Endogenous Cell Surface Lectin in Dictyostelium: Quantitation, Elution by Sugar, and Elicitation by Divalent Immunoglobulin

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**ABSTRACT** The amount of total endogenous cellular and cell surface lectin in aggregating Dictyostelium purpureum was determined by a number of immunochemical techniques. The results show that of the $5 \times 10^6$ molecules of the lectin (called purpurin) per aggregating cell only about 2% ($1 \times 10^5$ molecules) is present on the cell surface. Cell surface purpurin can be specifically eluted by lactose, which indicates that it is held to the surface by its carbohydrate-binding site. The eluted purpurin is replaced on the cell surface within 45 min. Estimates of cell surface purpurin made by binding of specific immunoglobulin to the cells at 4°C indicate that a much larger amount, about $1 \times 10^6$ molecules, becomes associated with the cell surface in the presence of this divalent ligand. In contrast, univalent antibody fragments do not have this effect.

Cellular slime molds are simple eukaryotic cells that exist either in a unicellular vegetative form or a differentiated form in which they become adhesive and aggregate into a multicellular structure. As the cells become adhesive, they synthesize polyvalent carbohydrate-binding proteins (4, 13). These proteins are referred to as lectins, because they can be assayed as agglutinins of erythrocytes, and because this agglutination can be blocked by specific sugars that bind their active sites. The lectins have been detected on the surface of aggregating slime mold cells (8, 13, 14), and considerable evidence has been presented to indicate that they play a role in cell-cell adhesion (2, 12).

The purpose of the present study was to quantify the amount of cell surface lectin and to examine the mechanism of its association with the cell surface. The results indicate that about 2% of the total cellular lectin is displayed on the surface of aggregating cells and that this lectin is held on the surface by noncovalent association with cell surface carbohydrate. However, when cells are reacted with divalent anti-lectin immunoglobulin a much greater amount of the lectin becomes associated with the cell surface. This raises the possibility that a large fraction of the intracellular lectin is readily available for surface interactions if elicited or maintained by appropriate molecules.

**MATERIALS AND METHODS**

**Growth and Differentiation of Dictyostelium purpureum**

*Dictyostelium purpureum*, strain 2, was obtained from J. T. Bonner. Vegetative cells were grown on SM agar in association with Klebsiella aerogenes as described previously (15). Aggregating cells were obtained after their differentiation to the tight aggregate stage on filter pads maintained in a moist atmosphere at 24°C based on methods described previously (15). Washed cells ($1 \times 10^7$ cells in 0.5 ml H$_2$O) were added to a filter pad, consisting of a Millipore AABP 047 00 filter on an absorbent cellulose pad (APIO 047 00, Millipore Corp., Bedford, Mass.) soaked with 1.6 ml of a solution containing per liter, 1.5 g KCl, 1.0 g MgCl$_2$-6 H$_2$O, 0.5 g streptomycin sulfate, 0.3 g Na$_2$HPO$_4$ and 1.2 g KH$_2$PO$_4$, pH 6.2. After differentiation, the cells were harvested by vortexing the filters in cold 16.7 mM sodium-potassium phosphate pH 6.2 (SPS) and centrifuging the suspended cells. We used two methods of obtaining differentiated cell populations. In some cases the washed vegetative cells were directly plated onto the filter pads, and tight aggregates were harvested 5–6 h after plating. In other experiments the washed vegetative cells were first shaken for 16 h in SPS before plating on filter pads, in which case tight aggregates appeared 2.5–3 h after plating. No differences in results were noted between the two methods.

**Antigen and Antibody Preparation**

Purpurin, the lectin from *D. purpureum*, was purified by affinity chromatography as described previously (3). This purified lectin shows two protein bands.
Immune serum was raised in New Zealand white rabbits by initial intradermal injections of 0.5 mg purpurin homogenized in Freund’s complete adjuvant, followed, at monthly intervals, by injections of 0.05 mg purpurin in Freund’s incomplete adjuvant for a total of five cycles. Immunoglobulin G (IgG) was prepared from the serum by chromatography on DEAE-Affigel Blue (Bio-Rad Laboratories, Richmond, Calif.) using the procedure supplied by the manufacturer. Univalent antibody fragments (Fab fragments) were prepared from IgG by the method of Porter (11).

**Radioiodination**

Radioiodination of purpurin, IgG, and Fab was done with immobilized lactoperoxidase and glucose oxidase purchased as Enzymobead Radioiodination Reagent (Bio-Rad Laboratories) following the directions in the accompanying literature. Typically 1 mg carrier-free [125I]-lodosulfanilic acid from New England Nuclear Corp. (Boston, Mass.) was incubated at room temperature for 15 min with 25 µl Enzymobeads. 25 µl 1% β-glucose, and either 10–15 µg protein for purpurin preparations or 1 mg protein for IgG or Fab preparations, suspended in 60 µl 0.2 M Na phosphate buffer, pH 7.2. The reaction was stopped by centrifugation followed by separation of the iodinated protein on Sephadex G-25 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.). Purpurin preparations normally had a specific activity of 10,000–15,000 cpm/µg with 5–10% incorporation of added [125I]-IgG and Fab preparations typically had a specific activity of 500–1,000 cpm/µg with ~95% incorporation of added [125I]. In some binding studies which used [125I]-IgG, the radioactive product was diluted 10- or 20-fold with unlabeled IgG from the same batch. Protein A obtained from Pharmacia Fine Chemicals was iodiated using chloramine T by the procedure of Brown et al. (7) with a resultant specific activity of 1.2 x 10^6 cpm/µg.

