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Isolation and Characterization of Lectin Binding Proteins from Murine Lymphoid Cells†

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ABSTRACT: The major cell surface proteins with affinity for the dimeric and tetrameric forms of concanavalin A (Con A), and for lens culinaris hemagglutinin, phytohemagglutinin, the pokeweed mitogens Pa-1, Pa-2, Pa-4, and Pa-5, and for wheat germ agglutinin have been isolated from murine lymphoid cells. These receptor proteins were isolated from radioactively labeled and NP-40 solubilized cell proteins by affinity chromatography using agarose–lectin resins. The specifically bound and eluted lectin binding proteins were analyzed according to their mobilities by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and in certain cases by immunoprecipitation. Specific depletion of the concanavalin A binding proteins in the NP-40 lysates removed essentially all of the radioactive material which bound to each of the other lectins. The data from this depletion study and from the electrophoresis profiles indicate that each of the mitogens studied share a common set of cell surface glycoprotein acceptors. The nonmitogenic lectin wheat germ agglutinin bound mainly to H-2D and H-2K antigens. The rest of the lectins investigated bound in varying amounts to Ia antigens, surface immunoglobulins, and two T cell associated proteins (tentatively identified as the Thy-1 alloantigen) in addition to the two H-2 antigens. All lectins, except for wheat germ agglutinin, also bound a low molecular weight (20 000) protein in varying amounts. It was shown that antibodies directed against each of the above cell surface glycoproteins compete with Con A for each of these binding sites on intact spleen cells, indicating that these glycoproteins act as lectin acceptors on the cell surface as well as after NP-40 solubilization.

The mechanism of lymphocyte activation has been one of the oldest central problems in cellular immunology. The behavior of lymphocytes and their response to antigens and plant lectins provide a unique opportunity to investigate cellular and molecular events taking place in the induction of cellular differentiation and in expression of the different characteristics. While the recognition of the specific antigen involves only a fraction of the lymphoid cells (i.e., clonal selection), the mitogenicity of certain lectins gives rise to a proliferative response involving a large fraction of the treated cells. It is well known that many of the mitogens specifically produce proliferation

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of thymus derived (T)[1] lymphocytes. Among these mitogens are concanavalin A (Con A), succinyl-Con A (the dimeric form of the tetrameric Con A), lens culinaris hemagglutinin (lentil lectin), phytohemagglutinin, and the pokeweed mitogens Pa-2, Pa-4, and Pa-5. The cell receptor structure involved in the stimulation by the different mitogens apparently is located on the outer surface of the lymphocyte membrane (Andersson et al., 1972; Greaves & Janossy, 1972). In our previous studies (Waxdal et al., 1976; Nilsson & Waxdal, 1976), Con A binding proteins were isolated from murine T lymphocytes. The two major proteins were, by precipitation with specific antisera, identified as the H-2D and H-2K antigens. The major Con A binding proteins on bone marrow derived (B) lymphocytes were, in addition to H-2D and H-2K, Ia antigens and the surface immunoglobulins IgM and IgD.

In this report we describe the isolation and identification of the major acceptor proteins from murine lymphocytes for all the other T cell specific lectins mentioned above. We also report the isolation and identification of the binding proteins for a mitogen (Pa-1) with specificity for both T lymphocytes and B lymphocytes, and for the nonmitogenic lectin wheat germ agglutinin.

In this present study we were also able to immunoprecipitate and chemically characterize two T cell associated polypeptide chains (tentatively identified as the Thy-1 alloantigen(s)), which were minor mitogen binding components observed among the Con A receptor proteins in previous studies reported from our laboratory (Waxdal et al., 1976; Nilsson & Waxdal, 1976). The glycoprotein nature of the Thy-1 antigen(s) is discussed in light of previous reports which have indicated this alloantigen to be a glycolipid (Esselman & Miller, 1974; Vitetta et al., 1973). We also show in this paper that Con A binds to all the above mentioned glycoproteins when they are on the surface of intact spleen cells and we are thereby able to rule out the possibility that Con A interacts with these structures only when they are presented to the mitogen in a solubilized form.

Materials and Methods

Cell Preparation. BALB/c mice were purchased from Charles River Laboratories. Athymic mice bearing the *nu/nu* gene on a Swiss genetic background and C3H/HeN mice were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. The mice were sacrificed by cervical dislocation and the spleens were removed sterilely and forced through a sieve into Hanks balanced salt solution. Clumps were allowed to settle and the single cell suspension was transferred to a plastic centrifuge tube. The spleen cells were washed twice with Hanks balanced salt solution. Red blood cells were removed from the lymphocytes by using lymphoprep (Nyegard & Co., A.S., Oslo, Norway). The method used was a slight modification of that described by Boyum (1968). In brief, 5 mL of a cell suspension (2×10^7 cells/mL) was layered on the top of 5 mL of lymphoprep in a 17 × 100 mm plastic snap top culture tube (Falcon). The cell separation was achieved by centrifugation for 10 min at 400*g* at ambient temperature. The centrifugation time was reduced, compared with that recommended for human cells, since it was found that the mouse lymphocytes appeared to have a higher density. The cells obtained from salt solution/lymphoprep interface were washed three times with Hanks balanced salt solution after the separation. In some experiments the red blood cells were removed by lysis with 0.83% NH_4Cl in phosphate buffer (0.01 M, pH 7.4) at room temperature. T cells were obtained by passing spleen cells twice over nylon wool (Julius et al., 1973).

