MEMBRANE ANTIGENS ON AKR MICE LYMPHOCYTES

DISSERTATION

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This investigation is concerned with cell surface antigens present on murine AKR/J mice spleen and thymus cells which have been extracted with papain. Polyacrylamide gel isoelectric focusing analysis of surface proteins extracted from two to nine month-old AKR/J spleen and thymus lymphocytes indicate an alteration of cell surface proteins as a function of age, onset of leukemogenesis, and the sex of the mice. Surface proteins extracted from a congenic resistant murine strain, C3Heb/FeJ, and a non-Murine Leukemia Virus carrier strain, BALB/C, demonstrated age-related variations of several proteins common with the AKR/J spleen and thymus patterns.

Isolation of individual proteins was accomplished by granulated gel electrofocusing. Similar patterns recorded by both electrofocusing procedures identified several proteins limited to the AKR/J and C3Heb/FeJ spleen and thymus samples, which represent Murine Leukemia Virus-associated surface proteins. Three other proteins were shown common to all samples from all three strains and include differentiation-related antigens. One protein with a molecular weight of 31,700 Daltons was observed following the

granulated gel electrofocusing method of the surface proteins which were papain-extracted from leukemic AKR/J thymus cells. This protein may represent a leukemia-associated surface protein marker.

Passive hemagglutination and serological analysis verified the presence of three surface proteins common to all spleen and thymus samples and three surface proteins restricted to the Murine Leukemia Virus carrier strains, the latter reacting with anti-N type viral components p12, p30, and gp70.

Immunoelectron microscopic analysis substantiated the extracted proteins as thymocyte surface proteins. In addition, transmission electron microscopy studies have localized differentiation related antigens as well as Murine Leukemia Virus associated proteins on the surface of leukemia AKR/J thymocytes.

These studies indicate a preferential synthesis of Murine Leukemia Virus associated proteins rather than differentiation-related antigens in viral transformed AKR/J leukemic spleen and thymus cells. Differentiation-related antigens are synthesized in altered quantities compared to normal murine spleen and thymus cells as a result of viral transformation of AKR/J lymphocytes.

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INTRODUCTION

The AKR inbred strain of mice was characterized by Furth (1946) as demonstrating a spontaneous incidence of leukemia of greater than 96 per cent. Onset and development of the disease usually occurs between six and twelve months after birth (Furth, 1946). The predominant site of leukemia development is a localized focus in a single lobe of the thymus (Siegler and Rich, 1963; Metcalf, 1966; Furth and Furth, 1936). Metcalf (1966) has characterized resultant histological alterations in the thymus tissue as a thinning of the cortical region accompanied by enlargement of the medullary areas due to lymphoid follicle infiltration. In addition to spontaneous development of thymocyte type leukemias, AKR mice are characterized by chronic infection with Murine Leukemia Virus (MuLV) (Gross, 1951; Aoki et al., 1968; Hartley et al., 1969; Rowe and Pincus, 1972).

Gross (1951, 1957) first isolated MuLV and demonstrated vertical transmission of these leukemogenic agents from parents to offspring. The MuLV group of viruses are C-type RNA viruses of the Gross, Friend, Rauscher, Maloney and Graffi types (Fenner, 1974). Rowe and Pincus (1972) have characterized the MuLV infection in the AKR strain of mice using the UV-XC plaque assay method. Their findings showed

the initial appearance of detectable virus at the time of birth or within the first four days thereafter. These results indicated that the physiological conditions of immediate postnatal life initiates the spread of the infection which continues throughout the animal's existence. Distribution of the virus was shown by the same authors to be present in highest titers in bone, uterus, spleen, and lymphoid tissues in weanling mice. Bone marrow serves as the major source of infective virus during the early stages of infection. Schofield et al. substantiated these findings by electron microscopic observations of a large number of C type particles in osteocytes and osteoblasts obtained from bone samples taken from young AKR mice (1970). Recent studies performed by Watanabe and Nakakaki (1977) have demonstrated differences in the infectivity and leukemogenic activity of viruses isolated from the uterus compared to those obtained from thymus tissue. Their findings indicate that a common viral envelope antigen exists between thymus tissue and thymus-derived virus which permits a greater infectivity value and subsequent leukemogenic activity than uterus-derived virus.

AKR cells which are not producing virus have been shown to be susceptible to viral infection and can become active producers by either of two methods. The first requires activation of the endogenous viral genome which is

represented as two chromosomal loci in the host genome. The second method is explained by an actual infection of the susceptible cell with virus released from other infected cells (Rowe et al., 1971; Schofield et al., 1970; Rowe and Pincus, 1972). MuLV production reaches a plateau from three to four weeks after birth and does not increase again until onset of overt leukemia in the AKR mice (Rowe and Pincus, 1972). This plateau in the viral titer has been suggested to result from the production of a neutralizing antibody response as seen in the production of IgG and IgM antibodies to MuLV viral antigens by Oldstone and Dixon (1972). With the onset of leukemogenesis between the sixth and twelfth month, malignant undifferentiated T cells begin to replace the more highly differentiated thymocytes (Kawashima et al., 1976; Furth and Furth, 1936). These neoplastic cells are able to produce infectious virus, while the mature cells are less able to do so. Thus, an increase in the level of endogenous AKR virus accompanies thymic lymphoma development (Pincus and Rowe, 1972).

Recent studies by Pincus and Snyder (1975) provide information concerning the genetic aspects of the virus-cell interaction involved in the oncogenic transformation processes. Their proposed scheme for leukemogenesis is based on a minimum of four loci involved with the onset and development of the neoplastic condition in AKR cells. The AKV 1 and 2

loci have been identified in the control of initiation of viral replication in the bone marrow (Taylor et al., 1971; Rowe et al., 1971; Rowe et al., 1972a; Rowe et al., 1972b; Meier et al., 1973). This particular locus is a structural gene and is part of linkage group I. Subsequent to this stage, the virus replicates and infects susceptible osteoblastic cells. Spread of the virions to appropriate target cells in the host occurs via the circulatory system, thus exposing cells located anywhere in the body to possible infection. This spread is under the direction of a regulatory gene Friend Virus-1^b (FV-1^b), which is part of linkage group VIII (Pincus et al., 1971a; Lilly, 1970; Pincus et al., 1971b). Following infection of host cells, the viral genome is integrated into the host cell chromosome and neoplastic transformation begins as seen in the expression of specific virus-associated proteins. The transformed cell then begins an uncontrolled mitotic division state which results in the mass production of immature T lymphocytes which are characteristic of the disease. One other gene, the Resistance to Gross Virus-1 (Rgv-1) locus located within the H-2^d complex of linkage group IX has been identified with the regulation of the progressive spread of infectious virus. Lilly et al. (1964, 1966) have suggested that this specific locus controls the proliferation of both the virus and MuLV-infected cells by control of host immune responsiveness. The role of H-2

linked genes in controlling resistance by generation of a primary and a secondary cell-mediated response to AKR tumor cell antigenic determinants has been observed using F1 hybrid mice of AKR and C3H, C57BL/10 strains. These data were obtained by measuring cell-mediated immune responses and the responsiveness associated with the H-$2^{k/k}$ homozygous genotype (Meruelo et al., 1977). This scheme for the development of leukemogenesis in the AKR mouse strain as suggested by Pincus and Snyder (1975) may yet involve other loci not determined at the current time.

The leukemogenic transformation process has been characterized by the presence of several MuLV-associated proteins on the surface of MuLV-infected lymphocytes by immunofluorescence (Burk et al., 1977; Cloyd, 1977a, b), immunocytology (Old et al., 1965; Kawashima et al., 1976; Waksal et al., 1975; Aoki et al., 1966), and immunoelectron microscopy (Aoki and Takahashi, 1972; Aoki et al., 1970; Kennel et al., 1973; Kennel, 1975). These studies demonstrate a complicated set of surface proteins unique to the different leukemia viruses and cells (Aoki et al., 1972, Kennel, 1975). The MuLV-associated surface proteins present on the surface of AKR lymphocytes have been shown to represent specific viral proteins of the different virus types of the MuLV group (Geering, 1966; August et al., 1974). The GIX surface antigen represents a major envelope glycoprotein encoded for by the Gross

viral genome (Old et al., 1965; Stockert et al., 1971; Geering et al., 1966). This surface protein is related to MuLV infection and appears spontaneously on cells infected with MuLV. The GSCA surface protein is found on the surface of cells replicating MuLV and thus serves as an indicator of overt MuLV infection (Boyse et al., 1972; Old et al., 1965). Another MuLV-associated surface protein is p30. This represents a major core protein of the Friend virus (Yoshiki et al., 1974). Other MuLV surface proteins associated with the major MuLV envelope glycoproteins are p15 (Ikeda et al., 1975), gp45 (August et al., 1974), and gp70 (Yoshiki, 1974). The gp69/71 membrane-bound protein is encoded for by the Rauscher virus and was shown recently by Kennel (1973, 1975) to carry immunologic specificities responsible for leukemia typing, targets for antibody-mediated cytolysis, neutralization of virus, and other serological traits. Schafer et al. (1977) treated young AKR mice with goat anti-gp71 against the gp71 surface protein of Friend MuLV and noted a decrease in leukemogenesis. Three nucleoproteins of the MuLV core proteins also present on infected cells are denoted as p10, p12, and p17 (August et al., 1974).

In addition to these MuLV-associated surface proteins on murine T and B lymphocytes, several antigens differentiation-related have been described in the literature. Lymphoid cells originate from precursor cells in the bone marrow and

differentiate as a result of thymic hormones which result in specific phenotypic alterations of the surface proteins (Ford and Micklem, 1963; Gausser and Silvers, 1974; Bluestein, 1977; Goodman, 1977; Raff and Wortis, 1970; Weissman et al., 1975). Surface proteins normally associated with T-lymphocytes include the Theta, Thy 1.1 or 1.2, Ly 1-3, TL, I-a, and H-2 (Bluestein, 1977; Goodman, 1977; Boyse and Old, 1969; Boyse et al., 1968a,b). B lymphocytes demonstrate I-a, H-2, Ig, Ly 4, and MBLA surface proteins as the result of differentiation processes (Klaus, 1977; Bluestein, 1977; Goodman, 1977; Boyse and Old, 1969).

During differentiation stages, thymocytes undergo phenotypic changes from TL^-, Ly^-, Thy 1^- to TL^+, Ly^+, $Thy1^+$, which are characteristic of mature AKR murine lymphocytes (Kawashima et al., 1976; Waksal, 1975). Immunoadsorption and cytology studies performed on non-AKR strains of mice have shown distinct low levels of H-2 and high levels of Thy 1 alloantigens on the surface of lymphocytes (Kawashima et al., 1976; Waksal, 1975). This same ratio has been obtained for thymocytes from two-month old AKR mice. However, in six-month old leukemia AKR mice, the ratio reverses, i.e., high H-2/low Thy1, following assays of these surface phenotypes. These changes are not recorded on thymocytes from strains of mice which have a low incidence

of leukemia and which served as controls in these investigations (Kawashima et al., 1976; Waksal et al., 1975).

Thymocytes from the AKR strain demonstrate an altered pattern of surface antigens. Modifications in Thy 1 and H-2 alloantigens occur as a result of age, differentiation and leukemia development. In addition to these quantitative alterations, MuLV-associated proteins appear on the surface of AKR cells as a result of viral transformation (Old and Boyse, 1973; Kawashima et al., 1976). Immunofluorescence studies have localized the increase of MuLV-associated proteins in pre-leukemic AKR mice in the cortex region of the thymus. This is also the major site of leukemic development (Furth and Furth, 1936). From these studies, Kawashima and his colleagues (1976) suggest that the cell population which is undergoing age-dependent changes in surface phenotype also appears to be the cell group at prime risk for leukemic transformation.

The significance of these altered surface proteins on AKR lymphocytes increases with the observation of the importance of the plasma membrane in cell proliferation and interaction of the tumor cell with the host's immune system (Kennel, 1975). The loss of regulation of cell replication accompanies the neoplastic/malignant disease condition (Todaro et al., 1964). Of even greater importance, the surface of the cell represents that site of cellular interaction with the host's defense mechanisms. From this

particular perspective, the transformation of MuLV-infected lymphocytes as seen in alterations in normal age-dependent surface proteins and the addition of viral-associated proteins, dramatically affects the tumor cell and its interactions with the host. The control of aberrant cells and resulting malignancies is attributed to the immunological surveillance system (Burnet, 1957, 1970). The recognition of abnormal changes in the host cells is accomplished by I-a surface antigens on specific T lymphocytes known as "Killer" T cells (Cerottini and Brunner, 1977). This interaction of I-a surface receptors of such cells with viral-associated surface proteins, tumor specific antigens or "altered self antigens" on the surface of neoplastic cells forms the basis of Edelman's revised theory of immune surveillance (1976). As described earlier, MuLV transformation of AKR cells results in distinct viral-associated surface proteins. A possible explanation for the production of "altered self-antigens" has been offered by Edelman (1976). He suggests that the I-a surface receptors interact with the virus allowing initial infection. Following viral uncoating, the viral genome is incorporated into the host cell chromosome and affects the K and D loci of the H-2 region. This process results in the biosynthesis of an "altered self antigen" which appears on the cell surface. It is recognized by the immune system as foreign, and subsequently, an immune response is elicited.

Several possible mechanisms have been offered for explanation of how the viral genome affects the Major Histocompatability Complex (MHC). These include a derepression of silent genes in the MHC loci which results in the synthesis of "new" surface proteins (Ennis, 1977), a modification of H-2 transcription and translation such that incomplete or irregular H-2 molecules are produced (Ennis, 1977), or the synthesized viral-associated proteins present on the cell surface bind to the H-2 antigens in such a way that a modified protein or hybrid is formed (Cunningham, 1977).

The manner in which "Killer" cells of the immune surveillance system recognize abnormal surface proteins is the subject of recent controversy. The two most accepted theories are the dual recognition interaction (physiological interaction method) and the "altered self theory" described previously. Ennis (1977) suggests that the "Killer" cell has individual surface receptors--one which represents the H-2 surface protein on other cells, and a different receptor which interacts with the MuLV-associated surface protein. Zinkernagel and Daugherty (1974, 1976) suggest that cytolysis requires recognition of both the H-2 and the viral-associated surface proteins on the malignant cell. They also propose that the recognition of an abnormal H-2 antigen alone is enough to induce cytolytic action. Cunningham (1977) has developed an additional theory for

"Killer" cell interaction with malignant cells. He formulates a mechanism whereby the H-2 alloantigen binds to a viral-associated, membrane-bound protein which results in a hybrid or modified molecule. "Killer" cell recognition of the hybrid would then require a similar receptor to the hybrid molecule. Failure of the immune system to destroy the malignant cell allows it to mestastasize elsewhere in the host and form a new loci of neoplastic growth (Mitchison, 1977).

The significance of the I-a and H-2 alloantigens represent the importance of surface proteins encoded for by the MHC loci. The H-2 antigens are present on almost all of the tissues in the body and were first described by Gorer as a blood group antigen involved in transplantation rejection (1936). These glycoproteins have molecular weights of between 31,000 and 34,000 Daltons (Shreffler and David, 1975) and consist of 60-64% protein, 8.5% amino sugars, and 7% carbohydrate (Nathenson and Davies, 1966). Based on hemagglutination and cytotoxic testing, eighteen different H-2 antigens have been identified. These alloantigens are similar in action to the F_c fraction of immunoglobulins, and loss of antigenicity has been observed at low pH, low salt concentrations, and at elevated temperatures (Dausset, 1972; Gausser and Silvers, 1974). The H-2 antigens are embedded in the lipid bilayer of the cell membrane with

part of the molecule extending into the cytoplasm and part extending outward from the cell surface as based on the fluid mosaic model of the cell membrane (Nicolson, 1976; Cunningham, 1977). Distribution of these surface antigens over the cell membrane has been demonstrated by immunofluorescence to be in a random fashion (Boyse et al., 1968a,b). However, Boyse, Old, and Stockert (1968a,1969) have proposed a "gene-determined" sequencing of surface antigens using double antibody adsorption for a mapping of frequency and order of H-2 antigens on B and T cells. The sequence obtained is TL. 3-TL.2-TL.1-H-2(d)-Ly-B-Theta-Ly-A-H-2(k) on the surface of thymocytes. These authors further suggest a theta antigen bridge or backbone which is continuous in the membrane with the other antigens (H-2, Ly-A, Ly-B, and TL) attached to this network. This sequence describes not only the order of the antigens on the membrane surface, but also delineates the order in which the MHC genes are transcribed and translated (Boyse et al., 1968a). The MHC is composed of six loci located on chromosome 17 in mice. These loci have been mapped in the order t / K / I / S / D / Tl. The K and D loci are responsible for the production of the eighteen classical H-2 antigens. The I gene controls the immune response which has been described in detail previously in this review. The S loci is responsible for the levels of hemolytic complement and B2 serum globulin protein

levels in the blood stream. The T1 loci has recently been linked with the occurrence of specific antigen formation during the onset of leukemia and other neoplastic diseases (Gasser and Silvers, 1974; McKenzie and Snell, 1973; Shreffler and David, 1975). The H-2 alloantigens function as viral receptors, control mechanisms of humoral immune responses, indirectly regulate antibody production, affect transplant acceptance or rejection, and serve as a possible key to disease susceptibility (Daussett, 1972; Eisen, 1974; Gasser and Silvers, 1974).

Katz, Armerding, and Benacerraf (1975, 1974) have suggested that the surface antigens coded for by the I gene, in particular the Ir-1 loci, are responsible for the regulation of the humoral immune response. These findings suggest that the H-2 alloantigens on the T lymphocytes and B lymphocytes serve as receptors which are able to interact, thus stimulating antibody production with the latter type of cells following stimulation with the appropriate "Helper" molecule. Alterations within a cell due to viral transformation and subsequent appearance of viral-related proteins on the membrane surface of these cells (Oroszlan et al., 1970; Strand and August, 1973; Kennel et al., 1973; Stephenson et al., 1974), or age-related breakdown (Orgel, 1963; Dreyfus, 1977; Rubinson, 1976) of biosynthetic pathways could be recognized by the immune surveillance cells (Strand and August, 1973; Gilden et al., 1971). It has

been suggested by several research groups (Boyse et al., 1968a,b); Boyse et al., 1972, Dausset, 1972) that variations in types and frequency of the H-2 surface proteins or the appearance of tumor specific antigens (TSA) would be recognized and serve as an early warning system. This process would elicit macrophages, polymorphonuclear phagocytes, and T and B cells for the destruction of the altered cells (Feldman et al., 1975).

Failure of AKR mice to destroy abnormal cells has been studied. Doell et al. (1967) and Dent (1965) have demonstrated a depressed cellular immunity by measurement of skin graft survival in pre-leukemic AKR mice. In addition, macrophage migration inhibition, a correlate of cellular immunity, has been reported to be impaired throughout the life span of AKR mice (Wettstein and Hays, 1970). Changes in macrophage activity as studied by Jennings and Oates may be due to an increase in the number of immature macrophages or due to non-productive phagocytosis by peritoneal macrophages (1967).

In studies performed by Martig and Tribble (1974) on spleen cells from three to ten month old AKR mice, it was shown that many animals were capable of responding to alloantigens as assayed by Mixed Leukocyte Reactions, contact sensitivity to picryl chloride, and Graft vs. Host Reactions. In related findings, Metcalf and Moulds (1967) report intact

humoral immunity in pre-leukemic AKR mice with normal formation of hemolytic plaques by spleen cells. In addition, normal hemagglutination titers were observed after stimulation of these animals with sRBC (sheep red blood cells) unless obvious lymphoma was present. Quantitative gamma globulin production and humoral antibody testing performed in AKR mice of different age groups gave normal values (Wettstein and Hays, 1970). From these observations, it was concluded that AKR mice are free from lymphoma cells before four months of age, and only the viral infection is present. Martig and Tribble (1974) have found that a depression of cell-mediated immunity, i.e., immunological surveillance, is not a prerequisite for the pre-leukemic state in AKR mice. The fact that cellular immunity is impaired but intact humoral immunity has been demonstrated suggests that viral infection of thymus cells causes an alteration in thymic function and ultimate development of lymphomas. Based on this assumption, the virus takes on a dual role--one of immunosuppression and also one of malignant transformation (Martig and Tribble, 1974). The virus-transformed cell finding itself in an impaired cellular immunity environment is then free to develop into lymphoma (Wettstein and Hays, 1970). Although this theory provides a consistent view with data from other workers, other studies by Cremer and her colleagues (1967, 1966) and Peterson

et al. (1963) report a depression of antibody formation before the appearance of overt lymphoma and possible inhibition in the mechanism of humoral immunity.

A possible molecular explanation suggested by the author for the loss of cellular and/or humoral immunity described above could be attributed to the loss of specific proteins on the surface of B and T cells which are required for interaction. The leukemic condition is characterized as a flooding of the immune system with immature, i.e., undifferentiated lymphocytes and thymocytes (Furth and Furth, 1936). The absence of certain H-2 and I-a encoded surface alloantigens would result in a lack of cooperation between B and T cells required for production of an immune response (Katz and Benacerraf, 1974). Similarly, following viral transformation, the MuLV-associated surface proteins are produced and begin to appear on the lymphocyte membrane. The incorporation of these viral-associated surface antigens in turn may result in the loss or shedding of differentiation-related antigens from the cell surface to make room for these viral encoded proteins. Immunofluorescence studies have substantiated this idea (Barnett, 1974; Boyse et al., 1969; Eisen, 1974). One other possible explanation for the loss of cellular and humoral immunity is that the viral genome may affect the production of surface proteins normally expressed. These proteins may be modified to such an extent that the protein structure is altered and no longer bears

resemblance to normally synthesized differentiation surface proteins, or a hybrid molecule of viral-associated protein may bind to the age-related surface protein, as suggested by Cunningham (1977). The final result of each of these possible mechanisms is the failure of the host to produce an immune response capable of the destruction and control of the neoplastic disease which eventually culminates in the death of the animal.

The techniques of quantitative absorption, membrane immunofluorescence, cytoxicity, and immunoelectron microscopy have been used in the past to assay the interaction of antibodies with the leukemic cell surface. These studies describe a complex of antigenic sites specific to different leukemic cells, but fail to describe the actual molecular nature of the antigens involved. In this study, surface proteins have been extracted from lymphocytes from AKR mice ages two to nine months and characterized to provide quantitative and qualitative data as to the correlation of age-related variations with the onset and development of leukemia. Age-related changes refer to time related alteration in the appearance of lymphocyte surface proteins. In addition, the alterations of these surface proteins with regard to the sex of the animal and development of the disease are reported. This study does not, therefore, represent an investigation of aging.

MATERIALS AND METHODS

Mice

Inbred strains of AKR/J, BALB/C, and C3Heb/FeJ from different age groups of both sexes were obtained from Jackson Laboratories, Bar Harbor, Maine.

Differentiation of AKR Mice

Separation of the AKR mice was based on age, spleen and thymus weights, and WBC counts (Table I).

Preparation of Spleen and Thymus Extracts

Spleen and thymus tissue was removed and prepared according to Mann et al (1975). The extraction procedure involved centrifugation of pooled supernatants of 0.14 M - 0.01 M saline washes of minced tissue in a Beckman Ultracentrifuge (L3-40 multiple) at 80,000 xG for 90 min at 4°C. The crude membrane pellets were suspended in distilled water and heated in 2 - 10 ml aliquots for 5 minutes at 37°C prior to addition of papain (1.47 mg papain/70 mg membrane. Digestion was then carried out at 37°C for 30 minutes according to Turner et al. (1975). Iodoacetic acid solution (0.05 M) adjusted with 0.1 N NaOH to a pH 7.6 was added to the reaction. Centrifugation for 60 minutes at 80,000 xG was accomplished. The supernatant of extracted membrane

TABLE I

CLASSIFICATION OF MICE BY STRAIN, AGE, SPLEEN AND THYMUS WEIGHTS, AND LEUKOCYTE COUNTS

AKR/J STRAIN

Early or Pre-Leukemic

2 - 3 Months Old, Spleen Weight 60-80mg, Thymus Weight 70-140mg, 8,200-17,100/mm WBC Counts

5 - 6 Months Old, Normal Spleen and Thymus Weights, 5,000-8,000/mm WBC Counts

6 - 7 Months Old, Spleen Weight 100-400mg, Thymus Weight 80-140mg, 10,000-43,500/mm WBC Counts

7 - 8 Months Old, Spleen Weight 575-800mg, Thymus Weight 290-725mg, 40,000-100,000/mm WBC Counts

8 - 9 Months Old, Spleen Weight 600-810mg, Thymus Weight 290-400mg, 11,000-15,000/mm WBC Counts

C3Heb/FeJ STRAIN (CONGENIC RESISTANT LINE)

2 - 3 Months Old, Spleen Weight 100-140mg, Thymus Weight 40-110mg, 8,000-11,000/mm WBC Counts

7 - 8 Months Old, Spleen Weight 120-220mg, Thymus Weight 40-70mg, 12,000-14,900/mm WBC Counts

proteins was purified using a series of dialysis steps. The first involved dialysis against 5% sucrose in 0.14 M saline buffered to pH 8.6 with 0.01 M Tris HCl at 4°C for 120 hours. Additional dialysis against 5% sucrose and 0.38 M glycine (Sigma Chemical Co., St. Louis, Mo.) in distilled water for 48 - 72 hours followed. Samples were concentrated against carbowax (Fisher Scientific, Pittsburg, Pennsylvania) for 1 hour at 4°C to a final volume of 5 ml for each sample with 6 - 9 mg of protein/ml.

