Isolation and Identification of the Major Concanavalin A Binding Glycoproteins from Murine Lymphocytes†

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ABSTRACT: The major glycoproteins which bind concanavalin A have been isolated and identified from murine spleen cells, thymocytes, and purified thymus-derived (T) lymphocytes, and from the spleen cells of congenitally athymic (nude) mice. The cells were radiolabeled by lactoperoxidase catalyzed ^{125}I iodination or by culturing the cells in media containing [^{3}H]leucine or [^{3}H]fucose. The cell membrane was solubilized with Nonidet P-40 and the concanavalin A binding proteins were isolated by affinity chromatography and analyzed according to their mobility on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The major proteins from various lymphocyte preparations were identified by immunoprecipitation with specific antisera. The molecules coded by the histocompatibility-2 complex acted as concanavalin A binding proteins. H-2K and H-2D were isolated from T lymphocytes, thymocytes, and bone marrow derived (B) lymphocytes. The Ia antigens were identified from B lymphocytes and tentatively identified from T lymphocytes. In addition to these H-2 complex proteins, immunoglobulin M and D on B lymphocytes also bound concanavalin A binding. All these glycoproteins have previously been identified as cell surface molecules. The presence of certain minor unidentified concanavalin A binding proteins on lymphoid cells is indicated.

By culturing lymphoid cells together with mitogens in vitro, the cells can be triggered into a state of active growth and proliferation. The mechanism by which the mitogen concanavalin A (con A)[1] exerts its stimulatory action is still unknown, although its specificity for glucosyl and mannosyl residues is well documented (Goldstein et al., 1965). It has, however, been shown that the specific receptor(s) for the mitogen-mediated triggering reside(s) in the plasma membrane, since the overall effects are the same even when con A is immobilized to an insoluble matrix (Anderson et al., 1972). When added in soluble form to lymphoid cells, con A triggers thymus-derived lymphocytes (T cells) selectively, while the thymus-independent lymphocytes (B cells) remain unstimulated (Anderson et al., 1972). This finding is of great interest since there is ample evidence that the mitogen binds to both T and B cells in equal amounts (Stobo et al., 1972). Obviously, a more detailed picture of the interaction between con A, as well as other lectins, and the surface receptors from the different cell populations is necessary for elucidating the triggering mechanisms, in particular, if there are receptors specific for B and T cells, and if mitogenic and nonmitogenic lectins bind to the same receptors.

In this communication we report the isolation and identification of the major con A binding glycoproteins from lymphoid cells. Murine cells were chosen since this genetic system is far better defined than for any other species. The glycoproteins were isolated from purified T cells, thymocytes, normal spleen cells, and spleen cells from congenitally athymic (*nu/nu*) mice. The presence of these proteins on the cell surface was verified by using a lactoperoxidase-catalyzed ^{125}I iodination technique. To our knowledge this is the first report presented identifying the major con A binding cellular proteins.

Materials and Methods

Animals. The 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.

Cell Preparation. The mice were sacrificed by cervical dislocation and the spleens/thymuses were removed sterilely. The tissues were then forced through a sieve into Hanks' balanced salt solution (HBSS). The clumps were allowed to settle and the single cell suspension was transferred to a plastic centrifuge tube. The cells were washed twice with HBSS. The lymphocytes were then separated from the red blood cells by using lymphoprep (Nyegard & Co. A.S., Oslo, Norway). The method used was a slight modification of that described by Boyum (1968). Briefly, 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 separation of cells was achieved by centrifugation for 10 min at 400*g* at room temperature. The cells were obtained from the HBSS/lymphoprep interface and washed three times after the separation.

Cell Labeling. Spleen or thymus cells were suspended at 4×10^7 cells/ml in labeling medium and incubated at 37 °C, 5% CO_2. Leucine free RPMI 1640 (GIBCO) with freshly added glutamine (0.3 mg/ml), penicillin (100 units/ml), and streptomycin (0.1 mg/ml) was used when labeling with 0.25–0.5 mCi of [^{3}H]leucine (Schwarz/Mann, N.Y.) for 4 or 18 h. In some experiments cells were radiolabeled by culturing in complete RPMI 1640 and 0.5 mCi of [^{3}]fucose (New England Nuclear, Boston, Mass.) for 18 h. Radioiodination (^{125}I) of cells was performed essentially as described by Marchalonis et al. (1971). In some experiments, a modified method was used where lactoperoxidase (Calbiochem), attached to Affi-gel

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[1] The abbreviations used are; con A, concanavalin A; T cells, thymus-derived cells; B cells, bone-marrow-derived cells; HBSS, Hanks balanced salt solution; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; DFP, diisopropyl fluorophosphate; α-MM, methyl α-D-mannoside; BSA, bovine serum albumin; Ia, I-region associated antigens; LPS, lipopolysaccharide; PHA, phytohemagglutinin; IgD, immunoglobulin D; IgG, immunoglobulin G; IgM, immunoglobulin M.

10 (Bio-Rad Laboratories), 0.5 mg bound/mg dry gel, was used instead of the soluble enzyme. Briefly, 2–10 × 10^7 cells in 1 ml of phosphate-buffered saline (PBS, 310 mOsm, pH 7.2) were mixed with 100 μg of the matrix-bound lactoperoxidase in a 50-ml plastic tube at room temperature. The reaction was initiated by the addition of 2.5 mCi of carrier-free ^{125}I (New England Nuclear, Boston, Mass.) and 25 μl of an 0.015% H_2O_2 solution. The tube was shaken gently for 20 min at room temperature and three more additions of H_2O_2 were made during this period of time. The cell suspension was thereafter diluted with 50 ml of ice-cold PBS and matrix-bound lactoperoxidase was removed by sedimentation at 4 °C. The cells were washed two more times with ice-cold PBS.

