Analysis of Chicken Bile by Gel Precipitation Reactions Using a Lectin in the Place of Antibody

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ABSTRACT A lectin obtained from black turtle beans (BTB) was precipitated with IgA in chicken bile samples in various forms of agarose gel systems. Ouchterlony-type double-diffusion (ODD) precipitation patterns between the lectin, bile IgA, and heavy chain-specific antibody contained spurs of the type suggestive of partial immunologic identity. The immunoelectrophoresis precipitation patterns between the same three reactants were mirror images and fused on the cathodic side of the immunoelectrophoresis origin. In addition to use in ODD-type gels, BTB could also be incorporated into agarose gels suitable for Mancini (radial immunodiffusion) or Laurell-type rocket electrophoresis.

Bile samples obtained from Cornell lines OS and C, broiler breeder males, and University of California—Davis congenic lines were investigated using BTB- and antibody-based methods. The results of this study indicated that IgA was the most frequently detected isotype in bile, occurring in 139 of 156 (89%) samples. Most bile samples (128/156; 82%) also contained IgG, whereas fewer (19/156; 12%) contained IgM. Cornell lines appeared to differ from broiler breeders, having a higher frequency of IgM-positive samples. Of the total bile samples studied, 11% (17/156) of samples from broiler breeders and the Cornell lines appeared to be devoid of IgA; the bile of one broiler breeder was found to be devoid of all three isotypes. Instances were found in which bile samples shown to be negative for IgA by antibody-ODD were shown to be positive by BTB-ODD. Thus BTB appears to be a suitable adjunct to antibody for the study of IgA.

(Key words: bile, chickens, lectin, IgA, precipitin reactions)

INTRODUCTION

Many studies have demonstrated that an immunoglobulin class analogous to mammalian IgA also exists in chickens. Lebacq-Verheyden et al. (1972), Leslie and Martin (1972), and Orland and Rose (1972) found IgA to be abundant in chicken bile, saliva, and lachrymal secretions. Watanabe and Isayama (1973) found IgA in serum and in ascitic fluid. The immunoelectrophoresis (IE) methods used in these studies required the preparation of heavy chain-specific antisera. Generally, this preparation was accomplished by exhaustive absorption of antisera raised in rabbits to remove specificities for IgG, IgM, and light chains. Presently, commercially prepared antisera to chicken IgA are available. Although such material is claimed to be specific for chicken heavy (α) chains, it is costly, so alternatives may be welcome.

Several types of bean lectins shown to be capable of agglutinating chicken erythrocytes (Silver and Cotter, 1992) also developed precipitates when tested with bile (Cotter, 1994). Because IgA was already known to be the predominant antibody class in bile, such precipitates might represent complexes containing IgA. This response is possible because the heavy (α) chains of IgA are glycosylated (Mandikka, 1992), and lectins have a great affinity for carbohydrates (Sharon and Lis, 1989). If lectins precipitate IgA, they may replace costly antisera in certain instances. This replacement would allow for the development of lectin-based methods that parallel those requiring antibodies. Validation of such methods rests on demonstrating a similarity in the ways the lectin and specific antibody react with IgA.

The purpose of this research was to show evidence that the lectin prepared from black turtle beans (BTB) could act as a reasonable substitute for the antibody usually used to detect chicken IgA. Moreover, BTB-based methods can be formatted to provide both qualitative and quantitative information on chicken IgA.

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Abbreviation Key: BTB = black turtle bean lectin; IE = immunoelectrophoresis; ODD = Ouchterlony double diffusion; RID = radial immunodiffusion (Mancini); ROC = rocket immunoelectrophoresis (Laurell); UCD = University of California—Davis.
MATERIALS AND METHODS

Preparation and Analysis of Black Turtle Bean Lectin

Beans sold under the generic name black turtle were purchased from local grocery stores. These were ground to coarse flour and were suspended in PBS as a 10% wt/vol solution. Extraction took place over 24 h in the cold (4°C) with constant stirring. Coarse solids were removed by low speed centrifugation using an IEC clinical centrifuge, whereas fine solids were removed using a Beckman model J2-21 centrifuge at 10,000 × g for 30 min. This crude material was capable of agglutinating erythrocytes from several species, including chickens (Silver and Cotter, 1992; Cotter and Midura, 1994).