**Polyacrylamide Gel Electrophoresis in SDS and Immune Staining of Gels**

Samples were solubilized and electrophoresed on 12.5% polyacrylamide slab gels by the method of Laemmli (10) and stained with Coomassie Blue. Identification of antigens on gels was determined by reacting the gels with immune or control sera, washing and reacting with [125I]-protein A as described by Adair et al. (1). The location of the [125I]-protein A bound to the gels was determined by autoradiography.

**Radioimmunoassay for Purpurin**

All components of the assay were made up in phosphate-buffered saline (75 mM sodium, potassium phosphate, 75 mM sodium chloride), pH 7.2, containing 50 mM galactose and 0.2% bovine serum albumin (RIA grade; Sigma Chemical Co., St. Louis, Mo.). This mixture is referred to as PGB.

To 100 µl of purpurin dilution (unknown or standard at 10–150 ng/ml) was added 25 µl of a 1:30,000 dilution of anti-purpurin serum made up in a 1:30,000 dilution of normal rabbit serum, followed by 25 µl of [125I]-labeled purpurin containing approximately 20,000 cpm. After 20 min at room temperature, 50 µl of a 10% suspension of lyophilized, fixed Staphylococcus aureus (G.IgGorb, New England Enzyme Center, Boston, Mass.) in PGB was added, and the mixture was incubated at 4°C for 20 min. Finally 2 ml of PGB was added with vortexing, and the precipitate was pelleted by centrifuging at 800 g for 7 min. After careful removal of the supernate, the precipitate was counted in a gamma radiation counter. In this assay, 25 µl of IgG unknown or standard in 1:300 normal rabbit serum was added to 25 µl [125I]-labeled purpurin and 100 µl of PGB. After 20 min at room temperature, 50 µl of 10% IgGorb was added and the mixture incubated on ice for 20 min. After addition of 2 ml of PGB, the precipitate was collected by centrifugation at 800 g for 7 min. The supernate was removed and the pellet counted. A standard curve was constructed using known amounts of immune IgG. The unknown samples were all studied at dilutions giving results in the midrange of the linearity of the standard curve. Because the specific activity of the labeled purpurin was known, we could determine with the standard curve the amount of immune IgG required to precipitate a given number of purpurin molecules. When cells adsorbed the amount of IgG required to precipitate one molecule of purpurin, the cells were said to have adsorbed one molecule of “purpurin equivalents,” presumably indicating the presence of one molecule of purpurin on the cell surface. As indicated in the text, this method was found to underestimate the amount of available cell surface purpurin.

**Measurement of Specific IgG Binding to the Cell Surface by Radioimmunoassay**

Adsorption of anti-purpurin IgG by aggregating D. purpureum cells was quantified by radioimmunoassay. Differentiated cells were washed and resuspended to 2 x 10^6 cells/ml in cold SPS. Portions of cells (or controls containing no cells) were added to centrifuge tubes, centrifuged at 600 g for 5 min, and the pellet was resuspended in an equal volume of anti-purpurin IgG in SPS containing 2 mg/ml bovine serum albumin and 1.5 mg/ml normal rabbit IgG. After shaking on ice for 30 min, the cells were centrifuged at 600 g for 5 min and the supernate centrifuged again at 100,000 g for 1 h. These supernates were then assayed for specific IgG using a radioimmunoassay of antibody. In this assay, 25 µl of IgG unknown or standard in 1:300 normal rabbit serum was added to 25 µl [125I]-labeled purpurin and 100 µl of PGB. After 20 min at room temperature, 50 µl of 10% IgGorb was added and the mixture incubated on ice for 20 min. After addition of 2 ml of PGB, the precipitate was collected by centrifugation at 800 g for 7 min. The supernate was removed and the pellet counted. A standard curve was constructed using known amounts of immune IgG. The unknown samples were all studied at dilutions giving results in the midrange of the linear portion of the standard curve. Because the specific activity of the labeled purpurin was known, we could determine with the standard curve the amount of immune IgG required to precipitate a given number of purpurin molecules. When cells adsorbed the amount of IgG required to precipitate one molecule of purpurin, the cells were said to have adsorbed one molecule of “purpurin equivalents,” presumably indicating the presence of one molecule of purpurin on the cell surface. As indicated in the text, this method was found to underestimate the amount of available cell surface purpurin.