Cell Separation. Partial purification of T lymphocytes (T cells) was achieved by passing spleen cells over nylon wool (LP-1 Leuko-Pak leukocyte filters, Fenwal Laboratories, Morton Grove, Ill.) in RPMI 1640 (GIBCO) containing 10% heat-inactivated fetal calf serum (GIBCO) essentially as described by Julius et al. (1973). Three grams of nylon wool was used per column. The fraction of cells that did not adhere to the nylon wool was passed over a second column. Of the cells which passed through the second column 97–100% failed to bind fluorescent anti-mouse Ig antibodies, indicating that B cells had essentially been eliminated and that a high degree of T cell enrichment had been obtained. This population is referred to as purified T lymphocytes.

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

Isolation of Con A Receptors. Con A (Pharmacia, Uppsala, Sweden) was covalently attached to Affi-gel 10 (Bio-Rad Laboratories) according to instructions supplied by the manufacturer and used as an affinity matrix (0.22 mg of protein bound/mg of dry gel). The NP-40 extract described above was diluted with three volumes of TKM-buffer and mixed with 0.30 mg of the Affi-gel-attached con A and incubated at 37 °C for 30 min in a 12 × 75 mm plastic snap top culture tube (Falcon) on a rocking plate. The material was then transferred to a conical glass tube and centrifuged at 3000*g* for 2 min. The resin was resuspended in 0.5 ml of washing solution (0.5% NP-40 in TKM), mixed rapidly on a Vortex mixer, and diluted to 5 ml. The resin was centrifuged and the washing procedure repeated four times. Bovine serum albumin (BSA) attached to Affi-gel 10 at the same concentration as con A was used as a control resin in all experiments. In several experiments bovine immunoglobulin was also used. After the washing, aliquots were removed to measure amount of labeled protein bound. The material that bound to the resin was eluted at 4 °C overnight with 400 μl of 0.5 M α-MM (methyl α-D-mannoside, Calbiochem) in PBS containing 0.5% NP-40.

Indirect Precipitation of Cell Membrane Proteins. Labeled cell membrane proteins were precipitated from α-MM eluates by using an antibody "sandwich" technique. The specific immunoprecipitation experiments were always preceded by one–three preprecipitations by adding rabbit anti-human immunoglobulin G and an equivalent amount of human IgG. Flow charts from three typical precipitation series are depicted in Figures 7 and 8. After incubation with an excess of appropriate alloantiserum for 15 min at 37 °C, an equivalent amount of goat anti-mouse immunoglobulin serum was added. The incubation proceeded for another 15 min at 37 °C and thereafter for 10 min at 4 °C. 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, Maine). The alloantisera against the H-2K antigen (raised in an H-2^a mouse strain by injecting Meth-A tumor cells), and the I-region associated antigens (ATH anti-ATL) were generous gifts from Drs. S. Cullen and D. Sachs (NIH, Bethesda, Md).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The eluates from the affinity resins and the immunoprecipitates were dissolved by boiling in 50–100 μl of 2% sodium dodecyl sulfate for 2 min. 2-Mercaptoethanol was added in a final concentration of 5% to reduce disulfide bonds. The discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis system was used as described by Laemmli (1970). In order to maximize resolution of molecules in the mol wt range of 10 000–100 000, samples were applied to both 10 and 15% acrylamide gels. [^{14}C]Leucine-labeled mouse immunoglobulin μ, γ, and light chains were used as reference proteins. After electrophoretic migration, the gels (140 × 6 mm) were sliced in 1-mm segments and the radioactivity in each slice was determined. A Searle Model 1185 γ counter was used to measure the ^{125}I activity. When [^3H]leucine-, [^3M]-fucose-, or [^{14}C]leucine-labeled proteins were analyzed, the polyacrylamide gel slices were suspended in 4 ml of Hydromix (Yorktown Research, N.J.) and counted in a Beckman LS-250 liquid scintillation counter (Beckman Instruments Inc., Calif.). All data were statistically analyzed and graphed with the use of a PDP-11 computer (Digital Equipment Corp., Maynard, Mass.).

Results

Intact spleen cells and thymocytes from BALB/c mice were radiolabeled by culturing in media containing [^3H]leucine or [^3H]fucose, or by using a lactoperoxidase-catalyzed iodination procedure. In some experiments spleen cells from the C3H/HeN mouse strain or from nude (*nu/nu*) mice were subjected to radiolabeling. Between 50 and 70% of the added radioactivity was incorporated into the cells by the different labeling methods used. After labeling, the cell membrane proteins were solubilized by treatment with the nonionic detergent NP-40. The 15 000*g* supernatant from this lysate was incubated with matrix bound con A at 37 °C for 30 min. After extensive washing, approximately 4% of the NP-40 extracted radioactivity remained bound to the resin. No significantly higher percentage of radioactivity bound at increasing time of incubation. Since the washing procedure was found to be a critical step, resins substituted with BSA or IgG or containing no protein were used as control resins and included in all experiments. All resins were washed until no significant radioactivity remained in these control resins. The fraction of cell protein that bound to the con A resin could be eluted with 0.5 M methyl α-D-mannoside (α-MM) in PBS containing 0.5% NP-40. The recovery of the retained protein by elution with the sugar varied between 92 and 99%. When 0.5 M α-MM was added to the NP-40 extract, essentially all subsequent binding to the con A resin was abolished. However, addition of D-galactose to the incubation medium did not interfere with the binding of the receptor proteins. On the contrary, the presence of D-galactose resulted in somewhat "cleaner" protein pattern, presumably indicating that some nonspecific binding of ra-