Cell Labeling. The cells were suspended at 4×10^6 cells per mL in labeling medium and cultured for 4 h at 37 °C, under 5% CO_2. The medium consisted of leucine free RPMI 1640 (GIBCO) with freshly added glutamine (0.3 mg/mL), penicillin (100 units/mL), and streptomycin (0.1 mg/mL), containing 0.25–0.50 mCi of [^{3}H]leucine (specific activity, 5–80 Ci/mmol; Schwarz/Mann, N.Y.). In some experiments lymphoid cells were surface radiolabeled by using the lactoperoxidase catalyzed iodination technique (Marchalonis et al., 1971; Nilsson & Waxdal, 1976).

NP-40 Solubilization of Cell Membrane Proteins. After labeling, the cells were washed three times and resuspended at 2×10^8/mL in TKMC buffer (0.05 M Tris-HCl (pH 7.6), 0.025 M KCl, 0.005 M $MgCl_2$, and 0.005 M $CaCl_2$). Nonidet P-40 (NP-40, Particle Data Laboratories, Inc., Elmhurst, Ill.) was added to a final concentration of 0.5%. The cell suspension was mixed vigorously and incubated for 30 min at 4 °C. In order to inhibit enzymatic breakdown of the cell proteins, 10 μL of 1 M diisopropyl fluorophosphate (DFP, Calbiochem) was added. Cellular debris was removed by centrifugation at 15 000*g* for 15 min at 4 °C.

Lectins. Con A was purchased from Pharmacia, Uppsala, Sweden. A dimeric form of Con A was prepared by chemically derivatizing the tetrameric Con A molecule with succinic anhydride as described by Gunther et al. (1973). Lentil lectin and purified phytohemagglutinin were obtained from Wellcome Research Laboratories, Beckenham, England. The pokeweed mitogens (Pa-1, Pa-2, Pa-4, and Pa-5) were isolated by the procedure described by Waxdal (1974). The starting material was ground roots of *Phytolacca americana* collected from Montgomery County, Md. Wheat germ agglutinin was isolated from wheat germ lipase, essentially as described by Marchesi (1972) and was found to bind quantitatively to human red blood cells. All the lectins prepared in this laboratory gave rise to single bands when subjected to discontinuous polyacrylamide gel electrophoresis in $NaDodSO_4$ in the presence of 2-mercaptoethanol according to the method described by Laemmli (1970). The mitogenic activities for all lectins used in this study were tested on spleen cells from BALB/c mice.

Isolation of Lectin Binding Proteins. The different lectins were covalently attached to Affi-Gel 10 (Bio-Rad Laboratories) according to the instructions supplied by the manufacturer. Bovine serum albumin coupled to Affi-Gel 10 was used as a control resin in the subsequent experiments. All proteins were added at a concentration of 0.20–0.25 mg per mg of dry gel for the coupling reaction. The coupling efficiency varied between 45 and 60%. NP-40 extracts, prepared as described above, were diluted with three volumes of TKMC buffer, mixed with 0.30 mg of the different affinity resins and incubated for 30 min at 37 °C in 12 × 75 mm plastic snap top culture tubes (Falcon) on a rocking plate. After the incubation the materials were transferred to conical glass centrifuge tubes and centrifuged at 3000*g* for 2 min. The resins were resuspended in 0.5 mL of washing solution (0.5% NP-40 in TKMC buffer), mixed rapidly on a Vortex mixer, and diluted to 5 mL. The resins were centrifuged and the washing procedure was repeated four times. After washing, aliquots of the resins were removed and analyzed for the amount of radioactive protein bound. In some experiments the Con A resin, the succinyl-Con A resin and the lentil lectin resin were eluted with 400 μL of

[1] Abbreviations used: NP-40, Nonidet P-40; Ia antigens, I region associated antigens; T cells, thymus derived cells; B cells, bone marrow derived cells; Con A, concanavalin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IgD, immunoglobulin D; DFP, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate.

0.5 M methyl α-D-mannoside in phosphate-buffered saline, containing 0.5% NP-40. In all other experiments with the different lectins, and including the three above mentioned resins, the receptor proteins were eluted by adding $NaDodSO_4$ gel electrophoresis sample buffer (see below).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. In those experiments where methyl α-D-mannoside was used to elute receptor proteins, the eluates were mixed with equal volumes of $NaDodSO_4$ gel electrophoresis sample buffer (1 M Tris-HCl (pH 7.0), containing 2% $NaDodSO_4$, 5% 2-mercaptoethanol, and 5% glycerol). The sample was incubated for 2 min in a boiling water bath and was subjected to $NaDodSO_4$ gel electrophoresis. When the $NaDodSO_4$ sample buffer was used to elute the proteins bound to the affinity resins, the mixture was boiled as described above and applied on the top of the polyacrylamide spacer gel. Comparison of the $NaDodSO_4$ gel electrophoresis profiles of Con A resins eluted by these two procedures did not show any significant differences. The discontinuous $NaDodSO_4$ gel electrophoresis system described by Laemmli (1970) was used. Samples were applied on 7%, 10%, and 15% acrylamide gels in order to maximize resolution of molecules in the molecular weight range 10 000–100 000. [^{14}C] Leucine-labeled mouse immunoglobulin μ, γ, and light chains (70 000, 53 000, and 23 000 daltons, respectively) were used as reference proteins. After electrophoretic migration, the gels were minced in 1-mm segments and the radioactivity in each segment was determined.