Preparation of Thin Layer Polyacrylamide Gels

A modified Vesterberg (1973) method was used for preparation of the gels as described by Sayed and Hatten (1976). Each gel was cast on glass plates (125 mm x 180 mm x 1 mm) using a volume of 25 ml per plate. A syringe and needle were used to add: 4.8 ml of 30.5% acrylamide, 4.8 ml of 1% N-N' methylene Bis acrylamide, 13 ml of 10 M Urea, 2 ml of 1% mercaptoethanol, and 1.8 ml of ampholine solutions. LKB Bromo Ampholytes (Chicago, Ill.) were mixed into a stock solution with a pH range of between 3.5 - 10 as follows: 2.4 ml of ampholine pH 3.5 to 10, 2 ml of pH 3.5 - 5, 2 ml of pH 5 - 7, 2 ml of pH 5 - 8, .2 ml of pH 6 - 8, and 4 ml of pH 9 - 11. The constituents were mixed and deaerated in vacuo for 5 min. The following was then added: 2.0 ml of .004% riboflavin, 1.0 ml of 1% ammonium persulfate, and 3.0 ml of 10% Triton X-100. The gels were poured and

photopolymerized using a Polylite 2114 (LKB Instruments, Inc., Rockville, Md.) for 1 hr. The gels were stored overnight at 4°C which resulted in easier removal of the rubber gasket form and top glass plate. The gels were placed in thin plastic bags (Baggies) and stored in the dark at 4°C until used. LKB Ampholine PAG plates with a pH of 3.5 - 9.5 were purchased pre-made from LKB Instruments, Inc. (Rockville, Md.).

Sample Application and Isoelectric Focusing

Samples were soaked in Whatman #3 filter paper (5 mm x 10 mm) and applied (in double application form) at the cathode region and in the middle of each gel on the surface of the commercially prepared or on modified Vesterberg gels. In some instances, one-ml fractions of each sample were pre-treated with 0.5 ml of 10 M Urea and 0.05 ml of Triton X-100 prior to application of the latter type of gel.

Paper electrofocusing strips (LKB Instruments, Inc., Rockville, Md.) were soaked in a 1 M phosphoric acid solution for the anode and a 1 M sodium hydroxide solution for the cathode.

An LKB Multiphor 2117 and LKB 3371E power supply (LKB Instruments, Inc., Rockville, Md.) were used for electrofocusing of all samples. A freon coolant system maintained the temperature at 0 - 2°C for each run. The total time of each run was 2 hours with initial voltages of 200 and

47 mAmps. The voltage was slowly increased until a final 1160 volts and 18 mAmps were reached at the end of the run.

Electrolyte strips were removed and replaced after the first 55 min of the run with removal of the sample applicators 5 min later.

Fixing, Staining and Destaining

Focused gels were immediately removed from the apparatus and fixed in trichloroacetic-sulfosalicylic acid solution for 30 min with agitation. Fixed gels were then placed in a destaining solution containing alcohol-water-glacial acetic acid (3:8:1) for 10 min and agitated. The gels were stained in a solution of 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, Ca.) in 9 parts water: 9 parts alcohol: 1 part glacial acetic acid at 60^{o}C for 10 min. Finally, destaining in the previously mentioned alcohol-water-acetic acid solution continued until the background cleared. Frequent changes in the destaining solution increased the rate of destaining.

Destained gels were placed in a 10% glycerol solution for 1 hour and allowed to dry overnight at room temperature on plastic sheets (LKB Instruments, Inc., Rockville, Md.) for preservation.

Densitometer Analysis

A Helena Laboratories Corp. (Beaumont, Tx.) auto scanner Flur-Vis and Quick Quant II were used to scan each gel with a A570 filter.

Antisera

One-half milliliter of each of the protein samples obtained from spleen and thymus samples from the several strains of mice was injected with an equal volume of Freund's Complete Adjuvant (Difco Co.) and injected subcutaneously at a single site into individual laboratory stock rabbits. The spleen and thymus samples used include: male 5 - 6 months old AKR/J samples, male 7 - 8 month-old AKR/J samples, male 6 - 7 month old BALB/C samples, female 7 - 8 month old C3Heb/FeJ thymus sample, and male 2 - 3 month old C3Heb/FeJ spleen sample. At the end of 5 weeks, 25 ml of blood was drawn from each rabbit, and the antisera obtained was stored at -20°C. A booster dose of 0.5 ml of the respective protein was injected intravenously and the animals were bled by cardiac puncture 7 - 10 days later. The antisera obtained was stored at -20°C.

Antisera to specific Murine Leukemia Virus surface proteins were generously supplied to the author by Dr. Stephen J. Kennel of the Viral Carcinogenesis Unit of Oak Ridge National Laboratory. The antisera provided include: Goat

Anti-Rabbit IgG; Rabbit Anti-AKR p12, p30, gp70; Goat Anti-Moloney Virus p30, gp70; Goat Anti-Scripps Leukemia Virus to all Moloney Viral surface proteins; and Goat Anti-Scripps Leukemia Virus gp70 (Identical to Moloney gp70). Antisera to specific differentiation related surface proteins were obtained from the National Institute of Health. These include: Goat Anti-Thy1.1; Goat Anti-Thy1.2; Goat Anti-D11, D23, D25, D52; Goat Anti-D1, D3; Goat Anti-D11; and Goat Anti-D32.

Ouchterlony Immunodiffusion

Ouchterlony plates were prepared according to Burrell (1974). Membrane protein samples were diluted with sterile water such that a particular antisera was allowed to react with undiluted, 1:5, and 1:10 dilutions of each protein sample. Plates were incubated at 37°C for 36 - 48 hours and stored at 4°C. Observation of precipitin reactions followed at days 7 and 20.

Immunoelectrophoresis

Immunoelectrophoresis slide gels were prepared with a Barbital-sodium barbital-sodium acetate buffer mixture 0.06 M, pH 8.6 (Kallestad Labs, Inc., Minneapolis, Minn.). Undiluted membrane protein samples were placed in the wells and electrophoresis of the gels occurred in an electrophoresis chamber at 4°C, 150 - 200 V, for 25 min.

Specific antisera was then added to the trough region of each gel and the slide gels were then stored in a humidity chamber at 25°C. A Buchler Instruments DC Power Supply (Ill.) was used. A determination of R_f values was obtained using a Fowler caliber (Poland).

Titration of Antisera

The rabbit antisera obtained from injection of AKR/J, BALB/C, and C3Heb/FeJ spleen and thymus protein samples were titered by Microtiter^R Hemagglutination techniques. Sheep Red Blood Cells (Texas Biological Association, Fort Worth, Tx.) were prepared according to Hudson and Hays (1976). The sRBC were washed three times with Phosphate Buffered Saline (PBS). To the packed washed sRBC, 10 ml of tannic acid was added, and incubation followed for 15 min at 37°C. At the end of this time period, cell suspensions were centrifuged, and the resultant pellet washed with 20 ml of PBS. Following further centrifugation, one portion of the sRBC were suspended in 20 ml PBS containing 1% heat inactivated rabbit serum. This portion represented the uncoated controls.

A second set of sRBC was subdivided into different fractions and each fraction suspended in 10 ml of PBS containing 1% of a different protein sample (concentration 6 - 9 mg/ml). The preparations were incubated for 30 minutes at 37°C with gentle shaking. The suspensions were

then subjected to centrifugation for 10 min and the supernatant removed. The sRBC were recoated with the respective protein sample and incubated as described previously.

U-bottom plastic Microtiter^{R} plates (CECO, Alexandria, Va.) were prepared by filling all wells, except the first row with 0.025 ml of PBS. To the first row, 0.050 ml of a specific antisera for each protein sample was added. A 0.025 ml microdiluter (CECO, Alexandria, Va.) was used to make serial dilutions of each antisera according to the Microtiter^{R} manual (1969). An equivalent volume of protein sample coated sRBC (0.025 ml) was subsequently added to each row. This was performed for homologous testing, i.e., AKR/J thymus protein coated sRBC were allowed to react with anti-AKR/J thymus protein antisera, etc. The plates were incubated at 37^{o}C for 45 minutes and gently shaken. Following incubation, the plates were placed at 25^{o}C and read 24 hours later. The plates were placed at 4^{o}C for 18 hours and observed subsequent to this incubation period.

Separation of Individual Proteins

Granulated Gel Electrofocusing

A modified Winter et al. (1975) procedure was used for the flat bed granulated gel electrofocusing of three thymus and three spleen samples of extracted proteins. The protein samples subjected to this procedure include samples of:

female 7 - 8 month old AKR/J thymus. The sample was stored at 4°C until being applied to the granulated gel flat bed as described later in this procedure. Each granulated gel slurry was prepared in a plastic tray (LKB Instruments, Inc., Rockville, Md.) with a total volume of 100 ml.

Ultrodex^{R} (LKB Instruments, Inc., Rockville, Md.) was prepared according to Winter et al. (1975) by swelling 4 grams in 100 ml of deionized double distilled water and allowed to stand for 10 minutes. The slurry was poured on a glass filter with a porosity of 20 - 40 μm, and the resultant bed which formed was washed with a total of 500 ml of deionized double distilled water slowly under suction. The gel was dehydrated with multiple 10 - 15 ml washes of absolute ethanol (Sigma Chemical Co., St. Louis, Mo.). Following this step, the remaining white powder was dried overnight in a dessicator under vacuum.

An ampholine solution was prepared by mixing LKB Bromma Ampholytes (LKB Instruments, Inc., Rockville, Md.) of a pH range of 7 - 11 as follows: 5 ml of pH 7 - 9 and 5 ml of pH 9 - 11. Six paper electrofocusing strips (LKB Instruments, Inc., Rockville, Md.) were soaked in the ampholine solution, and three strips were placed along the widths or short sides of the plastic tray. The weight of

the tray and strips was obtained on a Harvard Triple Balance Ohaus , Union, N.J.). The dried white Ultrodex^{R} powder was resuspended by slowly adding it to a solution containing 5 ml of ampholine solution and 95 ml of double deionized distilled water. A metal spatula was used to stir the slurry. The beaker containing the slurry was weighed prior to the pouring of the slurry onto the center of the tray. A depth of 2.0 - 2.5 mm of slurry in the tray was obtained with the slurry coming into contact with the soaked strips along the ends. The empty beaker which contains any residual slurry mixture was weighed, and an initial volume of the tray and slurry was calculated. The plastic tray was placed in a Prime Aire Laminar Flow Hood (Van Nuys, Calif.) for maximum evaporation of the slurry gel. The evaporation limit for this specific batch of #4051 of Ultrodex^{R} gel was determined to be 34.5% (LKB Instruments, Inc., Rockville, Md.) and required 4 - 5 hours of evaporation for each slurry gel to reach crack point. A final weight of the tray and slurry was obtained and the tray was wrapped in a thin plastic sheet (Saran Wrap) and stored overnight at 4^{o}C.

A 1.0% solution of Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was poured over the surface of an LKB Multiphore 2117 (LKB Instruments, Inc., Rockville, Md.) prior to the lowering of the plastic tray onto the glass surface. Paper electrofocusing strips were soaked in a 1 M

Glycine solution (Sigma Chemical Co., St. Louis, Mo.) for the anode and a 1 M Sodium hydroxide solution for the cathode electrolyte as suggested by Edy (personal communication).

A metal sample applicator (LKB Instruments, Inc., Rockville, Md.) was used to make a trough in the slurry gel at a point approximately half way the length of the tray. The volume of slurry within this region was removed with a metal spatula and mixed with the remaining ampholine solution. A volume of three milliliters of protein sample was added to the slurry-ampholine mixture. The suspension was then poured back into the trough, and the sample metal applicator was removed. The slurry gel was allowed to equilibrate for 5 minutes prior to the start of the electrofocusing process.

A freon coolant system was used to maintain the temperature at 0 - 2^{o}C during each run. An LKB 3371E power supply (LKB Instruments, Inc., Rockville, Md.) was used for the electrofocusing of all samples. The total length of time for each run was 8 hours with initial voltage of 500 and 32 mAmps. The voltage was increased, so that a constant 16 Watts of power was maintained. A final 1140 volts and 15 mAmps was reached at the end of the run.

A thirty division metal fractionating grid (LKB Instruments, Inc., Rockville, Md.) was placed on the slurry

gel at the end of each run which divided the tray volume into equal proportions. During this process, the freon coolant system was used to maintain the temperature at 0 - 2°C. A metal spatula was used to scoop out the slurry within a single division, and the gel placed in a 6 cc disposable plastic syringe (Monoject Sherwood, Inc., Deland, Florida) which contained a small amount of glass wool. One ml of deionized double distilled water was added to resuspend the slurry gel until it formed a 2 ml packed column of Ultrodex^{R}. An equal volume of deionized double distilled water was carefully layered on top of the column, and the protein and ampholine on the column was allowed to be eluted off in a dropwise manner into a sterile scintillation vial which was in an ice bath. This process was repeated for each of the 30 subdivisions using individual syringe columns.

An Optical Density (O.D.) profile was obtained for each sample subjected to the granulated gel electrofocusing procedure by measuring A_{280} values on a Beckman 25K Spectrophotometer (Fullerton, California) for each division fraction. These values are plotted as fraction number vs. Optical Density values at A_{280}.

Ampholine Removal

Ampholines present in each fraction represented as the peak of each curve of the female 7 - 8 month old AKR/J thymus sample and the female 7 - 8 month old C3Heb/FeJ thymus sample were removed by gel filtration. The fractions involved are number 4, 8, 13, 16, 18, 21, and 24, which correspond with Bands 1 - 7 for the AKR/J thymus sample and numbers 17, 20, 22, and 26 for Bands 1a-1d for the C3Heb/FeJ thymus sample.

These fractions were applied to BioGel P-2 pre-packed columns (Bio Rad, Richmond, California) for the removal of the ampholines as described by Abraham and Bakerman (1975). A maximum of three fractions was passed over a single pre-packed BioGel P-2 column. Calibration of these columns was performed using 0.5 ml and 2.5 ml of Dextran Blue for calculation of the void volume and 0.5 ml of potassium dichromate for the internal volume (Figure39). These calibrations suggested that a maximum volume of 2.5 ml of each fraction as the actual amount applied to a column. Samples were eluted off the BioGel P-2 columns with deionized double distilled water at $4^{o}C$. One ml fractions were collected with an LKB 7000 UltroRac Fraction Collector (LKB Instruments, Inc., Rockville, Md.). Optical density values were obtained by measuring the O.D. at A_{280} for each elution fraction. These values were plotted as elution fraction vs. optical density at A_{280}.

Concentration of Individual Protein Samples

The elution proteins representing the major portion of each peak on the graphs were pooled and placed in dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, California). Each sample was concentrated against carbowax at 4°C until a final volume of 1.5 ml (6 - 10 µg/ml) was obtained. The elution fractions pooled for each AKR/J and C3Heb/FeJ thymus sample band is represented as follows:

AKR/J thymus Band 1-pooled elution fractions number 3-8
Band 2-pooled elution fractions number 3-8
Band 3-pooled elution fractions number 3-8
Band 4-pooled elution fractions number 4-9
Band 5-pooled elution fractions number 3-8
Band 6-pooled elution fractions number 4-9
Band 7-pooled elution fractions number 5-10

C3Heb/FeJ thymus Band 1a-pooled elution fractions number 3-7
Band 1b-pooled elution fractions number 3&4
Band 1c-pooled elution fractions number 3-8
Band 1d-pooled elution fractions number 4-9

Passive Hemagglutination

Quantitative values were obtained for the relative amount each protein represented as a constituent of the AKR/J, BALB/C, C3Heb/FeJ spleen and thymus samples by the technique of passive hemagglutination. The individual

proteins assayed for include those proteins separated by granulated gel electrofocusing as Band 1 - 7 from the AKR/J thymus sample and Bands 1a - 1d from the C3Heb/FeJ thymus sample.

Sheep Red Blood cells (Texas Biological Associates, Fort Worth, Texas) were washed and tanned as described previously. One portion was suspended in 20 ml PBS containing 1% heat-inactivated rabbit serum and served as the uncoated controls. A second set of sRBC was subdivided into eight different fractions, and each fraction suspended in 10 ml PBS containing 1% of a different antisera, i.e., anti-BALB/C spleen and thymus samples, anti-pre-leukemic AKR/J spleen and thymus samples, anti-leukemic AKR/J spleen and thymus samples, and anti-C3Heb/FeJ spleen and thymus samples, which had been previously diluted with PBS to four Hemagglutination Units below the respective end point of each antisera.

Following incubation at 37^{o}C for 30 min, the suspensions were centrifugated for 10 min and the supernatant was removed. The sRBC were recoated with the respective PBS diluted antisera and reincubated. U-bottom plastic Microtiter^{R} plates (CECO, Alexandria, Virginia) were prepared as described previously with 50 µl of a specific protein (Bands 1 - 7 from the AKR/J thymus sample or Bands 1a - 1d from the C3Heb/FeJ thymus sample) added to the first row.

Serial dilutions were made of each protein with a 25 µl microdiluter (CECO, Alexandria, Virginia) and 25 µl of antisera coated sRBC was subsequently added to each row. This was performed with each antisera such that Protein 1 was allowed to react with Anti-BALB/C spleen sample, anti-BALB/C thymus sample, anti-pre-leukemic AKR/J spleen sample, etc.

Plates were incubated at 37°C for 45 min, gently shaken, and placed at 25°C overnight prior to reading. Subsequent incubation at 4°C for 18 hours was conducted prior to second observation. Calculation of the amount of protein present in each sample was determined by multiplying the end point titer times the protein concentration of the antisera (Kabat, 1976).

Immunoelectron Microscopy

An immunoelectron microscopy study was conducted according to a modified Ostrand-Rosenberg procedure (1975, 1976a, 1976b). Thymus tissue was removed from five leukemic 8 - 9 month old male AKR/J mice, five pre-leukemic 3 - 4 month old male AKR/J mice, and five 8 - 9 month old male BALB/C mice. The thymus tissue ranged in weight of 40 - 710 mg for the leukemic AKR/J mice, 50 - 80 mg for the pre-leukemic AKR/J, and 20 - 40 mg for the BALB/C mice.

Thymic tissue was teased appart in 5 ml PBS and the cell suspension passed over a glass wool syringe column.

Centrifugation of the suspension on a Sero-Fuge II Centrifuge (Clay Adams, New Jersey) for 10 min at 4°C preceded washing the pellet twice more with PBS, E rosette formation (Hudson and Hays, 1976) was used to determine the approximate number of T cells present (5×10^6 cells/ml).

Antisera absorption of these T cells was conducted according to Ostrand-Rosenberg (1975, 1976a, 1976b). Heat inactivated antisera were diluted with physiological saline to two dilution values below the end point titer of each antisera; specifically, anti-BALB/C thymus 1:64, anti-preleukemic AKR/J thymus 1:2, anti-leukemic AKR/J thymus 1:4, and anti-C3Heb/FeJ thymus 1:31. One milliliter of specific antisera was added to an equal volume of T cell solution and incubated for 30 min at 4°C. Subsequent to incubation, the suspension was centrifugated at 8000 x G for 5 min, and the pellet resuspended in PBS. Absorption was repeated twice more with the same antisera. The final pellet of antisera absorbed T cells was resuspended in physiological saline and stored overnight at 4°C.

The Goat Anti-Rabbit Immunoglobulin G (GARI)-Hemocyanin complex was prepared according to Ostrand-Rosenberg (1975, 1976a, 1976b). 1.2 ml of GARI (Cappell Laboratories, Cochranville, Pa.) containing 10 mg/ml protein was added to an equal volume solution containing 70 mg Keyhole Limpet Hemocyanin (Calbiochem, Fullerton, California) in PBS.

The suspension was vortexed vigorously and .22 ml of .5% gluteraldehyde (Fisher Co.) in PBS was slowly added. The suspension was incubated at 25°C for 45 min with no agitation. .2 ml of a 2 M glycine (Sigma Chemical Co.) solution in PBS was added to the suspension and incubation followed for 15 min, 25°C. The suspension was dialyzed overnight against PBS at 4°C. The GARI-Hemocyanin complex solution was removed from dialysis and centrifugated at 20,000 g for 30 min, 4°C. The resultant supernatant was stored at 4°C until needed.

Equal volumes (.5 ml) of antisera coated T cells and GARI-Hemocyanin solutions were combined and incubated for 30 min, 4°C. This was repeated using each type of antisera coated T cells. Following centrifugation, the pellet was washed twice with PBS. One milliliter of 2% glutaraldehyde in Cacodylate buffer (Biochemical Laboratories, Inc., Redondo Beach, California) was added to fix the cells during incubation at 4°C, 45 min. The suspensions were subjected to centrifugation, the pellet was resuspended with PBS and 1 ml of 2% Osmium tetraoxide in Cacodylate buffer (Biochemical Laboratories, Inc., Redondo Beach, California) was added. Incubation for 30 min at 4°C preceded centrifugation. The pellet was resuspended in cold sterile deionized double distilled water.

A drop of antisera coated T cells bound GARI-Hemocyanin suspension was placed on a copper 200 mesh grid according to Ostrand-Rosenberg (personal communication). Following air drying, a second drop was placed on the grid. Dehydration of the sample occurred at concentrations of 25, 30, 50, 75, 90, 100 per cent ethyl alcohol for 5 min. The sample was placed in 100 per cent ethyl alcohol, 100 per cent ethyl alcohol-amyl acetate, and amyl acetate for 5 min. Following dehydration, the samples were critically point dried in a Bomar Critical Point CO_2 Drier Model SPC-900/EX (Tacoma, Washington) and stored overnight in a dessicator.

Carbon replicas were prepared according to Ostrand-Rosenberg (1975, 1976a, 1976b) with platinum-carbon pellets (Ladd Research Industries) at an eight degree angle on a Mikros Vacuum Evaporator VE-10 (Mikros Inc., Portland Oregon). The carbon replicas were carbon shadowed at a ninety degree angle using carbon rods (Pelco EM Supplies, Tustin, California). Digestion of the organic material was conducted with bleach (Chlorox) according to Ostrand-Rosenberg (personal communication) and rinsed in distilled water several times.

An RCA-EMU-3G electron microscope with a resolution of 25 - 30 Å was used for observation of all samples. Three grids of each sample were observed.

Molecular Weight Determination of Individual Proteins

Sodium dodecyl sulfate (SDS) disc gels were prepared in 5.6 and 10 per cent polyacrylamide concentrations according to Fairbanks et al. (1971). The mobilities of the standard proteins were plotted against the log of their subunit molecular weight.

RESULTS

PAGIF Age-Related Changes

Preliminary studies of electrofocusing on the commercially prepared gel showed only a faint single band in the cathode region of the gels (pH 3.5 - 9.5). The use of modified Vesterberg prepared gels with a higher pH range (3.5 - 10), containing 10% Triton X-100 and 10 M Urea, however, provided distinct band separation for each protein sample as seen in Figure 1. As many as six bands are observed in the pH 8 - 10 region of the gel.

Densitometer analysis of the spleen cell protein extracts indicated that band 2 in the pre-leukemic 6 - 7 month-old AKR sample is of greater density than the other two samples. Bands 3 and 4 in the early leukemic AKR spleen also appear to be denser than those in the leukemic 7 - 8 month-old AKR spleen sample (Figure 2).

The thymus protein samples showed the following results: a faint band before band 2 in the pre-leukemic AKR and 6 - 7 month-old BALB/C thymus sample; a marked increase in the density of band 3 in the leukemic AKR thymus sample; and separation of band 5 into separate regions in the latter sample (Figure 3).

Figure 1. Papain extracted membrane proteins separated by isoelectric focusing. A.5 Ml samples containing 6 mg/ml proteins were applied in double application form as described in Methods. Band formation occurs in the cathode pH 8 - 10 region of the pH 3 - 10 and 5% polyacrylamide slab gel. Total run time 2 hours, at 0-4°C, final voltage 1100.

Abbreviations for protein samples: CT - 6-7 month old female BALB/C thymus; CT - 6-7 month old male BALB/C thymus; LT - 7-8 month old leukemic female AKR/J thymus; LT - 7-8 month old leukemic male AKR/J thymus; CS - 6-7 month old female BALB/C spleen; CS - 6-7 month old male BALB/C spleen; LS - 7-8 month old leukemic female AKR/J spleen.