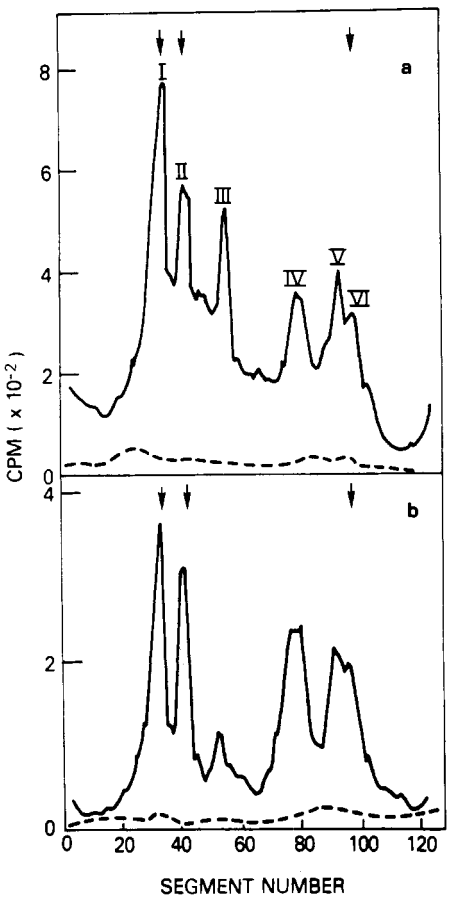

FIGURE 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of spleen cell proteins eluted with αMM from con A resin (—) and BSA resin (- - -). BALB/c spleen cells were labeled for 4 h (a) and 18 h (b) with [^{3}H]leucine. The acrylamide gel concentration used was 10%. The arrows indicate the migration of [^{14}C]leucine-labeled mouse immunoglobulin μ, γ, and light chain standards (70 000, 53 000, and 23 000 daltons, respectively). The direction of protein migration is from left to right. The gels were cut in 1-mm segments and analyzed for radioactivity.

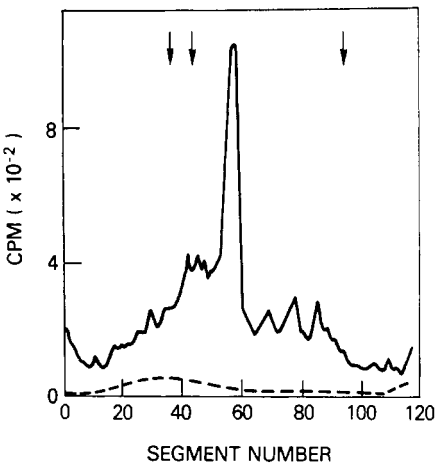

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of thymocyte proteins eluted with α-MM from con A resin (—) and BSA resin (- - -). BALB/c thymocytes labeled with [^{3}H]leucine for 4 h were used. The same experimental conditions and symbols are used as described in the legend to Figure 1.

TABLE I: Binding and Elution of Radiolabeled Proteins.

Labeling Procedures Used	% Bound[a]		% Eluted[b]		
	Con A Resin	BSA Resin	Con A Resin α-MM	Con A Resin Gal	BSA Resin α-MM
[^{3}H]Leucine	4.8	0.3	98	4.1	3.2
[^{3}H]Fucose	4.3	0.3	99	2.3	2.6
Lactoperoxidase	4.1	0.5	92	6.7	4.1

[a] Radioactivity remaining on the resins after washing with TKM buffer containing 0.5% NP-40. [b] Percent of bound radioactivity eluted from the resins with 0.5% NP-40 in TKM buffer containing 0.5 M α-MM or 0.5 M D-galactose.

diolabeled compounds to the galactosyl residues of the affinity matrix was eliminated. Table I summarizes some of the binding data obtained with the affinity resins. Typical results from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the material obtained from affinity chromatographies of [^{3}H]leucine-labeled BALB/c spleen cell lysates are presented in Figure 1a. Based on the migration of [^{14}C]leucine-labeled mouse immunoglobulin μ, γ, and light chain, the molecular weights obtained for peaks I–VI were 70 000, 53 000, 41 000, 31 000, 24 000, and 23 000, respectively. Peaks I, II, and VI coincided with the marker proteins (μ, γ, and light chains). The radioactivity peak migrating with the dye marker front on the 10% polyacrylamide gel could not be detected on a 15% gel. Surprisingly enough no additional small-molecular-weight protein peak was seen when the material was subjected to electrophoresis on the denser gel. Shorter time labeling (4 h) seemed to favor the incorporation of [^{3}H]leucine to peak III, relative to the protein pattern obtained after long time (18 h; Figure 1b) incubation.

The proteins isolated from BALB/c thymocytes by using the con A resin are shown in Figure 2. The cells, obtained from 4 week old mice, were labeled with [^{3}H]leucine for 4 h. The most pronounced difference in the protein pattern, as compared to that obtained from spleen cells, is that most peaks, except III, are suppressed. Essentially the same pattern was obtained when the cells were labeled for 18 h.

