Experimental Neonatal Colibacillosis in Cows: Immunoglobulin Classes Involved in Protection

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Pregnant cows were vaccinated with one of four vaccine preparations to induce passive immunity in their offspring against a homologous oral challenge with Escherichia coli strain B-44. Quantitative assays of specific antibody in colostral whey from both immunized and nonimmunized dams revealed that immunoglobulin G, immunoglobulin A (IgA), and immunoglobulin M (IgM) with anti-O (somatic) activity were present in whey of all dams tested, whereas a marked deficiency of IgA and IgM anti-K immunoglobulin was noted in the whey from control dams only. The degree of scours (neonatal colibacillosis) induced by oral challenge was evaluated clinically and reported by a semiquantitative scour index as 0 to 4+. Calf scour indexes showed an inverse relationship to the frequency of occurrence and to the levels of IgA and IgM in whey of dams vaccinated with killed vaccine, live vaccine, and culture supernatant, and from nonvaccinated controls. The data strongly suggested that IgA and colostral IgM anti-K immunoglobulins were important in passive immunity in experimental neonatal bovine colibacillosis.

In a previous report we have shown that various Escherichia coli vaccines induced the formation of antibodies in preparturient bovine which protected calves against experimental neonatal colibacillosis (scours) (15, 21). These data strongly suggested that protection against scours was afforded by both anti-O and anti-K antibodies acquired by the calf through the colostrum. The nature of the protective antigen and the immunoglobulin class of the protective antibody were not elucidated in these studies and are the subject of this report.

MATERIALS AND METHODS

Test animals. A 41-cow herd immunized with E. coli B-44 vaccine described previously (15) was used in these studies.

Preparation of immunoglobulins. Bovine immunoglobulin G, (IgG), and IgG were purchased in a purified form from Miles Laboratories (Research Division, Kankakee, Ill.). Bovine immunoglobulin A (IgA) was isolated from saliva obtained by suction from the buccal cavity of three cows. The saliva was clarified by centrifugation and dialyzed in the cold for 40 h against 0.03 M ammonium bicarbonate. The supernatant was concentrated threefold in a vacuum chamber and then dialyzed against 0.01 phosphate-buffered saline (PBS) in preparation for chromatographic separation. The concentrate was applied to Sephadex G-200 columns and eluted with PBS, and the fraction comprising the first major peak was pooled, concentrated, and again filtered through Sephadex G-200. The pooled fractions of the leading peak obtained after three cycles through G-200 were tested for the presence of IgG, IgA, and immunoglobulin M (IgM) using monospecific antisera in an immunodiffusion assay (17). A standard monospecific anti-IgA serum was obtained from J. P. Mach (Lausanne, Switzerland) to confirm the identity and purity of the IgA preparation.

Bovine IgM was isolated from the alpha-beta fraction of a serum concentrate separated by Pevikon block electrophoresis. IgM was purified by alternating molecular sieve and anion-exchange chromatographic procedures.

Preparation of monospecific anti-immunoglobulin. Rabbit antisera to bovine IgG, IgG, IgA, and IgM were developed by repeated injections of the purified immunoglobulins into adult albino rabbits. The first dose of 1 mg was emulsified in Freund complete adjuvant and three subsequent doses of 1 mg each were given subcutaneously with incomplete Freund adjuvant. The rabbits were bled 5 days before and 10 days after the fourth antigenization. Injections were given at 8- to 10-day intervals.

All antisera showed cross-reactivity to the four major immunoglobulins as a result of light-chain precipitating antibody. Therefore, light- and heavy-chain cross-reactive antibody was removed by absorbing the sera with immunoglobulin of the appropriate class bound to Sepharose 4-B with cyanogen bromide (4).