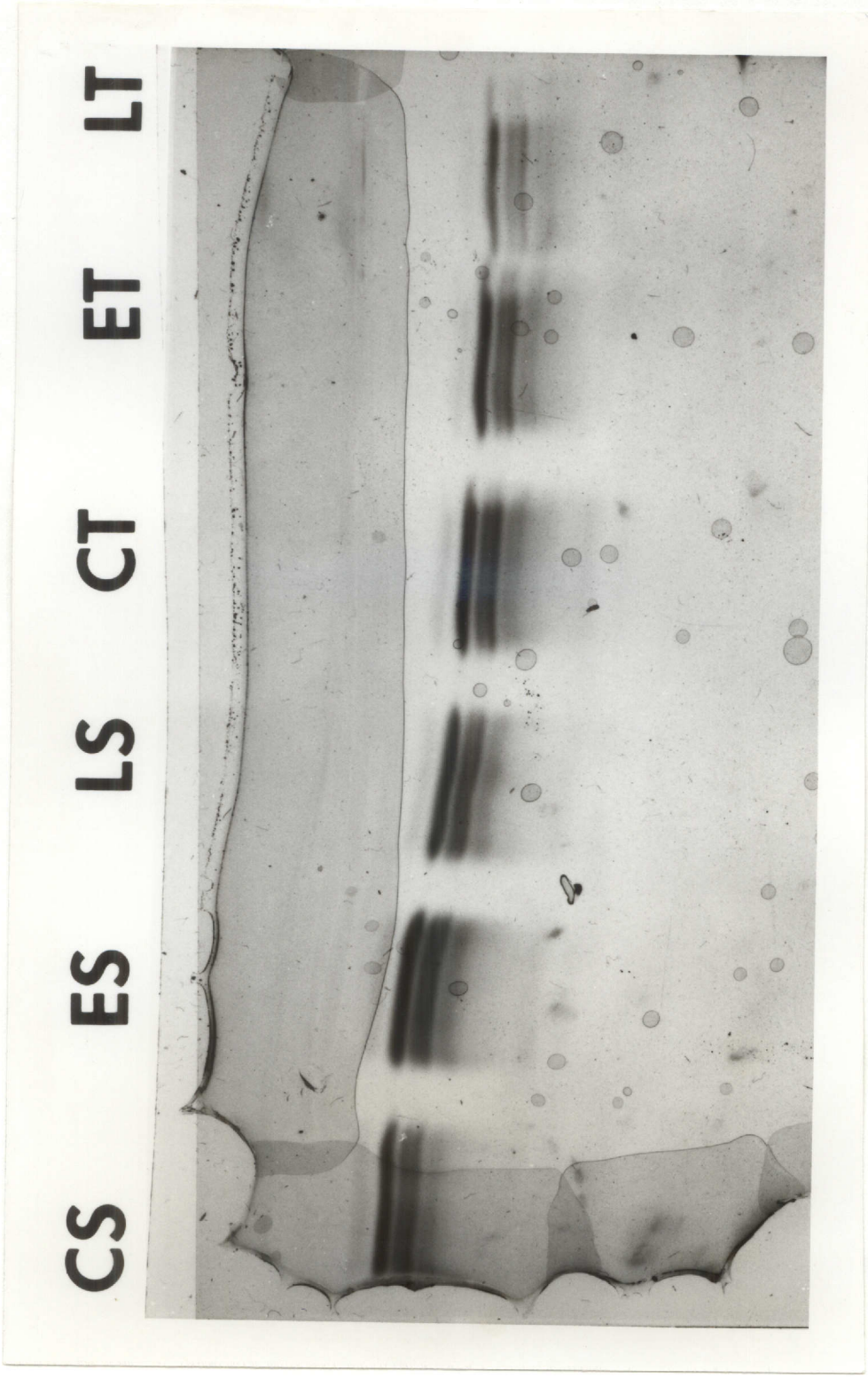

CS
ES
LS
CT
ET
LT

Figure 2. Densitometer scans with a A570 filter of papain extracted surface proteins from spleen cells obtained from AKR/J, BALB/C, and C3Heb/FeJ mice following polyacrylamide gel isoelectric focusing. Slab gels were stained in Coomassie Brilliant Blue, destained in 3:8:1 ethyl alcohol/distilled water/glacial acetic acid and preserved in 10% glycerol prior to air drying.

Abbreviations for protein samples: CS - 6-7 month old BALB/C spleen; ES - 6-7 month old AKR/J pre-leukemic spleen; LS - 7-8 month old leukemic AKR/J spleen.

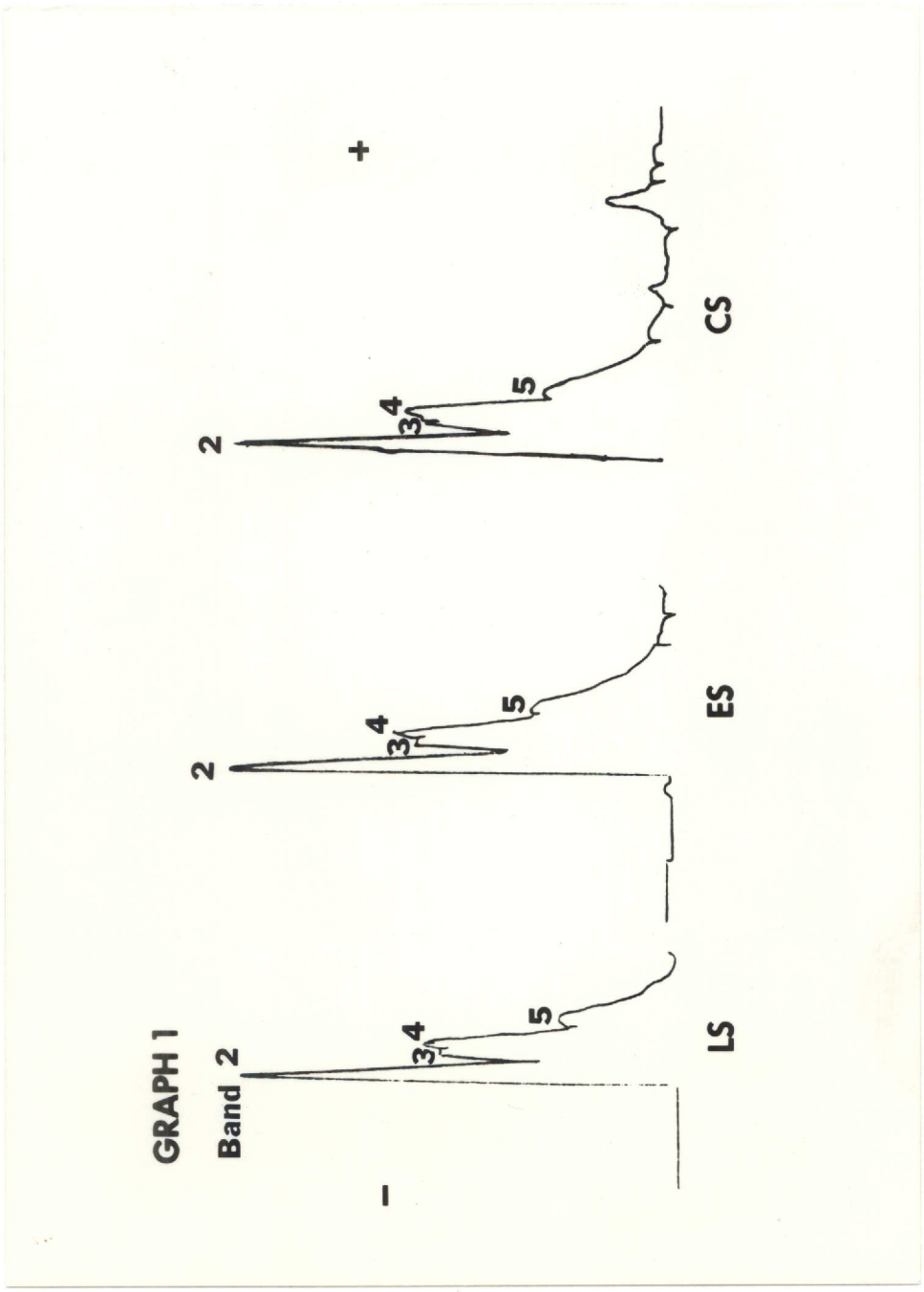

GRAPH 1
Band 2
−
+
2
3
4
5
LS
ES
CS

Figure 3. Densitometer scans of papain extracted surface proteins from thymus cells obtained from AKR/J, BALB/C, and C3Heb/FeJ mice following polyacrylamide gel isoelectric focusing. Slab gels were stained in Coomassie Brillian Blue, destained in 3:8:1 ethyl alcohol/distilled water/glacial acetic acid and preserved in 10% glycerol prior to air drying.

Abbreviations for protein samples: CT - 6-7 month old BALB/C thymus; ET - 6-7 month old AKR/J pre-leukemic thymus; LT - 7-8 month old leukemic AKR/J thymus.

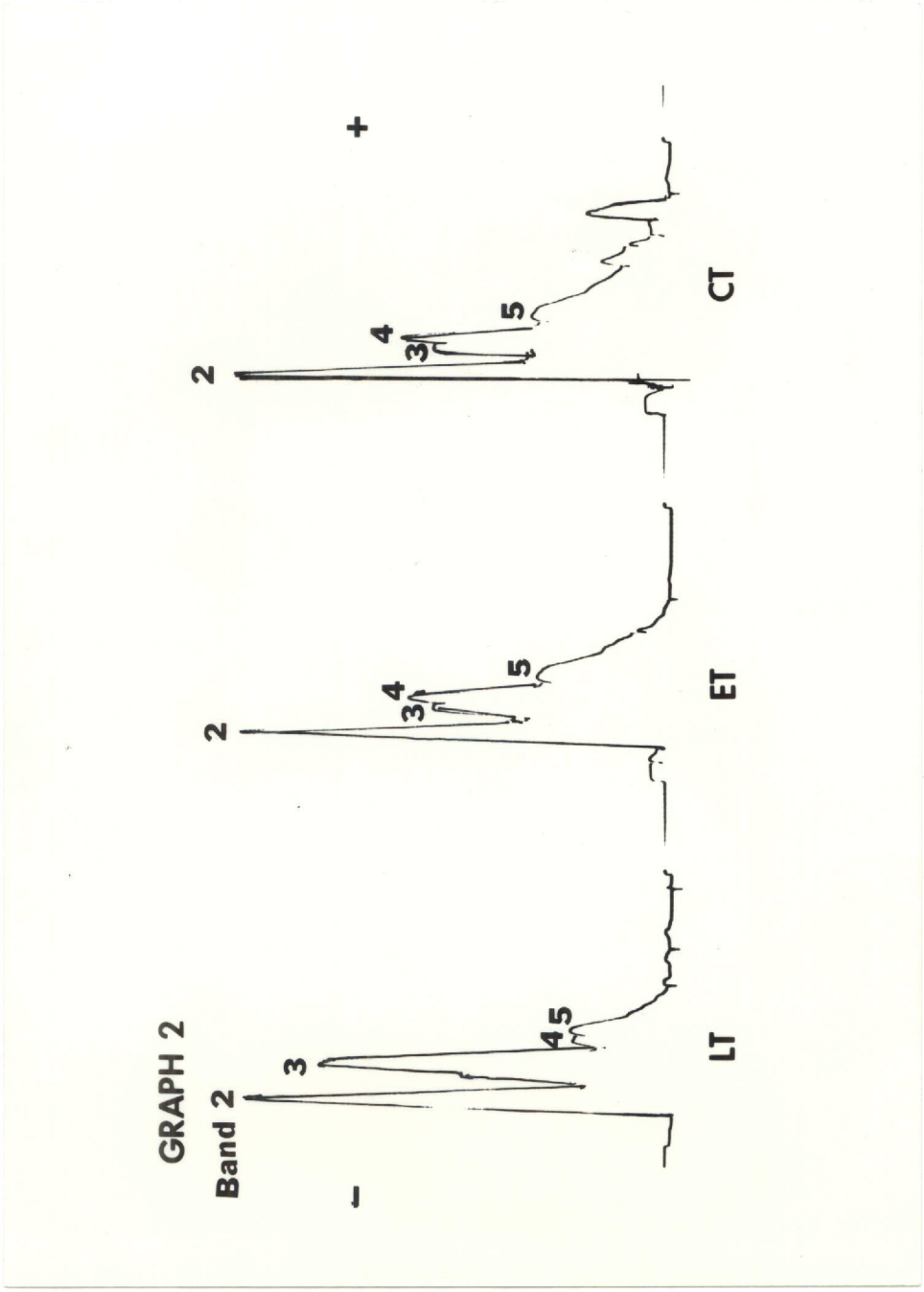

GRAPH 2
Band 2
-
+
2
3
4
5
LT
ET
CT

Figure 4 presents a photographic reproduction of pre-treated Triton X-100 and 10 M Urea samples run on modified Vesterberg gels (1976). Separation of sample proteins into 3 to 6 distinct bands are observed in these gels. As indicated in the densitometer analysis, bands 3 and 4 in the pre-leukemic and leukemic AKR spleen samples were of greater density than the BALB/C spleen sample. A fifth and sixth band was recorded for these two spleen samples. A study of the thymus samples showed a band of greater density prior to band 2 in the leukemic AKR thymus sample. Bands 3 and 4 in the leukemic AKR thymus were also shown to be of greater density in comparison to the other samples. Band 5 is recorded in the leukemic AKR and BALB/C thymus preparations as two distinct bands of equal density, while only a single band is demonstrated in the pre-leukemic AKR thymus sample.

Representative densitometer scans comparing separation of proteins in spleen and thymus samples are seen in Figures 5 and 6. It can be seen that greater protein band separation resulted from pre-treatment of the samples.

These preliminary studies demonstrated the increased resolution resulting from the pre-treatment procedure; thus, all subsequent studies involving slab gel polyacrylamide gel isoelectric focusing included samples which had been pre-treated prior to application.

Figure 4. Papain extracted membrane proteins separated by isoelectric focusing which had been pre-treated with Triton X-100 and 10 M urea prior to application on the pH 3 - 10 range 5% polyacrylamide slab gel surface. A .5 Ml of sample solutions containing 6 mg/ml protein were applied in double application form as described in Methods. Increased band separation of the protein bands are seen for each sample. Band formation in cathode pH 8 - 10 region. Total run time 2 hours, at 0 - 4°C, final voltage 1100.

Abbreviations for protein samples: CT - 6-7 month old female BALB/C thymus; CT - 6-7 month old male BALB/C thymus; LT - 7-8 month old leukemic female AKR/J thymus; LT - 7-8 month old leukemic male AKR/J thymus; CS - 6-7 month old female BALB/C spleen; CS - 6-7 month old male BALB/C spleen; LS - 7-8 month old leukemic female AKR/J spleen.


CS
ES
LS
CT
ET
LT

Figure 5. Densitometer scans with a A570 filter of papain extracted surface proteins from spleen cells obtained from AKR/J, BALB/C, and C3Heb/FeJ mice following polyacrylamide gel isoelectric focusing. Samples were pre-treated with Triton X-100 and urea prior to application on the slab gel surface. Slab gels were stained, de-stained, and presented as described in the Methods section.

Abbreviations for protein samples: CS - 6-7 month old BALB/C spleen; ES - 6-7 month old AKR/J pre-leukemic spleen; LS - 7-8 month old leukemic AKR/J spleen.

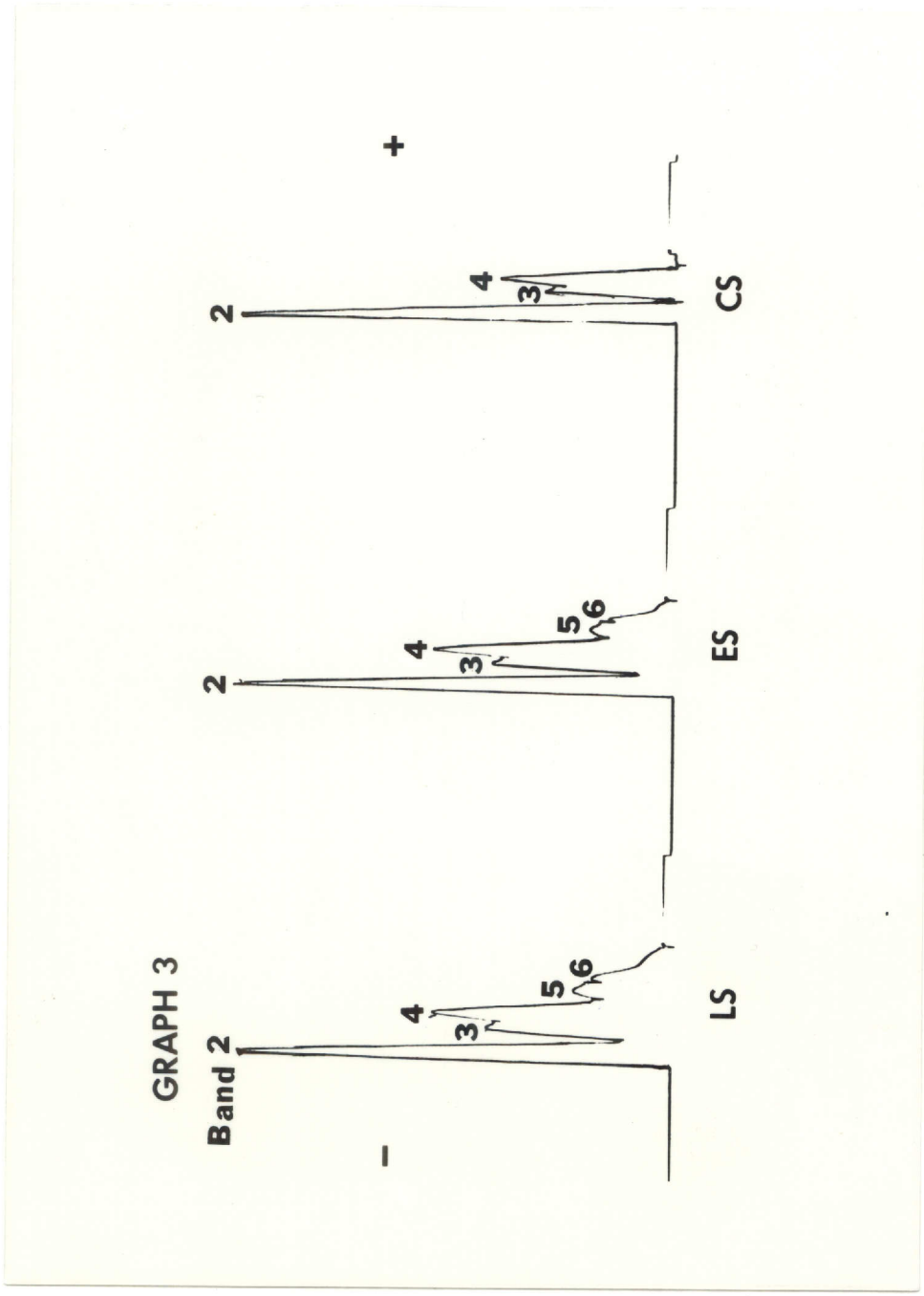
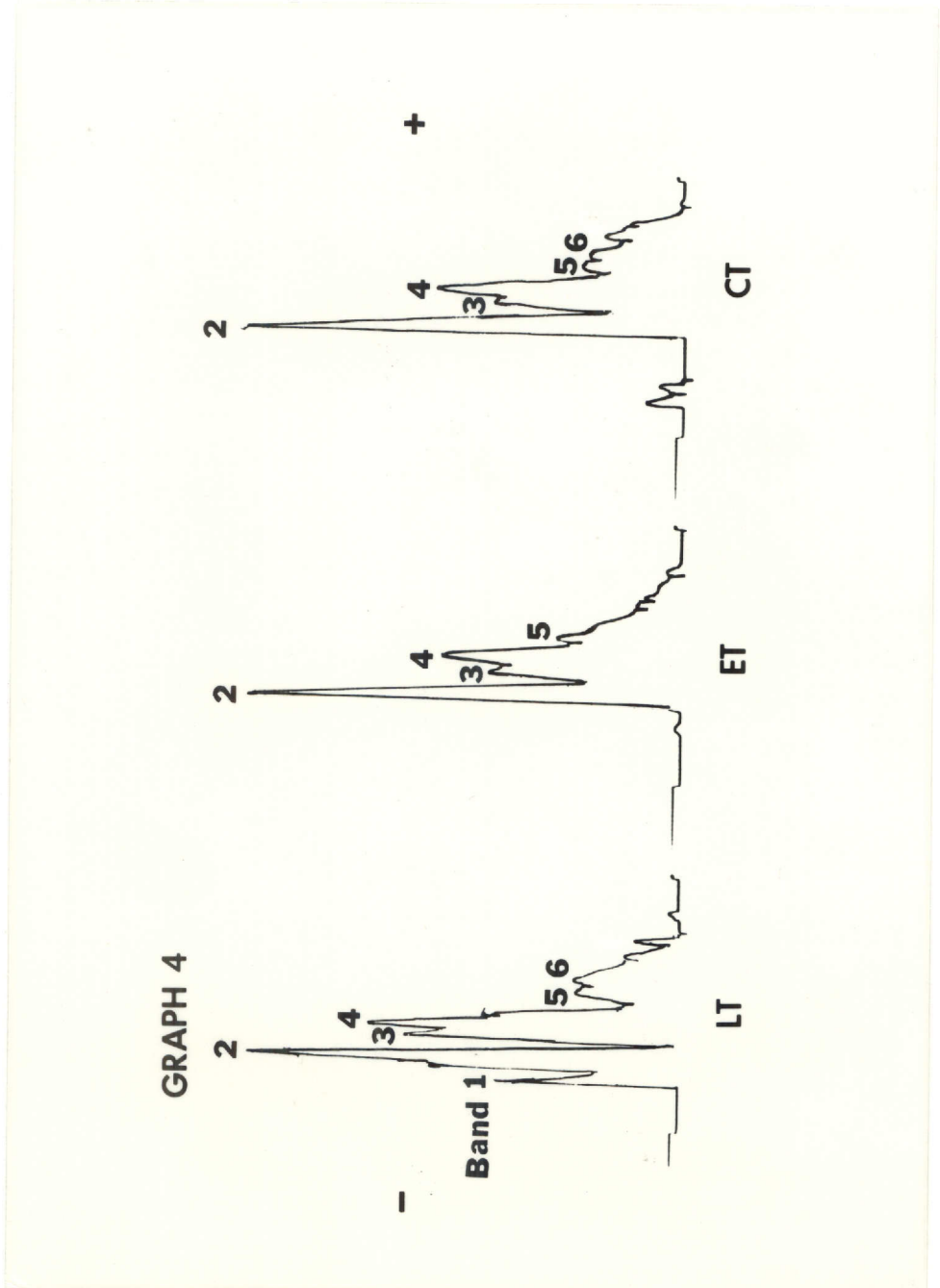

GRAPH 3
Band 2
−
+
LS
ES
CS
2
3
4
5
6

Figure 6. Densitometer scans with a A570 filter of papain extracted surface proteins from thymus cells obtained from AKR/J, BALB/C, and C3Heb/FeJ mice following polyacrylamide gel isoelectric focusing. Samples were pre-treated with Triton X-100 and urea prior to application on the slab gel surface. Slab gels were stained, destained, and preserved as described in Methods section.

Abbreviations for protein samples: CT - 6-7 month old BALB/C thymus; ET - 6-7 month old AKR/J pre-leukemic thymus; LT - 7-8 month old leukemic AKR/J thymus.


GRAPH 4
Band 1
–
+
2
3
4
5
6
LT
ET
CT

Subsequently, individual bands were observed for each spleen and thymus sample in the pH 7 - 10 region of the gels. As many as 6 to 8 bands were observed for some of the samples. The papain control sample upon observation following PAGIF and staining did not produce any visible bands.

Determination of the relative density of each protein present following PAGIF is by calculation of the area that each protein represents as a per cent of the total area of all of the protein bands in each sample. The per cent values calculated for the AKR/J, BALB/C, C3Heb/FeJ spleen and thymus samples are presented in Tables II, III, IV, V, VI, and VII.

The AKR/J spleen samples demonstrate a decrease in relative density as a function of age-related changes for Bands 3 and 5 and an increase in the relative density for Bands 2 and 6 (Figure 9). A similar age-related decrease in relative density is observed for Band 2 for the AKR/J thymus samples, while an increase is noted in Bands 1, 4 and 6 (Figure 10). An increase in Band 3 and a decrease in Bands 2, 5, and 6 are observed in the BALB/C female spleen samples (Figure 11). Similar age-related increases in relative density are observed in Bands 2, 4, and 5 in the male BALB/C spleen protein samples (Figure 12). The BALB/C thymus samples are characterized by a decrease in relative density for Band 4 (Figure 13).

Figure 7. Densitometer scan with a A570 filter of spleen samples from male and female 8 - 9 month old BALB/C mice following polyacrylamide gel isoelectric focusing. The male sample demonstrates a fourth protein band which is lacking in the densitometer scan of the female sample. Slab gels were stained, destained, and preserved as described in the Methods section.