In an attempt to investigate the con A receptors on thymus-derived cells, [^{3}H]leucine-labeled BALB/c spleen cells were passed twice over nylon-wool columns (Julius et al., 1973). No immunoglobulin bearing cells were detected when assayed by the binding of fluorescein labeled rabbit anti-mouse immunoglobulin antibody. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis the [^{3}H]leucine-labeled proteins from T cells gave rise to three peaks (Figure 3). The migration distance of the three peaks corresponded to peaks III, IV, and V, respectively, in Figure 1a.

Spleen cells from congenitally athymic (nude) mice were used as a source for B cells. This cell preparation is greatly depleted in thymus-derived cells, since no θ antigen bearing cells could be detected. Moreover, no stimulation of mitosis was seen by using the T cell mitogens con A and PHA. The cells respond normally to the B cell mitogen LPS. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 4), the material eluted with α-MM from the con A resin showed a radioactivity pattern similar to that obtained from BALB/c spleen cells (Figure 1), with the exception that peak II in Figure 1a is greatly reduced or absent in Figure 4.

No additional peaks could be seen even when the different cell preparations were incubated with [^{3}H]leucine for longer periods of time than 18 h. Since radioactively labeled amino acids are incorporated to both intracellular proteins and plasma membrane proteins, the next experiment was designed to de-

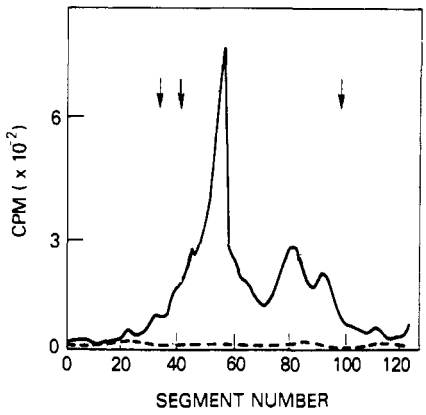

FIGURE 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of T cell proteins eluted with α-MM from con A resin (—) and BSA resin (- - -). The cells were obtained by passing BALB/c spleen cells twice over nylon-wool columns. The resulting T cells were labeled for 4 h in medium containing [^{3}H]leucine. For further information about symbols and experimental conditions see the legend to Figure 1.

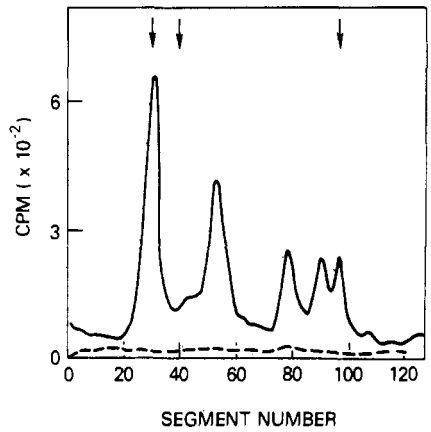

FIGURE 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of nude mouse spleen cell proteins eluted with α-MM from con A resin (—) and BSA resin (- - -). The cells were labeled with [^{3}H]leucine for 4 h. The same symbols and experimental conditions were used as described in the legend to Figure 1.

termine which of the protein peaks originated from the outer surface of the cell membrane. Spleen cells (2×10^8) from BALB/c mice were labeled with ^{125}I by using the lactoperoxidase-catalyzed reaction. In initial experiments the soluble form of lactoperoxidase was used. However, the enzyme autolabeled rather easily and, moreover, it apparently bound to the lymphocyte and had a rather high affinity for the con A resin. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis the enzyme itself displayed a broad radioactivity peak with the molecular weight around 75 000 and smaller amounts of lower molecular weight material. In order to circumvent these spurious peaks the cells were labeled by using matrix-bound enzyme. Disregarding the lactoperoxidase peaks, the protein patterns obtained from the two procedures were very much alike. As can be seen in Figure 5a, three components were iodinated preferentially. The molecular weights were calculated to be 70 000, 41 000 and 24 000. When compared to the protein pattern obtained in Figure 1a, it is evident that the migration of the iodinated compounds correspond to peaks I, III, and V. In some experiments when the α-MM eluate was subjected to 15% polyacrylamide gels containing sodium dodecyl sulfate, a small molecular weight component (11 000–13 000) was obtained in addition to the larger molecular weight components discussed above.

Since peaks I, II, and VI in Figure 1a comigrated with [^{14}C]leucine-labeled mouse immunoglobulin μ, γ, and light chain, the α-MM eluate from the affinity chromatography was tested by using goat anti-mouse immunoglobulin sera. A typical experiment on affinity chromatography purified con A receptors from [^{3}H]leucine-labeled spleen cells is depicted in Figure 6. A flow chart from the precipitation studies is shown in Figure 7. One aliquot of the sample, pretreated by adding human IgG and rabbit anti-human IgG serum, was subjected to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 6a). Goat anti-mouse immunoglobulin serum was added to another portion of the material also pretreated as described above. Precipitation was obtained by subsequently adding an equivalent amount of BALB/c serum to provide carrier protein. (In total, three sequential precipitations were performed.) When subjecting the combined precipitates to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, three distinct peaks were seen (Figure 6b). These peaks comigrated with the ^{14}C-labeled reference proteins. The ^{125}I-labeled material (Figure 5a) was also precipitated with a number of anti-mouse immunoglobulin sera. Certain of these sera (notably anti-κ chain) indicated the presence of a 62 000-dalton component in addition to the μ chain (Figure 5b). On the basis of its molecular weight it has been tentatively identified as the δ chain from cell surface IgD (Melchers et al., 1974; Finkelman, et al., 1975a). To the 15 000g supernatant (Figure 7) was added antisera directed against H-2D and H-2K. The mouse alloantibodies were subsequently precipitated by adding a goat anti-mouse immunoglobulin serum. It can be seen from Figure 6c that a protein peak with the molecular weight 41 000 appeared when the precipitate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The migration of this protein peak corresponds to peak III in Figures 1a and 6a. Three sequential precipitations using these antisera were performed. The resulting supernatant, which was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis,