The protein content of BTB was determined by the Lowry et al. (1951) method to be 3.75 mg/mL. Black turtle beans so prepared were different from commercial preparations of the lectins phytohemagglutinin and concanavalin A as judged by native polyacrylamide gel electrophoresis, but shared several major bands with them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Precipitation Methods

Ouchterlony-type double diffusion reactions (ODD) were performed in 60-mm plastic petri dishes containing 4 mL of agarose gel at either 0.75 or 1%. Wells were made by hand using a 3-mm diameter skin biopsy punch after placing the petri plate over a paper template that depicted a standard, seven-well ODD format. Diffusion of the reactants was allowed to proceed overnight at room temperature. The plates used for photography were carefully dehydrated by overlaying the agarose with absorbent paper and pressing with a force of 50 g. The dehydrated plates were dried to a thin film in a hot air stream. Precipitated materials were stained for protein with Coomassie brilliant blue R.

Antibodies against chicken Ig of the following specificities were purchased from a commercial source: goat-Anti-IgA (α-chain specific), rabbit-anti-IgG (heavy + light chain specific), and goat anti-IgM.

Gel casting for Mancini-type radial immunodiffusion (RID) was performed by adding BTB to liquefied agarose held at 45°C that was poured onto glass plates of varying dimensions. After solidification, 1-mm diameter wells were punched by hand after placing the gels over a template. The proportions of BTB to 1% agarose were typically 1:4 so that diffusion took place in 0.75% agarose.

RESULTS AND DISCUSSION

An initial survey of the antibody isotypes found in chicken bile was made using standard (antibody) ODD methods. The results shown in Table 1 are those obtained after staining of the dehydrated gels. A + indicates that precipitin lines were visible between the bile sample and each of the isotype-specific antibodies, and a − represents their absence. In some samples, two distinct precipitin lines were visible with anti-IgG or anti-IgA. Figure 1 shows typical ODD precipitates formed between bile and antibody (goat anti-chicken IgA) or between bile and BTB using the same samples.

The demonstration of cross reactivity between IgA, goat anti-chicken IgA, and BTB was established in several ways. First, an ODD gel was prepared in which the center well contained heavy chain specific (anti-α) antibody. The peripheral wells contained samples of chicken bile (positions 2, 4, 6), whereas the remaining positions contained BTB. Precipitin lines of the partial identity type (showing spurs) are clearly visible between each of the bile samples and the two reagents (Figure 2).

Secondly, additional validation was established using IE procedures. The precipitation patterns produced be-
TABLE 1. Isotypes present in chicken bile as demonstrated by Ouchterlony double diffusion

<table>
<thead>
<tr>
<th>Line(^2)</th>
<th>Sex(^3)</th>
<th>n</th>
<th>+(^4)</th>
<th>−(^4)</th>
<th>+</th>
<th>−</th>
<th>+</th>
<th>−</th>
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<tr>
<td>BB</td>
<td>1</td>
<td>55</td>
<td>50</td>
<td>5</td>
<td>53</td>
<td>2</td>
<td>0</td>
<td>55</td>
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<td>62</td>
<td>57</td>
<td>5</td>
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<td>2</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>COS</td>
<td>1</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>COS</td>
<td>2</td>
<td>19</td>
<td>13</td>
<td>6</td>
<td>18</td>
<td>1</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>CC</td>
<td>2</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>11</td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>156</td>
<td>139</td>
<td>17</td>
<td>135</td>
<td>21</td>
<td>19</td>
<td>137</td>
</tr>
</tbody>
</table>

\(^1\)A 2.0-µL sample was reacted against goat anti-chicken IgA, IgM, or rabbit anti-chicken IgG.
\(^2\)Line: BB = broiler breeder, COS = Cornell OS, CC = Cornell C.
\(^3\)Sex: 1 = male, 2 = female.
\(^4\)A (+) indicates the presence of a precipitin line, and a (−) indicates that no precipitate was observed.

Between BTB and bile were compared with those produced between bile and antibody. Figure 3 shows the results of a representative run. The center trough was charged with antibody, whereas the top and bottom troughs were charged with BTB. A Cornell-OS strain bile sample (no. 69) is shown, as well as a UCD Leghorn bile (no. 7174) sample. The qualitative and quantitative differences between these two specimens are clearly indicated, but both precipitation patterns show the gull-like features typical of chicken IgA. Moreover, the precipitin lines produced by BTB appear as mirror images to those produced by antibody. Additionally, they are fused on the cathodic side of the origin. The latter observation lends further support to the immunologic identity of the material (IgA) precipitated by both reagents.

