AN IMMUNOLOGICAL STUDY OF SOLUBLE HUMAN LEUKEMIA ASSOCIATED ANTIGENS

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by

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ABSTRACT

The aim of this research work was to isolate and immunochemically characterize the soluble leukemia associated antigen in human leukemic liver tissues.

Soluble antigens were obtained from the liver of myelocytic and congenital human leukemia cases by hyaluronidase treatment of tissues, extraction with 1M KCl, purification by isoelectrofocusing and prolonged gel electrophoresis.

The leukemia associated antigen (LAA) was identified in the electrofocusing fraction corresponding to the pI 4.85. The antiserum produced against this fraction, on absorption with pooled extracts of normal liver, was found on immunodiffusion to react with 6 out of 9 preparations, four of which were derived from myeloid and one from congenital leukemia. In contrast, the absorbed antiserum did not react with lymphoid leukemia extracts or normal extracts.

The pI 4.85 fraction containing the LAA was purified further by using a prolonged technique of electrophoresis in agarose-gel.

The LAA was located in the β_1 position. The purified LAA was eluted from the cut out portion of gel in 0.01M PBS at pH 7.4.

A physico-chemical analysis of the purified antigen preparation by chromatography and by means of an Amino Acid Autoanalyzer has revealed the presence of galactose and of thirteen different amino acids.

The results have therefore demonstrated the presence of a unique water soluble antigen closely associated with human leukemia in liver extracts obtained from four cases of myelocytic leukemia and one congenital leukemia.

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INTRODUCTION

Human leukemia is a condition characterized by a neoplastic change in blood forming tissues. In this disease there are many more leucocytes than normal and they tend to grow in large deposits in various parts of the body.

The occurrence of tumors in experimental animals is often associated with the appearance of "new" antigens. Such new antigens will be referred to as tumor antigens in the following material. This may reflect the results of gene activation or mutation or infection with oncogenic virus. Virus-induced tumors in animals have been shown to possess tumor antigens (specific to the virus although not necessarily part of the virus) and in contrast, the tumor antigens from chemically induced tumors rarely cross-react, even if induced by the same chemical agent in the same animal genotype, and in cases where multiple primary tumors have been tested, even if induced in the same individual animal.

In most of the investigations on tumor antigens, the identification of a constituent as tumor-specific will depend entirely on the nature and composition of the normal tissue used for comparison, i.e., the controls. Any tissue component may appear as tumor-specific, if the normal tissue does not contain it, or contains it in a

concentration insufficient for recognition by the antibodies available or by the immune system of a non-tolerant animal. It has been shown that tumor antigens, including those of viral origin may indeed be present in very low concentrations on normal tissues and some in embryonic tissues as is the case of the carcino-embryonic antigens first described by Gold in 1965.

If tumor associated antigens are found to be characteristic of cancer cells, the elucidation of the mechanisms underlying their appearance de novo or in higher concentrations on tumor cells will contribute to the understanding of oncogenesis.

The detection of leukemia associated antigens in afflicted humans may provide a useful tool in the early diagnosis of the disease. The study of human leukemia antigens may also yield information about the etiology of human leukemia. This knowledge may eventually lead to improved means of prevention and treatment.

Unlike animal leukemias, where the causative agents and immunological methods for the detection of leukemic antigens have been extensively studied, the immunobiological properties of human leukemia are scarcely known. One of the prime objectives of the immunological study of human leukemia is the recognition of leukemia associated antigens, their genetic origin and chemical nature. This will provide us with a deeper appreciation of the events

leading to the emergence of the malignant phenotype and perhaps with more rational approaches to immunotherapy.

Evidence for the occurrence of soluble antigens associated with human leukemia thus far has been obtained by examination of crude extracts obtained from white blood cells, plasma and bone marrow or of partly purified materials obtained from human leukemia sources, all of which were produced in insufficient quantities for a thorough immunological examination. Therefore, the main emphasis in my approach in this work on leukemia associated antigen is to obtain larger amounts of leukemic tissues than had previously been worked with and to use more extensive purifications procedures.

A. REVIEW OF LITERATURE ON IMMUNOLOGY OF LEUKEMIA

Despite the lack of sufficient experimental evidence of an immunological analogy between the leukemic process in man and in other mammals or birds, the experience derived from immunological studies on the latter leukemias appear as pertinent to my studies; therefore, these data are being reviewed.

I. General Consideration on Animal Tumor

Under appropriate conditions, tumor viruses cause cells to grow in an uncontrolled, unregulated fashion and to loose their response to contact. In their relationship to one another, normal cells are orderly and respond to contact with their neighbours, while malignant cells do not respond. Normal cells are said to be "contact inhibited".