**Immunofluorescent Studies**

Immune or normal rabbit IgG was labeled by reaction with fluorescein isothiocyanate by the method of Brandzaeg (6). Fluorescent material not incorporated into IgG was removed by gel filtration on Sephadex G-50 followed by chromatography on Whatman DE-52 cellulose. The fluorescein-to-protein molar ratio for both samples was approximately 2.8. Portions containing up to 0.3 mg fluorescein labeled IgG/ml diluted in SPS containing 1% heat-inactivated goat serum and 2 mg/ml BSA were reacted with dissociated aggregating D. purpureum (5 x 10^6 cells/ml at 4°C for 20 min. The cells were then washed by centrifugation through 10% Ficoll (Sigma Chemical Co.) in SPS and resuspended in SPS at a concentration of 3 x 10^6 cells/ml. A droplet was added to a glass cover slip and observed with an inverted Leitz microscope through phase and fluorescence optics, using a high intensity silicon intensified tube video camera (RCA 1030/H RCA Closed-Circuit Video Equipment, Lancaster, Pa.).

**RESULTS**

**Properties of Antibody**

Antisera of high titer were obtained and could readily be used in a radioimmunoassay at dilutions of 1:30,000. Immunodiffusion tests, in which the antisera was reacted against adjacent wells containing either purified purpurin or 0.3 M lactose extracts of whole aggregating D. purpureum, showed sharp immunoprecipitation arcs without spurting. Because all the antigens that reacted with specific antibodies to purpurin was present in these lactose extracts (as shown below), this relatively crude procedure suggests that there were no obvious immunologically cross-reactive proteins in D. purpureum. This was also evaluated by reacting immune or normal serum with polyacrylamide gels in which SDS extracts of whole aggregating D. purpureum had been electrophoresed. After incubation with the immune or control sera, the bound immunoglobulin was visualized with [125I]-protein A as described by Adair et al. (1). The only bands on the gels that reacted specifically with...
the immune serum were the two protein bands of purpurin (Fig. 1). Other evidence for the specificity of this antiserum and the absence of significant cross-reactive material will be presented below.

**Determination of Cell Surface and Total Cellular Purpurin by Radioimmunoassay**

Aggregating *D. purpureum* cells contained, on the average, 4.7 \( \times 10^6 \) molecules of purpurin per cell, as determined by radioimmunoassay of combined supernates of disrupted cells (Table I). Several additional washes of the residual pellet were no longer significant amount of additional purpurin. Solubilization of the resultant washed pellet with 1% Triton X-100 and assay of this detergent extract after dilution with 10 or more volumes of assay buffer showed only traces of purpurin (Table I). Because the detergent did not significantly affect the radioimmunoassay at concentrations as high as 0.1%, failure to detect substantial purpurin in the Triton extract was not caused by the detergent.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Molecules released per cell ( \times 10^{-5} )</th>
</tr>
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<tbody>
<tr>
<td>Disrupted cells</td>
<td></td>
</tr>
<tr>
<td>Freeze-thaw, no sugar</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Freeze-thaw, 10 mM lactose</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>Freeze-thaw, 150 mM lactose</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>1% Triton X-100 after freeze-thaw</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>with 150 mM lactose and three</td>
<td></td>
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<tr>
<td>washes</td>
<td></td>
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<tr>
<td>Intact cells</td>
<td></td>
</tr>
<tr>
<td>Elution without sugar</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>Elution with 10 mM cellobiose</td>
<td>0.25 ± 0.01</td>
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<tr>
<td>Elution with 10 mM lactose</td>
<td>1.0 ± 0.06</td>
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<tr>
<td>Elution with 150 mM lactose</td>
<td>0.85 ± 0.15</td>
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Aggregating *D. purpureum* cells were eluted to determine total cellular purpurin with various media to strip off cell surface purpurin. For cellular disruption 5 \( \times 10^7 \) cells/ml were suspended in SPS containing the indicated sugar, frozen in liquid nitrogen, and then thawed. The soluble extract was obtained by centrifugation at 100,000 g for 1 h. The procedure was then repeated. The two soluble extracts were then combined and purpurin was estimated by radioimmunoassay. For the experiments in which Triton X-100 extraction was done, the pellets were washed three times after freezing and thawing twice in 150 mM lactose, and the resultant pellet was solubilized in 1% Triton X-100 in SPS. Elution of purpurin from intact cells was done by shaking 5 \( \times 10^7 \) cells/ml of SPS containing the indicated sugar at 115 rpm on a gyratory shaker (model C-2; New Brunswick Scientific Co., Inc., Edison, N.J.) at 4°C for 10 min. The cells were then centrifuged at 600 g for 5 min, and the supernate was reincubated at 100,000 g for 30 min to remove any traces of particulate material, then used for purpurin determination by radioimmunoassay. Results are the mean ± SEM for the number of independent experiments indicated by n.