Quantification of IgA was accomplished using RID and ROC procedures. Figure 4 shows the RID patterns from bile samples obtained from 10-wk-old chickens representing several UCD congenic lines (Abplanalp et al., 1992). Each of the chickens providing these samples had survived a neonatal challenge with *Salmonella enteritidis*, and each was asymptomatic at the time their bile was collected. Thus, the sample population was composed of five animals from lines 003 and 254, and six from line 342. The remaining four lines had only one representative (Table 2). The diameters of the precipitin rings were measured with a Vernier caliper to the nearest 0.1 mm. Because three of the lines had five or six samples, these could be compared with each other. The remaining lines, having only one sample each, were pooled to construct a fourth group, and then all of the data were re-analyzed. The congenic line (254) showing the highest susceptibility to neonatal salmonella challenge (Cotter et al., 1998) also had the lowest average ring diameter.

The ROC patterns from Cornell lines OS and C are shown in Figure 5. Calculation of the area of the resulting patterns was determined algebraically by measuring their height and width (mm) and by assuming that each form represented a triangle. Bile samples obtained from females appeared to contain less IgA than those from males \((P < 0.001)\), but line differences were not apparent \((P \approx 0.8)\). This observation contrasts somewhat with that made by Luster et al. (1977), who found that chickens from the...
OS strain were (serum) IgA deficient. However, those same workers found that the serum IgA levels were lower in females, as is the case here with bile IgA. There was a noticeable difference in the intensity of the stained arcs among the samples reported here, presumably reflecting differences in the glycosylation patterns of IgA.

The results of the survey study shown in Table 1 indicate that IgA was the most frequently detected isotype in bile occurring in 139 of 156 (89%) samples. Most bile samples (128/156, 82%) contained IgG in addition, whereas fewer (19/156, 12%) contained IgM. In regard to the latter isotype, Cornell lines appeared to differ from broiler breeders in having a higher frequency of IgM-positive samples, but it is uncertain whether this has any true physiological significance. More important, however, was the observation that there were a number (17/156, 11%) of chickens in broiler breeder lines, Cornell lines, and at least 1 UCD sample that appeared to be devoid of bile IgA. This observation was surprising given the generally held belief that gall bladder bile represents an approximate 10-fold concentration of liver bile. Furthermore, the bile of one broiler breeder was found to be devoid of all three isotypes.

The methods described here were useful for detecting bile IgA, because it can be precipitated by BTB in various gel matrix systems, and also due to its predominance in bile fluid (Table 1). Tears, saliva, cyst fluids, and seminal plasma are other examples of fluids for which this expectation is also reasonable. For example, IgA was detected by BTB in cyst fluid taken from a hen found at autopsy to contain multiple abdominal adenocarcinomas (data not shown). The utility of BTB in detecting IgA in serum samples where levels are expected to be lower than those found in bile has not as yet been established.

The possibility that BTB reacts with other immunoglobulin classes contained in bile may be addressed by the present results. Examination of the IE patterns shown in Figure 3 suggests that BTB does not react with IgG. This isotype was present in both specimens 69 and 7174 as determined by precipitation with anti-IgG using ODD (data not shown). If BTB also reacted with IgG, an additional large cathodic precipitin arc occupying the usual IgG place should have been expected. Such an arc does not appear in Figure 3, nor has one been seen in any of the other IE results obtained thus far. This argument cannot be extended to include IgM, however, because that arc is typically small, and it usually occupies a place near the electrophoretic origin. Thus, it might easily be obscured by the presence of the larger IgA arc. IgM may be found in bile, but more frequently it is not precipitated from this secretion by specific antibody (Table 1). The question of BTB-IgM cross reactivity is being examined in detail by experiments currently in progress; however, the Cornell samples may be exceptional in that 18 of 31 (58%) of the females of OS and C lines contained IgM. This high detection of IgM may be related to the immune dysfunction that is especially characteristic of females of these lines (Dietrich et al., 1999).

The BTB-ODD system described here may be useful in survey work with large sample sizes. It could provide qualitative information such as the presence or absence of IgA in bile or other fluids. For example, there have been several instances in which bile samples that were found to be IgA-negative by antibody-ODD were found to be positive using BTB-ODD. Conversely, there has not
yet been an instance in which IgA that was detected by antibody was not also detected by BTB, which suggests that the latter may be a more sensitive reagent in certain instances.

