, the bacterial colonies were counted, and in vitro growth was expressed as log10 colony-forming units per milliliter. The minimum detection limit of the assay was 1.3979 cfu log10/ml. The control medium for optimal growth of coliform bacteria was Bacto Synthetic Broth® (BSB; Difco Laboratories) supplemented with dextrose (2 g/L). The iron-restricted medium was SDCS or BSB containing 0.5 mg of apoLf/ml. The final concentration of sterile bovine IgG in iron-restricted synthetic medium or SDCS was 4 mg/ml. Ferric chloride (50 μM) was added to the medium to determine whether exogenous iron would override the inhibitory effects of antibodies.

**Statistical Analysis**

Differences between bacterial species in different media were analyzed by least squares analysis of covariance; growth in the control medium (BSB for growth in the synthetic medium and SDCS for growth in the SDCS) was the covariant. Mean comparisons among different media within species were tested by ANOVA. Multiple comparisons of the means of unequal sample size were analyzed by the Tukey-Kramer method. All statistical analyses were performed using MINITAB® software (15).

**RESULTS**

**IgG Purification**

The method of ammonium sulfate precipitation combined with Protein G affinity chromatography resulted in highly purified bovine IgG (12). The majority of albumin was removed by the ammonium sulfate precipitation. The SDS-PAGE was run under reducing conditions that resulted in a typical IgG profile appearing as its constituent heavy chains (∼55 kDa) and light chains (∼25 kDa).

**Growth Responses to Bovine IgG in BSB**

The effects of purified IgG on the in vitro growth of coliform bacteria were initially tested by culturing the isolates in the synthetic medium BSB, which contained 0.5 mg/ml of apoLf and 4 mg/ml of IgG from cows immunized with FepA or *E. coli* J5 (Table 1). Compared with the optimal growth in BSB, both *E. coli* and *K. pneumoniae* isolates had reduced (*P < 0.05*) growth in the presence of 0.5 mg/ml of apoLf. The addition of 4 mg/ml of purified bovine IgG directed against FepA in BSB supplemented with apoLf resulted in additional growth inhibition (*P < 0.05*) of both *E. coli* and *K. pneumoniae* isolates. Supplementation of BSB with bovine anti-FepA IgG resulted in greater (*P < 0.05*) growth reduction of *E. coli* than that of *K. pneumoniae*. Purified IgG from cows immunized with *E. coli* J5 had no inhibitory effect on the growth of *E. coli* isolates (*P > 0.05*) but had a slight inhibitory effect (*P < 0.05*) on the growth of *K. pneumoniae* isolates. In vitro growth of *E. coli* AN193B213 in the presence of IgG from a cow immunized with FepA or *E. coli* J5 did not differ (*P > 0.05*) from the growth in BSB plus apoLf. Supplementation with 50 μM of ferric chloride to the medium completely reversed the inhibitory effects of the lactoferrin and antibodies.

**Growth Responses to Bovine IgG in SDCS**

Table 2 shows the effects of purified IgG from cows immunized with FepA or *E. coli* J5 on the growth of *E. coli* and *K. pneumoniae* isolates in SDCS. The addition of 4 mg/ml of purified anti-FepA IgG to SDCS resulted in a growth reduction (*P < 0.05*) of *E. coli* isolates. Contrary to the growth responses in synthetic medium, the growth of *K. pneumoniae* was not inhibited (*P > 0.05*) by the addition of purified IgG from cows immunized with FepA. Only 9 of 21 *K. pneumoniae* isolates (43%) experienced >0.2 cfu log10/ml of growth reduction in the presence of anti-FepA IgG in SDCS. The supplementation with 50 μM of ferric chloride to SDCS completely reversed the inhibitory effects of the antibodies. The purified IgG from cows immunized with *E. coli* J5 had no inhibitory effect (*P > 0.05*) on the growth of *E. coli* isolates but had an enhancing effect (*P < 0.05*) on the growth of *K. pneumoniae*. The enhancing effect of anti-J5 IgG was >0.2 cfu log10/ml for 14 of 21 *K. pneumoniae* isolates in SDCS. The growth of only two *E. coli* isolates was enhanced (>0.2 cfu log10/ml) by the addition of IgG from cows immunized with *E. coli* J5.