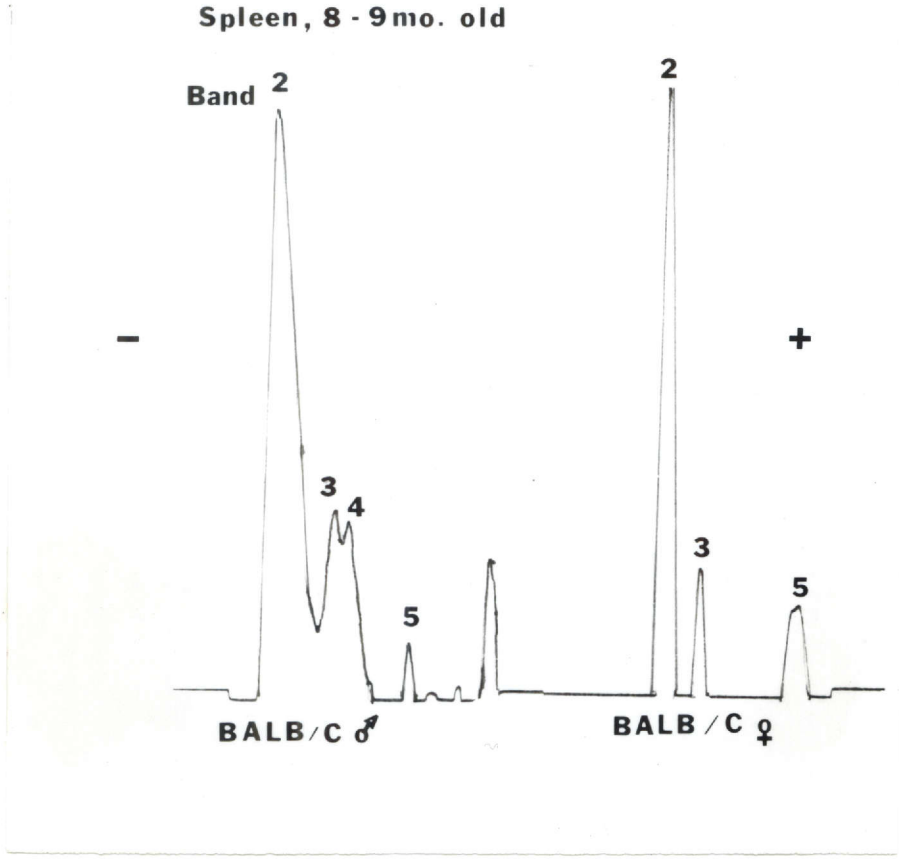

Spleen, 8 - 9 mo. old
Band
2
3
4
5
2
3
5
−
+
BALB/C ♂
BALB/C ♀

Figure 8. Densitometer scans with a A570 filter of papain extracted surface proteins from thymus cells obtained from AKR/J and BALB/C mice following polyacrylamide gel isoelectric focusing. The leukemic female AKR/JAKR/J thymus does not show a distinct band r which is present in the leukemic AKR/J male thymus. Slab gels were stained, destained, and preserved as described in the Methods section.

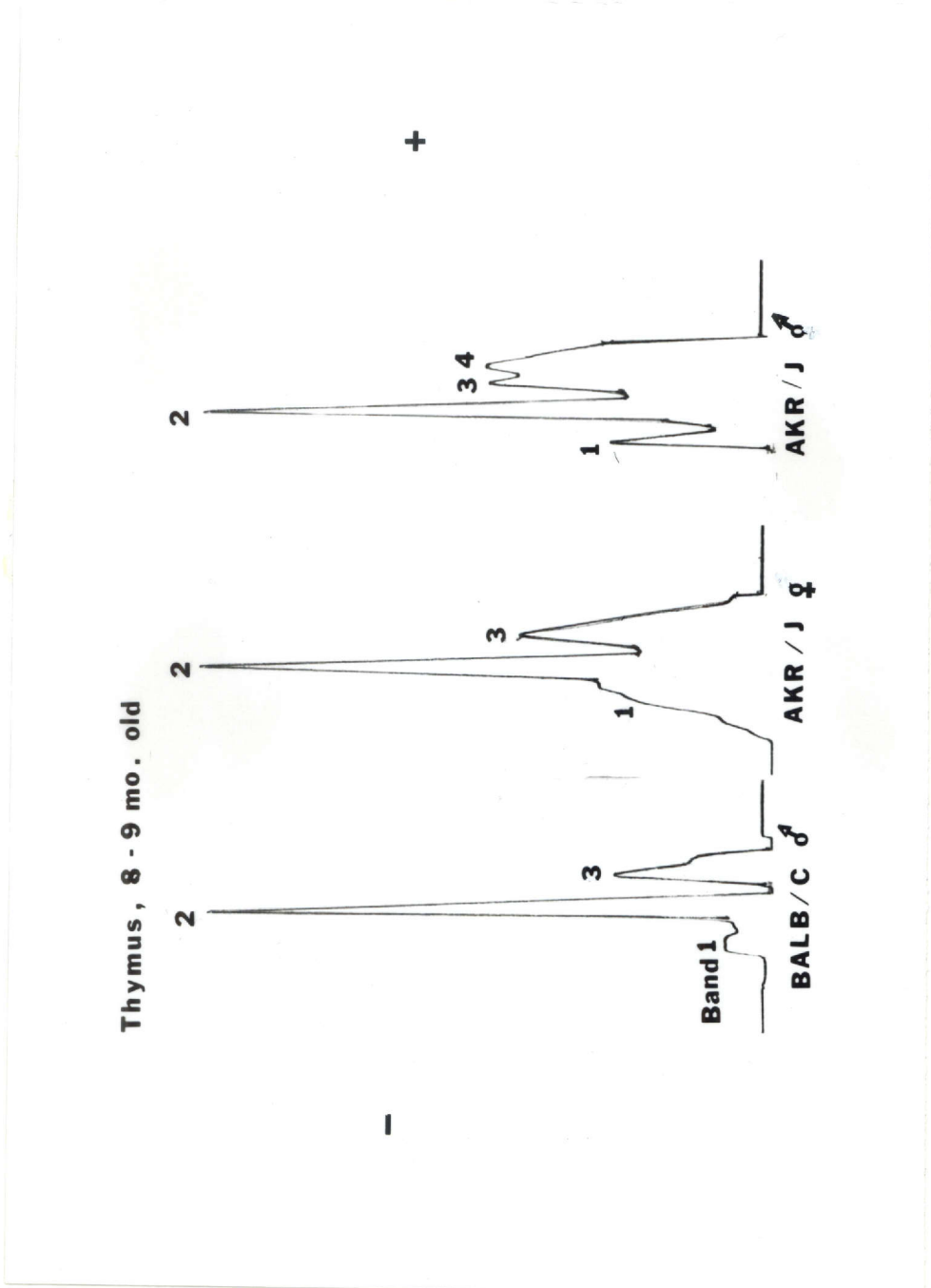
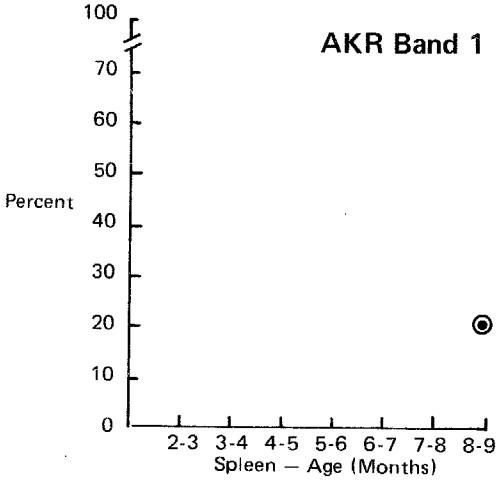
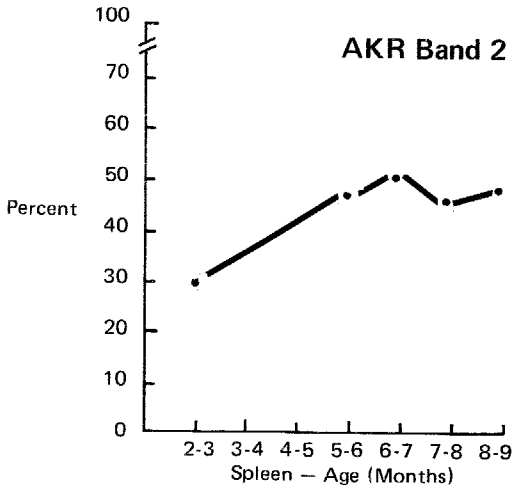
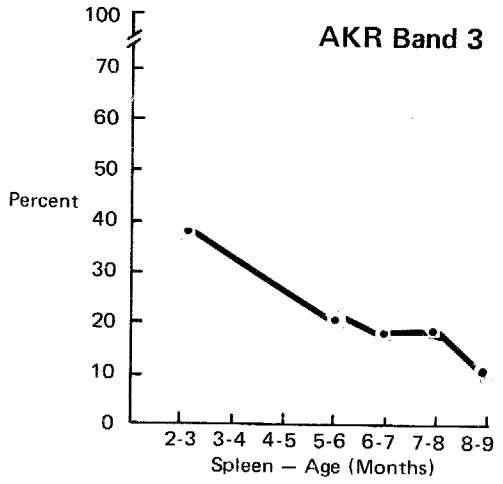
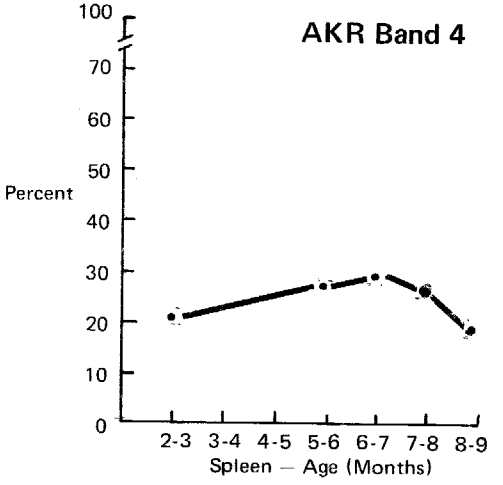

Thymus, 8-9 mo. old
−
+
Band 1
2
3
2
1
3
2
1
3 4
BALB/C ♂
AKR/J ♀
AKR/J ♂

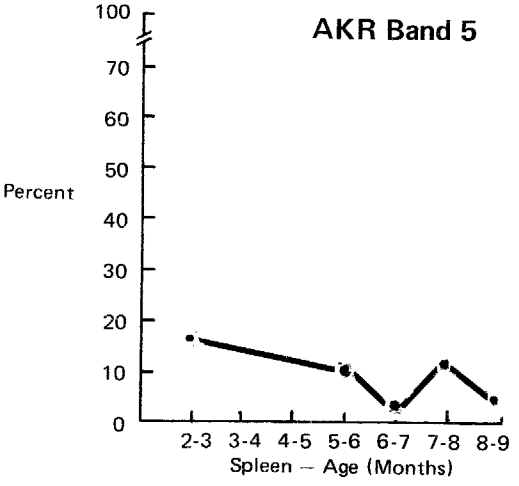
Figure 9. Age-related changes in Triton X-100 and Urea pre-treated AKR/J spleen samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis of polyacrylamide slab gels. Per cent values are plotted against age (in months) of AKR/J mice when tissues were obtained. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.


AKR Band 1
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen – Age (Months)


AKR Band 2
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen – Age (Months)


AKR Band 3
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen – Age (Months)


AKR Band 4
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen – Age (Months)


AKR Band 5
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen – Age (Months)

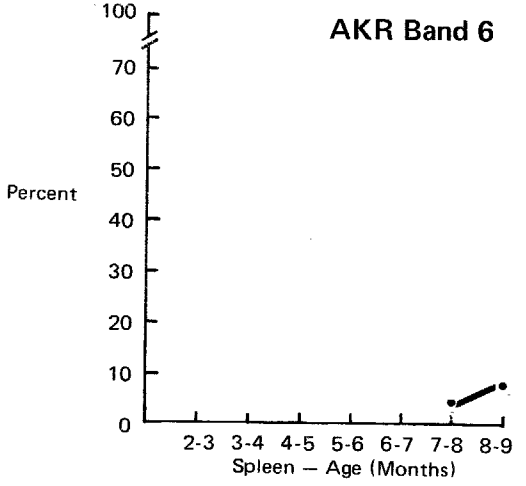
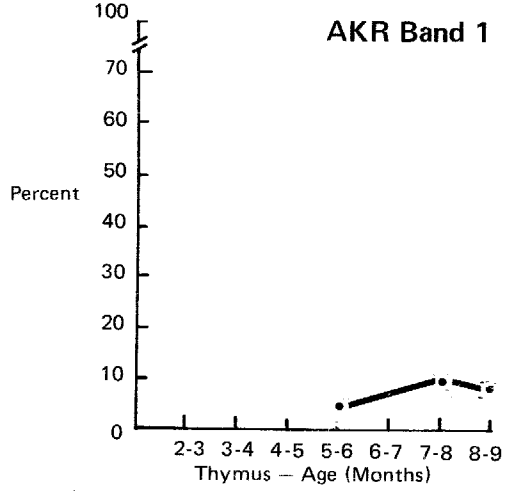
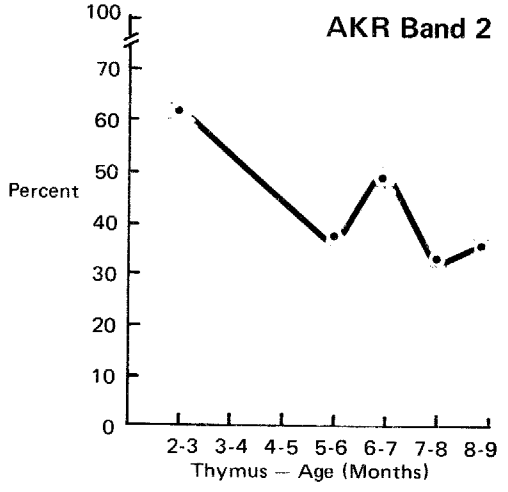
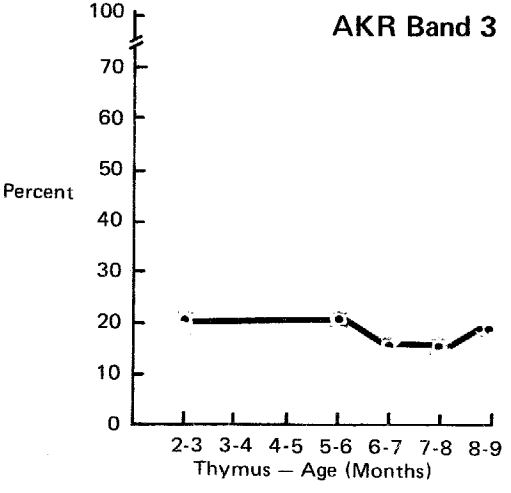
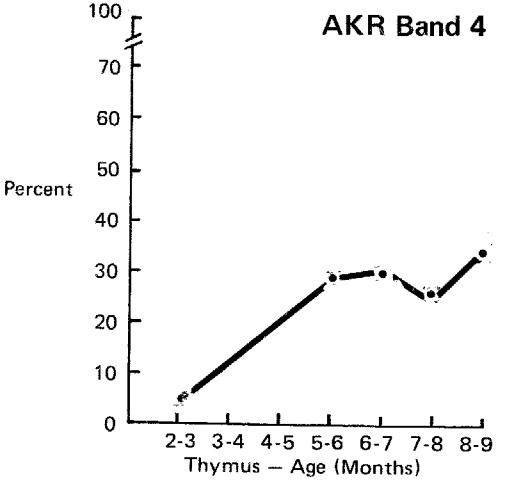

AKR Band 6
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen – Age (Months)

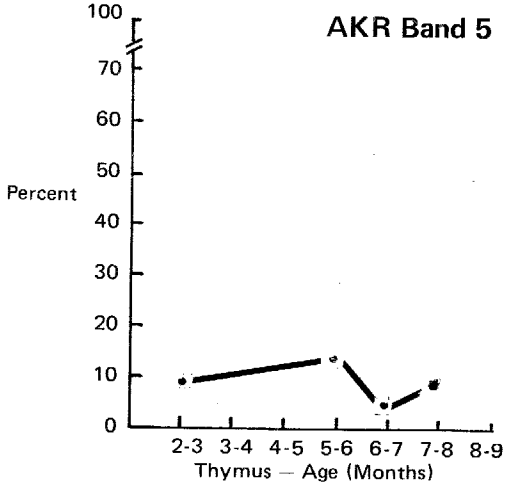
Figure 10. Age-related changes in Triton X-100 and Urea treated AKR/J thymus samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis of polyacrylamide slab gels. Per cent values are plotted against age (in months) of AKR/J mice when tissues were obtained. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.


AKR Band 1
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


AKR Band 2
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


AKR Band 3
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


AKR Band 4
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


AKR Band 5
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)

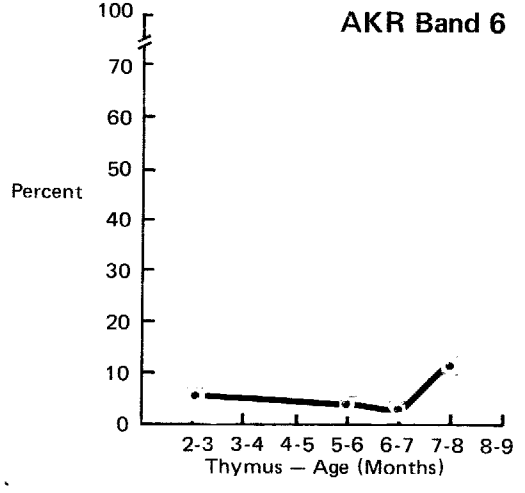
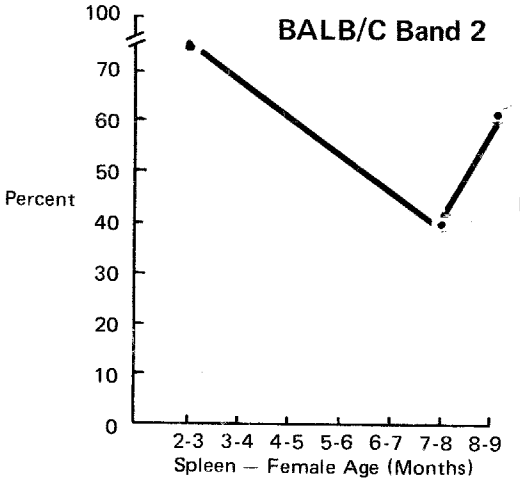
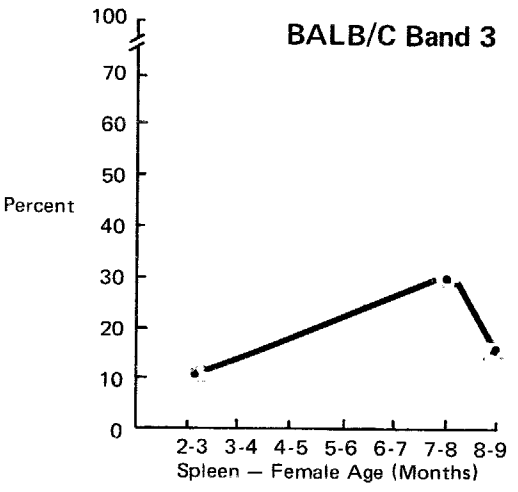
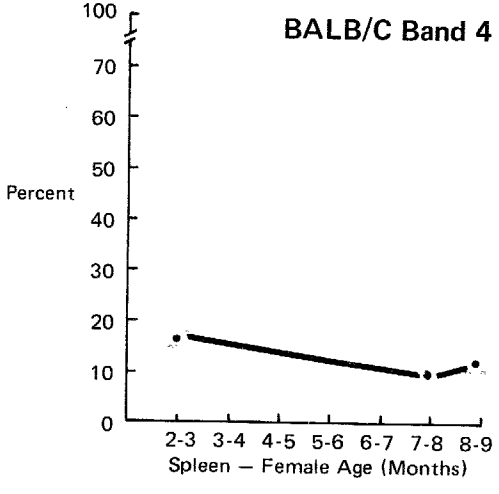
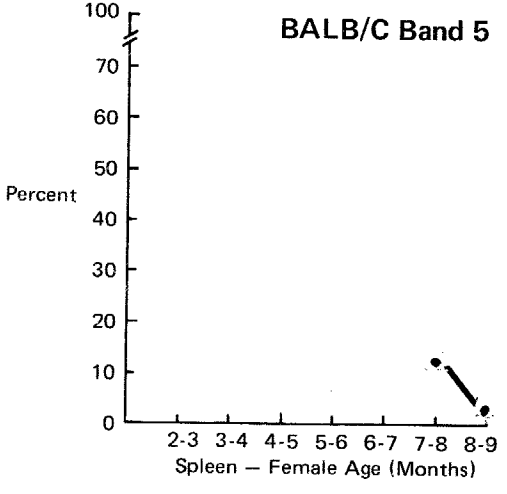

AKR Band 6
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)

Figure 11. Age-related changes in Triton X-100 and Urea pre-treated female BALB/C spleen samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis of polyacrylamide slab gels. Per cent values are plotted against age (in months) of BALB/C since when tissues were obtained. Eavh value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.


BALB/C Band 2
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Female Age (Months)


BALB/C Band 3
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Female Age (Months)


BALB/C Band 4
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Female Age (Months)


BALB/C Band 5
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Female Age (Months)

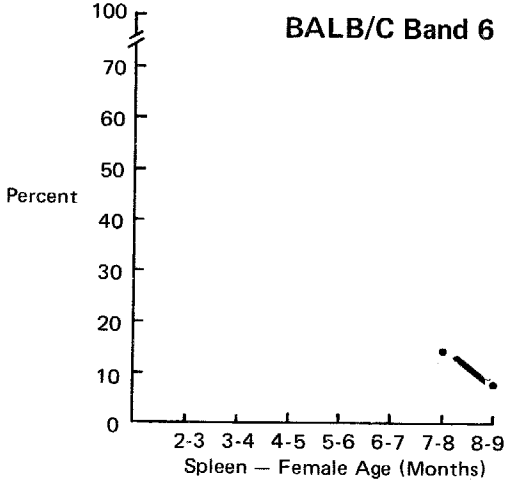
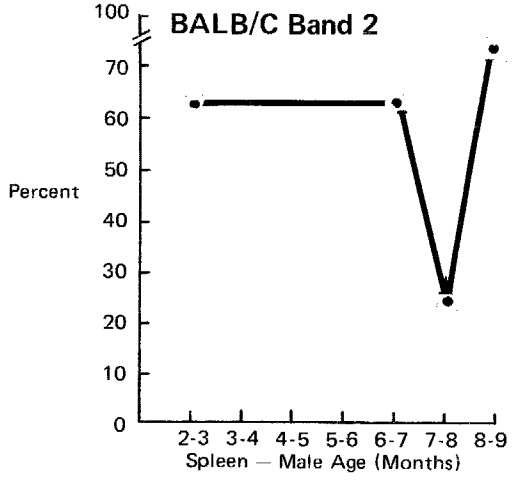
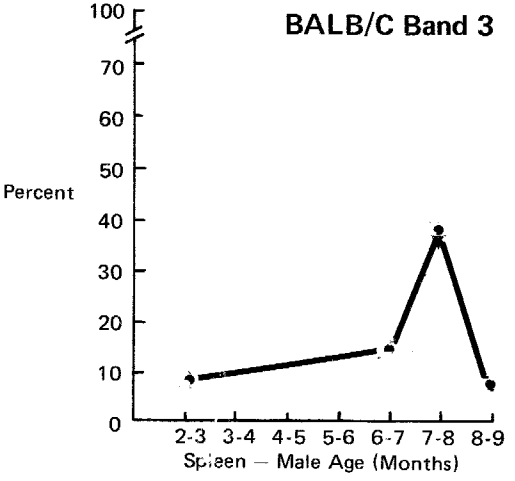
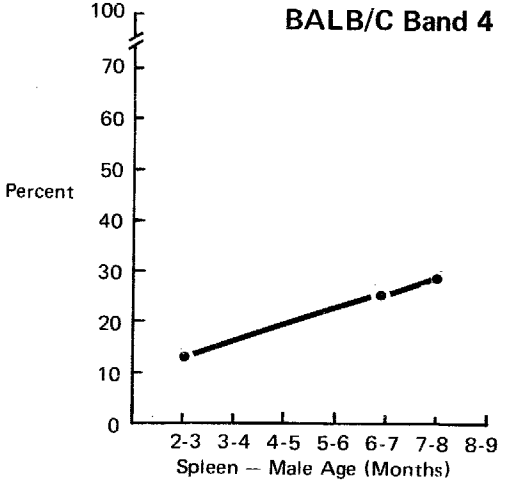
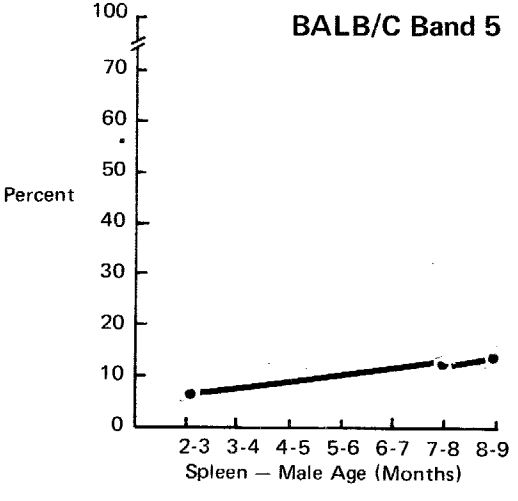

BALB/C Band 6
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Female Age (Months)

Figure 12. Age-related changes in Triton X-100 and Urea pre-treated male BALB/C spleen samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis in polyacrylamide slab gels. Per cent values are plotted against age (in months) of BALB/C since when tissues were obtained. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.