The precipitin lines formed by commercial antibody were more easily seen in the unstained gels because of their greater sharpness. BTB precipitin lines appeared more diffuse in these gels, and this property carried over into gels stained by Coomassie brilliant blue R (Figure 1). Perhaps this difference is a consequence of less cross-linking associated with BTB or is due to the lectin’s affinity for glycosylated amino acids (asparagine, serine, and threonine) that may not be located at precisely the same place as the epitopes recognized by antibody.

Although quantification of IgA might be expected to be accomplished by RID procedures using either lectin- or antibody-containing gels, complications may arise due to the heterogeneous nature of IgA. Watanabe and Kobayashi (1974) estimated the molecular weights of serum IgA to be between 800 and 900 kDa, whereas that of intestinal secretions were in the 300- to 500-kDa range. Such size estimates were obtained by precipitating IgA from pooled samples, followed by separation using column chromatography. Molecular weights were then determined from sucrose density gradient sedimentation coefficients using ultracentrifugation. Moreover, Hadge and Ambrosius (1988) state that all biliary samples investigated by them contained IgA in the form (H2L2)n, each having a different molecular weight.

It is clear that several types of bile IgA can be detected by observing ODD patterns alone. Careful examination of the positions occupied by single sample precipitates shows that some IgA types diffused more slowly than others (compare the positions of the precipitates shown in Figure 1A). Presumably such samples represent the higher molecular weight forms (Samples 4 and 5, Figure 1A). Moreover, two distinct precipitin lines were seen in some samples by antibody-ODD, suggesting the presence of different allotypes or subclasses such as IgA1 and IgA2 (data not shown) as are found in mammals. In RID procedures, such multiple forms would lead to the formation of double rings or to the underestimation of the amount of IgA in the sample. The latter would be due to the inverse relation between molecular weight and diffusion rate (Allison and Humphrey, 1960). Thus, if one sample contained only dimers (H2L2)n, whereas another contained only tetramers (H2L2)2, then the latter would appear to contain less IgA than the former, when in reality that might not be the case.

The results obtained with ROC procedures suggest that this method provided some additional sensitivity over that of RID for quantifying differences among bile samples. In a study using bile specimens obtained from turkeys, differences in IgA levels were associated with dietary treatments using BTB-ROC that were not detectable using BTB-RID (Savage et al., 1996).

Lectins have been shown to react with human IgA, IgA myeloma proteins, the secretory component, and the J-chain. Wold et al. (1994) found differences between IgA1 and IgA2 with lectins. The former was more reactive with the mannose-specific lectin concanavalinA, whereas the latter was more reactive with galactose-specific lectins. These authors suggested that such differences might be important in the interactions of IgA with microbial flora such as *Escherichia coli*, and endogenous lectins, which act as phagocytic receptors.

Given the importance of IgA in the maintenance of mucosal surface integrity, methods facilitating its study such as those described here, should provide a valuable

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**TABLE 2.** Black turtle bean lactin-radial immunodiffusion (BTB-RID) diameters using 1-µL bile samples obtained from 10-wk-old chickens representing seven University of California—Davis congenic (UCD) lines challenged as neonates with *Salmonella enteritidis*

<table>
<thead>
<tr>
<th>Item</th>
<th>Line susceptibility score</th>
<th>No.</th>
<th>Mean ring diameter (mm)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>003</td>
<td>17</td>
<td>0</td>
<td>5</td>
<td>5.6</td>
</tr>
<tr>
<td>254</td>
<td>15</td>
<td>−3</td>
<td>5</td>
<td>4.4</td>
</tr>
<tr>
<td>342</td>
<td>C</td>
<td>+4</td>
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</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remainder</td>
<td></td>
<td>3 lines</td>
<td>F = 2.8</td>
<td>P = 0.09</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>NA</td>
<td>5</td>
<td>5.1</td>
</tr>
<tr>
<td>Remainder</td>
<td></td>
<td>4 lines</td>
<td>F = 1.6</td>
<td>P = 0.2</td>
</tr>
</tbody>
</table>

*0 = neutral; −3 = susceptible; +4 = resistant (see Cotter et al., 1998).*
adjunct to those requiring antibodies. Because BTB can be prepared easily from bean flour, its use as a substitute for antibody produced in rabbits or goats has obvious additional advantages.

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REFERENCES