Malignant cells may also acquire an ability to divide without limitation on the number of generations, in contrast to normal cells, which seem to have a "built-in" predetermined limit to the number of divisions they can undergo (Hayflick, 1968).

Virus-induced tumor cells acquire one or more new antigens with specificities determined by the virus. The new antigens may be related to the changed morphology of the tumor cells and perhaps may be related to new enzymes. Metabolic patterns of the cells may also be changed, as evidenced by an increased production of lactic acid,

although the increased acid production may only be a reflection of a greater metabolic rate. In addition, some tumor viruses cause cells in culture to differentiate or to dedifferentiate.

In the usual context, a tumor is any abnormal growth. a benign tumor, such as a common wart, is localized and the cells are relatively regulated in their behaviour. In contrast, a malignant tumor, frequently called a cancer, usually grows rapidly, and its cells tend to invade adjacent tissues. The tumor may erode through blood vessel walls, allowing malignant cells to be carried by the blood to distant sites in the body.

Malignant tumors are called sarcomas if they arise from meso-dermal derivatives and carcinomas if they arise from ecto-dermal or entodermal derivatives. Leukemia is usually considered to be a form of malignancy in which the malignant cells are elements of the white blood cell genes.

II. General Considerations on Animal Tumor Viruses

Tumor viruses are divisible into those that contain RNA and those that contain DNA. The RNA-containing tumor viruses (see Table I) seem to fall into a natural group resembling the myxovirus-paramyxovirus group, whereas the DNA-containing tumor viruses are in the pox, adeno, and polyoma virus groups.

(i) Tumor viruses that contain RNA

These RNA containing tumor viruses are commonly

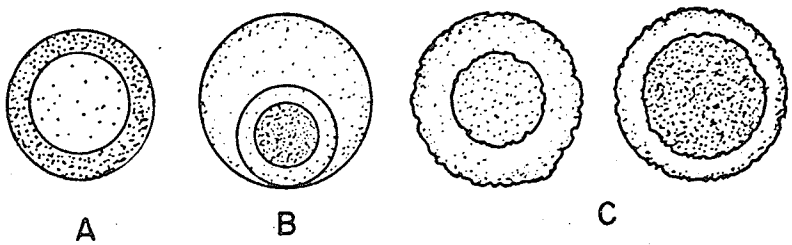


Figure 1. Morphological types of RNA tumor viruses as they appear in sectioned material examined by electron microscopy.

TABLE I. REPRESENTATIVE RNA-CONTAINING TUMOR VIRUSES

TYPE OF VIRUS	KINDS OF TUMORS	ANIMALS FOR IN VIVO TUMORS	ANIMALS FOR IN VITRO TRANSFORMATION
Chicken Viruses			
Avian Leukosis Complex	Leukemia; erythroblastosis; osteopetrosis; lymphomatosis; kidney, ovarian, and other carcinomas and sarcomas	Chicken	Chicken
Rous Sarcoma Virus	Fibrosarcoma	Chicken, hamster, turkey	Chicken
Bryan Strain,	Fibrosarcoma	Chicken, rat, hamster, mouse, guinea pig, rabbit, marmoset, Chinese hamster	Chicken, rat, guinea pig, mouse, human, monkey
Schmidt-Ruppin Strain			
Carr Strain	Fibrosarcoma	Chicken, cotton rat, monkey	
Mouse Viruses			
Murine Leukemia			Rat
Gross (Graffi, Moloney, etc.)	Lymphoid leukemia	Mouse, rat	
Friend (Rauscher, etc.)	Erythroblastosis, reticulum cell sarcoma	Mouse	
	Lymphoid leukemia	Rat	Mouse, hamster, rat
Murine Sarcoma Virus	Sarcomas, rhabdomyosarcomas	Mouse, other rodents	Mouse
Mammary Tumor Virus (Bittner)	Adenocarcinoma	Mouse	
Cat Virus			
Feline Leukemia Virus	Leukemia	Cat	
Guinea Pig Virus			
Guinea Pig Leukemia	Leukemia	Guinea pig	

referred to according to their morphology by electron microscopy as type A, B, or C particles (see Figure 1).

All RNA tumor viruses seem to mature at the cell membrane by budding, as do the myxoviruses, and all have many structural features common to the myxoviruses. However, unlike the myxoviruses, no RNA tumor virus causes visible cytopathic effect. The production of virus becomes a new "excretory" function of a transferred cell, which it performs without dying in the process.

The RNA tumor viruses are transmitted "vertically", e.g., the lymphomatosis virus (Rubin, 1962b; Rubin et al., 1961, 1962), the Gross leukemia virus (Gross, 1951b, 1952, 1961, 1962a), the Moloney leukemia virus (Ida et al., 1965) and the mammary tumor virus (Bittner, 1936) are transmitted from parent to offspring through the egg, sperm or milk. They can also be transmitted "horizontally" in some cases by the method of contact with excreta containing viable virus.