BALB/C Band 2
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Male Age (Months)


BALB/C Band 3
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Male Age (Months)


BALB/C Band 4
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Male Age (Months)


BALB/C Band 5
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Male Age (Months)

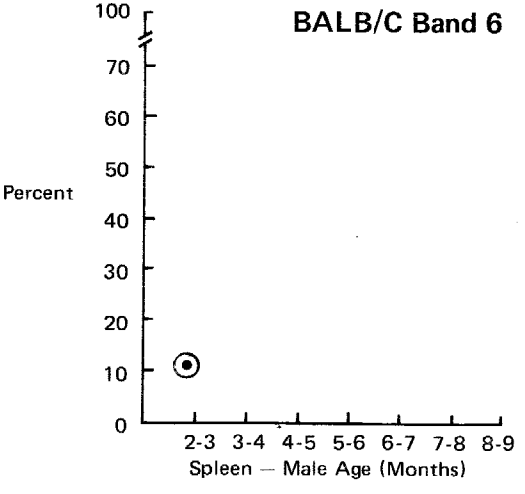
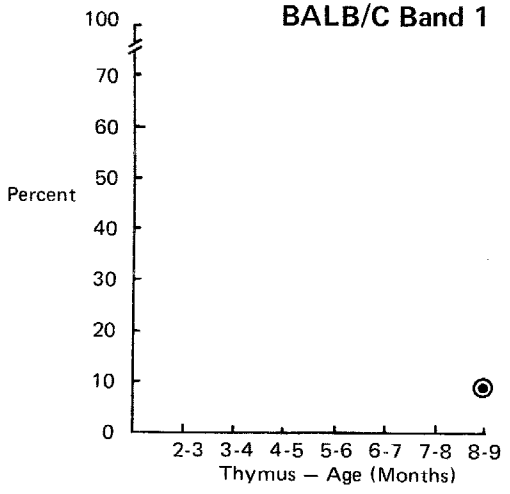
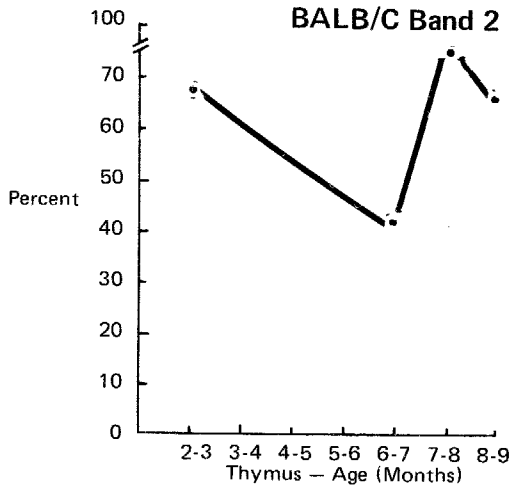
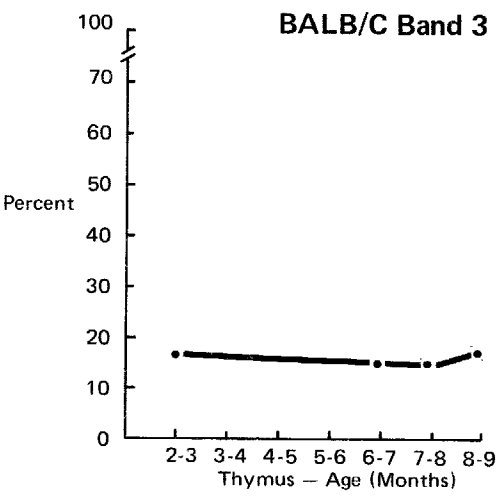
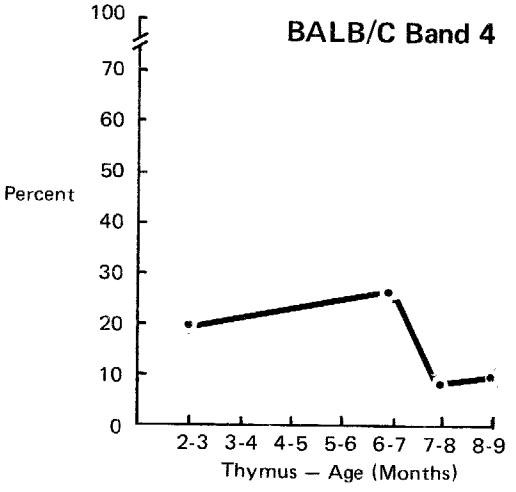

BALB/C Band 6
Percent
100
70
60
50
40
30
20
10
0
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Spleen — Male Age (Months)

Figure 13. Age-related changes in Triton X-100 and Urea pre-treated BALB/C thymus samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis of polyacrylamide slab gels. Per cent values are plotted against age (in months) of BALB/C since when tissues were obtained. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.


BALB/C Band 1
100
70
60
50
40
30
20
10
0
Percent
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


BALB/C Band 2
100
70
60
50
40
30
20
10
0
Percent
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


BALB/C Band 3
100
70
60
50
40
30
20
10
0
Percent
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)


BALB/C Band 4
100
70
60
50
40
30
20
10
0
Percent
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)

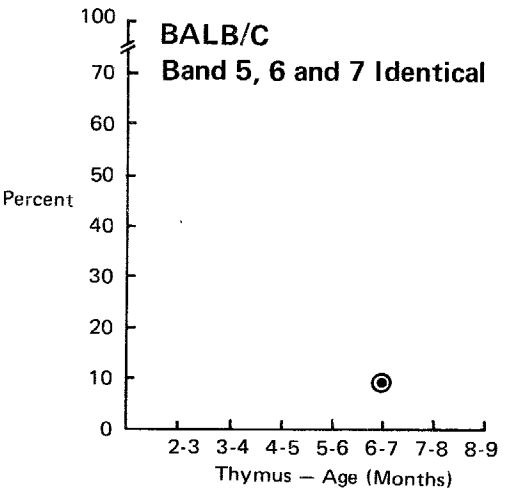

BALB/C
Band 5, 6 and 7 Identical
100
70
60
50
40
30
20
10
0
Percent
2-3 3-4 4-5 5-6 6-7 7-8 8-9
Thymus — Age (Months)

TABLE II

COMPARISON OF PAGIF RESULTS FOR PRE-TREATED SPLEEN MEMBRANE SURFACE PROTEINS
BY CALCULATION OF AREA IN EACH BAND FROM DENSITOMETER SCANS

AKR/J STRAIN

	Female 2-3 Month Old		Male 2-3 Month Old		Male 5-6 Month Old		Male 6-7 Month Old		Male 7-8 Month Old		Female 8-9 Month Old	
	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%
Band 1	--	--	--	--	--	--	--	--	--	--	118	19.4
Band 2	95	27.5	114	48.7	262	44.3	318	48.8	248	41.6	285	46.9
Band 3	123	35.7	58	24.8	115	19.5	109	16.7	101	16.9	67	11.0
Band 4	74	21.4	30	12.8	155	26.2	185	28.4	153	25.7	113	18.6
Band 5	53	15.4	32	13.7	59	10.0	20	3.1	60	10.1	25	4.1
Band 6	--	--	--	--	--	--	19	5.0	34	5.7	--	--
TOTAL	345	100.0	234	100.0	591	100.0	651	100.0	596	100.0	608	100.0

TABLE III

COMPARISON OF PAGIF RESULTS FOR PRE-TREATED THYMUS MEMBRANE SURFACE PROTEINS BY CALCULATION OF AREA IN EACH BAND FROM DENSITOMETER SCANS

AKR/J STRAIN

	Female 2-3 Month Old		Male 2-3 Month Old		Male 5-6 Month Old		Male 6-7 Month Old		Female 7-8 Month Old		Female 8-9 Month Old		Male 8-9 Month Old	
	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%
Band 1	--	--	--	--	4	0.1	--	--	72	8.3	50	8.2	32	7.4
Band 2	317	40.5	302	62.4	238	37.0	318	48.8	282	32.6	222	36.6	185	42.8
Band 3	210	26.8	106	21.9	130	20.3	109	16.7	138	16.0	125	20.6	215	49.8
Band 4	208	26.6	17	3.5	180	28.1	185	28.4	210	24.3	210	34.6	--	--
Band 5	48	6.1	40	8.3	72	11.3	20	3.2	60	6.9	--	--	--	--
Band 6	--	--	19	3.9	20	3.2	19	2.9	102	11.9	--	--	--	--
TOTAL	783	100.0	484	100.0	644	100.0	651	100.0	864	100.0	607	100.0	432	100.0

TABLE IV

COMPARISON OF PAGIF RESULTS FOR PRE-TREATED SPLEEN MEMBRANE SURFACE PROTEINS
BY CALCULATION OF AREA IN EACH BAND FROM DENSITOMETER SCANS

BALB/C STRAIN

	Female 2-3 Month Old		Male 2-3 Month Old		Male 6-7 Month Old		Female 7-8 Month Old		Male 7-8 Month Old		Female 8-9 Month Old		Male 8-9 Month Old	
	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%
Band 1	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Band 2	239	73.1	447	61.5	242	61.4	160	38.8	126	22.2	420	67.3	210	76.9
Band 3	33	10.1	71	9.8	55	14.0	116	28.1	219	38.2	91	14.6	25	9.2
Band 4	55	16.8	92	12.7	97	24.6	35	8.5	165	28.8	63	10.1	--	--
Band 5	--	--	41	5.5	--	--	41	10.0	63	11.0	10	1.6	38	13.9
Band 6	--	--	76	10.5	--	--	60	14.6	--	--	40	6.4	--	--
TOTAL	327	100.0	727	100.0	394	100.0	412	100.0	412	100.0	624	100.0	273	100.0

TABLE V

COMPARISON OF PAGIF RESULTS FOR PRE-TREATED THYMUS MEMBRANE SURFACE PROTEINS BY CALCULATION OF AREA IN EACH BAND FROM DENSITOMETER SCANS

BALB/C STRAIN

	Female 2-3 Month Old		Male 2-3 Month Old		Male 6-7 Month Old		Female 7-8 Month Old		Male 8-9 Month Old	
	mm^2	%	mm^2	%	mm^2	%	mm^2	%	mm^2	%
Band 1	--	--	--	--	--	--	--	--	28	9.3
Band 2	163	65.2	80	48.8	292	39.6	751	79.7	197	65.2
Band 3	40	16.0	39	23.8	110	14.9	126	13.4	52	17.2
Band 4	47	18.8	45	27.4	167	22.7	65	6.9	25	8.3
Band 5	--	--	--	--	65	8.8	--	--	--	--
Band 6	--	--	--	--	55	7.5	--	--	--	--
Band 7	--	--	--	--	--	--	--	--	--	--
TOTAL	250	100.0	164	100.0	737	100.0	942	100.0	302	100.0

TABLE VI

COMPARISON OF PAGIF RESULTS FOR PRE-TREATED SPLEEN MEMBRANE SURFACE PROTEINS BY CALCULATION OF AREA IN EACH BAND FROM DENSITOMETER SCANS

C3Heb/FeJ STRAIN

	Female 2-3 Month Old		Male 2-3 Month Old	
	mm^2	%	mm^2	%
Band 1a	--	--	20	2.1
Band 1b	96	12.3	260	27.3
Band 1c	100	12.8	92	9.7
Band 2	236	30.3	242	25.5
Band 3	188	24.1	164	17.2
Band 4	77	9.9	83	8.7
Band 5	82	10.6	90	9.5
TOTAL	729	100.0	951	100.0

TABLE VII

COMPARISON OF PAGIF RESULTS FOR PRE-TREATED THYMUS MEMBRANE SURFACE PROTEINS BY CALCULATION OF AREA IN EACH BAND FROM DENSITOMETER SCANS

C3Heb/FeJ STRAIN

	Male 2-3 Month Old		Female 7-8 Month Old	
	mm^2	%	mm^2	%
Band 1a	--	--	35	2.3
Band 1b	--	--	65	4.3
Band 1c	83	8.3	319	20.9
Band 2	354	35.6	507	33.2
Band 3	172	17.2	178	11.7
Band 4	248	24.9	154	10.1
Band 5	138	13.9	92	6.0
Band 6	--	--	175	11.5
TOTAL	995	100.0	1525	100.0

Comparisons of AKR/J, BALB/C, and C3Heb/FeJ spleen samples from 2 - 3 month old male and female samples demonstrate the following: Band 1a - 1c restricted to C3Heb/FeJ strain samples; Bands 2 and 6 present in greater relative density in BALB/C strain sample than the MuLV carrier strains; Band 3 present in least relative density in BALB/C samples (Figures 14 and 16).

Similar strain-related differences were observed between 7 - 8 month old female AKR/J, BALB/C, and C3Heb/FeJ strain thymus samples. The results show: Bands 1a - 1c were present in the MuLV carrier strains; Bands 2 in greater relative density in the BALB/C sample; Bands 3 and 4 in greater relative density in the AKR/J sample and Bands 5 and 6 are restricted to the C3Heb/FeJ samples (Figure 15).

Sex-related differences are observed for the 2 - 3 month old AKR/J spleen samples (Figure 17) and the 2 - 3 month old C3Heb/FeJ spleen sample (Figure 18). The male AKR/J sample demonstrates an increase in relative density as compared to the female sample for Band 2 and lower values for Bands 3 - 5. The female C3Heb/FeJ is characterized by a greater relative density for Bands 1c - 5. An additional band is observed in the male 8 - 9 month old spleen densitometer scan as compared with the

Figure 14. Strain-related differences in Triton X-100 and Urea pre-treated 2 - 3 month old female AKR/J, C3Heb/FeJ, and BALB/C spleen samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis. Per cent values are plotted against specific bands present. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.

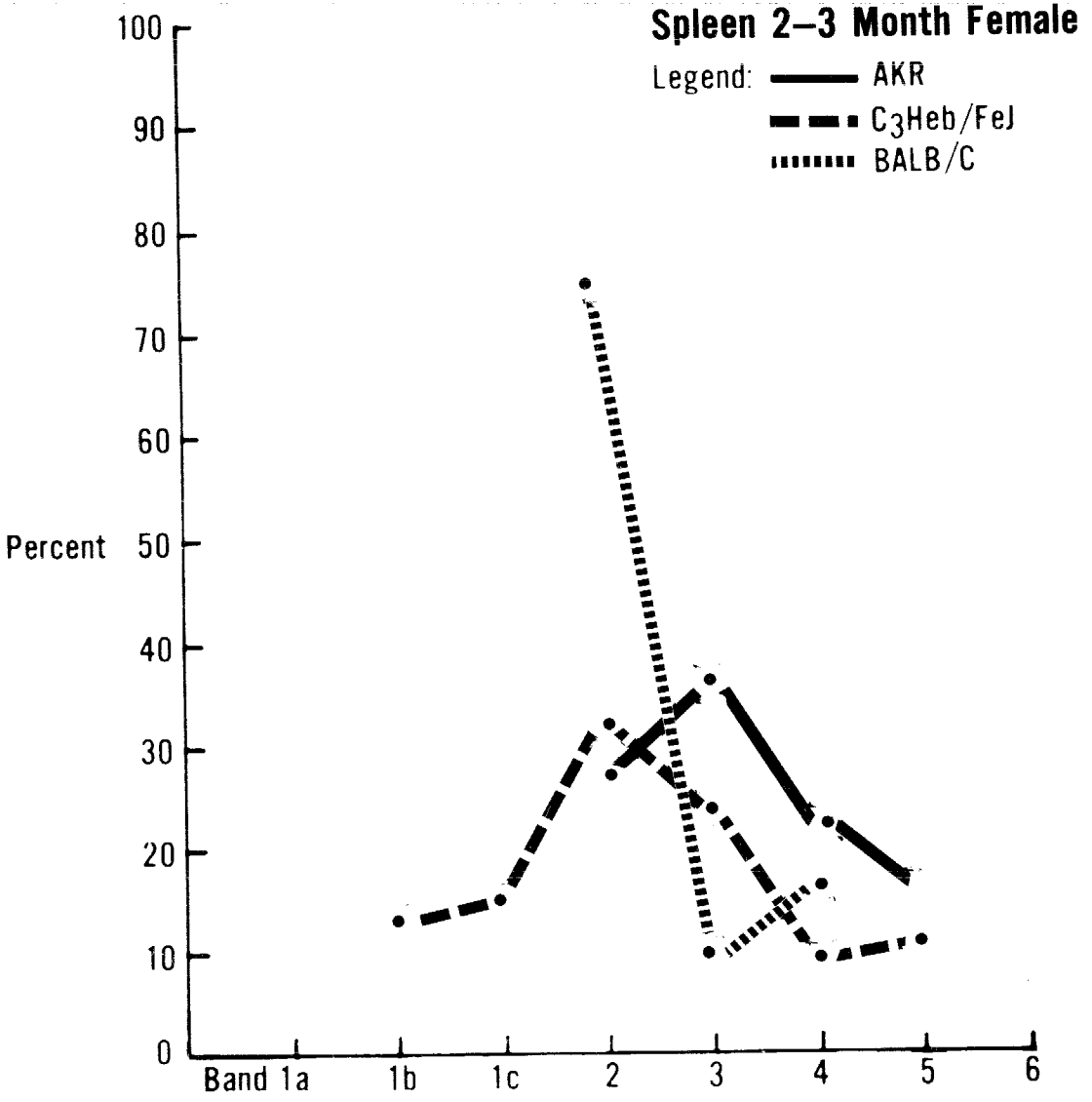

Spleen 2–3 Month Female
Legend:
AKR
C3Heb/FeJ
BALB/C
Percent
100
90
80
70
60
50
40
30
20
10
0
Band 1a
1b
1c
2
3
4
5
6

Figure 15. Strain-related differences in Triton X-100 and Urea pre-treated 7 - 8 month old female AKR/J, C3Heb/FeJ and BALB/C thymus samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis. Per cent values are plotted against specific bands present. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.

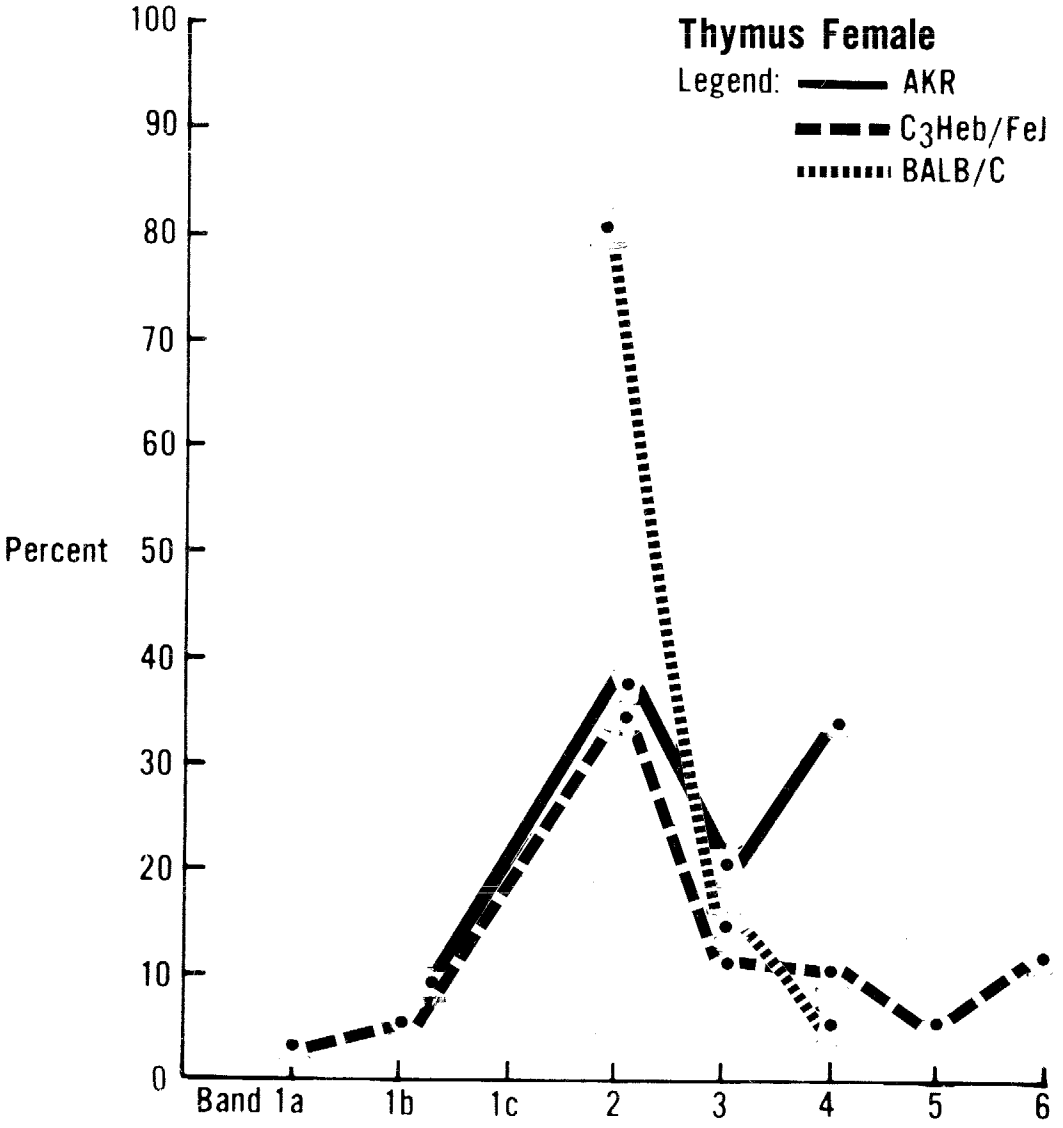
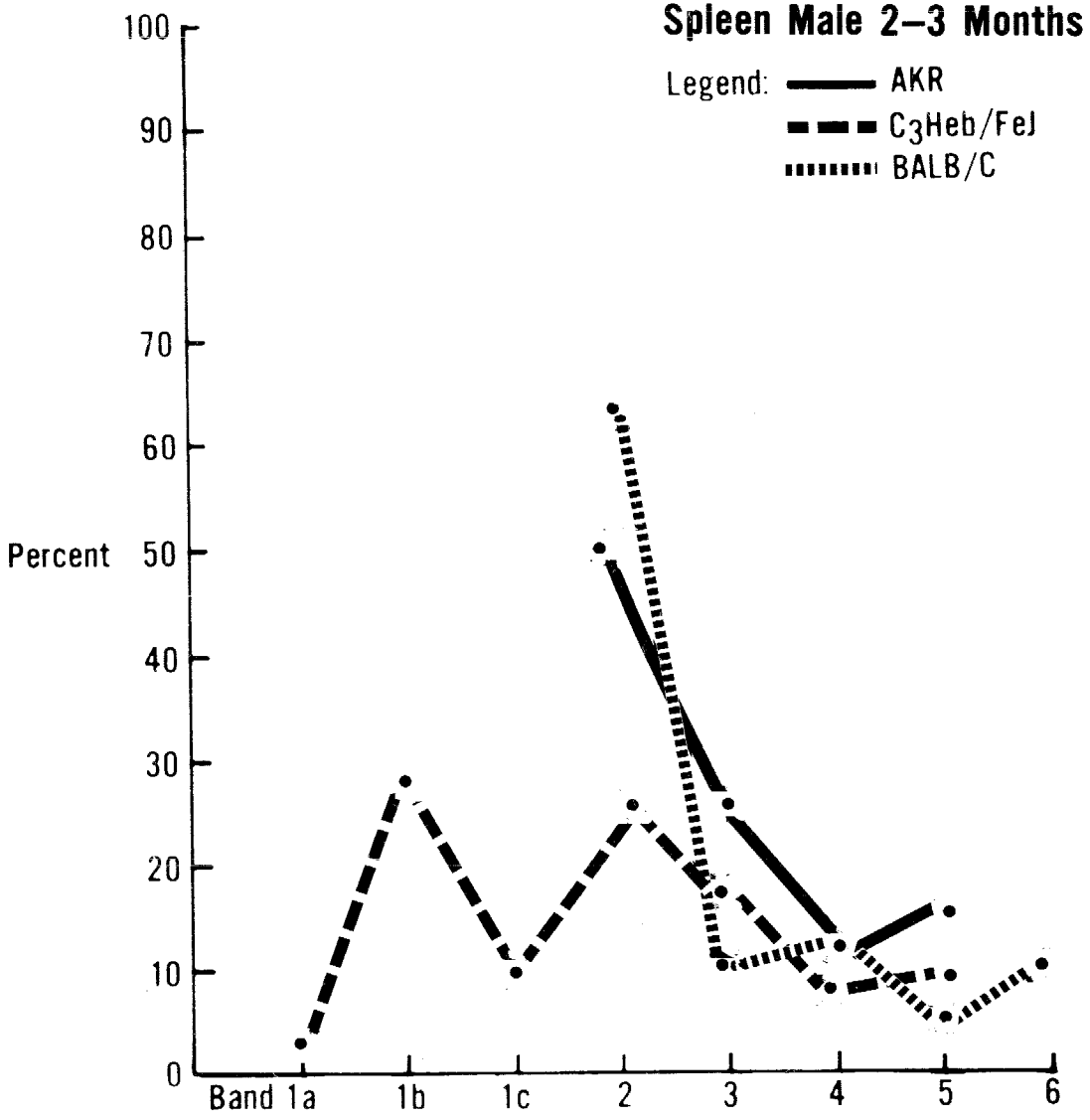

Thymus Female
Legend: AKR
C3Heb/FeJ
BALB/C
Percent
100
90
80
70
60
50
40
30
20
10
0
Band 1a
1b
1c
2
3
4
5
6

Figure 16. Strain-related differences in Triton X-100 and Urea pre-treated 2 - 3 month old male AKR/J, C3Heb/FeJ, and BALB/C spleen samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis of polyacrylamide slab gels. Per cent values are plotted against specific bands present. Each value represents the average value obatined after repeating the procedure twice with similarly prepared tissue samples.