Quantitation of immunoglobulins. Immunoglobulins in serum and colostral whey were measured by a modified Massieyff-Zisswiler technique (13). Ten-millimeter disks were cut from 1% agarose layered in Hyland diffusion trays. Antisera were added to...
RESULTS

Immunological studies. (i) Quantitative assays of immunoglobulins in the dam. Serum specimens from two cows in each group were analyzed by single radial diffusion techniques to determine the total concentrations of serum IgG1, IgG2, IgA, and IgM. The results of these studies are shown in Table 1. The number of samples tested was too small to apply a statistical evaluation; therefore, the differences that were noted were viewed on a relative basis.

Serum levels of IgG1 were not markedly different between the control and vaccine groups. On the other hand, the mean values obtained for serum IgG2 showed some difference between control and vaccinated dams as well as among the different vaccine groups. The mean concentration of IgG2 in the sera of the control supernatant (CS) dams was nearly three times greater than control values and two times as high as levels in the serum of killed bacteria (KB) dams. The sera from live bacteria (LB) dams were not assayed for IgG2.

Serum levels of IgA, ranging from 5 to 13 mg/100 ml, were highest in the sera of dams antigenized with LB. Serum IgA in the LB dams was nearly three times greater than serum levels detected in the CS group.

The levels of serum IgM in control dams were lower than the values obtained in sera of the dams in the vaccine groups. These data would mildly suggest that subcutaneous and intramammary antigenization had a greater influence on IgG2, IgM, and serum IgA levels (Table 1) than on serum concentrations of IgG1.

Intramammary administration of antigenic material has been shown to affect the levels of immunoglobulins in lacteal secretions (11). Additionally, it has been noted that selective concentration of immunoglobulins can occur in the mammary glands of the bovine (7). Therefore, a random selection of whey specimens acquired from each vaccine and the control group was

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (2)</td>
<td>1,846</td>
<td>706</td>
<td>8</td>
<td>148</td>
</tr>
<tr>
<td>CS (2)</td>
<td>1,437</td>
<td>2,008</td>
<td>5</td>
<td>629</td>
</tr>
<tr>
<td>LB (2)</td>
<td>1,482</td>
<td>ND</td>
<td>13</td>
<td>457</td>
</tr>
<tr>
<td>KB (2)</td>
<td>1,575</td>
<td>1,041</td>
<td>8</td>
<td>644</td>
</tr>
</tbody>
</table>

* C, Adjuvant only. Parentheses indicate number of animals tested.
* Concentration of IgG1, IgG2, IgA, or IgM reported as milligrams per 100 ml of fluid.
* ND, Not done.
assayed for IgG₁, IgG₂, IgA, and IgM. The results of these studies are shown in Table 2. Little or no significant difference between the levels of whey immunoglobulins of vaccinated and control dams was observed. The elevated IgG₁ in the colostrum of the control dams was due primarily to an inordinately high level (9, 200 mg/100 ml) of 7S immunoglobulin in the right front quarter of one of the control dams used in the current study.

The variation between quarters of a cow may exceed variation between different groups of cows, as was seen in the assays for colostral IgG₁. Thus, the mean concentration of each group clusters about a value of 400 mg/100 ml, whereas the standard deviation for each group is extremely large. Variance is notably high (one standard deviation). Similarly, the data for IgA and IgM in the colostrum of control and vaccine groups show considerable variation among groups and among cows within groups.

(ii) Quantitative assays of immunoglobulins in the calf. The sera of neonatal calves, obtained soon after birth and before intake of colostrum, did not have detectable levels of IgG₁, IgG₂, or IgA (Table 3). Of particular interest, therefore, was the finding that neonatal calves did have detectable levels of IgM in their sera. Finding higher levels of this early immunoglobulin in calves in two of three vaccine groups would indicate that antigenization may have influenced the immunological status of the fetal calves.

It is apparent that neonatal calves acquired a considerable quantity of IgG₁, IgA, and IgM from the colostrum of the dam (Table 4). Detectable levels of IgG₂ had not been passed to neonatal sera by day 4. Considerable amounts of IgA and IgG₁ were detected in the sera of all 4-day calves studied. The total concentration of IgG₁ was about one-half the levels measured in adult cow sera at parturition, whereas IgG levels exceeded adult serum levels by nearly twofold.