![Binding of anti-purpurin IgG and normal IgG to *D. purpureum* protein solubilized in SDS and separated by polyacrylamide gel electrophoresis.](image)

Cell surface lectin could be removed by shaking the cells in 10 mM lactose (Fig. 2A). Higher concentrations, up to 150 mM, were no more effective under the conditions used (Fig. 2A). The elution was specific in that celllobiose, a disaccharide that does not react with the active site of purpurin, eluted relatively little lectin (Fig. 2A). Elution was complete within 5 min of shaking (Fig. 2B). Based on a large number of studies in which we compared the amount of lectin eluted from the cell surface and the total amount of intracellular lectin, we conclude that about 2% of the total cellular purpurin was eluted by one exposure to lactose (Table I). A second elution done immediately after the first yielded only a small additional amount of cell surface lectin (Fig. 3). Slightly more lectin was eluted if the cells were first shaken at room temperature for 10 min before a second elution. With continued incubation for 45 min after the first elution, the amount of elutable cell surface lectin returned to the starting level (Fig. 3).

The material that was solubilized by this elution procedure actually came from the cell surface and did not result from fracture of a small percentage of the cells because polyacrylamide gel electrophoresis of the eluted material showed only purpurin (Fig. 4 C). In contrast, on electrophoresis of 10 mM lactose extracts of *D. purpureum* cells containing the same amount of purpurin as in Fig. 4 C, we found a multitude of proteins (Fig. 4 D). This was true whether the soluble extracts of whole cells were obtained by freeze-thawing, sonication, or homogenization. Were the eluted material actually released by cell breakage, one would expect to see many other proteins in Fig. 4 C.

![Table I](image)
Evidence That all the Available Cell Surface Purpurin Can be Eluted with Sugar

The previous results indicated that only a small fraction of the total cellular purpurin could be eluted with sugar, but it remained possible that there was additional purpurin that stayed on the cell surface and that was not susceptible to elution because it was either more tightly bound or held by some other type of bond. To test for this possibility, we labeled the surface of aggregating *D. purpureum* cells with the diazonium salt of [*125*I]-iodosulfanilic acid, an iodinating reagent that does not penetrate membranes (5). After carefully washing the cells, we obtained the three fractions described in Table II by (a) eluting the cell surface purpurin with 10 mM lactose, (b) then obtaining the supernate by freezing and thawing the cells in 10 mM lactose, and (c) then solubilizing the residual particulate material with 1% Triton X-100. These three samples were then precipitated with an excess of anti-purpurin IgG followed by goat anti-rabbit gamma globulin. The immune precipitates were electrophoresed on SDS polyacrylamide gels, and the radioactive purpurin was identified and counted. Of the total iodinated purpurin, about 93% was eluted from the cell surface with 10 mM lactose (Table II). In another experiment, we reacted one portion of cells with the diazonium salt of [*125*I]iodosulfanilic acid after elution with 10 mM cellobiose and another portion after elution with 10 mM lactose and then compared total labeled cellular purpurin. The lactose-eluted cells had only 9% as much labeled purpurin as the cellobiose-eluted cells. Therefore, with both of these approaches, the results show that more than 90% of the cell surface purpurin, identified by its availability to surface iodination, was removed by a single elution with 10 mM lactose. These results are consistent with the results in Fig. 3, which show that little elutable cell surface purpurin remained after the initial elution.

The cell surface labeling experiments (Table II) and the elution experiments with radioimmunoassay (Table I and Fig. 3), when taken together, suggest that all the cell surface purpurin was held there by a lactose-sensitive bond and was not integrated into the membrane bilayer. The fact that total cellular lectin was solubilized in lactose solutions and did not require detergent extraction (Table I) is consistent with this conclusion.

In cell surface iodination experiments, we also compared the immunoprecipitates obtained at each stage using immune IgG and normal IgG. Some protein other than purpurin was precipitated by either immune IgG or normal IgG followed by goat anti-rabbit gamma globulin (Table II). Autoradiographs of SDS gels of the precipitates indicated that only purpurin was specifically immunoprecipitated, providing further evidence for the specificity of the immune IgG.

**Cell Surface Binding of Anti-purpurin Fab**

To quantitate cell surface purpurin in an alternative way, we sought to determine how much antibody could bind to aggre-
In the two conditions, cellobiose respectively eluted 0.4 purpurin-treated cells and 1.1 x 10^5 lactose eluted 3.6 then reacted with the radioimmunoassay. The three portions of cells were without sugar. The amount of purpurin eluted was measured eluted either with 10 mM lactose or 10 mM cellobiose or buffer three times to remove unbound purpurin, and portions were purified lectin. After shaking for 15 min, the cells were washed by mixing 10
purpurin into the Fab. To evaluate these possibilities, we reacted the Fab for purpurin or to relative inaccessibility of cell surface purpurin. To our surprise, the 125I-Fab gave a much lower estimate of cell surface purpurin than we expected from the elution experiments, approximating saturation after binding of <2 x 10^5 Fab molecules per D. purpureum cell (Fig. 5). Most of the binding appeared to be specific in that 125I-Fab binding to D. purpureum cells, which had first been eluted with 10 mM lactose, was reduced by about 70% (Fig. 5).