Spleen Male 2–3 Months
Legend:
AKR
C3Heb/FeJ
BALB/C
Percent
100
90
80
70
60
50
40
30
20
10
0
Band 1a
1b
1c
2
3
4
5
6

female sample (Figure 7). Similar sex-related differences are observed in the leukemic 8 - 9 month old AKR/J female thymus scans (Figure 8). The leukemic AKR female sample does not demonstrate Band 4, which is present in the male sample. A comparison of spleen samples from 2 - 3 month old C3Heb/FeJ samples demonstrates Band 1d in the male samples which are lacking in the female.

In summary, the results of the calculation of density of each band as a per cent of the total area of all proteins in a sample show for the thymus samples -- Bands 1a - 1c, 5, and 6 limited to the MuLV carrier strains, and Bands 2, 3, and 4 in all three strains with the AKR/J demonstrating a greater relative density for Bands 3 and 4.

Antisera Titers

Titration of antisera was determined following the injection procedure of individual rabbits by Microliter^{R} hemagglutination techniques. Homologous antisera-protein sample assays were conducted, and the values obtained are presented in Table VIII.

Ouchterlony Immunodiffusion

Ouchterlony analysis showed several precipitation bands resulted when the undiluted, 1:5, and 1:10 dilutions of the protein samples reacted with each of the antisera. As seen in Figure 19, these bands show complete identity

Figure 17. Sex-related differences in Triton X-100 and Urea pre-treated 2 - 3 month old AKR/J samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis. Per cent values are plotted against specific Bands present. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.

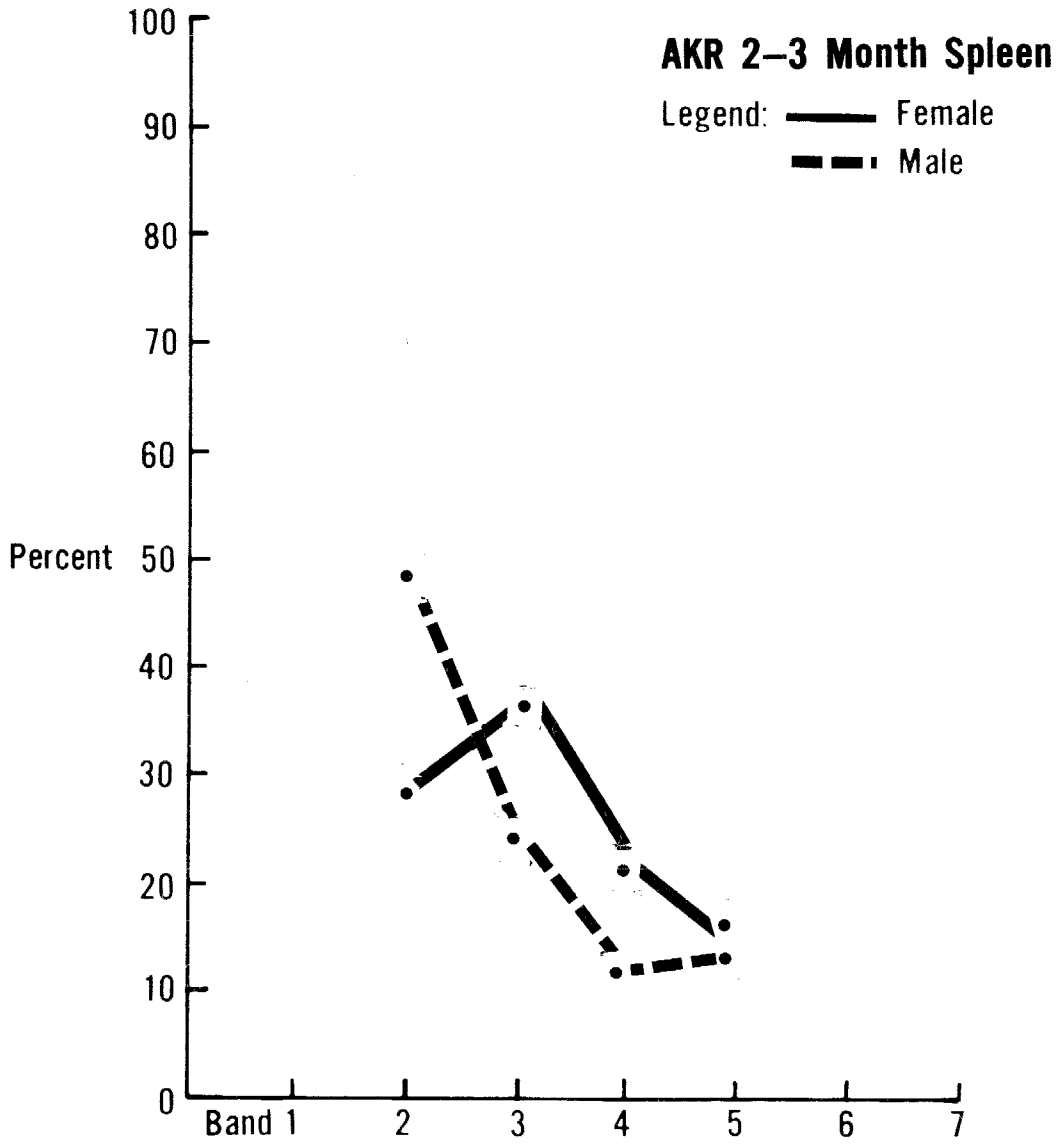

AKR 2–3 Month Spleen
Legend: Female
Male
Percent
100
90
80
70
60
50
40
30
20
10
0
Band 1
2
3
4
5
6
7

Figure 18. Sex-related differences in Triton X-100 and Urea pre-treated 2 - 3 month old C3Heb/FeJ spleen samples as determined by per cent of total area represented by all bands in each sample following densitometer analysis. Per cent values are plotted against specific Bands present. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.

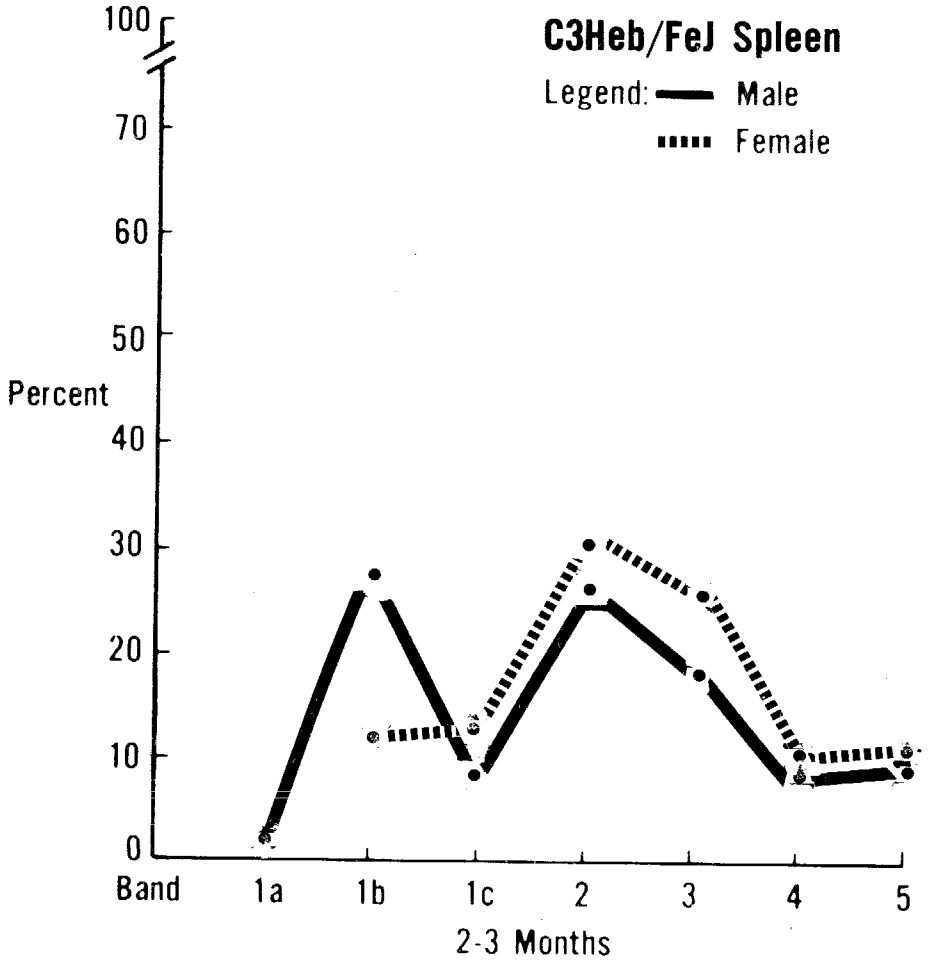

C3Heb/FeJ Spleen
Legend: Male
Female
100
70
60
50
Percent
40
30
20
10
0
Band
1a
1b
1c
2
3
4
5
2-3 Months

Figure 19. Ouchterlony analysis for papain extracted lymphocyte protein samples with anti-control spleen antisera. Complete identity shown for three different proteins in each sample.

Abbreviations: CS - 6-7 month old BALB/C spleen; ES - 5-6 month old early leukemic AKR/J spleen; LS - 7-8 month old leukemic AKR/J spleen; CT - 6-7 month old BALB/C thymus; ET - 5-6 month old early leukemic AKR/J thymus; LT - 7-8 month old leukemic AKR/J thymus; C - 6-7 month old BALB/C spleen sera.

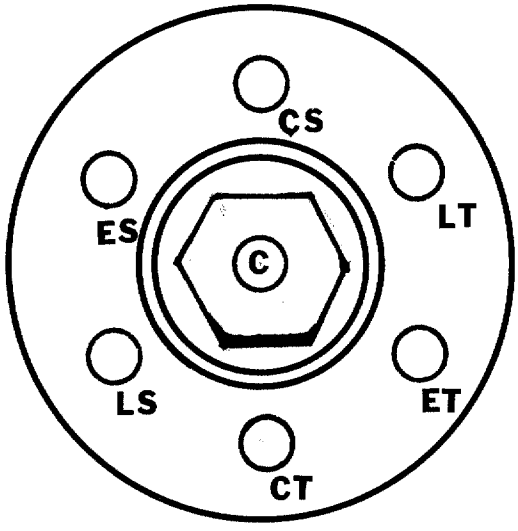

CS
ES
LT
C
LS
ET
CT

TABLE VIII

DETERMINATION OF TITER FOR ANTISERA PREPARED TO AKR/J, BALB/C, AND C3Heb/FeJ SPLEEN AND THYMUS EXTRACTED SURFACE PROTEINS

Homologous Testing

Antisera	Extracted Protein Source	Titer
Anti-BALB/C spleen	BALB/C spleen	1:128
Anti-pre-leukemic AKR/J spleen	Pre-leukemic AKR/J spleen	1:64
Anti-leukemic AKR/J spleen	Leukemic AKR/J spleen	1:4096
Anti-C3Heb/FeJ spleen	C3Heb/FeJ spleen	1:64
Anti-BALB/C thymus	BALB/C thymus	1:256
Anti-pre-leukemic AKR/J thymus	Pre-leukemic AKR/J thymus	1:8
Anti-leukemic AKR/J thymus	Leukemic AKR/J thymus	1:16
Anti-C3Heb/FeJ thymus	C3Heb/FeJ thymus	1:128

for all of the spleen and thymus membrane extracted protein samples with anti-BALB/C spleen (anti-CS) sera. Similar results were obtained for either two or three proteins in each of the samples with the different antisera. The total number of precipitation bands recorded for each antisera is the cumulative number of bands from the different dilutions for a particular protein sample.

Immunoelectrophoresis

R_f values obtained from the immunoelectrophoresis slide gels are presented in Table IX. The R_f value was obtained by measuring the distance from the center of the antigen well to the center of the precipitin arc. Testing each protein samples with its homologous antisera, as well as heterologous antiserum show different distinct precipitin arcs were obtained. The number of arcs is dependent upon the specific antisera used. As many as three different proteins are shown to be present in each sample and are similar in each of the presentations as evidenced by the heterologous testing.

Granulated Gel Electrofocusing

The technique of flat bed granulated gel electrofocusing was applied as a means of separation of the individual proteins from three spleen and three thymus samples. A pH gradient of 7 to 11 was used in the preparation of the slurry gels. The division fractions were collected with

TABLE IX

ANALYSIS BY IMMUNOELECTROPHORESIS OF ANTISERUM TO PAPAIN EXTRACTED SPLEEN AND THYMUS CELL SURFACE PROTEINS

Surface of Membrane Extracted Proteins	Reacted with Antisera Type	R_f Values Obtained*
Control BALB/C spleen	Anti-control spleen	.39 cm
	Anti-early leukemic spleen	.38-.39 cm, .41 cm
	Anti-leukemic spleen	.43 cm
Early leukemic AKR spleen	Anti-early leukemic spleen	.27-.29 cm, .41 cm
Leukemic AKR spleen	Anti-control spleen	.35 cm, .38 cm
	Anti-leukemic spleen	.39 cm
Control BALB/C thymus	Anti-control thymus	.34 cm, .41 cm
	Anti-early thymus	.35 cm, .41 cm
	Anti-leukemic thymus	.38 cm, .41 cm
Early leukemic AKR thymus	Anti-early leukemic thymus	.36 cm
Leukemic AKR thymus	Anti-control thymus	.36 cm
	Anti-leukemic thymus	.34 cm, .41 cm

*R_f = distance measured from center of antigen well to center of precipitation arcs.

division 1 located near the positive electrode and division 30 at the negative electrode. Optical density profiles were obtained for each division fraction and plotted as fraction number vs. O.D. units at A_{280}. Seven distinct bands were observed from the AKR/J thymus sample profiles (Figure 20).

Comparison of Results from PAGIF and Granulated Gel Electrofocusing Techniques

A comparison of these O.D. profiles with densitometer scans of the same samples run on a polyacrylamide slab gel subjected to isoelectric focusing (PAGIF) shows a large degree of similarity.

Comparison of the thymus samples subjected to PAGIF and the granulated gel technique demonstrates the following results: (Figure 21); the 7 - 8 month old female AKR thymus is represented by Bands 1 - 6 on PAGIF and Bands 1 - 7 by the slurry gel assay.

The observation of additional protein bands from the O.D. profile for the granulated gel electrofocusing method such as Band 7 in the AKR/J thymus sample compared to the densitometer scans on PAGIF runs indicates an increased sensitivity for the Ultrodex^{R} as suggested by Peterson (personal communication).

Further quantitative analysis of the data was made by calculation of the per cent area of each protein

Figure 20. Optical Density profile obtained following flat bed granulated gel electrofocusing of papain extracted AKR/J thymus cell surface proteins. Range of granulated gel was pH 7 - 11 and prepared as described in the Methods section. Electrofocusing was for a total of 8 hours at 0 - 4°C. Final voltage was 1140 volts. Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.

O.D. PROFILE OF 7-8 MONTH OLD MALE AKR/J THYMUS SAMPLE FOLLOWING GRANULATED GEL ELECTROFOCUSING

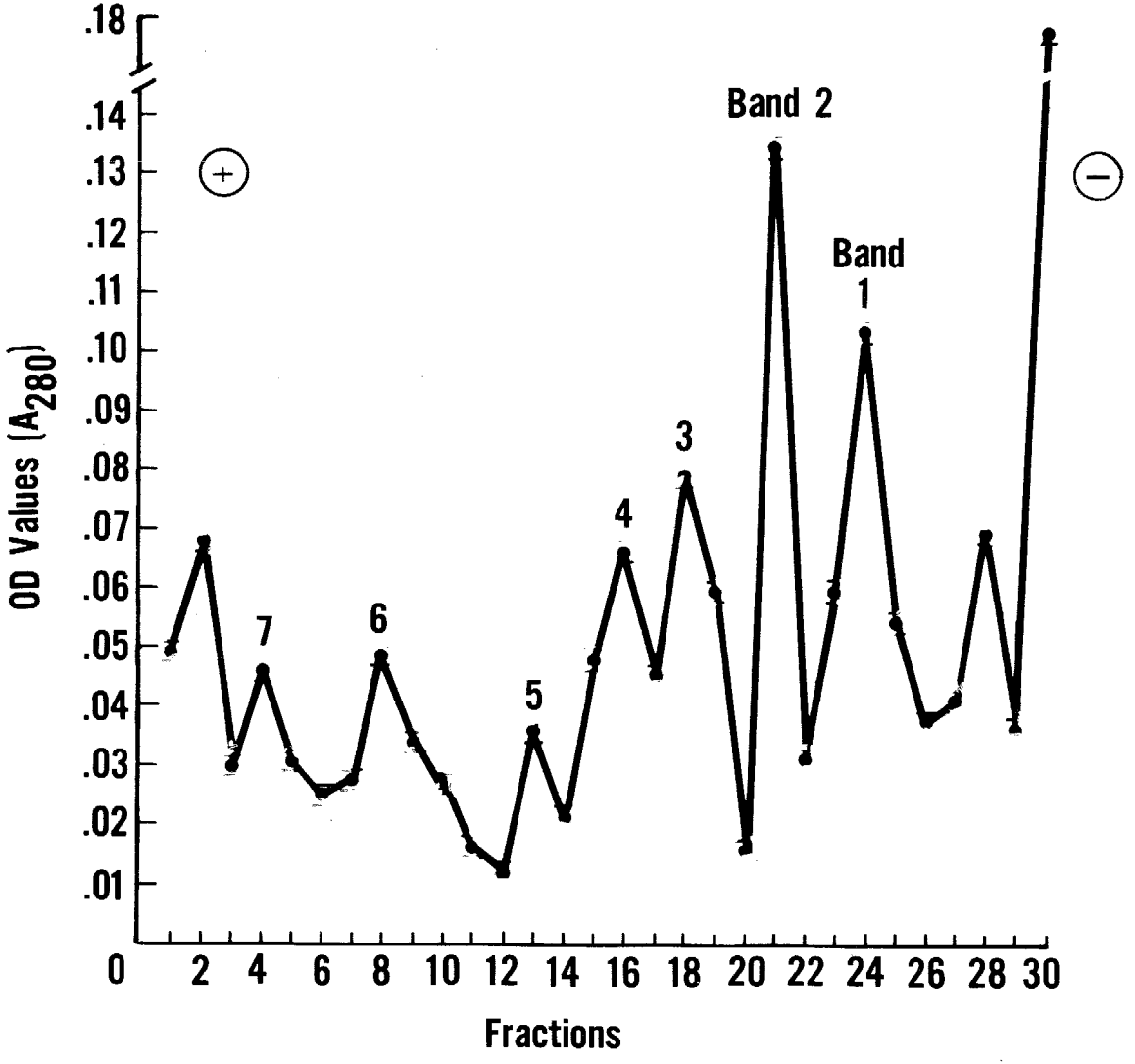
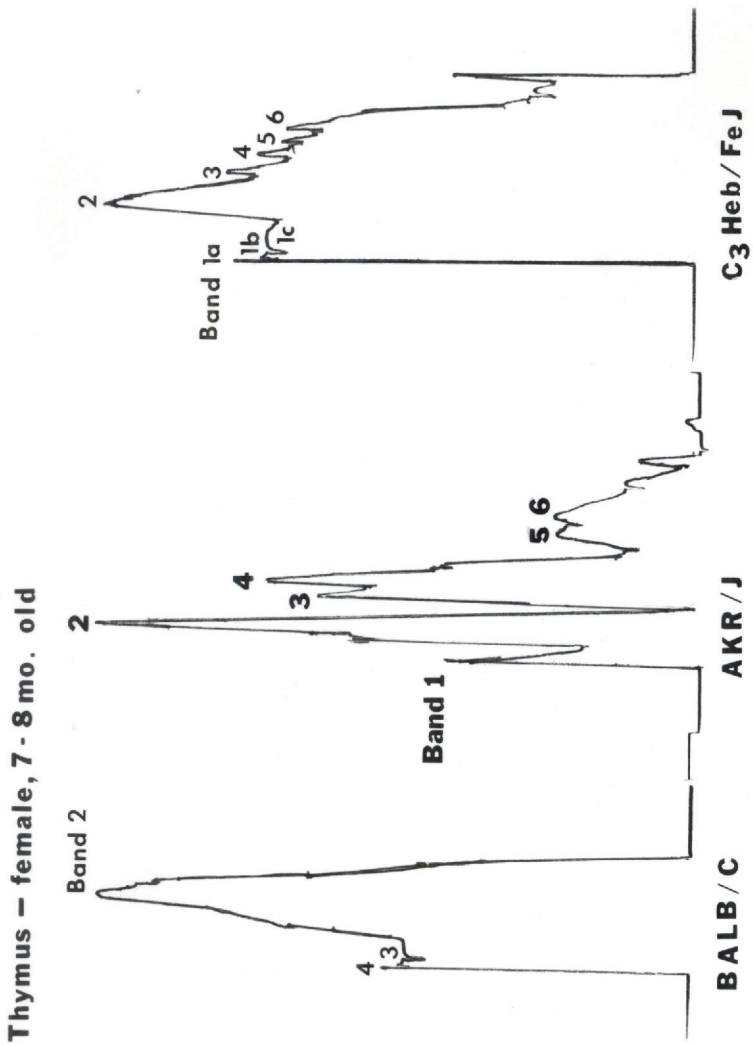


Figure 21. Densitometer scans with a A570 filter following polyacrylamide slab gel isoelectric focusing of AKR/J, BALB/C, and C3Heb/FeJ thymus samples following polyacrylamide gel isoelectric focusing. Samples were pre-treated with Triton X-100 and Urea prior to application on the slab gel surface. Slab gels were stained, destained, and preserved as described in the Methods section.


Thymus — female, 7 - 8 mo. old
Band 2
2
4
3
Band 1
5 6
Band 1a
1b
1c
2
3
4
5 6
BALB/C
AKR/J
C3 Heb/FeJ

represented in a sample. The area under each band from the densitometer scan was calculated for the PAGIF results, while the A_{280} values for each band were totalled for the O.D. profiles of the sample subjected to the granulated gel technique. These values are presented in Tables X and XI.

BioGel Filtration

The increase of the O.D. profiles from the baseline results from the presence of ampholines in the individual fractions. Two and one-half milliliters of those fractions representing the peak of Bands 1 - 7 from the AKR/J thymus sample and Bands 1a - 1d from the CeHeb/FeJ thymus sample were subjected to gel filtration on BioGel P-2 columns and the protein eluted off within the first 10 ml elution fractions. Applying the techniques of difference spectroscopy to these collected fractions, it was determined that an average of .04 mg/ml of ampholine was present in each division fraction (Figures 22 and 23).

TABLE X

VALUES OBTAINED BY CALCULATION OF AREA FROM DENSITOMETER SCANS OF PAGIF GELS FOR AKR/J THYMUS SAMPLES

	7 - 8 Month Female AKR/J	
	mm^2	%
Band 1	72	8.3
Band 2	282	32.6
Band 3	138	16.0
Band 4	210	24.4
Band 5	60	6.9
Band 6	102	11.8
TOTAL	864	100.0

TABLE XI

VALUES OBTAINED BY CALCULATION OF AREA FROM O.D. PROFILES FOLLOWING GRANULATED GEL ELECTROFOCUSING FOR AKR/J THYMUS SAMPLES

	7 - 8 Month Female AKR/J	
	A_{280} units	%
Band 1	.290	25.0
Band 2	.185	16.0
Band 3	.103	8.9
Band 4	.183	15.8
Band 5	.071	6.1
Band 6	.194	16.8
Band 7	.132	11.4
TOTAL	1.158	100.0

Figure 22. Elution profile for calibration of BioGel P-2 columns with Dextran Blue and potassium dichromate ($K_2Cr_2O_7$) (O.D. values at A_{255} vs. fraction number).