Antisera. The specific antiserum directed against the H-2D antigen [(B10.AKM × 129) anti-B10.A] was provided by NIAID Transplantation Immunology Branch and produced by Jackson Laboratory (Bar Harbor, Me.). The alloantisera against the H-2K antigen (raised in the A mouse strain by injecting Meth-A tumors cells), and the I region associated antigens (ATH anti-ATL) were gifts from Drs. S. Cullen and D. H. Sachs (NIH, Bethesda, Md.). The goat anti-mouse immunoglobulin serum was purchased from Meloy (Springfield, Va.) The alloantiserum directed against Thy-1 (AKR anti-C3H) and the T cell specific rabbit antiserum were gifts from Drs. C. Janeway, Jr., and R. Hodes (NIH, Bethesda, Md.).

Indirect Immunoprecipitation of Labeled Proteins. Radiolabeled T cell proteins were precipitated from methyl α-D-mannoside eluates from Con A resins by adding specific rabbit anti-mouse T cell serum and thereafter protein A bearing, formaldehyde fixed staphylococci (*Staphylococcus aureus,* Cowan I strain). The washing of the precipitate and further treatment were according to the procedure by Cullen & Schwartz (1976).

^{51}Cr Release Cytotoxicity Assay. In order to determine if Con A bound to known antigens on the cell surface antibody-complement mediated cell lysis experiments were performed. Spleen cells (30×10^6) were incubated in 0.9 mL of RPMI 1640 (GIBCO) containing 10% heat-inactivated fetal calf serum (GIBCO) and 100 μCi of ^{51}Cr (185 μCi/μg; Amersham/Searle, Des Plaines, Ill.) for 45 min at 37 °C. The cells were then washed twice with RPMI 1640 containing bovine serum albumin (1 mg/mL) and resuspended in four equal portions, each at a concentration of 10×10^6 cells/mL. The temperature of the cell suspension was adjusted to 4 °C and Con A was added at different concentrations (0, 5, 10, and 25 μg/5×10^6 cells). The incubation was allowed to proceed for 30 min and the cells were washed thrice with cold RPMI 1640 containing 10% heat inactivated fetal calf serum. Fifty microliters of the cell suspension (5×10^6 cells/mL) was then incubated with serial dilutions of different antisera (50 μL) and guinea pig complement (GIBCO) (50 μL; diluted 1:5 with media) for 30 min at 37 °C as described by Shevach et al. (1973). After centrifugation the radioactivity in the supernatant was determined. The amount of radioactivity in the sample was compared with the radioactivity present in an equal volume of the supernatants from tubes containing pretreated cells (previously incubated with 0, 5, 10, and 25 μg of Con A per 5×10^6 cells) in medium plus complement only, and with that present in tubes containing cells which were frozen and thawed five times.

Antibody Absorption. Varying numbers of spleen cells that had previously been incubated (for 30 min at 4 °C) with Con A (50 μg/5×10^6 cells) or medium only were, after washing, incubated with a single dilution (1:30) of heat inactivated antiserum for 1 h at 4 °C. The absorbed antiserum was thereafter incubated with ^{51}Cr-labeled spleen cells plus guinea pig complement according to the procedure described above. The percentage of lysis was determined and graphically presented with respect to number of cells used to absorb the antiserum.

Results

Spleen cells from BALB/c mice were cultured for 4 h in the presence of [^{3}H]leucine. The cells always showed greater than 95% viability before and after the culturing period as judged from their ability to exclude trypan blue. Between 60 and 70% of the added radioactively labeled leucine was incorporated into the cell proteins. After extensively washing the cells with Hanks balanced salt solution the cell membrane proteins were solubilized by adding the non-ionic detergent NP-40 to a final concentration of 0.5%. The insoluble material, including the nuclei, was removed by centrifugation. The supernatant was then diluted with TKM buffer and aliquots were incubated at 37 °C with resins to which different lectins had been covalently coupled. The binding of [^{3}H]leucine-labeled proteins with respect to the length of the incubation time was investigated. In a typical experiment six separate portions of resin-bound lectin or bovine serum albumin (0.30 mg each) were mixed with aliquots of the NP-40 lysate (originating from 4×10^7 cells). The six samples were each incubated for a different length of time (5–90 minutes), washed thoroughly, and analyzed for the amount of radioactivity bound. As can be seen from Figure 1 the graphs reach a plateau of optimal binding after 20 min. Between 2 and 7% of the radioactivity in the NP-40 lysate remained bound to the different lectin resins after extensive washing. The binding of cell proteins to the bovine serum albumin resin, which served as a control resin, was negligible compared with other resins. The washing procedure for the resins was found to be a critical step since the agarose matrix of the Affi-Gel exhibited an affinity for many of the labeled cell proteins. These nonspecifically bound proteins would be eluted by the subsequent treatment with the $NaDodSO_4$ buffer and, consequently, give rise to spurious peaks in the electrophoresis profiles. By using the bovine serum albumin resin as a control, the washing procedure was optimized to remove these proteins so that the elution with the $NaDodSO_4$ solution recovered only specifically lectin bound cell proteins. Between 90 and 99% (average 95) of the bound radioactivity could be eluted from the resins with the $NaDodSO_4$ buffer. The same high degree of elution was also seen when methyl α-D-mannoside was used in some experiments with the Con A, succinyl-Con A, and lentil lectin resins. A typical pattern obtained when analyzing methyl α-D-mannoside eluted Con A receptors on $NaDodSO_4$ gel electrophoresis is shown in Figure 2A. When eluting the proteins from the Con A resin with the $NaDodSO_4$ buffer instead of methyl α-D-mannoside, essentially the same electrophoretic radioactivity pattern was