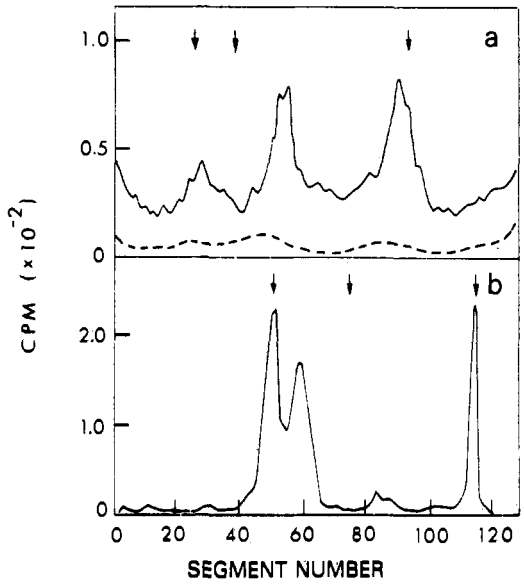

FIGURE 5: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of ^{125}I-labeled BALB/c spleen cell proteins, eluted with α-MM from con A resin (—) and BSA resin (- - -). The same experimental conditions and symbols were used as described in the legend to Figure 1. (a) Eluted proteins (10% gel). (b) Immunoprecipitation with goat antimouse κ sera (7% gel).

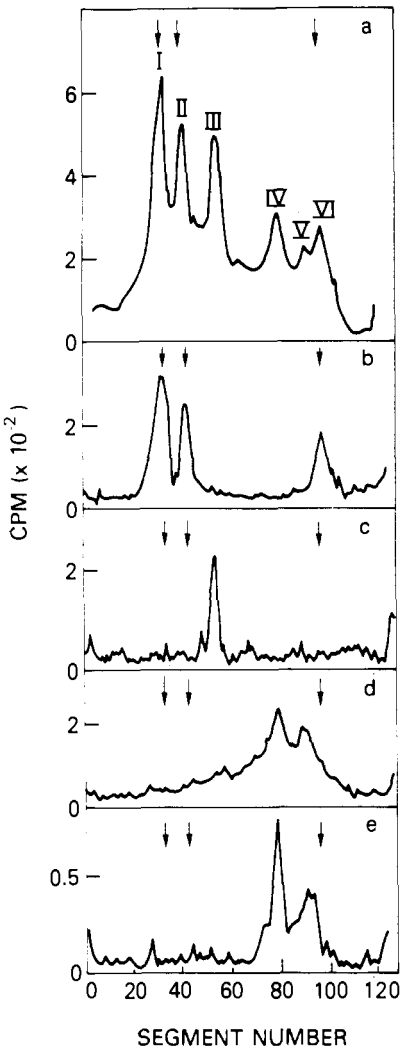

FIGURE 6: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of [^{3}H]leucine labeled BALB/c spleen cell proteins, eluted with α-MM from con A resin (a). The α-MM eluate had previously been subjected to three sequential preprecipitations by adding an aliquot of human IgG and an equivalent amount of rabbit antihuman IgG. (b) Immunoprecipitation of mouse immunoglobulin. (c) Immunoprecipitation of H-2 antigens by using a mixture of antisera directed against H-2D and H-2K. (d) Proteins remaining in the α-MM eluate after above mentioned immunoprecipitations. (e) Specific precipitation of Ia from [^{3}H]leucine-labeled C3H/HeN spleen cell proteins isolated by affinity chromatography on con A resin. The same experimental conditions and symbols were used as described in the legend to Figure 1.

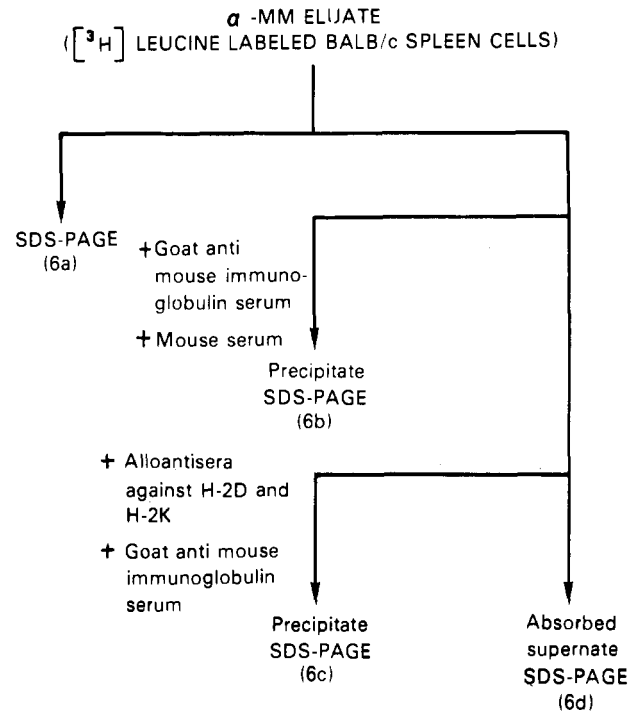

FIGURE 7: Flow chart of the procedure used to precipitate immunoglobulins and H-2 antigens from affinity isolated [^{3}H]leucine-labeled con A binding proteins from BALB/c spleen cells. The antiserum used against H-2 antigens was a mixture of an anti H-2D serum and an anti-H-2K serum. The α-MM eluate had previously been subjected to a "nonspecific" precipitation by adding human IgG and an equivalent amount of a rabbit antihuman IgG serum. Three sequential precipitations were performed with each antiserum. The materials were subsequently analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The numbers in brackets refer to the figures showing the electrophoretic pattern obtained.