ELUTION PROFILE FOR CALIBRATION OF BIO-GEL P-2 COLUMNS

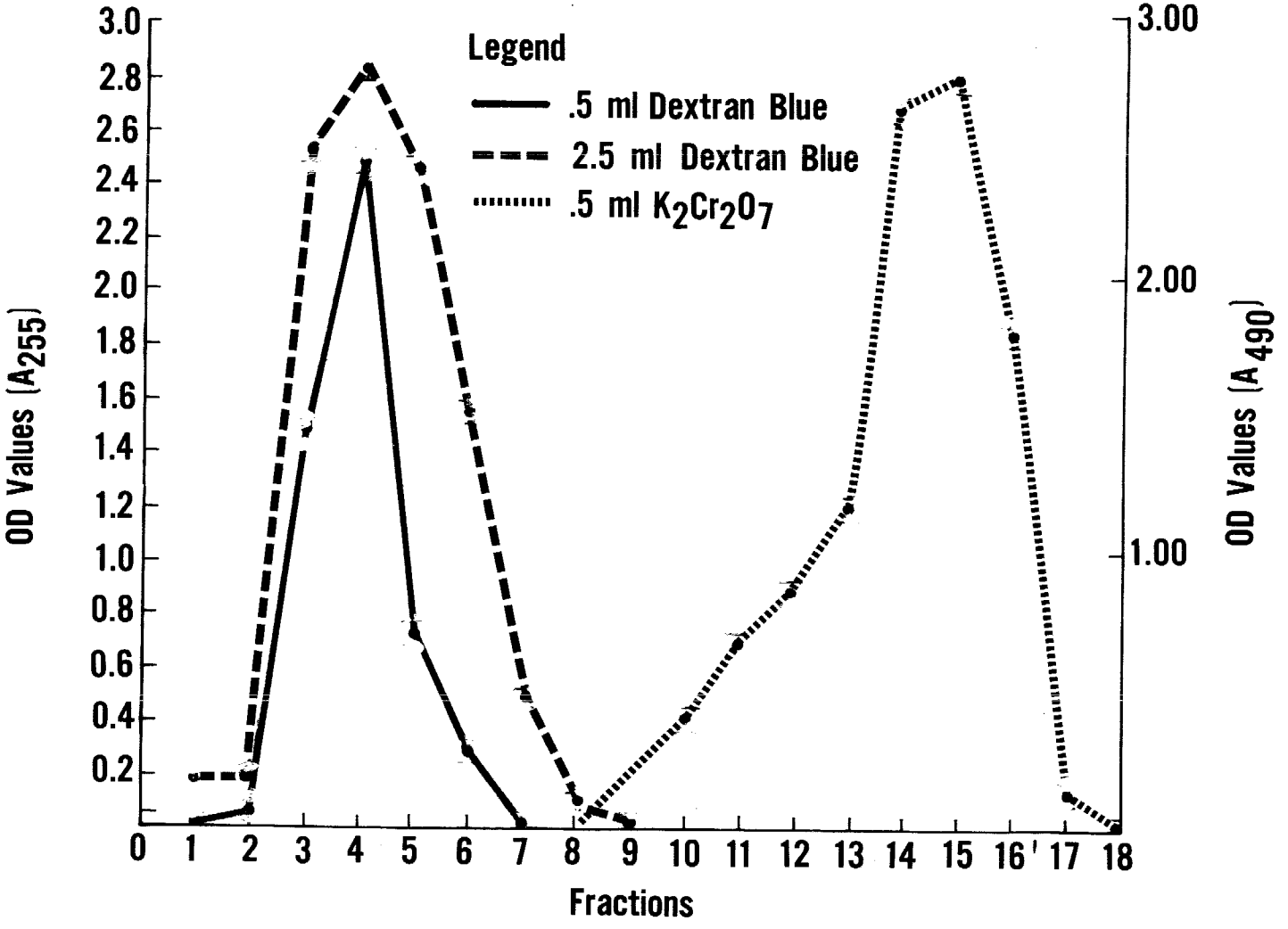
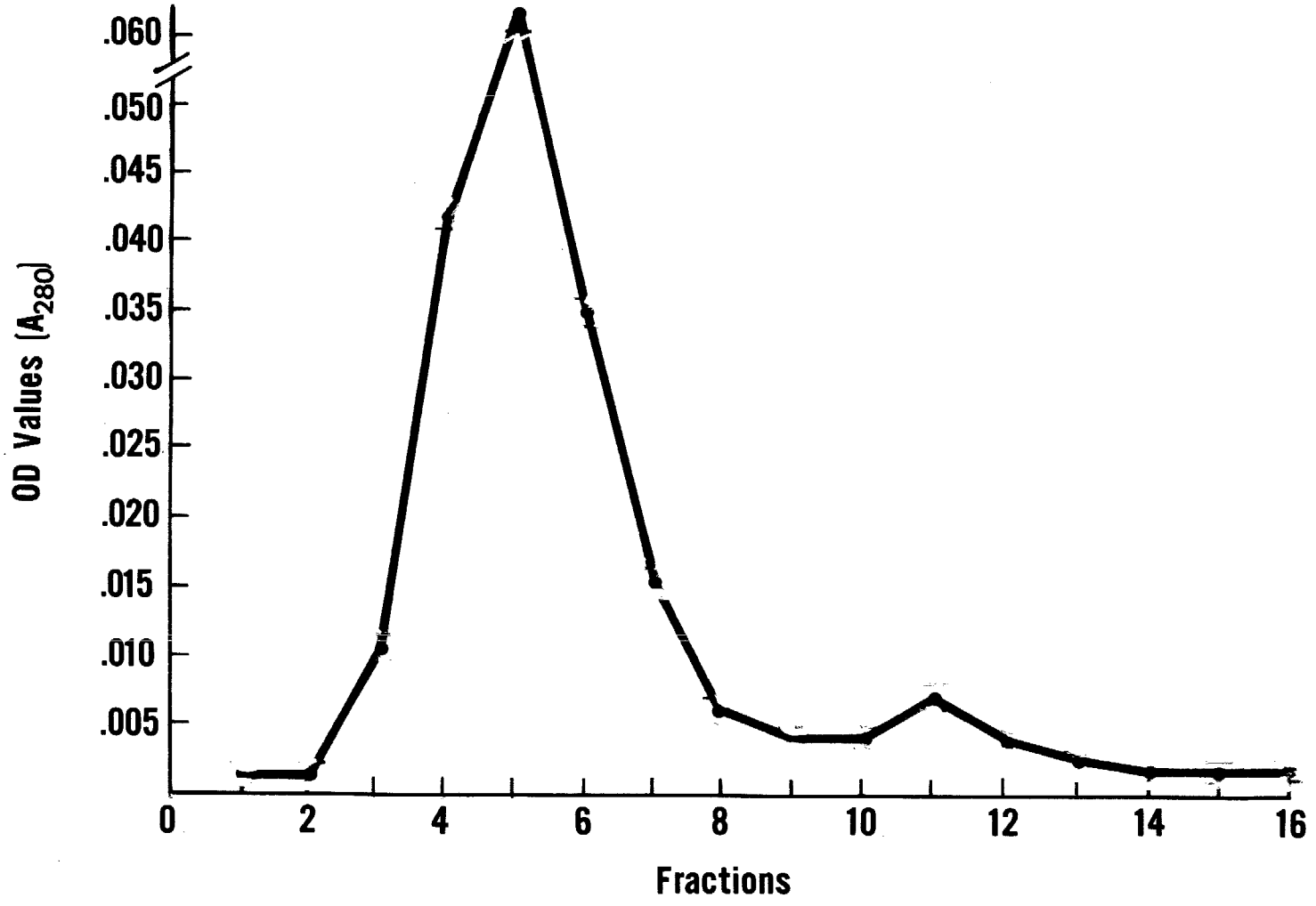


Figure 23. Elution profile following filtration on a BioGel P-2 column for Band 1 from 7 - 8 month old male AKR/J thymus sample separated by granulated gel electrofocusing (O.D. profile at A_{280} vs. elution fractions). Each value represents the average value obtained after repeating the procedure twice with similarly prepared tissue samples.

ELUTION PROFILE AFTER BIO-GEL P-2 FILTRATION OF BAND 1 FROM 7-8 MONTH OLD MALE AKR/J THYMUS SAMPLES



The six milliliter elution fractions containing a common protein were pooled and concentrated against carbowax to a final volume of 1.5 ml with 6 - 10 µg/ml concentration.

Passive Hemagglutination

The findings of the passive hemagglutination techniques for Proteins 1 - 7 and 1a - 1d are presented in Table XII. This assay system demonstrated Proteins 1 and 5 to be limited to the spleen and thymus samples from the MuLV carrier strains, AKR/J and C3Heb/FeJ, with Protein 6 limited to thymus samples in these two strains. Proteins 2, 3, and 4 are present in all spleen and thymus samples from the AKR/J, C3Heb/FeJ, and BALB/C strains with the concentration of Protein 2 present to a larger degree in the pre-leukemic and leukemic AKR/J spleen and thymus samples. Protein 7 is observed in the leukemic and pre-leukemic spleen and thymus samples only, with a greater concentration obtained for the leukemic samples. These findings substantiate the results obtained following the granulated gel electrofocusing procedure, in regard to the presence of Proteins 1, 5, and 6 identifiable only in the MuLV carrier strains and the presence of Band 7 limited to the AKR/J leukemic spleen and thymus samples.

TABLE XII

CALCULATION OF INDIVIDUAL PROTEIN CONCENTRATION
BY PASSIVE HEMAGGLUTINATION

Antisera	Protein						
	1	2	3	4	5	6	7
BALB/C Spleen	Neg*	.00001	.00006	.00003	Neg	Neg	Neg
Pre-Leukemic AKR/J Spleen	.002	.469	.0005	.234	.234	Neg	.000007
Leukemic AKR/J Spleen	.004	.117	.0001	.0005	.0005	Neg	.002
C3Heb/FeJ Spleen	.01	.01	.234	.00001	Neg	Neg	Neg
BALB/C Thymus	Neg	.059	.469	.0005	.00001	Neg	Neg
Pre-Leukemic AKR/J Thymus	.0009	.469	.00006	.0001	.007	.00001	.00001
Leukemic AKR/J Thymus	.0001	.469	1.00000	.00006	.00003	.0018	.0018
C3Heb/FeJ Thymus	.004	.000007	.00003	.01	.117	.117	Neg

*Neg = Negative.

Ouchterlony Analysis of Individual Proteins

The individual proteins Bands 1 - 7 from the AKR/J thymus and Bands 1a - 1d from the C3Heb/FeJ thymus did not react with antisera to the differentiation related antigens or to the Murine Leukemia Virus components. Several dilutions of these antisera (1:5, 1:10, 1:50, 1:100) also did not result in precipitin band formation in Ouchterlony gel diffusion studies. The failure of attempting a partial identity of the individual proteins with these antisera may be explained by the fact that the antisera to the differentiation related antigens only included the H-2^D loci encoded surface proteins, and not the H-2^K, Theta, T1 or MBLA surface antigen markers.

Capillary precipitin reactions were set up using the individual proteins and these same antiserum were tested. The results show positive reactions of Proteins 1, 5, 6, 7, 1a, 1b, 1c and 1d with antisera to the N type AKR Viral components; specifically, p12, p30, and gp 70. This indicates these specific isolated proteins to be Murine Leukemia Virus associated proteins. Negative results were recorded for these same proteins with the Goat anti-Moloney Virus p30, gp 70, Goat anti-Scripps Leukemia Virus to all Moloney Virus surface proteins, and Goat anti-Scripps Leukemia Virus gp 70. These latter three antisera represent Moloney Viral components.

Immunoelectron Microscopy

Observation of over thirty fields by Transmission Electron Microscopy (TEM) per grid of each sample demonstrates the lymphocytes appear in clusters composed of two or more cells. The diameter of the T cells range from 3.5 to 5.3 μm. Surface topography of the thymocytes derived from 8 - 9 month old leukemic AKR/J male mice which had not been absorbed with antisera are characterized as smooth to slightly irregular or uneven in nature (Figure 25).

Thymocytes derived from 8 - 9 month old AKR/J male mice absorbed with anti-leukemic AKR/J surface proteins exhibit a large number of dark "knobs". These "knobs" represent antisera molecules which have bound to a specific surface protein as seen in Figure 26. The "knob" is the more clearly delineated than the rest of the cell surface as a result of carbon shadowing of the carbon replica.

A comparison of the recognition or interaction of the anti-BALB/C, anti-C3Heb/FeJ, and anti-pre-leukemic AKR/J with the leukemic AKR/J thymocytes surface proteins was determined by the number of "knobs" present following the IEM procedure. The number of "knobs" observed with the anti-pre-leukemic AKR/J sera coated T cells was similar to the number recorded with the anti-leukemic AKR/J sera (Figure 27). Fewer "knobs" were noted anti-C3Heb/FeJ and less can be seen with the anti-BALB/C (Figure 28).

Figure 25. Carbon replica of thymocytes obtained from leukemic 8 - 9 month old male AKR/J mice not absorbed with antisera which serve as controls. Cell surface is rough and uneven in appearance. (Total magnification 75,680X).

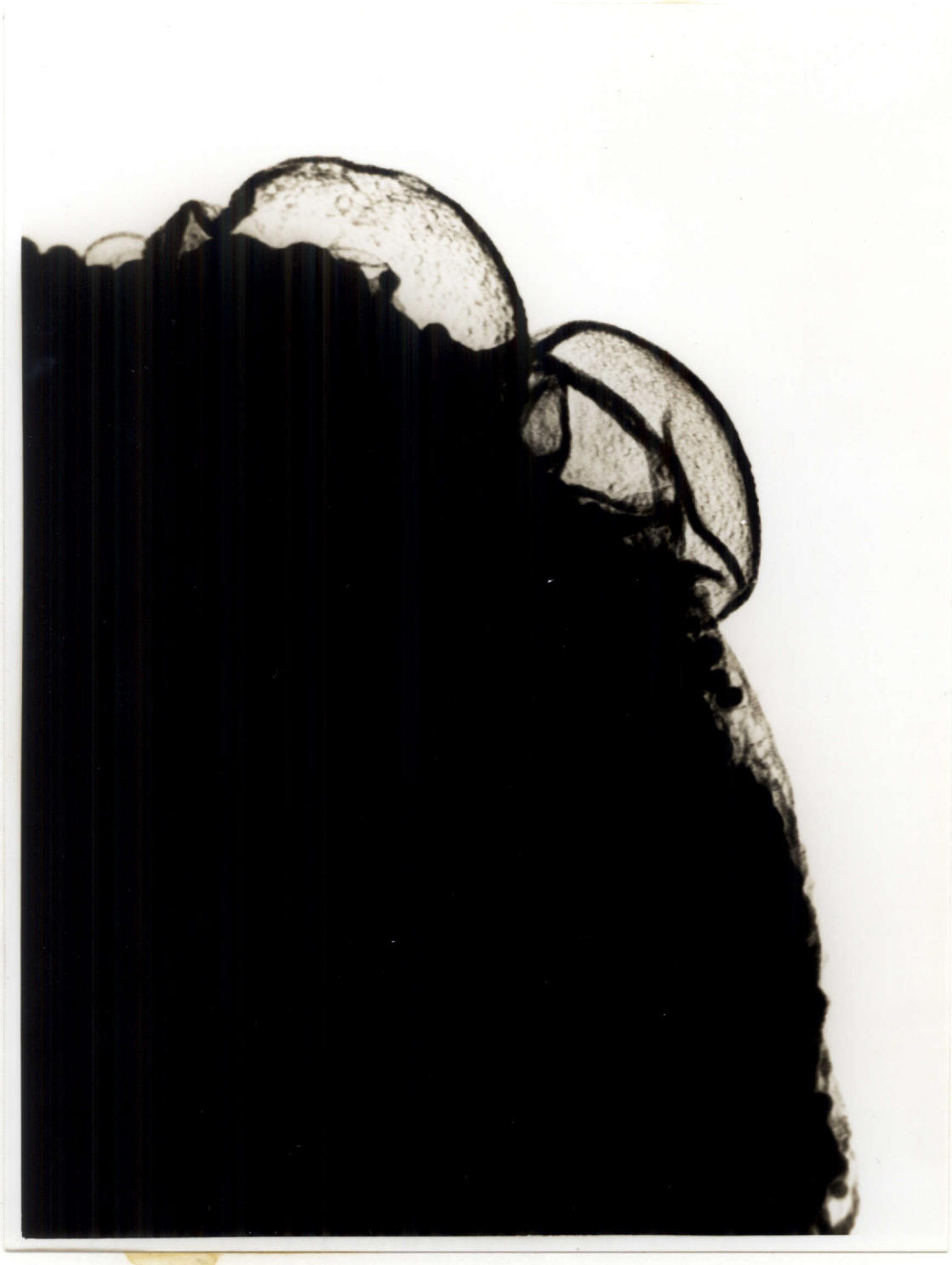


Figure 26. Carbon replica of thymocytes obtained from leukemic 8 - 9 month old male AKR/J mice absorbed with anti-leukemic AKR/J surface proteins as described in the Methods section. Black knobs on the cell surface represent interaction of the antisera with surface proteins. (Total magnification, 44,320X).

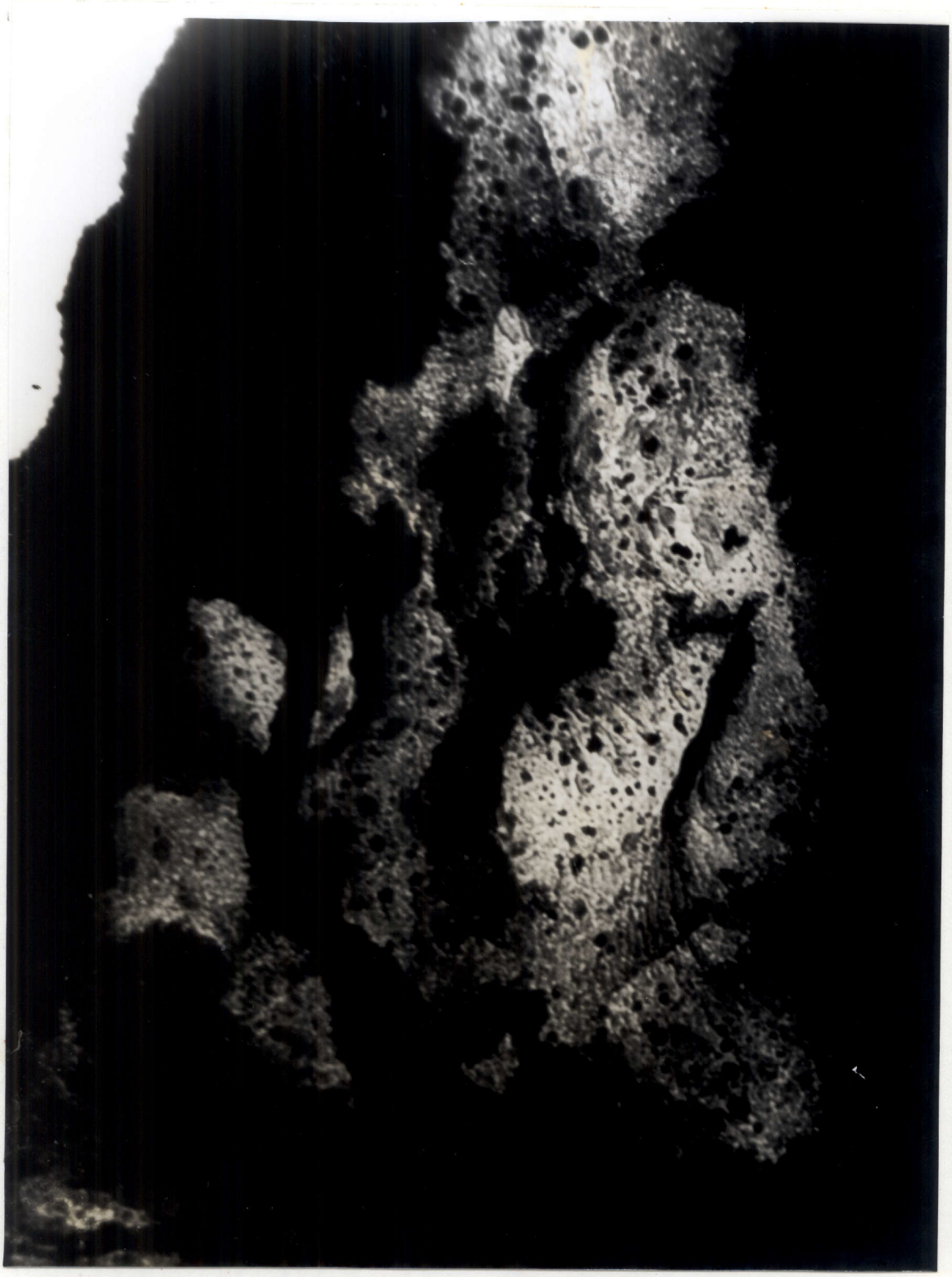


Figure 27. Carbon replica of thymocytes obtained from leukemic 8 - 9 month old male AKR/J mice absorbed with anti-pre-leukemic AKR/J surface proteins as described in the Methods section. Black knobs on the cell surface represent interaction of the antisera with surface proteins. (Total magnification, 96,000X).