Specific immunoglobulins with activity

**Table 2. Total whey immunoglobulin levels in vaccinated and control cows**

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>IgG₁</th>
<th>IgG₂</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (6)</td>
<td>4,176 (3,147)</td>
<td>401 (405)</td>
<td>383 (336)</td>
<td>322 (292)</td>
</tr>
<tr>
<td>CS (8)</td>
<td>1,749 (900)</td>
<td>415 (434)</td>
<td>135 (26)</td>
<td>290 (240)</td>
</tr>
<tr>
<td>LB (9)</td>
<td>2,190 (1,384)</td>
<td>390 (110)</td>
<td>183 (102)</td>
<td>502 (251)</td>
</tr>
<tr>
<td>KB (6)</td>
<td>2,721 (1,832)</td>
<td>401 (202)</td>
<td>207 (114)</td>
<td>495 (290)</td>
</tr>
</tbody>
</table>

* C, Adjuvant alone. Parentheses indicate number of animals tested.

**Table 3. Immunoglobulin levels in calf serum at time of birth (precolostral)**

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>IgG₁</th>
<th>IgG₂</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (13)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.4</td>
</tr>
<tr>
<td>CS (4)</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>LB (5)</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>502 (251)</td>
</tr>
<tr>
<td>KB (2)</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>502 (251)</td>
</tr>
</tbody>
</table>

* C, Adjuvant alone. Parentheses indicate number of animals tested.

**Table 4. Immunoglobulin levels in the sera of calves at 4 days of age**

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Immunoglobulin conc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG₁</td>
</tr>
<tr>
<td>C (4)</td>
<td>649</td>
</tr>
<tr>
<td>CS (6)</td>
<td>949</td>
</tr>
<tr>
<td>LB (8)</td>
<td>649</td>
</tr>
<tr>
<td>KB (4)</td>
<td>583</td>
</tr>
</tbody>
</table>

* C, Adjuvant only. Parentheses indicate number of animals tested.

**Table 3. Immunoglobulin levels in calf serum at time of birth (precolostral)**

(i) Immunoglobulin class of anti-O antibodies. Data tabulated for control and KB vaccine groups only are presented (Tables 5 and 6) to demonstrate the degree of variation between cows within groups. A summary of data relating to the average concentration of specific colostral antibody in whey is found in Table 7. Twenty-eight colostral specimens from dams in the control group were assayed for the immunoglobulin class of anti-O antibody classes (Table 5). IgG₂, anti-O was detected in all of the control whey specimens tested, whereas an occasional whey sample from vaccinated dams was negative (Table 6). The mean concentration of specific colostral IgG in the vaccine groups formed a tight cluster about a derived mean of 17 mg/100 ml (Table 7). The IgG₂, anti-O level was nearly three times greater in the control dams than in the vaccinated dams (Table 7).

Specific antibody activity in whey and sera against the O and K antigens of strain B-44 was not detected in the IgG₂ class when assayed by the techniques employed.

The varied levels of colostral IgA anti-O seen in the control (Table 5) and KB dams (Table 6) are indicative of the variation seen among udders and between groups throughout the study. Values from 2.6 to 19 mg/100 ml (mean = 4) were noted in the LB group in contrast to a
range of 1.7 to 3.9 mg/100 ml in controls. IgA anti-O was found in more than 90% of the udders tested, including control whey samples (Table 8). The mean levels of IgA elutable from the O somatic antigens were not significantly different between control and immunized groups (Table 7).

Not unlike the levels of IgG, and IgA, colostral IgM antibody directed against the O antigens was detected in nearly all of the udders tested (Table 8). The mean value of 28 mg/100 ml obtained in control whey compares favorably with mean levels of the whey of immunized dams (Table 7). It was noted that the marked variation among quarters and between cows in the control group of dams was not observed in the LB nor the KB group.

A comparison of the mean values of immuno-globulins possessing anti-O activity failed to detect a significant relationship between whey anti-O antibodies and protection from scouring. In fact, O-specific immunoglobulin levels of whey from vaccinated dams were generally lower than those observed in the controls (Table 7).