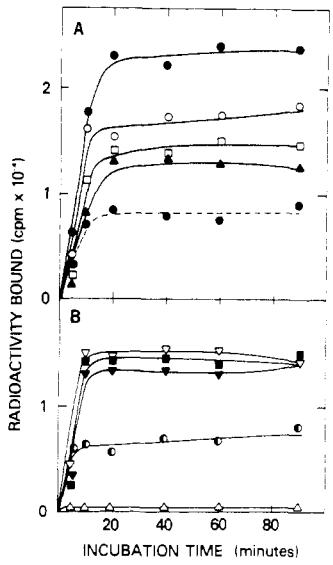

FIGURE 1: Binding of [^{3}H]leucine-labeled and detergent solubilized spleen cell proteins to lectin resins with respect to length of incubation at 37 °C. (A) Con A resin (— ● —), lentil lectin resin (— ○ —), phytohemagglutinin resin (— □ —), Pa-1 resin (— ▲ —) and succinyl-con A resin (- - ● - -). (B) Pa-2 resin (— ▽ —), Pa-3 resin (— ■ —), Pa-4 resin (— ▼ —), and wheat germ agglutinin resin (— ◐ —). Bovine serum albumin resin (— △ —) was used as a control.

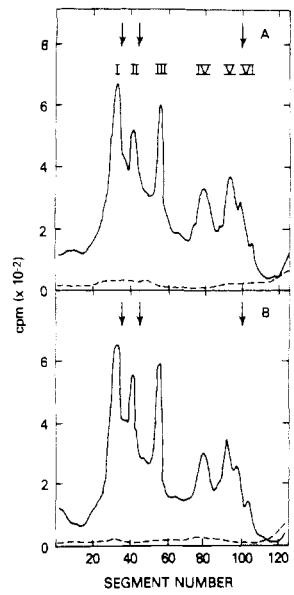

FIGURE 2: NaDodSO$_4$ gel electrophoresis analyses of [^{3}H]leucine-labeled spleen cell proteins eluted with (A) α-MM and (B) NaDodSO$_4$ buffer from Con A resin (—) and bovine serum albumin resin (- - -). The acrylamide gel concentration used was 10%. The arrows indicate the migration of ^{14}C-labeled mouse immunoglobulin μ, γ, and light chain (70 000, 53 000, and 23 000 daltons, respectively). The direction of migration is from left to right. The gels were cut in 1-mm segments and analyzed for radioactivity.

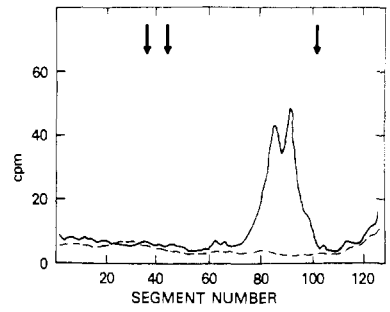

FIGURE 3: NaDodSO$_4$ gel electrophoresis analysis of Thy-1 antigen (—), immunoprecipitated from [^{3}H]leucine-labeled and detergent solubilized T cell proteins. Normal rabbit serum was used as a control (- - -). The same experimental conditions and symbols are used as described in the legend to Figure 2.

obtained (Figure 2B) retaining the high degree of specificity of this technique. By using concomitantly electrophoresed [^{14}C]leucine-labeled mouse immunoglobulin μ, γ, and light chain as molecular weight standards, the molecular weights for peaks I through VI were calculated to be 70 000, 53 000, 41 000, 31 000, 24 000 and 23 000, respectively. In a previous paper (Nilsson & Waxdal, 1976) data were presented showing that peaks I, II, and VI in the Con A acceptor profile represent respectively immunoglobulin μ, γ, and light chain. Of these polypeptide chains, the γ chain and part of the light chains were shown to originate from an intracellular pool of IgG. The μ chain and part of the light chains are present on the outer surface of thymus-independent lymphocytes. Immunoglobulin D, which is also present on the cell surface of these lymphocytes, exhibits a high affinity for Con A. The δ chain of this molecule which, compared with μ chain and γ chain, is harder to label with [^{3}H]leucine gives rise to a small radioactive peak between peaks I and II. It is therefore sometimes rather hard to detect this protein using this method. However, when cell surface proteins are labeled by using the lactoperoxidase catalyzed iodination procedure, the δ chain is easier to detect in the methyl α-D-mannoside eluate from the Con A resin, especially when analyzing immunoglobulin immunoprecipitates on polyacrylamide gels of lesser density (e.g., 7%). Peak III contains H-2D and H-2K cell surface antigens, present on both B and T cells. Peaks IV and V in Figure 2A represent mainly two Ia antigen polypeptide chains present on B cells (and on certain T cells).

Preliminary evidence suggesting that Con A has affinity for the Thy-1 alloantigen on the surface of thymus derived cells prompted us to further investigate this molecule. T cells, purified by passing BALB/c spleen cells twice over nylon wool columns, and thymocytes from 4 week old BALB/c mice were labeled with [^{3}H]leucine as described above. The cell membrane proteins were then solubilized with NP-40 and the proteins with affinity for Con A were isolated by affinity chromatography on Con A–Sepharose. The methyl α-D-mannoside eluate was first pretreated by adding formaldehyde fixed *S. aureus*. Thereafter the methyl α-D-mannoside eluate was divided in two equal portions, to which were respectively added rabbit anti-mouse brain serum (tentatively called rabbit anti-mouse Thy-1 serum) and normal rabbit serum. After incubation for 1 h at 4 °C, the added antibodies were precipitated by adding formaldehyde fixed *S. aureus*. The washed precipitates were subjected to NaDodSO$_4$ gel electrophoresis on 10% gels. Figure 3 depicts the results obtained when using splenic T cells. As can be seen from the figure, two protein components with the molecular weights of 28 000 and 26 000 were specifically precipitated. The same protein components were also observed from thymocytes, but were not detected on spleen cells from nude mice (i.e., non-T cells). Among the Con A binding proteins in Figure 2A these two polypeptide chains migrate between peaks IV and V and do not give rise to distinct separate peaks.