The 125I-Fab binding observed was less than 20% of that expected. This could be caused either by the poor affinity of the Fab for purpurin or to relative inaccessibility of cell surface purpurin to the Fab. To evaluate these possibilities, we reacted aggregating D. purpureum cells with a large excess of purpurin by mixing 10^6 cells in a 1-mL solution containing 1 mg of the purified lectin. After shaking for 15 min, the cells were washed three times to remove unbound purpurin, and portions were eluted either with 10 mM lactose or 10 mM cellobiose or buffer without sugar. The amount of purpurin eluted was measured by the radioimmunoassay. The three portions of cells were then reacted with the 125I-Fab as in Fig. 5. We found that lactose eluted 3.6 x 10^5 molecules of purpurin/cell from the purpurin-treated cells and 1.1 x 10^5 from the untreated cells. In these two conditions cellobiose respectively eluted 0.4 x 10^5 and 0.2 x 10^5 molecules of purpurin/cell. When we compared 125I-Fab binding using Fab and cell concentrations as in Fig. 5, we found that the cells with added purpurin bound 2.1 x 10^5 molecules of 125I-Fab whereas the normal cells bound only 1.8 x 10^5 molecules/cell. Apparently, the exogenous purpurin bound to the cells is much more available to Fab than the endogenous cell surface purpurin.

Cell Surface Binding of Anti-purpurin IgG

To examine antibody binding to D. purpureum in another way, we reacted cells with iodinated IgG. In initial studies in which we used up to 0.4 mg/ml of 125I-IgG and only 5 x 10^6 cells/ml, we obtained linear binding curves with no evidence of saturation. This was extremely surprising because we were using 10 times the saturating concentration of 125I-Fab and only 1/10 as many cells as used in the Fab experiments. By diluting the 125I-IgG with unlabeled immune IgG, thereby allowing for the addition of much larger concentrations of this reagent, we eventually approximated saturation (Fig. 6B). To our surprise, we found that the binding of 125I-anti-purpurin IgG per D. purpureum cell was about two orders of magnitude greater than the number of molecules of 125I-anti-purpurin Fab bound per cell. Some of this binding of IgG appeared to be nonspecific, because considerable iodinated normal rabbit IgG also bound to D. purpureum cells under identical conditions. To determine the amount of specific anti-purpurin IgG bound, we compared the binding of 125I-anti-purpurin IgG, after sequential adsorptions with denatured purpurin (Fig. 6A). We found that, under the conditions used, three adsorptions with denatured purpurin removed about 40% of the 125I-IgG that bound to the D. purpureum cells and an additional adsorption had no further effect. We then compared the binding of 125I-anti-purpurin IgG that had been adsorbed three times with denatured purpurin with another portion of this 125I-IgG that had been adsorbed with denatured bovine serum albumin (Fig. 6B). Binding of the 125I-IgG that had been adsorbed with purpurin reached saturation at concentrations of 1-2 mg/ml of IgG, at which concentration approximately 1 x 10^5 molecules
mixture we could eliminate nonspecific binding of the specific adding at least 1 mg/ml of normal rabbit IgG to the reaction with this IgG, and there are no cross-reactive antigens. By shown that purpurin is the only cellular antigen that reacts binding of specific anti-purpurin IgG, because we had already measured the residual specific antibody in the supernate with portions of immune IgG with D. purpureum cells and then measured the purpurin IgG binding to the cells. In this approach we adsorbed also used an alternative approach to measure specific anti-purpurin IgG binding to aggregating D. purpureum cells with what was expected from the Fab binding experiments, we dissociated aggregating D. purpureum bound more than 6 x 10^6 molecules of purpurin equivalents per cell at IgG concentrations approximating saturation (Fig. 8). In a total of four experiments using those concentrations, the average binding was 5.7 x 10^5 molecules of purpurin equivalents per cell. However, the exact meaning of this number is difficult to determine, because the concentrations of antibody and antigen are different in the radioimmunoassay and in the

of IgG were bound per cell. In contrast, 125I-IgG that had been adsorbed with denatured bovine serum albumin reached saturation at approximately 2 x 10^6 molecules of IgG bound per cell. Based on the difference between purpurin-adsorbed and control 125I-IgG, we conclude that approximately 1 x 10^6 molecules of specific anti-purpurin IgG were binding to these cells under these conditions.