(II) O antibodies of the IgG1, secretory (S-) IgA, and IgM class in the calf. Quantitative analysis of the serum of newborn calves failed to show any immunoglobulins with activity against O or K antigens. Assays of serum from 4-day-old calves yielded very low values for one class of immunoglobulin (IgG1) with specific antibody activity. The results of these studies are shown in Table 9. Only four calves in each group were tested.

However, the data indicated that all of the
Parentheses show one standard deviation.

Antibodies none detected.

Extremely low immunoglobulins, anti-O ble

Average concentration of immunoglobulin in colostral whey.

Vaccine groupa No. of quarters tested Anti-Ob Anti-Kc Calf scour index

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>No. of quarters tested</th>
<th>Anti-O</th>
<th>Anti-K</th>
<th>Calf scour index</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>20</td>
<td>48d (19)</td>
<td>2.3 (1.0)</td>
<td>28 (18)</td>
</tr>
<tr>
<td>CS</td>
<td>28</td>
<td>19 (17)</td>
<td>3 (1.6)</td>
<td>21 (15)</td>
</tr>
<tr>
<td>LB</td>
<td>25</td>
<td>18 (23)</td>
<td>4 (3.7)</td>
<td>22 (13)</td>
</tr>
<tr>
<td>KB</td>
<td>21</td>
<td>16 (8)</td>
<td>5 (2.4)</td>
<td>17 (8)</td>
</tr>
</tbody>
</table>

a C, Adjuvant alone.
b O-a, Antibodies eluted from somatic antigens of E. coli B-44 cells heated at 118 C, 18 lb/in², 3 h. Parentheses show one standard deviation.
c K-a, Antibodies eluted from capsular antigens of E. coli B-44 cells treated with sodium azide.
d Average concentration of immunoglobulin in colostral whey.

Table 8. Number of quarters with detectable immunoglobulins with specificity to the somatic or capsular antigens

Vaccine groupa No. of animals | O-a | K-a | Calf scour index
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG₁ (mg/100 ml)</td>
<td>S-IgA (mg/100 ml)</td>
<td>IgM (mg/100 ml)</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>13/13³</td>
<td>13/13</td>
</tr>
<tr>
<td>CS</td>
<td>9</td>
<td>21/23</td>
<td>21/23</td>
</tr>
<tr>
<td>LB</td>
<td>9</td>
<td>16/19</td>
<td>20/20</td>
</tr>
<tr>
<td>KB</td>
<td>7</td>
<td>13/15</td>
<td>15/16</td>
</tr>
</tbody>
</table>

a C, Adjuvant alone.
b O-a, Antibodies eluted from somatic antigens of E. coli B-44 cells heated at 118 C, 18 lb/in², 3 h. K-a, Antibodies eluted from capsular antigens of E. coli B-44 cells treated with sodium azide. Number of quarters with detectable immunoglobulin/number of quarters tested.

calfs acquired about the same quantity of IgG₁, anti-O immunoglobulins, whereas no detectable O- or K-specific IgA or IgM was present. Extremely low values of IgG₁, anti-K were found in the sera of 4-day-old calves. The concentrations were so low that it precludes making any judgment on the significance of their presence in the infant calves.

(iii) Anti-K antibodies of the IgG₁, S-IgA, and IgM classes in the dam. Colostral IgG₁, anti-K antibody was found in 10 of 15, 15 of 28, 19 of 25, and 15 of 21 quarters tested from the adjuvant, CS, LB, and KB dams, respectively (Table 8). The mean level of IgG₁ in the controls was twice as high as the means measured in whey from the vaccinated dams (Table 7). It was interesting to find that, with an overall group frequency of 53%, at least one-quarter of
each udder tested in the CS group had detectable IgG, anti-K. With the exception of cows 439, 407, and 442, this observation holds for the other vaccine groups as well. Again, considerable variation in the mean udder levels was noted. The levels of IgG, anti-K did not appear to correlate to the scour indices.