**DISCUSSION**

The purpose of the in vitro growth assays in BSB and in SDCS was to investigate the interaction be-
between coliforms and host defenses during bacterial multiplication within iron-restricted environments. The current study showed that polyclonal IgG from cows immunized with FepA successfully inhibited both *E. coli* and *K. pneumoniae* isolates in synthetic medium. These results differ from an earlier finding in which a MAb directed against FepA inhibited the growth of *E. coli* but had no significant effect on the growth of *K. pneumoniae* because of antigenic variation in the enterobactin ligand-binding site (13). Unlike polyclonal antiserum that is expected to contain a large quantity of different antibodies that recognize various epitopes, MAb is an epitope-specific antibody. Cows immunized with FepA may produce antibodies directed against the epitopes at the ligand-binding site or in the other regions of FepA that are important for FepA conformation. In the current study, anti-FepA IgG was demonstrated to inhibit the growth of

**TABLE 1.** Effects of purified IgG from cows immunized with FepA or *Escherichia coli* J5 on the growth of *E. coli* and *Klebsiella pneumoniae* in a chemically defined medium.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th><em>E. coli</em> (n = 21)</th>
<th><em>K. pneumoniae</em> (n = 21)</th>
<th><em>E. coli</em> AN193B4J3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>SE</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td>BSB</td>
<td>8.91$^{a,x}$</td>
<td>0.04</td>
<td>9.00$^{a,x}$</td>
</tr>
<tr>
<td>BSB + apoLf</td>
<td>6.98$^{b,y}$</td>
<td>0.16</td>
<td>7.53$^{c,x}$</td>
</tr>
<tr>
<td>BSB + apoLf + α-FepA</td>
<td>6.14$^{a-z}$</td>
<td>0.19</td>
<td>6.99$^{a-x}$</td>
</tr>
<tr>
<td>BSB + apoLf + α-J5</td>
<td>6.85$^{b,x}$</td>
<td>0.19</td>
<td>7.17$^{c,x}$</td>
</tr>
<tr>
<td>BSB + apoLf + α-FepA + FeCl₃</td>
<td>8.95$^{a-x,y}$</td>
<td>0.03</td>
<td>9.07$^{a-x,x}$</td>
</tr>
<tr>
<td>BSB + apoLf + α-J5 + FeCl₃</td>
<td>8.92$^{a-x,y}$</td>
<td>0.03</td>
<td>9.03$^{a-x,x}$</td>
</tr>
</tbody>
</table>

$^{a,b,c}$Means within a column with no common superscript differ ($P < 0.05$).

$^{x,y,z}$Means within a row with no common superscript differ ($P < 0.05$).

1BSB = Bacto Synthetic Broth® (Difco Laboratories, Detroit, MI), apoLf = apolactoferrin, α-FepA = purified IgG from cows immunized with FepA, and α-J5 = purified IgG from cows immunized with *E. coli* J5. Concentration of apoLf = 0.5 mg/ml, concentration of α-FepA = 4 mg/ml, concentration of α-J5 = 4 mg/ml, and concentration of FeCl₃ = 50 μM.

2Number of isolates.

3Results are means of six independent determinations.