Proteins migrating with the same mobility as each of the Con A binding proteins described above were found to bind in variable amounts to all lectins investigated in this study. By analogy in migration mobility, these components are hereafter referred to as the previously identified proteins. Evidence was

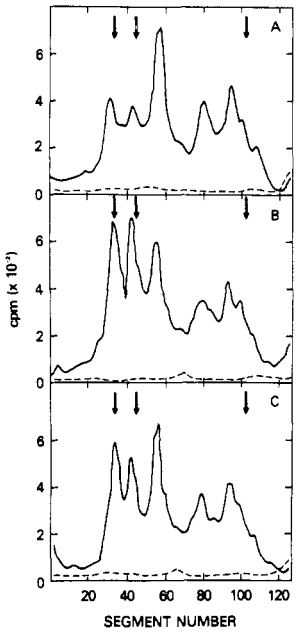

FIGURE 4: $NaDodSO_4$ gel electrophoresis analyses of [^{3}H]leucine-labeled BALB/c spleen cell proteins specifically bound to and eluted with $NaDodSO_4$ buffer from (A) succinyl-Con A resin; (B) lentil lectin resin; (C) phytohemagglutinin resin. The solid line represents protein eluted from lectin resins and the broken line represents protein eluted from bovine serum albumin resin. The same experimental conditions and symbols are used as described in the legend to Figure 2.

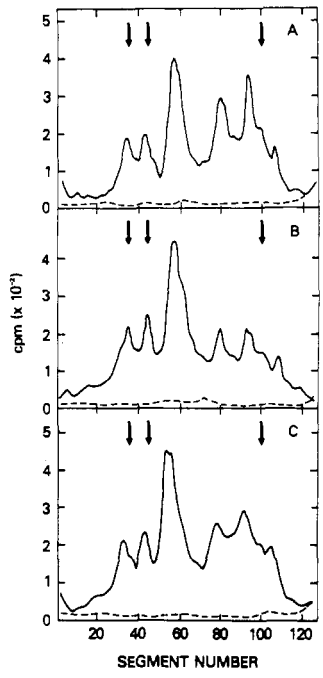

FIGURE 5: $NaDodSO_4$ gel electrophoresis analyses of BALB/c spleen cell proteins eluted with $NaDodSO_4$ buffer from (A) Pa-2 resin; (B) Pa-4 resin; (C) Pa-5 resin. For further information about symbols and experimental conditions see legend to Figure 2.

obtained (see below) that the acceptor proteins for all the other lectins investigated were indeed identical with those for Con A.

The radioactivity pattern obtained when subjecting $NaDodSO_4$-eluted spleen cell proteins specific for the dimeric form of Con A, succinyl-Con A, to $NaDodSO_4$ gel electrophoresis is shown in Figure 4A. As judged from the profiles of the eluted proteins, the most marked difference between the succinyl-Con A resin and the Con A resin (Figure 2) is that the dimeric form of the lectin binds less immunoglobulin. The contrary possibility that succinyl-Con A exhibits an extremely high affinity for the immunoglobulins and that it therefore would be more difficult to elute these proteins with $NaDodSO_4$ from the resin can be ruled out since the radioactivity initially bound to the resin could almost be eluted quantitatively (>95%).

The result from the analysis of the binding proteins for lens cularinus hemagglutinin (lentil lectin) is depicted in Figure 4B. As can be seen from this figure, this mitogen binds immunoglobulins, H-2 and Ia antigens. However, when compared with the rest of the proteins, IgG seems to bind in somewhat larger quantities to lentil lectin than to Con A (Figure 2). The binding of all the receptor molecules to the resin could be quantitatively abolished by adding 0.5 M methyl α-D-mannoside, a sugar for which lentil lectin shows affinity. However, the addition of D-galactose in the same concentration did not qualitatively or quantitatively alter the receptor pattern, indicating that all polypeptide chains represent specifically bound protein.

The $NaDodSO_4$ gel electrophoresis profile obtained (Figure 4C) when analyzing the acceptor proteins for phytohemagglutinin is also quite similar to that obtained for Con A binding proteins (Figure 2).

The pokeweed mitogens Pa-2, Pa-4, and Pa-5 were isolated from the plant *Phytolacca americana*. Of these three T cell mitogens, Pa-2 is the most potent. Pa-2 and Pa-5 are in addition potent hemagglutinins. Despite certain differences in biological behaviour (Waxdal & Basham, 1974; Basham & Waxdal, 1975), all three mitogens bind to the same set of acceptor proteins on murine lymphocytes as judged from the $NaDodSO_4$ gel electrophoresis profiles (Figure 5). The specifically bound proteins were eluted with $NaDodSO_4$ sample buffer because no suitable inhibitor was available. When compared with the proteins eluted from the Con A resin (cf. Figure 2A), the pokeweed mitogens apparently bound less immunoglobulins.