Because of the large amount of nonspecific IgG binding and the surprisingly large amount of specific IgG bound compared with what was expected from the Fab binding experiments, we also used an alternative approach to measure specific anti-purpurin IgG binding to the cells. In this approach we adsorbed portions of immune IgG with D. purpureum cells and then measured the residual specific antibody in the supernate with a radioimmunoassay. With this technique we only measured binding of specific anti-purpurin IgG, because we had already shown that purpurin is the only cellular antigen that reacts with this IgG, and there are no cross-reactive antigens. By adding at least 1 mg/ml of normal rabbit IgG to the reaction mixture we could eliminate nonspecific binding of the specific IgG (Fig. 7). In subsequent binding experiments we therefore added an excess of normal rabbit IgG. To calculate the binding of specific anti-purpurin IgG we defined a unit called purpurin equivalents, which is the amount of antibody that immunoprecipitates a molecule of purpurin in the radioimmunoassay. By this measure, dissociated aggregating D. purpureum bound more than 6 x 10^6 molecules of purpurin equivalents per cell at IgG concentrations approximating saturation (Fig. 8). In a total of four experiments using those concentrations, the average binding was 5.7 x 10^5 molecules of purpurin equivalents per cell. However, the exact meaning of this number is difficult to determine, because the concentrations of antibody and antigen are different in the radioimmunoassay and in the

![Figure 6](https://example.com/figure6.png)

**Figure 6** Binding of 125I-anti-purpurin IgG with and without adsorption by purpurin. The reaction mixture consisted of 5 x 10^6 D. purpureum cells per milliliter of SPS containing 2 mg/ml of bovine serum albumin and varying concentrations of 125I-anti-purpurin IgG, some of which had been adsorbed with purpurin as described below. After reaction for 30 min on ice, the cells were separated from the reaction mixture by layering over cold 10% Ficoll and centrifugation. The pellet was counted with a gamma scintillation counter. For the adsorption experiments, 1-ml portions containing 0.3 mg of either purpurin or bovine serum albumin were heated in a boiling water bath for 10 min, cooled to 4°C, and the denatured precipitated protein was removed by centrifugation and used for a single adsorption. For each adsorption, a denatured protein pellet was mixed with 1 ml of a solution containing 2.2 mg of 125I-anti-purpurin IgG in SPS containing 2 mg/ml bovine serum albumin and incubated on ice for 30 min. The mixture was then centrifuged, and in some cases a portion was saved for use in binding studies. The adsorption process was repeated with the residual supernate. (A) Binding of 125I-anti-purpurin IgG to aggregating D. purpureum cells after zero to four adsorptions with denatured purpurin. (B) Binding of various concentrations of 125I-anti-purpurin IgG that had been adsorbed three times with either denatured bovine serum albumin (○) or denatured purpurin (●).

![Figure 7](https://example.com/figure7.png)

**Figure 7** Effect of concentration of normal IgG on cell surface binding of specific anti-purpurin IgG. Reaction mixtures containing 1 x 10^7 D. purpureum cells per milliliter of SPS containing 2 mg/ml BSA, 0.2 mg/ml of immune anti-purpurin IgG, and the indicated concentrations of normal IgG were shaken for 30 min at 4°C. The cells were removed by centrifugation and the amount of residual anti-purpurin IgG in solution was determined by radioimmunoassay. The data shows the molecules of purpurin equivalents bound per cell, calculated as described in Materials and Methods.

![Figure 8](https://example.com/figure8.png)

**Figure 8** Relationship between amount of immune IgG added to the reaction mixture and the molecules of purpurin equivalents bound per cell. The experiment was performed as in Fig. 7 except that the normal IgG concentration was 1.5 mg/ml in all cases, and the immune IgG concentration was varied as indicated. The points marked cellobiose and lactose represent binding to portions of cells that were first shaken for 10 min at 4°C with either 10 mM cellobiose or lactose, then centrifuged, resuspended, and reacted with immune IgG as with the untreated cells. All results are the average of triplicate determinations.
adsorption experiment. Furthermore, the radioimmunoassay measures a reaction in solution, whereas the binding assay measures a cell surface interaction.

In an attempt to evaluate further the purpurin equivalents measure, we determined the effects of elution of cell surface purpurin with 10 mM lactose before reacting the cells with IgG. We found that cells treated in this way showed a decrease in purpurin equivalents of about $0.4 \times 10^2$ per cell (Fig. 8). The average decline after elution in four experiments was $0.44 \times 10^2$ purpurin equivalents per cell, and the average number of purpurin molecules eluted from the cell surface by treatment with 10 mM lactose, as measured by radioimmunoassay of the eluate, was $0.95 \times 10^2$ molecules per cell. Therefore, a purpurin equivalent measured by the binding assay corresponds with about two molecules of purpurin measured directly in solution. By this reasoning the purpurin equivalents binding method underestimates cell surface purpurin by a factor of about 2. Multiplying the observed result (5.7 $\times 10^5$ molecules purpurin equivalents per cell) by 2 gives about $1 \times 10^6$ molecules of purpurin per cell surface. This is in close agreement with the results found with $^{125}$I-anti-purpurin IG binding (Fig. 6), if one assumes that under saturating conditions only one molecule of $^{125}$I-IG binds per molecule of purpurin. The difference in the saturating IG concentration found in Figs. 6 and 8 could be caused, in part, by our use of different preparations of anti-purpurin IG for these experiments.