was depleted in peaks I, II, III, and VI (cf. Figures 1a and 6a). Two radioactivity peaks with molecular weights of 31 000 and 24 000 remained (Figure 6d).

In an attempt to identify the [^{3}H]leucine-labeled protein components (IV and V) with the molecular weight 31 000 and 24 000 (cf. Figures 1a and 6a) an antiserum directed against the I-region associated (Ia) antigens was used. These Ia precipitation experiments are summarized in a flow chart in Figure 8a. Spleen cells from the C3H/HeN mouse strain were labeled for 4 h with [^{3}H]leucine, solubilized with NP-40, and passed over an affinity column. The α-MM eluted material was subjected to three precipitations by adding a goat anti-mouse immunoglobulin serum and thereafter an equivalent amount of serum from C3H/HeN mice. After these preprecipitations, another precipitation was performed by using a mouse alloantiserum directed against I-region associated antigens and a goat anti-mouse immunoglobulin serum. The molecular weights for the two specifically precipitated proteins were 31 000 and 24 000.

The fourth precipitation study was designed to try to answer the question if both H-2D and H-2K antigens bind to con A. A flow chart of the procedure used is shown in Figure 8b. In most experiments [^{3}H]leucine-labeled BALB/c spleen cells were used. However, in some studies these cells were radiolabeled by culturing in media containing [^{3}H]fucose. The radiolabeled BALB/c spleen cells were lysed and passed over a con A resin. The α-MM eluate was then treated with a goat-anti mouse immunoglobulin serum and an equivalent amount of BALB/c serum. The 15 000g supernatant was then precipitated by using a mouse alloantiserum directed against H-2D and a goat anti-mouse immunoglobulin serum. The same "sandwich technique" was thereafter used on the resulting 15 000g supernatant to precipitate H-2K. Figure 9 depicts the results obtained from a typical study using the [^{3}H]fucose-labeled proteins. The results show that, indeed, con A binds both H-2D and H-2K antigens. The same results were obtained when [^{3}H]leucine cells were used. The molecular weights obtained for H-2D and H-2K antigens were 40 000 and 41 000, respectively.

Discussion

Table II presents the molecular weights of all the identified proteins which specifically bound to the con A resin. Immunoglobulin μ chain was serologically identified as being one of the [^{125}I]- or [^{3}H]leucine-labeled BALB/c spleen cell surface proteins with affinity for con A. This polypeptide chain was also found on spleen cells from nude mice, homozygous for the

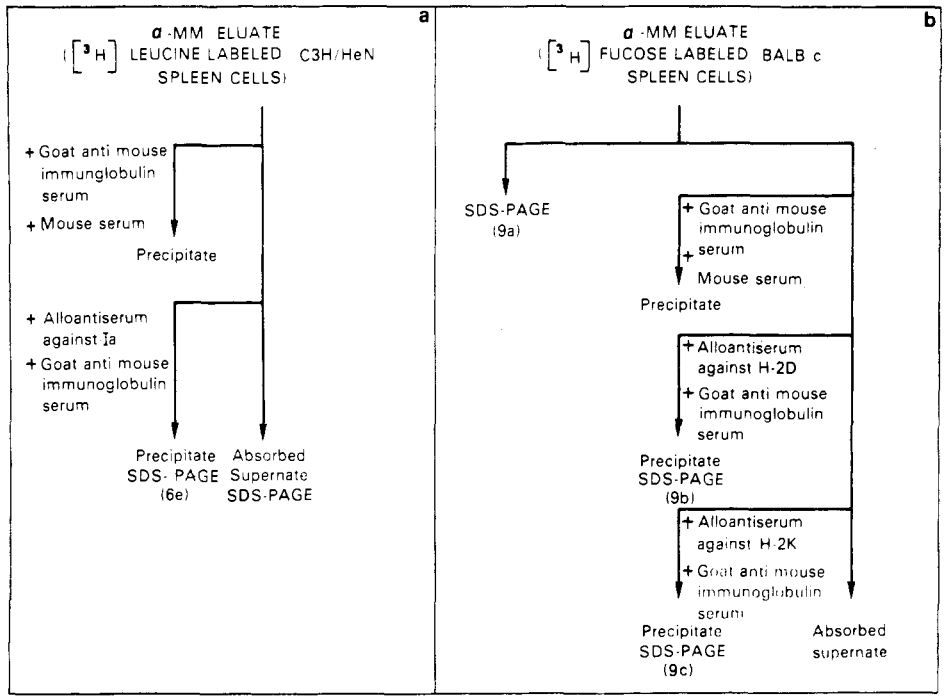

FIGURE 8: Flow chart of the procedure used: (a) to precipitate the Ia gene products from [^{3}H]leucine labeled con A binding proteins from C3H/HeN spleen cells. (b) Procedure used to precipitate H-2D and H-2K from [^{3}H]fucose labeled con A receptor proteins from BALB/c spleen cells. The materials were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The numbers in brackets refer to the figures showing the results from the electrophoretic analyses.