All seven lectins mentioned above are monospecific mitogens in that they stimulate only T cells. In order to characterize acceptor proteins for a mitogen which stimulates both T and B cells, Pa-1 (Waxdal & Basham, 1974; Basham & Waxdal, 1975) was used. This lectin was isolated in the same manner as the rest of the pokeweed mitogens described above and coupled to an insoluble resin. The cellular proteins with affinity for this resin were eluted with $NaDodSO_4$ sample buffer. When analyzed on $NaDodSO_4$ gel electrophoresis (Figure 6A), only minor differences were apparent compared with the profiles obtained for the other pokeweed mitogens, indicating that Pa-1 also binds immunoglobulin, H-2 antigens, and Ia antigens.

Our next experiment was designed to try to answer the question if wheat germ agglutinin, a nonmitogenic lectin, binds to the same set of acceptors as the mitogenic lectins, or if a different set of acceptors is involved. Matrix bound wheat germ agglutinin was incubated with an NP-40 lysate from BALB/c spleen cells at the same concentration as the previously described lectins. The protein fraction bound to the resin was eluted with the $NaDodSO_4$ sample buffer. As can be seen from the result from the $NaDodSO_4$ gel electrophoresis (Figure 6B) wheat germ agglutinin showed a weak binding of protein, with the H-2 antigens as the major components.

When analyzing ^{125}I-labeled spleen cell proteins, IgD and β-2-microglobulin could be identified among the lectin acceptors by using 7 and 15% $NaDodSO_4$ gel electrophoresis,

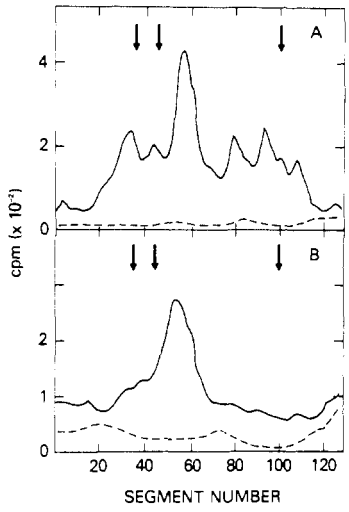

FIGURE 6: $NaDodSO_4$ gel electrophoresis analyses of ^3H-labeled BALB/c spleen cell proteins specifically bound to and eluted with buffer from (A) Pa-1 resin; (B) wheat germ agglutinin resin. The same experimental conditions and symbols are used as described in the legend to Figure 2.

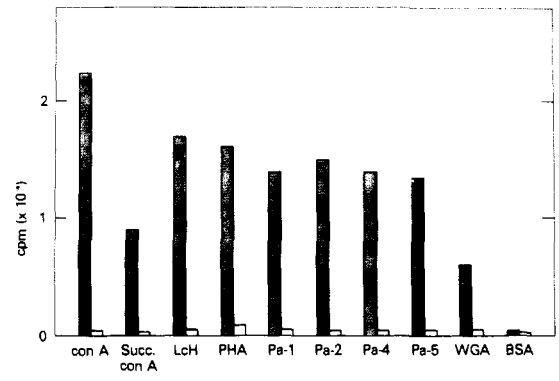

FIGURE 7: Amount of radioactivity specifically bound to various lectin resins and the bovine serum albumin resin on incubation with portions of the NP-40 lysate preincubated sequentially seven times with Con A resin (□) or bovine serum albumin resin (■).

respectively. Only β-2-microglobulin was found bound to wheat germ agglutinin, apparently indirectly via noncovalent association to the H-2 antigens.

The following experiment was performed to try to determine if some of the lectins used in this study bound ^3H-labeled proteins in addition to those bound by Con A, and if so whether these other acceptors for the different lectins co-migrated with the Con A binding proteins. BALB/c spleen cells (4×10^8) were labeled with [^3H]leucine for 4 h and the cell membrane proteins were extracted with NP-40. After centrifugation, the supernatant from the lysate was divided in two portions of equal volumes. To one portion was added 0.30 mg of the Con A resin. To the other portion was added an equivalent amount of the bovine serum albumin resin. After 30 min of incubation at room temperature, the resins were removed and the same amount of the respective resin was again added to the two supernatant NP-40 lysates. This serial depletion procedure was repeated seven times. Room temperature was chosen rather than 37 °C for these incubations to eliminate the possible risk of some cell protein denaturation during this long (4 h) procedure. The resins were washed immediately after they had been removed from the protein solutions. Aliquots of the resins were analyzed for amount of radioactivity bound. Almost no radioactivity bound to the bovine serum albumin resin during the sequential incubations. The Con A resin, however, initially bound significant amounts of radiolabeled protein. After the seventh incubation no more binding to the Con A resin was seen, indicating a total depletion of Con A binding proteins from the lysate. $NaDodSO_4$ gel electrophoretic analysis of material bound to the Con A resins and eluted with $NaDodSO_4$ sample buffer did not indicate a change in composition between the initial and latter incubations. The number of incubations required fits with previous results showing that 0.30 mg of Con A resin depletes about one-tenth of the lysate from this number of cells. The two resulting depleted NP-40 lysates from the Con A and bovine serum albumin resin incubations were each divided in 10 equal portions (each equivalent to the material from 2×10^7 cells) and tested with each of the different insolubilized mitogens. As an example, resin-bound succinyl-Con A (0.30 mg) was added to two portions of the lysates, one portion pretreated as described above with Con A resin and the other portion pretreated with bovine serum albumin resin. The two succinyl-Con A incubations were allowed to proceed for 30 min at 37 °C. After washing the resins the amount of radioactively labeled protein bound was measured. As can be seen from Figure 7, essentially no protein bound to the succinyl-Con A resin when incubated with the NP-40 extract previously treated with Con A resin. The depletion of receptor proteins was specific since the pretreatment of the NP-40 lysate with bovine serum albumin resin did not affect the amount of proteins bound. Con A resin, used as a control, was also reincubated with two other pretreated portions of the lysate, exactly in the same way as described for succinyl-Con A (see Figure 7). Evidence was obtained that the material previously pretreated with bovine serum albumin resin still contained all Con A receptor proteins since the $NaDodSO_4$ gel electrophoresis (not shown) obtained on the Con A binding proteins from this material was identical with that depicted in Figure 2B.