**TABLE 2.** Effects of purified IgG from cows immunized with FepA or *Escherichia coli* J5 on the growth of *E. coli* and *Klebsiella pneumoniae* in standard dry cow secretion.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th><em>E. coli</em> (n = 20)</th>
<th><em>K. pneumoniae</em> (n = 21)</th>
<th><em>E. coli</em> AN193B4J3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>SE</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td>SDCS</td>
<td>3.92$^{b,y}$</td>
<td>0.32</td>
<td>5.35$^{c,d,x}$</td>
</tr>
<tr>
<td>SDCS + α-FepA</td>
<td>3.17$^{c,x}$</td>
<td>0.37</td>
<td>5.23$^{d,a}$</td>
</tr>
<tr>
<td>SDCS + α-J5</td>
<td>3.69$^{b,y}$</td>
<td>0.32</td>
<td>5.67$^{c,b,x}$</td>
</tr>
<tr>
<td>SDCS + FeCl₃</td>
<td>8.44$^{a,x,y}$</td>
<td>0.13</td>
<td>8.75$^{a,x,x}$</td>
</tr>
<tr>
<td>SDCS + α-FepA + FeCl₃</td>
<td>8.41$^{a,x,y}$</td>
<td>0.12</td>
<td>8.75$^{a,x,x}$</td>
</tr>
<tr>
<td>SDCS + α-J5 + FeCl₃</td>
<td>8.59$^{a,x,y}$</td>
<td>0.08</td>
<td>8.78$^{a,x,x}$</td>
</tr>
</tbody>
</table>

$^{a,b,c,d}$Means within a column with no common superscript differ ($P < 0.05$).

$^{x,y,z}$Means within a row with no common superscript differ ($P < 0.05$).

1SDSC = Standard dry cow secretion, α-FepA = purified IgG from cows immunized with FepA, and α-J5 = purified IgG from cows immunized with *E. coli* J5. Concentration of α-FepA = 4 mg/ml, concentration of α-J5 = 4 mg/ml, and concentration of FeCl₃ = 50 μM.

2Number of isolates.

3Results are means of six independent determinations.
all coliform isolates in the chemically defined medium containing apoLf. In SDCS, the growth of all *E. coli* isolates and 43% of *K. pneumoniae* isolates were inhibited by the addition of anti-FepA IgG. Supplementation of exogenous iron reversed the inhibitory effects of the antibodies. Therefore, bovine IgG directed against FepA apparently inhibited the growth of coliform bacteria by interfering with the binding of ferric enterobactin complex to its cell surface receptor FepA, because Gram-negative bacteria can use a low affinity iron acquisition system when free iron is available in the medium (6). Consequently, after antibodies migrate into the mammary gland, these anti-FepA antibodies may prevent iron uptake of heterologous coliforms by blocking FepA, thereby reducing the ability of coliforms to replicate and cause IMI during the nonlactating period.

The mechanism by which immunization with LPS core-antigen vaccines protect the mammary gland from clinical mastitis is not entirely understood. Previously proposed (8, 29) mechanisms of action for crossreactive immunity to Gram-negative bacterial infections include enhanced opsonization of bacterial cells and lipopolysaccharide (LPS) and detoxification of LPS by blocking active lipid A epitopes. However, the ability of the dry cow secretion to support phagocytosis is decreased during involution of the mammary gland (22). The decreased ability of opsonization was due to the formation of immune complexes that blocked Fc receptors on polymorphonuclear leukocytes (27). The high concentration of Lf, an iron-binding protein, dramatically increased from 0.1 to 0.3 mg/ml in bovine milk to 30 mg/ml in dry cow secretion (1). The bactericidal effect of anti-FepA IgG differed in the presence of bovine anti-FepA IgG in the chemically defined medium BSB, a low percentage of *K. pneumoniae* isolates was inhibited by anti-FepA IgG in SDCS. Three possibilities may explain this finding. Murphy (16) reported that ferric enterobactin-binding domains of FebA of *E. coli* are free of LPS steric hindrance. No information is currently available concerning the molecular structure of FepA expressed by the *K. pneumoniae* strain. The polysaccharide moiety of LPS might have shielded FepA of some *K. pneumoniae* isolates from the interaction of the antibodies and concomitantly have decreased the antibacterial effect of purified IgG from cows immunized with FepA in SDCS. In addition, the presence of a capsule surrounding *K. pneumoniae* in vivo (33) might have prevented access of antibodies to their target, the bacterial outer membrane in SDCS. Consequently, anti-FepA IgG could not easily bind to the FepA receptor in SDCS. The third possibility is that some *K. pneumoniae* isolates possibly used other iron-uptake systems in addition to the ferric enterobactin system, such as an iron-uptake system mediated by aerobactin.