(a) The avian leukosis complex. The first tumor virus to be discovered (Ellermann and Bang, 1908) causes leukemia in chickens. It is now known that chicken leukemias and related tumors are caused by a group of viruses, the avian leukosis complex. Serologically, these viruses are difficult to distinguish from one another, as they share common antigens.

This fundamental observation (Ellermann and Bang,

1908, 1909) did not at the time attract sufficient attention, neither was its importance immediately realized. Amply confirmed, and soon accepted as a fact, this unexpected discovery was considered as indicative of the infectious nature of chicken leukemia, thus clarifying this disease of the fowl apart from leukemias and related tumors in other species. Only gradually it became apparent that transmission of chicken leukemia by filtrates was the first break in the fundamental approach to the problem of tumors.

Beard et al. (1963) reviewed the properties in which he recognizes at least three strains of viruses that differ chiefly in their biological behavior. The BAI strain A causes myeloblastic leukemia in chickens, but occasionally also causes visceral lymphomatosis, osteopetrosis, and kidney tumors. Which type of tumor develops depends on the virus dose inoculated. At high doses leukemia results. At low doses, only a transient disturbance of blood cells develops, sometimes followed later in life by kidney tumors and tumors of other organs (Baluda and Jamieson, 1961). The RPL 12 strain (Beard et al., 1963) causes chiefly erythroblastosis in chickens (erythroblastosis is a proliferation of young and immature forms of red blood cells in the circulating blood). The virus also transforms bone marrow cells to myeloblasts in tissue culture (Beaudreau et al., 1960).

(b) <u>The Rous sarcoma virus</u>. In 1911, Rous described the cell-free transmission of a tumor-producing agent.

This discovery, which followed the earlier discovery of the leukosis viruses, showed that solid tumors, in addition to tumors of blood cells, can be caused by viruses.

Manaker and Groupé (1956) noticed that Rouse sarcoma virus caused cells in tissue cultures to grow into localized foci. This observation served as a starting point for the development of a tissue culture assay for the virus (Temin and Rubin, 1958), which made it possible to do more detailed experiments at the level of individual cells.

Most of the work with Rous sarcoma virus has been with two strains (Rubin, 1964a). The Bryan strain grows to high titers but may be defective, requiring another of the avian viruses as a helper in order to replicate (Rubin and Vogt, 1962). The Schmidt-Ruppin strain does not seem to be defective and can replicate without helper virus.

The Bryan strain produces tumors almost exclusively in fowl (chickens and turkeys: Spencer and Groupé, 1962; Japanese quail: Pienta and Groupé, 1967), although exceptions include production of tumors in hamsters (Rabotti et al., 1962; Eidinoff et al., 1965). In contrast, the Schmidt-Ruppin strain produces sarcomas in fowl and in many mammalian species (Deinhardt, 1966; Ahlström and Forsby, 1962; Ahlström et al., 1964). It also causes malignant change and chromosomal abnormalities in human cells in culture (Jensen et al., 1964; Nichols et al., 1964).

The Carr strain produces sarcomas in cotton rats (Svet-Moldavsky and Svet-Moldavskaya, 1964) and monkeys (Munroe and Windle, 1963).

(c) Murine leukemia viruses: All work with leukemia viruses before 1951 was done using chickens, and it was generally believed that the avian model had little or no relevance for mammalian or human leukemia. In that year, however, Gross (1951, 1952) reported his success in passing leukemia from one mouse to another with cell-free extracts, thus providing a mammalian analogy to the chicken leukemia virus. The virus he isolated has been studied extensively in mice. The Gross leukemia virus has been shown by electron microscopy to resemble the Rous sarcoma virus (Parsons, 1963).

The murine leukemia viruses include many separate isolates, which seem to fall into two groups on the basis of their biological properties. Gross passage A virus induces many different forms of leukemia in mice or rats. The most common form of leukemia is lymphatic, but other forms also occur, depending largely on the genetic susceptibility of the host. Many of the other strains of mouse leukemia viruses resemble Gross' virus in the form of pathology produced. At least one causes proliferation of immature red blood cells rather than white blood cells and is called erythroblastosis virus (Kirsten et al., 1967). It relationship to other murine viruses is not clear.

The other major groups of strains resembles the virus isolated by Friend (1959) and causes erythroblastosis as well as solid tumors, such as reticulum cell sarcoma.

Some workers (Gross, 1965a) believe that only two different murine leukemia viruses have been isolated and that all other isolates represent reisolation of one of these.