In the same manner the amount of radiolabeled protein which bound to the other lectins after preincubation with Con A resin and bovine serum albumin resin was investigated. Results similar to those described for the succinyl-Con A resin and the Con A resin were obtained with lentil lectin, phytohemagglutinin Pa-1, Pa-2, Pa-4, Pa-5, and wheat germ agglutinin (see Figure 7). These data, in conjunction with the $NaDodSO_4$ gel electrophoresis profiles for each of the different lectins suggest that all these mitogens interact with immunoglobulins, H-2 antigens, and Ia antigens on murine lymphocytes, while the nonmitogenic lectin wheat germ agglutinin binds primarily to H-2 antigens. Due to technical problems it was, however, not possible to directly and positively determine if the T cell specific antigens (Thy-1) with affinity for Con A also were present among the specifically eluted proteins from all the other lectins investigated.

In an attempt to determine if any or all of the solubilized and identified Con A binding proteins served as acceptor molecules on the intact cell surface, the two following experiments were undertaken:

1. In the first study spleen cells from BALB/c mice were labeled with ^{51}Cr and thereafter divided in four equal portions and coated with different amounts of Con A (see Materials and Methods). The cytotoxicity of antisera directed specifically against each of the previously identified Con A binding proteins was then tested on the pretreated cells. As can be seen from Figure 8, the Con A coated cells were more resistant to the cytotoxic effect of each of the antisera tested than were the cells that had been preincubated with medium only.

2. In another approach to the same problem, portions of each antiserum tested (diluted 1:30) were first absorbed with

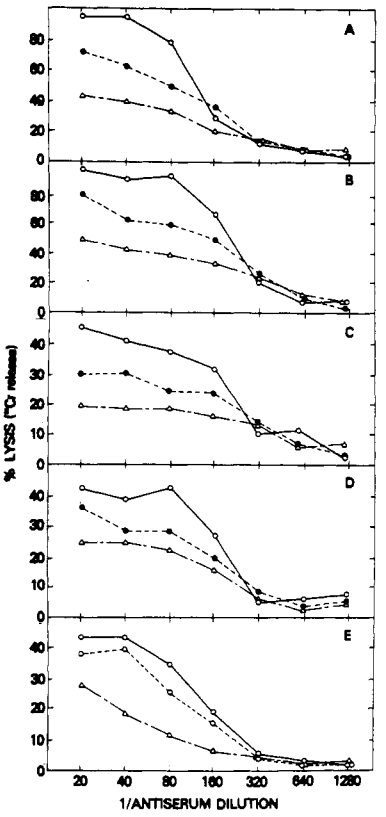

FIGURE 8: Antibody-complement mediated lysis of ^{51}Cr-labeled spleen cells, previously incubated with various concentrations of Con A [0 (○), 10 μg (●), and 25 μg (△) per 5 × 10^6 cells]. The antisera tested were (A) anti-H-2D; (B) anti-H-2K; (C) anti-Ia; (D) anti-Ig, (E) anti-Thy-1. All antisera used were alloantisera except for the goat anti-mouse Ig. BALB/c spleen cells were used in experiments A, B, D, and E. In the experiment using antiserum directed against Ia antigens spleen cells from the C3H/HeN mouse strain were used. Five × 10^5 cells (50 μL) were added to serially diluted antisera (50 μL) and guinea pig complement (50 μL, 1:5 dilution). After incubation (30 min at 37 °C) the cells were centrifuged down and 100 μL of the supernatant was analyzed for radioactivity. For further information about the calculation of percentage of lysis, see Materials and Methods. All values represent the mean from four experiments.

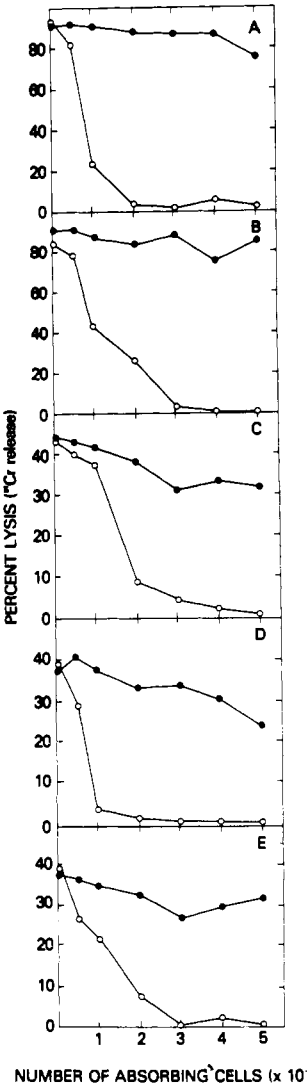

FIGURE 9: Antibody-complement mediated cytolysis of ^{51}Cr-labeled spleen cells by antisera previously absorbed with untreated (○) or Con A coated (●) spleen cells (see Materials and Methods). The antisera tested were directed against (A) H-2D; (B) H-2K, (C) Ia; (D) Ig; (E) Thy-1. The antiserum dilution used was 1:30. The absorbing cells and target cells used for the different antisera were the same as described in the legend to Figure 8. All values represent the mean from four experiments.