The data (Table 8) show that only 2 of 20 control dams had detectable levels of IgA anti-K. This is in contrast to 8 out of 28 (34%), 13 out of 25 (48%), and 11 out of 21 (52%) of the quarters assayed among CS, LB, and KB dams, respectively, which contained measurable levels of specifically immune IgA. Data not included in Tables 7 and 8 showed six of nine and six of seven dams in the CS, LB, and KB groups, respectively, had detectable levels of IgA anti-K in at least one-quarter of the udders tested. This compares to the finding that only two-quarters of seven dams in the control group had detectable levels of IgA anti-K (Table 8).

Of signal importance is the fact that all of the whey specimens obtained from control dams lacked detectable levels of IgM anti-K immunoglobulins (Tables 7 and 8). Furthermore, all but one of the calves born in this group attained a 4+ diarrhea condition (Table 5). Although 8 of 28, 8 of 25, and 7 of 21 of the quarters assayed from dams in the CS, LB, and KB groups, respectively, contained measurable levels of specifically immune IgM, 8 of 28, 13 of 25, and 11 of 21 quarters in the CS, LB, and KB groups, respectively, had large quantities of IgM anti-K activity. Data not included in Tables 7 and 8 showed eight of nine, eight of eight, and five of seven dams in the CS, LB, and KB groups, respectively, had detectable levels of IgM anti-K activity. Thus, the apparent lack of this immunoglobulin and IgA with specific anti-K activity provides evidence for its role in protection, especially when correlated with the clinical data.

DISCUSSION

The four immunoglobulin classes were quantitated in sera of two cows each from the vaccine and the control groups. Only an indication of the magnitude of the levels of the various immunoglobulins can be derived from this data since the number of observations in each group is small. Very little variation was noted in levels of IgG, and IgM in serum of dams in the vaccine groups. IgG, levels were tightly clustered about a mean level that did not differ between vaccine and control groups. These data further indicated that levels of serum IgA probably were not markedly influenced by the vaccination regimes. On the other hand, the IgM levels were four to six times greater in the vaccinated dams than levels measured in controls.

The increased levels of serum IgG, did not correlate well with the IgG2 levels in the colostrum. Evidently the transfer of IgG2 is much less efficient as evidenced by low concentrations of this immunoglobulin in the colostrum in the serum of the calf. It has been clearly shown by Porter et al. (18), Murphy et al. (14), and Dixon et al. (5) that IgG, is selectively transferred from serum to mammary glands.

The study of immunoglobulin levels and classes in calves revealed substantial serum levels of IgG, IgA, and IgM, but not IgG2. Specific activity against O and K antigens was detected in the IgG, fraction of all calves tested. Thus, these data fail to establish a relationship between specific immunoglobulin levels in calf serum and protection from scouring.

Results of the quantitative measurements of immunoglobulins in colostrum show marked variation of the globulin levels between quarters and cows. Since the results were not normalized with the aid of a marker in the whey, the effect of varying fluid secretion (in volume and composition) among individual quarters cannot be evaluated. The marked variance observed among quarters in these studies, however, is not unprecedented. Butler et al. (3) observed marked variation in immunoglobulin concentrations between quarters and cows.

The mean concentrations of colostral IgG, in all groups (except the control) corresponded well to the values reported in the literature by Wilson et al. (20) and Butler et al. (3); the values are considerably less than the values reported by Mach and Pahud (12) and Porter et al. (18). Besides the different techniques employed by different laboratories, it would appear that the breed of cattle used in the experiments may have had an effect upon the results of these investigators.

Colostral IgA values were not significantly different among the vaccine groups, although a relatively lower value is seen in the whey from dams antigenized with culture supernatant. Interestingly, the variance among cows in this particular group is also extremely low. After intramammary antigenization, a pronounced effect upon the levels of secretory immunoglobulins would be expected. However, a significant difference in the total IgG, level was not found among vaccinated and control dams. Recent work by Wilson et al. (20) indicated that significant differences in the levels of IgA and IgG, in the whey from E. coli antigenized and nonvaccinated quarters were not apparent until 3 to 4
days postpartum. Their data indicated that S-IgA and IgG, remained significantly higher over a 28-day period in whey from vaccinated quarters, whereas the values in the nonvaccinated declined.