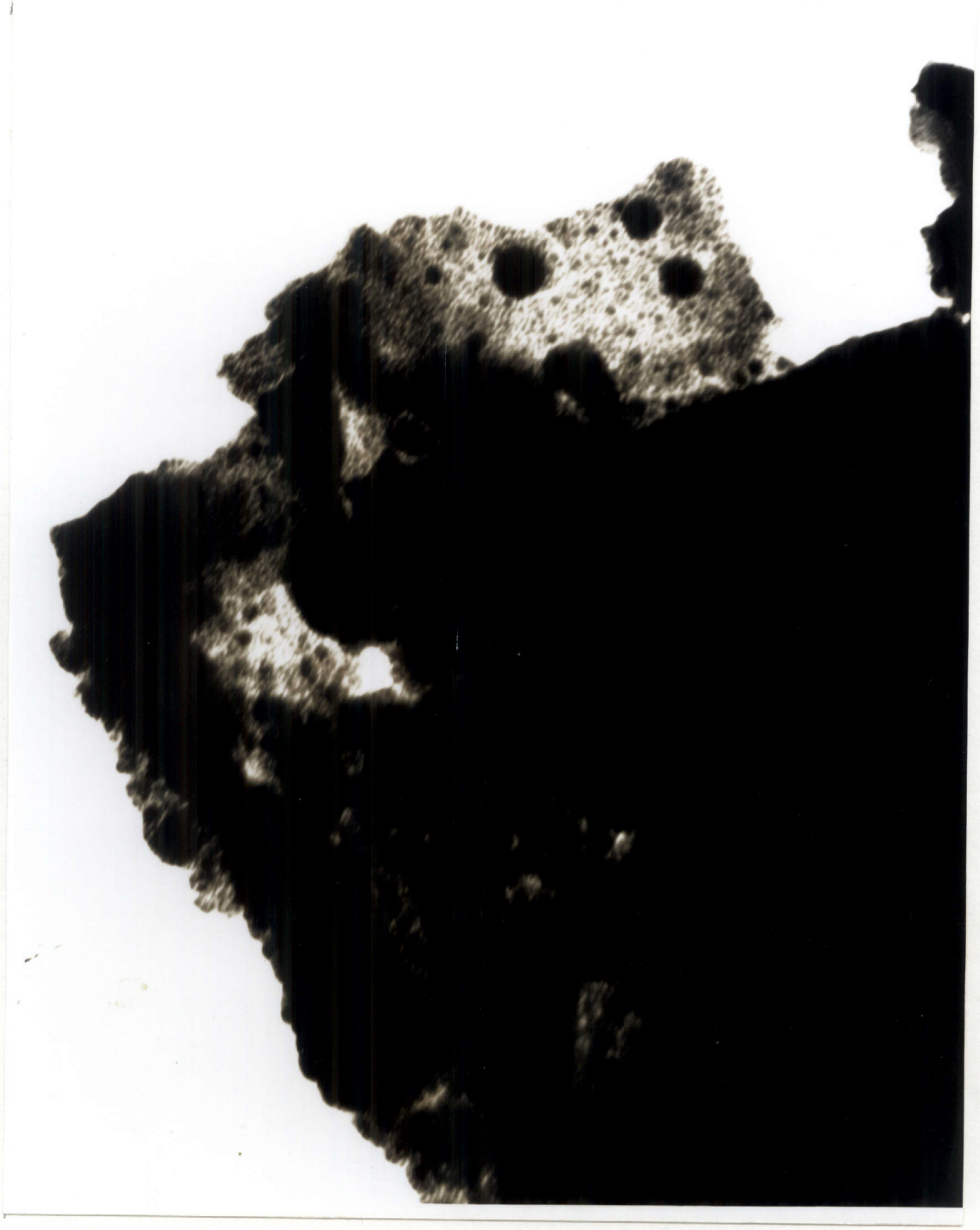
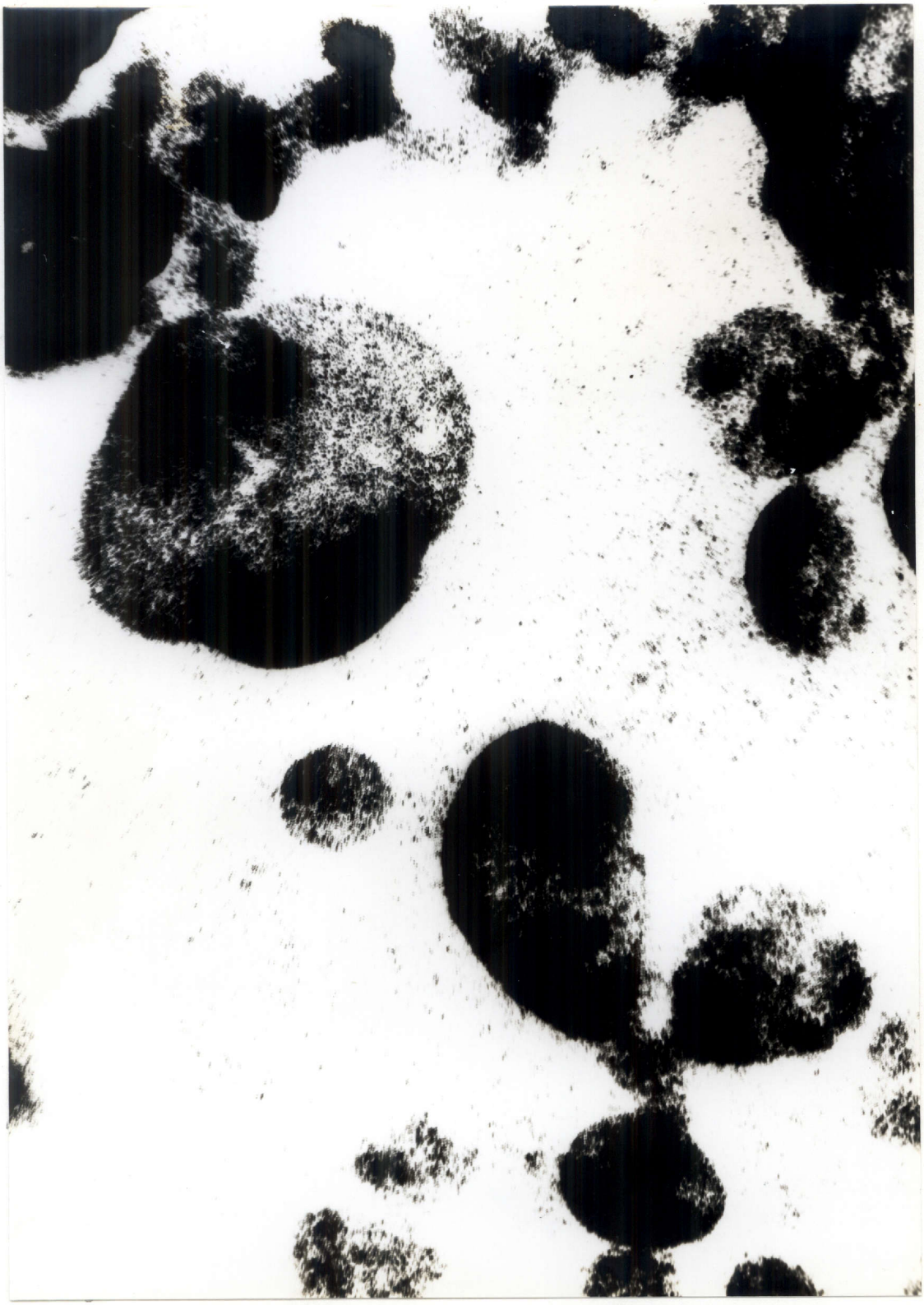


Figure 28. Carbon replica of thymocytes obtained from leukemic 8 - 9 month old male AKR/J mice absorbed with anti-BALB/C surface proteins as described in the Methods section. Black knobs on the cell surface represent interaction of the antisera with surface proteins. (Total magnification, 62,650X).



Thymocytes derived from 8 - 9 month old male BALB/C mice were absorbed with anti-BALB/C and these T cells demonstrated the most number of "knobs" as seen in Figure 29, as compared with BALB/C T cells coated with anti-leukemic AKR/J (Figure 30), anti-pre-leukemic AKR/J (Figure 31), or anti-C3Heb/FeJ (Figure 32).

Molecular Weight Determination

Polyacrylamide disc gel electrophoresis was performed according to the method of Fairbanks et al. (1971). A single band was observed for protein 7 on both 5.6% and 10% polyacrylamide concentration. Calculation of a molecular weight of 31,700 was made from mobility of standards and plotted in Figure 24.

Figure 29. Carbon replica of thymocytes obtained from 8 - 9 month old male BALB/C mice absorbed with anti-BALB/C surface proteins as described in the Methods section. Black knob on the cell surface represent interaction of antisera with cell surface proteins. (Total magnification, 62,650X).

Figure 30. Carbon replica of thymocytes obtained from 8 - 9 month old male BALB/C mice absorbed with anti-leukemic AKR/J surface proteins as described in the Methods section. Black knobs on the cell surface represent interaction of antisera with proteins on the cell surface. (Total magnification, 44,320X).

Figure 31. Carbon replica of thymocytes obtained from 8 - 9 month old male BALB/C mice absorbed with anti-preleukemic AKR/J surface proteins as described in the Methods section. Black knobs on the cell surface represent antisera interaction with proteins on the cell surface. (Total magnification, 62,650X).

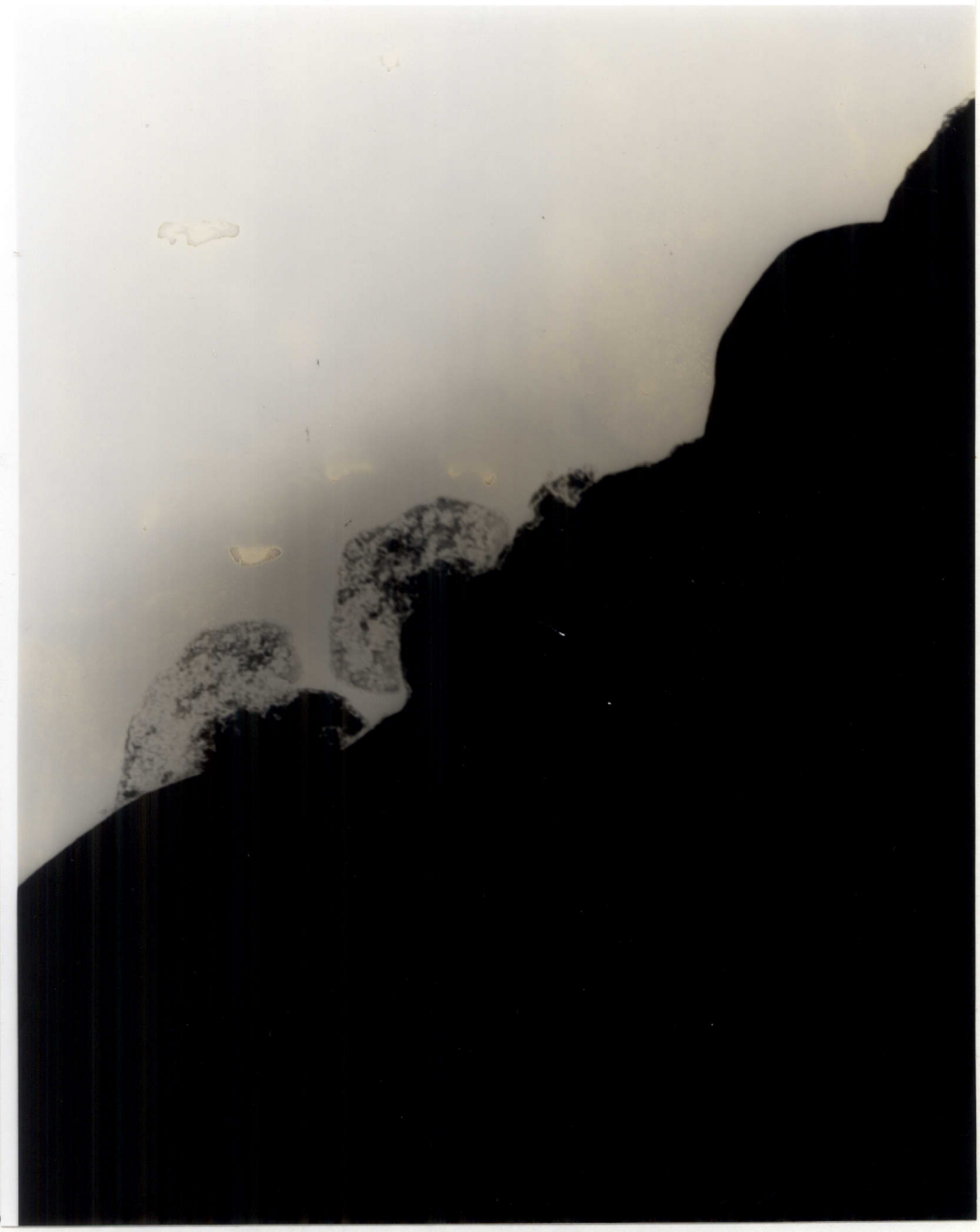
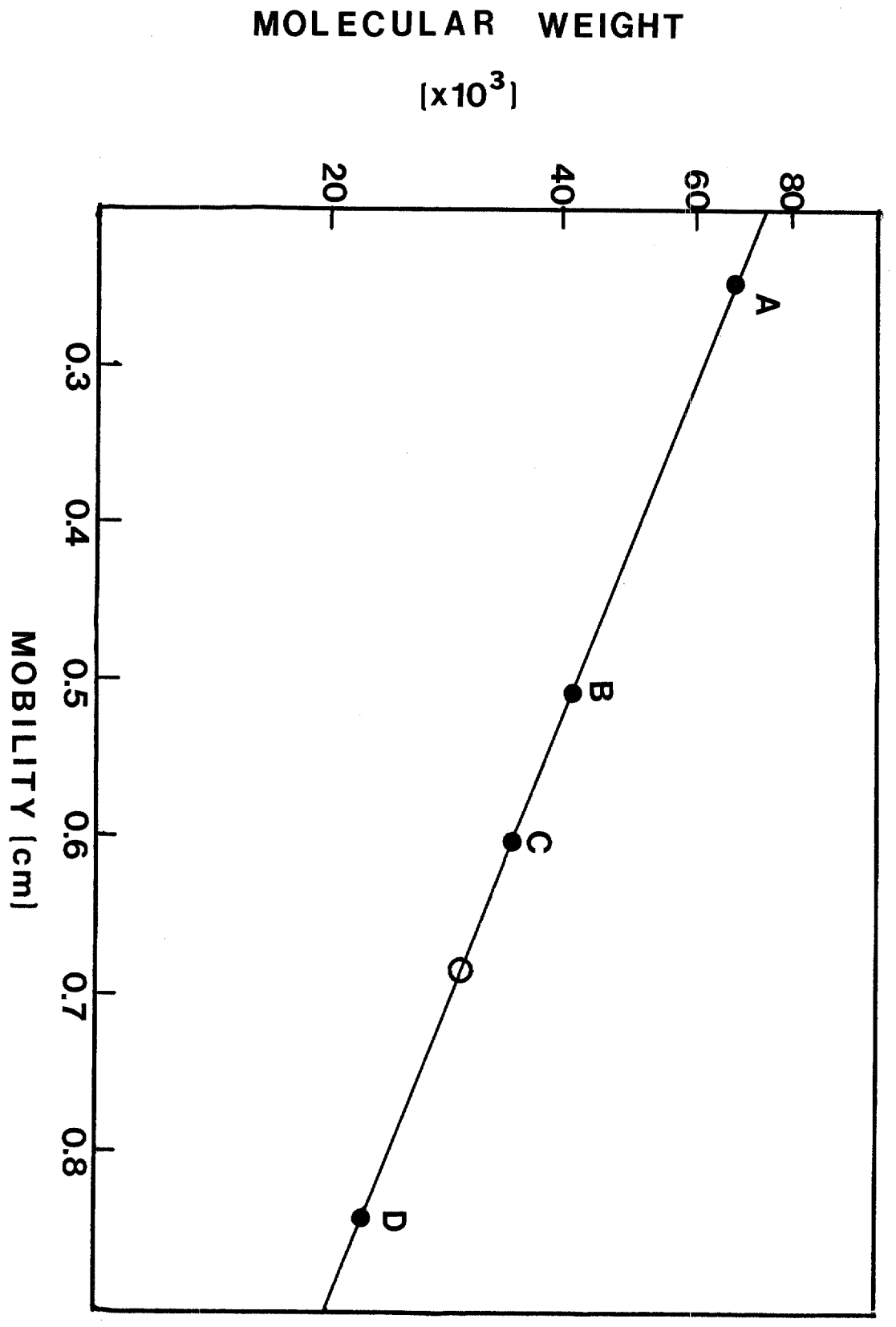


Figure 32. Carbon replica of thymocytes obtained from 8 - 9 month old BALB/C mice absorbed with anti-C3Heb/FeJ surface proteins as described in the Methods section. Black knobs on the cell surface represent interaction of antisera with proteins on the cell surface. (Total magnification, 87,400X).

Figure 24. Sodium dodecyl sulfate gel electrophoresis of protein 7. Protein 7 and standard proteins were prepared as described in "Methods" and subjected to electrophoresis (5.6%, 3 hours; 10%, 5 ma, 5 hours). The mobility of each protein was plotted against the log of its molecular weight. Albumin, molecular weight 68,000, is represented by a; ovalalbumin, molecular weight 43,000, is represented by b; pepsin, molecular weight 35,000, is represented by c; and trypsin, molecular weight 23,000, is represented by d. Protein 7 is shown as an open circle.


MOLECULAR WEIGHT
[x10^3]
20
40
60
80
A
B
C
D
0.3
0.4
0.5
0.6
0.7
0.8
MOBILITY [cm]

CONCLUSIONS

Analysis of papain extracted proteins isolated from the surface of murine lymphocytes obtained from AKR/J, BALB/C, and C3Heb/FeJ mice by polyacrylamide gel isoelectric focusing has demonstrated distinct differences in the level of certain proteins as a function of age, onset of leukemogenesis, strain, and sex of the mice.

These studies describe alterations in the surface protein expression on spleen and thymus cells from 2 - 9 month AKR/J strain of mice. An age-related decrease is noted for AKR/J spleen cells in Bands 3 and 5 with an increase recorded for Bands 2 and 6. A decrease in relative density is observed for protein Band 2 for the thymus cells which coincides with the onset of leukemogenesis at 6 - 7 months of age. Similarly, an increase is noted in the relative density of Bands 1, 4, and 6 at this age. These findings concur with Kawashima et al. (1976), who report from immunofluorescence studies elevated levels of viral antigens on the cell surface of AKR/J T-cells as a function of aging.

Alterations of surface proteins on BALB/C spleen cells from 2 - 9 month old female mice demonstrate a decrease in Bands 2, 5, and 6, while an increase in relative density in

Band 3 is recorded. The BALB/C thymus samples are characterized by a decrease in Band 4. The BALB/C strain is a non-Murine Leukemia Virus-carrying strain as shown by Peters et al. (1972) and Lilly et al. (1964); thus, it can be concluded that the surface proteins present on BALB/C spleen and thymus cells are representative of differentiation and sex-related antigens only. These findings (Eisinger and Hatten, 1977a) are similar to the reported changes in differentiation antigens as a function of aging by immunocytology analysis by Kawashima et al. (1976) and Waksal et al. (1975).

Age-related comparison of alterations in common bands which were shown by serological assay to be present in both AKR/J and BALB/C thymus samples can be concluded. The AKR/J sample shows a decrease in relative density in Band 2, with an increase in Bands 1, 4, and 6, while the BALB/C is characterized by decreases in Band 4. Similar differences were seen in the spleen samples as a function of age: AKR/J spleen denotes a decrease in Bands 3 and 5, an increase in Bands 2 and 6; female BALB/C sample show a decrease in Bands 2, 5, and 6, and and an increase in Band 3. This alteration in production of probable differentiation-related antigens in the AKR/J strain suggests a preferential synthesis among these type of antigens.

A third strain, C3Heb/FeJ, which is a congenic resistant murine strain (Green, 1968) was compared with the two mouse strains to determine what affect carrying MuLV, but not developing leukemia, would be expressed in the surface antigen profile. The results indicate the expression of Bands 1 - 6, which represent differentiation, sex, and MuLV associated surface proteins.

Comparison of thymus samples from 7 - 8 month old female AKR/J, BALB/C, and C3Heb/FeJ mice demonstrates Bands 2, 3, and 4 common to all samples. Bands 3 and 4 were present to a larger extent in the AKR than the other strains. Band 2 was present in greater concentration in the BALB/C sample. Bands 1a - 1c, 5, and 6 are limited to expression in the MuLV carrier strains only. These findings (Eisinger and Hatten, 1978a) concur with the immunofluorescence studies of Cloyd et al. (1977a,b) and immunocytology findings of Kawashima et al. (1976) and Waksal et al. (1975), which suggest a preferential synthesis of MuLV antigens in AKR/J T-cells rather than in differentiation related antigens as a function of aging.

Sex-Related Differences

Differences in protein patterns have been observed as a function of the sex of the mice for AKR/J, BALB/C, and C3Heb/FeJ derived spleen and thymus tissues. In those age groups in which both sexes of mice were available, the

protein patterns obtained following polyacrylamide gel isoelectric focusing and ensitometer scanning demonstrated an additional protein band in the male samples. These findings follow those proposed by Wachtel (1978) and reviewed by Gasser and Silvers (1974) in which a male specific cell surface molecule was present on cells which was immunologically identifiable. These authors suggest the cell surface protein to be encoded for the Histocompatability Y gene. The H-Y surface molecule has been shown to be responsible for male-to-female skin grant incompatability and rejections, and thus represents the sex genetic differences at the level of cell surface interaction with the other host cells (Wachtel, 1978; Gasser and Silvers, 1974).

Separation of Individual Proteins

Isolation of individual proteins from the extracted group of surface proteins in each sample was accomplished by flat bed granulated gel electrofocusing. Optical density profiles obtained from the individual fractions substantiated the number of surface proteins recorded by the densitometer scans following PAGIF of the 7 - 8 month old sample from female AKR/J mice. Similar findings obtained from both procedures demonstrate six surface proteins (Bands 1 - 6) are present in the AKR/J thymus sample. In adddition, one protein

(Band 7) was recorded by the flat bed granulated gel electrofocusing technique to be present only on the leukemic AKR thymus cell surface (Eisinger and Hatten, 1978b). Band 7 has a molecular weight of 31,700 Daltons and could possibly represent a leukemia-associated surface protein.

An increased sensitivity for the Ultrodex^{R} as suggested by Peterson (1977) was found compared to the polyacrylamide gel. The application of the granulated gel technique for the separation of proteins for more quantitative investigation of the molecular nature of the individual surface proteins present on normal and neoplastic cells has been suggested (Eisinger and Hatten, 1978b).

Passive hemagglutination findings confirm the presence of Proteins 1, 5, and 6 to be limited to the MuLV carrier strains -- AKR/J and C3Heb/FeJ. Proteins 2, 3, and 4 were shown to be shared by all three strains with the greatest concentration of Protein 2 on leukemic and pre-leukemic AKR/J spleen and thymus cells. The presence of Protein 7 was again shown to be limited to the pre-leuekmic and leukemic AKR/J spleen and thymus samples as suggested by the results of the granulated gel electrofocusing.

Immunoelectron microscopy studies demonstrated the AKR/J and BALB/C thymocytes to be spheroid in shape with a smooth

to slightly irregular cell surface. These findings concur with studies performed by Polliack et al. (1973) and Matter et al. (1972) which described digitations on T-cells lending to a slightly rough or stublike appearance to the outer cell membrane. This observation compared to B cells which are "woolly" in appearance as a result of large microvilli on the cell surface. The surface morphology was demonstrated by SEM studies to vary from smooth to more irregular in nature, depending on the stage of differentiation of the particular thymocyte (Polliack et al., 1973). Similarly, de Harven et al. (1973) have reported from scanning electron microscopy analysis of murine leukemia virus infected erythroleukemia cells the presence of small "knobs" approximately 100 nm in diameter on the surface of these cells. These "knobs" are randomly scattered on the cell surface and represent budding MuLViruses. In addition to the entire virus being present on the cell surface, other viral-related components and differentiation-related antigen determinants can be expressed on the surface as proposed by Nicolson (1976).

Localization of specific surface proteins by appropriate antisera coupled with Goat anti-Rabbit IgG and Hemocyanin have been observed in this study by transmission electron microscopy. Observation of "knobs" following carbon replication and carbon shadowing procedures allow the following conclusions to be made. The first is the verification that

the proteins isolated and extracted with papain earlier in this investigation were indeed surface proteins on the spleen and thymus cells. This conclusion can be drawn, since the proteins isolated were injected into rabbits and the antisera obtained is localized by the IEM procedure on the cell membrane of spleen and thymus cells. A second conclusion verified by the immunoelectron microscopy study is the existence of several proteins common to surface of thymus cells from AKR/J, BALB/C, and C3Heb/FeJ strains of mice. Specifically, this was shown by the absorption of AKR/J leukemic thymocytes with anti-BALB/C or C3Heb/FeJ antisera, and the observation of "knobs" following IEM procedures. A cross-check of the appearance of common proteins was conducted by absorbing BALB/C thymocytes with antileukemic AKR/J or C3Heb/FeJ antiserum. Again, several "knobs" were observed. These common proteins probably represent Proteins 2, 3, and 4 as seen from the PAGIF and granulated gel electrofocusing findings and may be differentiation related surface proteins.

A third conclusion from the IEM findings is the decrease in the relative number of "knobs" recorded for the AKR/J thymocytes coated with anti-BALB/C serum as compared to anti-leukemic AKR/J, pre-leukemic AKR/J, or C3Heb/FeJ

sera. This finding suggests the presence of surface proteins common to the AKR/J and C3Heb/FeJ cell surface which are lacking from the BALB/C thymocyte surface. These proteins must, therefore, be the Murine Leukemia Viral-associated membrane proteins which have been observed by others using immunofluorescence (Burk et al., 1977; Cloyd, 1977a,b), immunocytology (Waksal et al., 1975; Kawashima et al., 1976), and immunoelectron microscopy techniques (Kennel, 1975; Aoki, et al., 1970).

In summary this study demonstrates differentiation-related and Murine-Leukemia Virus-related protein molecules on the cell surface of murine leukemic AKR/J lymphocytes. Quantitative results show a preferential synthesis of the Murine Leukemia Virus associated antigens rather than differentiation-related antigens with the onset of cell neoplastic transformation in six to seven month-old AKR/J cells. This theory has recently been proposed by Waksal et al. (1975), Pincus and Snyder (1975) and Kawashima et al. (1976) and is reported in this present study by polyacrylamide gel isoelectric focusing of spleen and thymus extracted surface proteins from different age mice. In addition, a preferential synthesis among the differentiation related antigens is reported. This alteration in surface antigens on the lymphocyte cell membrane interfere with direct cell to cell contact and this offers a possible explanation for the immunosuppression associated with

malignancy, as suggested by Stutman (1975) and Kolb et al. (1977). The exact role the virus associated surface proteins play in immunosuppression is not known (Cimprich et al., 1978), but an effect on B and T cell cooperation or lack thereof is proposed.

The isolation of a specific Protein 7 (Molecular weight 31,700) from AKR/J spleen and thymus cells suggests the possibility of the identification of a leukemia-associated surface protein on murine lymphocytes. The correlation of specific cell surface proteins with malignant and non-malignant diseases has been previously suggested by Dausset (1972). The isolation and characterization of tumor specific transplantation antigen (TSTA), carcinoembryonic antigen (CEA), and fetal embryonic antigen (FEA) has been important for the serodiagnostic detection of human carcinomas and sarcomas (Mitchison, 1977). Cloyd and his colleagues (1977 a,b) have recently suggested a significant immunological role for the gp69 major MuLV glycoprotein on murine, feline, and Gibbon age lymphoma cell lines. The complete characterization and identification of the specific antigenic moiety isolated in this study may provide an "early warning" marker for rapid diagnosis of murine and human leukemic cells. These speculations are offered by the author for the consideration of others working in the area of leukemia research. The characterization of

alterations of cell surface proteins can lead to a better understanding of the interaction between neoplastic and normal cells and neoplastic cells and the host immune system. This would answer some of the questions dealing with the cell, the neoplastic disease process, and its eventual control.

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