varying amounts of spleen cells. The absorbing cells were either pretreated (coated) with Con A (the incubation concentration of Con A was 50 μg/5 × 10^6 cells) or with medium only. After the absorptions the cytotoxicities of the antisera were tested on untreated ^{51}Cr-labeled BALB/c spleen cells. The results from the experiments are shown in Figure 9. From the figure it is evident that Con A coated cells absorbed less specific antibodies from the sera tested than did untreated cells. This second approach rules out the possibility that Con A, due to interaction with immunoglobulins and complement factors, interferes with the antibody mediated cytotoxicity in the first of these two experiments.

The results from these two experiments suggest that Con A and specific antibodies directed against each of the identified acceptors compete for the same sites on the intact cell surface and therefore provide indirect evidence that all cell surface molecules identified in this study serve as acceptor molecules on the intact cell.

Discussion

The mitogen Con A, covalently attached to an insoluble matrix, can quantitatively deplete spleen cell NP-40 lysates of [^{3}H]leucine-labeled proteins with affinity for succinyl-Con A, lentil lectin, phytohemagglutinin, wheat germ agglutinin, and the pokeweed mitogens Pa-1, Pa-2, Pa-4, and Pa-5. In a previous paper reported from our laboratory (Nilsson & Waxdal, 1976), data were presented identifying the major Con A binding glycoproteins from the surface of murine spleen lymphocytes. These were H-2D, H-2K, and Ia antigens, all coded for by the major histocompatibility complex, and IgM and IgD. In addition other glycoproteins in the molecular weight range 25 000–29 000 showed affinity for Con A.

In this present report we show that the proteins in the molecular weight range 25 000–29 000 are two T cell specific proteins. These two proteins were also detected when analyzing ^{125}I-labeled cell surface proteins and could be immunoprecipitated from the methyl α-D-mannoside eluate of splenic T cells and thymocytes by using a rabbit anti-mouse brain serum. The antiserum exhibits a specificity for thymus derived cells that closely parallels that of alloantisera against the Thy-1 antigen. Letarte-Muirhead et al. (1975) have reported the isolation and characterization of Thy-1 alloantigen from rat thymocytes by using a rabbit anti-rat brain serum. The conclusions reached from their data are in close agreement with the data obtained on the tentatively identified Thy-1 alloantigen isolated from

mouse in our study. The Thy-1 antigen is very poorly labeled with [^{3}H]leucine and the structures recognized by alloantisera seem to be easily destroyed during the detergent solubilization procedure. These two parameters may represent part of the reason for suggesting a glycolipid like structure of the Thy-1 antigen in previous studies (Esselman & Miller, 1974; Vitetta et al., 1973).

Evidence was obtained, as shown in the present paper, that all lectins investigated, except for wheat germ agglutinin, bind in varying amounts to all the above mentioned Con A binding cell surface glycoproteins. Data reported by Ray & Simmons (1973) show that Con A and antibodies against H-2 antigens compete for the same acceptor site on murine lymphocytes. In the present investigation we obtained data indicating that in addition to H-2K and H-2D, antibodies also directed against Ia antigens, immunoglobulins, and Thy-1 are able to compete with Con A for the binding sites on murine spleen cells, thereby giving indirect evidence that the cell surface glycoproteins interacting in solubilized form with Con A also serve as acceptor molecules on the intact cell surface. However, although the rest of the lectins apparently are able to bind these cell surface components after solubilizing the cell membrane, we cannot rule out the possibility that some of these interactions may, for sterical reasons, not take place on the intact cell. The lectins investigated in this study also interact with certain glycolipids from the spleen cell membrane (Nilsson & Waxdal, unpublished results).

Data reported by Sela et al. (1975) show that phytohemagglutinin and wheat germ agglutinin compete for at least some of the receptor sites on intact mouse splenic lymphocytes. However, Con A and wheat germ agglutinin were not found by these authors to compete for the same receptors on the cells, suggesting binding to different sets of major surface receptors. This might consequently also indicate that Con A and phytohemagglutinin do not share the major receptors on the intact cell. However, results presented in the present paper show that Con A shares its solubilized glycoprotein receptors with phytohemagglutinin and many other lectins. It has also been shown by Henkart & Fisher (1975) that the $NaDodSO_4$ gel electrophoresis profiles of the Con A and phytohemagglutin binding cell surface components are nearly identical. In the light of the results obtained by different investigators, at least two possible interpretations can be made:

1. As suggested above, certain cell surface glycoproteins may for sterical reasons be unable to interact with some of the different lectins while on the cell surface, but may be able to bind to these lectins after NP-40 solubilization.

2. Some lectins may interact with different structures of the glycolipid portion of the cell surface or with glycoproteins with slow turnover times, and thus not be detected in our experiments. These interactions may make it difficult to detect differences in the interaction of lectins with the known surface glycoproteins on intact cells.

Functional studies (such as investigation of the lymphocyte responses to the different lectins in presence of excess amount of antibodies directed against the lectin binding proteins) are currently under way in order to evaluate if any or all of the glycoproteins identified as cell surface lectin acceptors are essential to the mitogen induced stimulation of lymphocytes or if still unidentified receptors are necessary for proper functioning of the activation mechanism.

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