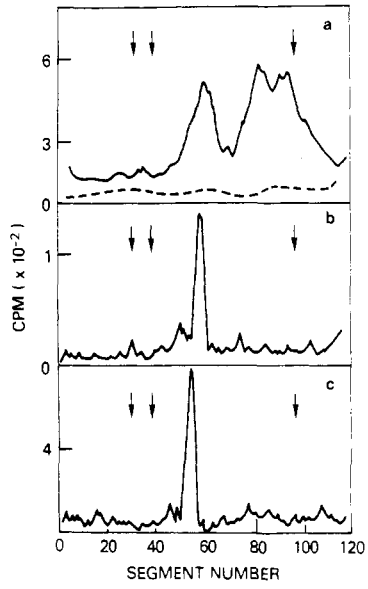

FIGURE 9: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses. (a) [^{3}H]Fucose-labeled BALB/c spleen cell proteins eluted with α-MM from con A resin (—) and BSA resin (- - -). Immunoprecipitation of con A binding proteins with (b) anti-H-2D serum and (c) anti-H-2K serum. For further information about symbols and experimental conditions see the legend to Figure 1.

TABLE II: Identification and Molecular Weights of the Proteins with Affinity for Con A.

Peak Number[a]	Identified As[b]	Mol Wt Found
I	μ Chain	70 000 (±5000)
	δ Chain	62 000 (±5000)
II	γ Chain	53 000 (± 4000)
III	H-2D	40 000 (±3000)
	H-2K	41 000 (±3000)
IV	Ia	31 000 (±2000)
V	Ia	24 000 (±2000)
VI	Light chain	23 000 (±2000)
—	$\beta_2\mu$ globulin[c]	12 000 (±1000)

[a] See Figures 1a and 6a. [b] Identified by using immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. [c] Tentative identification of component seen when subjecting ^{125}I-labeled con A binding proteins to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

gene *nu*, and generally considered to form a convenient source of B lymphocytes essentially free from T cells (Kindred, 1971; Pantelouris, 1971; Feldmann et al., 1972). However, no significant amount of μ chain was detected on the thymocyte or the T cell surface.

The presence of IgD among the cell surface con A receptors was indicated in the ^{125}I labeling studies, where a 62 000-dalton component was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the precipitation by certain anti-mouse immunoglobulin sera. The molecular weight and immunological cross-reactivity suggest that this is δ chain (Melchers et al., 1974; Finkelman, et al., 1975a). The lack of specific antisera precludes its positive identification. It should also be noted that not all polyvalent anti-mouse immunoglobulin sera detect this protein. The reason we were unable to detect this protein when analyzing [^{3}H]leucine-labeled spleen cells may be explained by the fact that this molecule is poorly labeled by this method (Finkelman, F. D., personal communication).

The immunoglobulin γ chain detected in the α-MM eluate from con A resin when using [^{3}H]leucine-labeled BALB/c spleen cells presumably originated from an intracellular pool of IgG, since little or none could be detected after exclusive labeling of the cell surface proteins of a separate portion of the cell preparation. The reason no γ chain could be detected from

the nude mouse spleen cells even after labeling with [^{3}H]leucine may be explained by a low rate of IgG synthesis. Moreover, it has been shown by Pantelouris (1971) that the serum concentration of IgG is lower in nude mice than in normal mice. By combining these data, it seems evident that, of the three immunoglobulins detected with affinity for con A, only IgM and IgD are exposed on the cell surface, and, consequently, are theoretically available for interaction with the mitogen. Our findings that IgM is present in significant amounts only on B cells are in accord with results obtained by Vitetta et al. (1971).

Both H-2D and H-2K antigens found on the surface of T cells, thymocytes, and spleen cells from the BALB/c and nude mouse strain showed molecular weights in the same range as those previously reported for mouse (Schwartz and Nathensen, 1971) and guinea pig (Finkelman et al., 1975b). However, it has previously been noted that the molecular-weight determinations for histocompatibility antigens vary somewhat with different buffers used in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis system (Schwartz, B. D., personal communications). It has recently been shown (Rask et al., 1974) that $\beta_{2\mu}$-globulin, a protein with the molecular weight 12 000, is noncovalently associated with histocompatibility-2 antigens. We were not able to detect a component in that molecular weight range by the immunoprecipitation of H-2D and H-2K [^{3}H]leucine-labeled con A binding proteins. However, when the iodination procedure was used, a distinct peak with the molecular weight 11 000–13 000 was detected on 15% polyacrylamide gels in sodium dodecyl sulfate. The explanation to this discrepancy is still unknown. If murine $\beta_{2\mu}$-globulin, like its human analogue, lacks carbohydrate (Berrgård and Bearn, 1968), this protein is most likely still associated with the heavy polypeptide chain of the H-2 gene products in the NP-40 solution and, consequently, indirectly bound to the con A resin.