To evaluate the effects of cellular metabolic activity on IG binding we compared the results of IG binding at 4°C and at room temperature. We found binding of 1.2 $\times 10^5$ purpurin equivalents per cell at room temperature in an experiment in which binding at 4°C was $5.4 \times 10^5$ purpurin equivalents per cell. In 2.5 mM sodium azide, a potent metabolic inhibitor, binding at room temperature was reduced to $6.4 \times 10^5$ purpurin equivalents per cell.

**Immunofluorescence Studies**

Given the large discrepancy between the amount of anti-purpurin IG bound per cell surface and other measures of cell surface purpurin, we considered the possibility that the IG was becoming associated with the cell, not only by binding to cell surface lectin molecules but also by pinocytosis. One argument against this possibility was that specific IG binding was saturable whereas fluid pinocytosis would not be saturable. To evaluate further this possibility we prepared fluorescein-labeled anti-purpurin IG, added it at saturating concentrations, and directly observed the distribution of bound antibody with a fluorescence microscope. Details of the experiment are described in Materials and Methods. From observations of the cells with a highly sensitive fluorescence-video microscopy system, we concluded that the only fluorescent antibody we could see was located on the cell surface either diffusely or in patches. The cells settled on the substrate and moved about normally. Under appropriate conditions, the cell surface antibody was cappled within several minutes by unlabeled goat anti-rabbit immunoglobulin added to the washed cells containing bound fluorescent IG. As already observed in previous studies with *D. discoideum* (8) capping was very prominent, and there was no obvious ingestion of the fluorescent antibody. These experiments support the conclusion that the anti-purpurin IG is actually bound to purpurin on the cell surface rather than ingested. Attempts to elute bound $^{125}$I-anti-purpurin IG with lactose were, unfortunately, unsuccessful, presumably indicating that the cell surface purpurin IgG complex is too firmly bound, perhaps as a result of cross-linking.

**Studies with Dictyostelium discoideum**

Although technical considerations led us to use *D. purpureum* for these studies, we examined the generality of our findings with a more widely studied species, *D. discoideum*. Using dissociated aggregating cells of strain NC-4 harvested from pads and a potent antiserum raised against both lectins from *D. discoideum* (discoidin-I and discoidin-II), we measured total cellular lectin and elutable cell surface lectin by methods like those used for *D. purpureum*. We solubilized the cellular and cell surface lectin, using N-acetylgalactosamine rather than lactose because this is the most potent inhibitor of discoidin (13). The control sugar was N-acetylglucosamine instead of cellobiose. Otherwise, conditions were the same as with *D. purpureum*. In two experiments, we found an average of 4.7 $\times 10^5$ molecules of discoidin per aggregating cell and 5.2 $\times 10^5$ molecules of cell surface discoidin elutable by 10 mM N-acetylgalactosamine. We then measured cell surface binding with $^{125}$I-Fab prepared from IgG that had been raised against discoidin. We found 3.7 $\times 10^4$ Fab molecules bound per cell at a concentration approximating saturation, a relatively higher number than we found with *D. purpureum*. Cell surface IgG binding was also estimated but in this case $^{125}$I-Fab from goat anti-rabbit IG was used to detect the binding of rabbit anti-discoidin IgG, as described elsewhere. With this method, saturation was achieved with binding of approximately $1 \times 10^5$ $^{125}$I-Fab molecules per cell. Whereas this certainly overestimates the number of molecules of cell surface discoidin, the result shows that, as with *D. purpureum*, a large amount of cell surface lectin is detected after exposing the cells to divalent antibody directed against the lectin.

**DISCUSSION**

The results of the experiments with lactose elution of intact cells and with $[^{125}]$iodosulfanilic acid labeling, when taken together, indicate that only a small fraction of the total endogenous lectin of aggregating *D. purpureum* cells is present on the cell surface. The vast majority, about 98% of the total, is apparently intracellular. The 2% on the surface is by no means a negligible amount, representing about $1 \times 10^5$ tetramer molecules per cell. All the cell surface purpurin is apparently held there by its carbohydrate binding site, presumably by association with cell surface oligosaccharides because elution requires lactose. None is integrated into the plasma membrane because detergent is not needed to solubilize it. Display of this amount of purpurin on the cell surface is not limited by availability of complementary cell surface oligosaccharides, since severalfold more exogenously added purpurin can specifically bind to the cell surface by a lactose-sensitive bond.

In view of the evidence that there are only $10^5$ purpurin molecules on the cell surface, how can we explain the results from anti-purpurin IgG binding that indicate that there are about $10^6$ molecules of purpurin on, or accessible to, the cell surface? One possible explanation is that the latter experiments are measuring nonspecific IgG binding. There is indeed substantial nonspecific cell surface binding of IgG. However, with the alternative IgG binding methods we used, nonspecific binding of specific anti-purpurin IgG was either eliminated by

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competition with a large excess of nonimmune IgG or was distinguished from specific binding by adsorption of the specific IgG with denatured purpurin. Binding of the specific anti-purpurin IgG is also saturable, suggesting that what is being measured is cell surface binding rather than internalization by fluid pinocytosis. Direct microscopic observation of fluorescent anti-purpurin IgG bound to cells supported the conclusion that there was no internalization. The studies with fluorescent IgG also exclude the possibility that the antibody is binding to intracellular lectin in a subpopulation of broken cells because these were not seen.