The concentration of colostral IgM was not significantly different between vaccine groups and the controls. However, IgM was notably lower in the whey of the dams antigenized with CS. The degree of variation observed with all of the immunoglobulin studies precludes analyzing the data for the effectiveness of different vaccine preparations.

The present evaluation of the immune mechanisms involved in the protection of neonatal calves from experimental scouring revealed the importance of specific colostral anti-K antibody of the IgA and IgM immunoglobulin classes. The significance of this finding was enunciated by the fact that little or no IgA or IgM anti-K immunoglobulins were present in the whey of the control dams. These results strongly imply that intramammary antigenization stimulated in situ production of both colostral S-IgA and IgM. It was not infrequently noted that either IgA or IgM anti-K would be detected in only one or two quarters of a cow, whereas IgG anti-K was detected more frequently, even in the quarters of the control dams. The extreme variation in the levels of IgA and IgM anti-K among quarters would indicate an autonomous response by the udders. It was of interest to note that the lowest concentrations of IgA in the CS group corresponded to the highest scour index in their calves, whereas the highest concentrations of IgA in the KB group corresponded to the lowest scour index in calves.

The values for IgM anti-K also related inversely to the scour indices in that the lowest concentration was noted in the whey of CS dams (scour index 1.3), whereas the highest concentration was found in the whey of the LB dams (scour index 1.9). IgG anti-K immunoglobulin was not detected in any of the colostral whey samples included in these assays. This may be explained by an inability to elute the antibody from whole cells by the procedures employed.

Since IgG, IgA, and IgM anti-O immunoglobulins were found in high concentrations in the control dams as well as in the vaccine groups, it was not possible to establish any role for these immunoglobulins in passive protection. The relative values of the immunoglobulin classes are not markedly different between groups; further, they did not have a relationship with the scour indexes.

In field studies and in controlled experiments, the importance of K agglutinins in colostrum and calf serum has been established (1, 2, 6, 8, 9). The immune status of the colostrum and serum, however, was based upon the agglutinin activity and did not include direct assay for either the incomplete (natural) IgM antibodies nor for the IgA immunoglobulins, which are not a highly efficient agglutinating antibody. Low levels of these immunoglobulins could play a significant role in passive immunity to E. coli intestinal infections under field conditions.

Outbreaks of scouring in the field could be explained by the emergence of an E. coli strain characterized by new capsular antigenic determinants, which could overcome the effectiveness of low avidity, cross-reactive colostral antibody (8). There are data that indicate that certain K antigens are plasmid controlled and, further, that the plasmid can be transferred between strains of E. coli under laboratory conditions (16, 19).

There is adequate evidence in the literature to indicate that the K antigen is involved with the adhesive qualities of certain enteric strains of microorganisms. The role of K antigens in neonatal diarrhea of piglets was recently investigated by Jones and Rutter (10). Their results showed that the K-88 antigen of E. coli is responsible for attachment of K-88 positive bacteria to the wall of the small intestine and that adhesion was essential for the virulence of K-88 positive bacteria in conventionally reared piglets.

In recent studies by Smith and Linggood (19), an inability to produce diarrhea in lambs with a Kco- form of the O8 enteropathogenic lamb strains indicated that common K antigen may be involved with the pathogenic process. Their results suggested that the adhesion properties may be specific in nature since the pig strain possessing K-88 was unable to adhere properly to calf or lamb intestine, whereas the common K antigen was unable to bring about adhesion to pig intestine.

The results of the present study clearly indicate the importance of IgM and IgA in passive immunity of neonatal calves in experimentally induced colibacillosis. Moreover, IgM and IgA with specific activity against the capsular antigens when present in the colostrum of the dam conferred solid immunity to the neonatal calves.

ACKNOWLEDGMENTS

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ture of these enterotoxins and of a K antigen pos-
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