The concentration of Lf, an iron-binding protein, in SDCS, the growth of all *E. coli* isolates and 43% of *K. pneumoniae* isolates was significantly inhibited in the presence of bovine anti-FepA IgG in the chemically defined medium BSB, a low percentage of *K. pneumoniae* isolates was inhibited by anti-FepA IgG in SDCS. Three possibilities may explain this finding. Murphy (16) reported that ferric enterobactin-binding domains of FebA of *E. coli* are free of LPS steric hindrance. No information is currently available concerning the molecular structure of FepA expressed by the *K. pneumoniae* strain. The polysaccharide moiety of LPS might have shielded FepA of some *K. pneumoniae* isolates from the interaction of the antibodies and concomitantly have decreased the antibacterial effect of purified IgG from cows immunized with FepA in SDCS. In addition, the presence of a capsule surrounding *K. pneumoniae* in vivo (33) might have prevented access of antibodies to their target, the bacterial outer membrane in SDCS. Consequently, anti-FepA IgG could not easily bind to the FepA receptor in SDCS. The third possibility is that some *K. pneumoniae* isolates possibly used other iron-uptake systems in addition to the ferric enterobactin system, such as an iron-uptake system mediated by aerobactin.

The concentration of Lf, an iron-binding protein, dramatically increased from 0.1 to 0.3 mg/ml in bovine milk to 30 mg/ml in dry cow secretion (1). The high concentration of Lf can be attributed in part to the enhanced bacteriostatic properties of secretion from the involuted bovine mammary glands (1). Lactoferrin has bacteriostatic properties because of its ability to chelate iron (1, 28). In addition, Lf can bind to the bacterial surface and directly kill bacteria by damaging the outer bacterial membrane, subsequently changing its permeability (3, 34). Damage to the bacterial membrane involves the binding of Lf to LPS molecules and releasing them from the outer membrane of Gram-negative bacteria (34). More recently, Naidu et al. (17) showed a correlation between Lf binding to porins and a bactericidal effect mediated by Lf. Erdei et al. (4) further showed that the binding of Lf to porins caused changes to the permeability of *E. coli*. In the current study, two different media were used to determine bacterial growth. In SDCS, bacterial growth was strongly inhibited compared with growth in BSB plus apoLf. The inhibitory property of SDCS wa attributed to Lf, Ig, and other unknown components (28). Bactericidal function of Lf may play a dominant role in the inhibitory properties of SDCS because the Lf concentration in the growth media was critical for the antibacterial effect (17). Alternatively, the low concentration of apoLf used in the synthetic medium BSB was suffi-
cient to create an iron-restricted condition but possibly not enough to induce a strong bactericidal effect.

A surprising finding in the SDCS assay system was the growth of K. pneumoniae, which was enhanced in the presence of purified IgG from cows immunized with E. coli J5. The possibility of iron carryover in purified IgG that could overcome the inhibitory effect caused by Lf was minimized. To exclude iron carryover in the growth assay, affinity-purified bovine IgG was used. The small amount of extracellular iron in serum is bound to transferrin (21). Protein G affinity chromatography resulted in highly purified bovine IgG (12) that apparently did not contain any iron-binding protein. Therefore, some other mechanisms may contribute to the enhanced growth of K. pneumoniae in the presence of anti-J5 IgG in SDCS.

CONCLUSIONS

The purified IgG from cows immunized with ferric enterobactin receptor FepA inhibited the growth of all coliform isolates derived from naturally occurring bovine IMI in the chemically defined medium containing apoLf. In SDCS, the growth of all E. coli isolates and 43% of K. pneumoniae isolates was inhibited by the addition of anti-FepA IgG. The supplementation with exogenous iron to the medium completely reversed the inhibitory effects of antibodies, which suggests that bovine IgG directed against FepA apparently inhibited the growth of coliform bacteria by interfering with the binding of ferric enterobactin complex to its cell surface receptor FepA. Therefore, FepA is a possible antigen for use in vaccine formulation to control coliform mastitis during the nonlactating period.

ACKNOWLEDGMENTS

The authors greatly appreciate the technical assistance of Pamela Schoenberger, Sue Roming, and Lisa Thompson.

REFERENCES