Two protein components were specifically precipitated when analyzing the α-MM eluates from [^{3}H]leucine-labeled C3H/HeN spleen cells using a mouse alloantiserum directed against Ia (Figure 6e). The molecular weights found (31 000 and 24 000) correspond closely to those recently obtained by Cullen et al. from mouse spleen cells (Cullen, S. E., personal communication) and by Schwartz et al. (1975) from guinea pig lymphocytes. However, due to technical problems it was not possible to perform several sequential precipitations with the anti-Ia serum to see if the two peaks with the molecular weights 31 000 and 24 000 could be quantitatively removed from the α-MM eluate. Although a significant reduction of the two protein peaks occurred, the possibility still remains that some minor components, other than Ia antigens, were present.

It was found that the low molecular weight (24 000) Ia polypeptide chain was more easily labeled with ^{125}I and detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis than was the 31 000-dalton component. The reason for this is still unknown. One possible explanation is that the tyrosine and histidine residues are more buried in the polypeptide chain of the larger component and, as a consequence, less susceptible to iodination. Another explanation could be that the smaller polypeptide chain has a higher tyrosine and/or histidine content than the heavier chain. Using a ^{125}I-labeling technique, molecular weights of about 30 000 for the alloantigens coded by the cistrons in the Ir region of the mouse MHC have also been reported recently (Cullen et al., 1974; Vitetta et al., 1974). The two Ia polypeptide chains were also present on spleen cells from BALB/c mice and athymic mice.

The question of whether Ia antigens are present on T cells has been controversial for some time (for review see, e.g., Hämmerling et al., 1975). When analyzing the sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern obtained on the con A binding proteins from the purified T lymphocytes, protein peaks in the molecular weights corresponding to Ia antigens are visible. This would indicate that Ia antigens, identical or not identical with those present on B cells, indeed are expressed on T cells. However, although there is no significant contamination of our T cells by Ig bearing cells, we have to be aware of the possibility that these polypeptide chains may originate from undetected contaminants of the T cell population used, and that they may also contain other minor unidentified con A receptors.

Occasionally we have also observed a minor component with the molecular weight of ca. 20 000. The material is apparently associated with the B cell, but appears only spuriously. The identity of this material and the reason for its behavior are unknown. Preliminary studies in our laboratory have shown that in addition to the cell surface proteins mentioned above con A also binds to the glycolipid portion of the cell membrane. This could be one possible explanation to the fact that the total number of con A receptors on T cells is almost the same as on B cells (Stobo et al., 1972), despite the finding that T cells have fewer receptor proteins expressed on its surface.

A few articles have been published dealing with the isolation of con A binding proteins from lymphoid cells. Allan et al. (1972) purified con A binding cell surface proteins from hog lymph node cells. The protein fraction eluted from their affinity column contained most of the membrane glycoproteins. However, an enrichment of two protein components with the molecular weight of 33 000 and 27 000 was observed. Choi and Jenson (1974) isolated a con A binding protein from chicken spleen cells. The protein was shown to dissociate into two components with the molecular weights 30 000 and 20 000, respectively. During the preparation of this manuscript, Henkart and Fisher (1975) reported a thorough study of the characterization of con A binding components from human peripheral blood cells. They found three components with molecular weights of approximately 68 000, 58 000, and 43 000. In addition, radioactive peaks corresponding to molecular weights of 30 000 and 25 000 are visible in their sodium dodecyl sulfate–polyacrylamide gel electrophoresis profiles.

Given allowances for the different species, different methods, and the determination of molecular weights on impure mixtures, all of these observations are consistent with the molecular weights of the con A binding components isolated and identified in our studies (Table II). Furthermore, our preliminary experiments with human peripheral lymphocytes suggest the same type of con A binding glycoproteins are present on human cells as on murine cells. Evidence supporting our finding that H-2 antigens compose con A receptors on intact lymphocytes has been presented by Ray and Simmons (1973). The results obtained showed that con A and alloantibodies against H-2 antigens compete for the same receptor site on the cell surface.

The finding that B cells and T cells both have H-2D and H-2K as the major con A binding proteins and that B cells in addition possess Ia antigens, IgD, and IgM to which the mitogen can bind may indicate that lack of mitogen responsiveness can not be explained by the absence of the proper receptors. Indeed, stimulation of B cells may be achieved by attaching T cell mitogens (such as con A and PHA) to a solid matrix (Anderson et al., 1972; Greaves and Janossy, 1972) or by chemically cross-linking certain T cell mitogens (Waxdal et al., 1975; Basham and Waxdal, manuscript in preparation). It could be a distribution or spatial difference in the receptors

that is responsible for the specific behavior of B and T cells when binding the mitogen, or that the different cell types require different interactions. However, we do have to bear in mind that several assumptions must be made before concluding that the critical receptors on the lymphocyte surface are among the proteins identified in this study (e.g., that all mitogen receptors are solubilized with NP-40, and that the critical receptor(s) is (are) labeled by the different methods used and are in sufficient number to be detected by our assays).

It is our hope that this report will help to shed some light over the still unknown mechanism by which mitogens exert their effects on cells. Several other mitogens have also been found to interact with the receptors reported in this article (Waxdal et al., 1975; Nilsson and Waxdal, manuscript in preparation). Experiments are currently under way in our laboratory attempting to modulate the mitogenic responses with alloantisera directed against various antigens coded for by the histocompatibility-2 complex, in an attempt to determine whether there is only one critical receptor, or if mitogen binding to any or all receptors is a prerequisite for triggering the cell.

Acknowledgment

The authors wish to express their appreciation to Dr. B. Schwartz for many helpful discussions regarding the histocompatibility-2 alloantigens. We also wish to acknowledge the generous gifts of antisera from Drs. S. Cullen and D. Sachs.

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