Another possibility is that much of the material that binds anti-purpurin IgG to the cell surface is actually an immunologically cross-reactive antigen. There are a number of lines of evidence against this. First, total cell extracts showed no evidence of a cross-reactive antigen when studied by the technique of Adair et al. (1), as shown in Fig. 1. Autoradiographs of the immunoprecipitates of cell surface iodinated proteins also failed to reveal a cross-reactive antigen. In addition, 125I-Fab prepared from anti-purpurin IgG did not show the marked cell surface binding that might be expected if there were cross-reactive materials displayed on the cell surface.

In view of these results, we conclude that the extensive cell surface binding of anti-purpurin IgG is caused by its association with authentic purpurin that is actually on the cell surface. Yet the lactose elution experiments, the cell surface iodination experiments, and the 125I-Fab binding experiments indicate that there is much less cell surface purpurin than is found in the IgG binding studies. One possible explanation for the discrepancy is that all the reagents except IgG may be incapable of detecting a large fraction of the cell surface purpurin. Yet this seems unlikely because IgG is the largest of these molecular probes and is the most likely to be excluded from inaccessible sites on the cell surface.

Another possibility is that purpurin is continuously cycling between the interior and surface of the cell. This cycling purpurin might be associated with oligosaccharide receptors on the inner surface of membrane vesicles, which are capable of fusing with the plasma membrane and evertting their contents. Cycling would occur by alternating exocytosis and endocytosis. As new purpurin appeared on the cell surface, IgG already bound to a cell surface purpurin molecule also might bind to the newly exposed purpurin with its other valence. This might, in turn, trap the newly exposed purpurin on the cell surface. However, were this hypothesis correct, one would expect that some of the cycling purpurin would be elutable by continuous exposure of the cells to lactose. Yet both brief exposure to lactose and sustained exposure for as long as 45 min released the same amount of purpurin. One would also expect that 125I-Fab would become associated with the exposed cycling purpurin and would either remain associated with it or be internalized. However, we have found that (a) very little immune Fab becomes associated with the cells, and (b) adding exogenous purpurin to the cell surface leads to considerable additional 125I-Fab binding. Therefore, this hypothesis seems unlikely.

A more likely possibility is that the IgG elicits the appearance of cell surface purpurin to which it can then bind. This could result from the cross-linking by IgG of cell surface purpurin as well as the oligosaccharide receptors that bind it to the cell membrane. Such cross-linking could, in some manner, signal the insertion of more purpurin and oligosaccharide receptor into the plasma membrane. The mechanism for this elicitation could be the same as that proposed for the cycling hypothesis, that is fusion of vesicles containing purpurin and receptor. The fact that complete release of intracellular purpurin requires extensive disruption of the cellular contents by freezing and thawing in lactose is consistent with this type of intracellular localization of the lectin. How cross-linking of cell surface purpurin and its receptor would elicit erosion of intracellular vesicles is not known.

A precedent for the induced display of cell surface receptors by divalent immunoglobulin has been reported (9) in studies with mouse plasmacytoma cells. The surface of these cells contains an IgA, which binds trinitrophenyl bovine serum albumin (TNP-BSA). There are about 8 x 10^5 such IgA molecules per cell but only 6 x 10^4 TNP-BSA molecules bind to the surface of each cell. However, the amount of TNP-BSA receptors on the cell surface increased eightfold when the cells were preincubated with a divalent ligand, anti-mouse IgA (9).

One difference between the present experiments and those with the myeloma cells is that most of our binding studies were performed at 4°C whereas most of theirs were done at 37°C. In our work increasing the temperature to about 24°C produced an additional twofold increase in IgG binding, and this increase was blocked by sodium azide, a metabolic inhibitor. In the experiments with myeloma cells, the eightfold increase seen in TNP-BSA binding in the presence of anti-mouse IgG at 37°C was reduced to a twofold increase at 4°C. In both systems the partial temperature dependence may indicate that metabolic processes or the state of fluidity of membrane lipids may play some role in the response to cross-linking of cell surface proteins. The fact that slime molds are poikilothermic, existing at ambient temperatures, may account for the smaller effect of low temperature on this apparently related phenomenon.

The finding that divalent ligands elicit appearance of cell surface molecules may reflect mechanisms that are physiologically significant. With the myeloma cells, progressive association of cell surface IgA with a polyvalent antigen could be related to a process that is important in development of the immune response. In slime molds, elicitation of additional cell surface purpurin might result from cross-linking of the purpurin molecules already on the cell surface by association with available complementary molecules on the surface of another cell. This could strengthen lectin-mediated adhesion of slime mold cells, for which there is considerable evidence (2, 12).

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