(d) Other leukemia viruses. Leukemia is a common disease of mammals. In addition to the virus-induced leukemias already discussed, it has also been shown that guinea pig leukemia is virus-induced (Opler, 1967a, 1967b).

Lymphosarcoma is quite common in cats. It has been estimated that leukemia lymphosarcoma and related tumors of the hematopoietic system represent about 9% to 15% of the total of all malignant tumors in that species (Cotchin, 1952, 1957; Jarrett, 1966).

Jarrett et al. (1964) first reported successful transmission of lymphosarcoma in cats by a centrifuged, presumably cell-free extract. The extract was prepared from a field case of spontaneous lymphosarcoma which developed in an 8 1/2-year-old female cat. Tissue fragments from the mediastinal tumor were removed aseptically, stored first at -40°C for 5 days, then placed in 50% glycerin at -10°C for 66 days. The extract was prepared from the stored tissues by grinding in a mortar, and then centrifuged at 2000 X g for 20 minutes.

The supernatant fluid was inoculated subcutaneously into 4 kittens of a litter less than 12 hours old. After 6 months, the inoculated cats had palpable lymph nodes, and 2 of them also developed large spleens. All 4 cats had disseminated lymphosarcoma including large thymic tumors, large spleens, and large mesenteric tumors. The predominant leukemic cells revealed on microscopic examination of the unfiltrated organs was of the primitive stem-cell type.

A second cell-free passage was then made with a tumor removed from one of the experimental animals in which lymphosarsoma had been induced. In the second passage, the centrifuged extract induced lymphosarcoma in the inoculated kittens after 8 weeks (Jarrett, 1966). A further passage was obtained with the original field material using a centrifuged cell-free extract. In this transmission experiment, two inoculated kittens developed, and died from acute blast-cell leukemia after a latency of 6 months (Jarrett, 1966).

In another similar study, Rickard and his colleagues (1967) prepared a leukemic cell suspension from a large mediastinal lymphosarcoma which developed spontaneously in a one-year-old male Siamese cat.

Kawakami et al. (1967) also demonstrated transmission of leukemia in cats by cellular and by cell-free extracts.

It is apparent, therefore, that cat lymphosarcoma could be readily transmitted by cell-graft or by cell-free

centrifuged extracts to newborn cats.

Transmission of lymphosarcoma by cell-graft to irradiated puppies was reported (Moldovanu et al., 1966). All three recipients were newborn mongrel dogs of unknown origin. As a preliminary treatment, the dogs received 85 to 128 R total body irradiation. Repeated inoculations of leukemic cell suspensions were made after the irradiation.

Progressively growing disseminated lymphosarcomas developed in the inoculated dogs after a latency varying from 1 to 3 months. One inoculated dog developed generalized lymphosarcoma after 26 days, and the second dog after 90 days, following inoculation of cell suspensions prepared from donor dogs with spontaneous lymphosarcomas. The third animal developed generalized lymphosarcomas after 44 days, following inoculation of a canine lymphosarcoma cell line, LS No. 30, which had been carried in tissue culture for almost one year. In previous attempts, 35 adult dogs had been inoculated with dog lymphosarcoma cells, and none developed symptoms of the disease.

In more recent studies, two lymphosarcomas which developed spontaneously in adult dogs were carried serially through 6 transplantations in irradiated newborn pups (Moldovanu et al., 1968). The inoculated dogs developed lymphosarcomas at the site of inoculation after a short latency of only two weeks.

Kakuk et al. (1968) also reported two serial

passages by cell-graft of a malignant lymphoma in dogs. Cell suspensions prepared from a spontaneous lymphoma which developed in a 20-month-old Doberman Pinscher female were inoculated into 2 litters of newborn Beagles. Three out of 15 inoculated dogs developed lymphomas 2 to 3 months after inoculation.

As more animal leukemias are studied, it is found that many have virus-like particles associated with them. Although virus-like particles have been found by electron microscopy, even in human cases of leukemia (e.g., Viola et al., 1967; Moore et al., 1966), it cannot be determined whether the particles have biological activity and therefore it is impossible to say whether they have any association with the disease. Furthermore, mycoplasma and even cell debris can be indistinguishable from leukemia viruses. The investigator pursuing this type of search must be alert to the many artifacts that can be misleading.

(e) Mammary tumor virus. A model system for human breast cancer became available with the discovery that at least some kinds of mammary tumors in mice are caused by an agent secreted in the mother's milk (Bittner, 1936).

Recently the virus was isolated and purified (Lyons and Moore, 1962, 1965; Moore et al., 1959; Nowinski et al., 1967). Electron microscopy of the isolated virus shows it to have an envelope and an internal core or nucleoid. Typically, the core is eccentrically located in the envelope.

The envelope has many radial spikes or projections on the outer surface, somewhat similar to influenza virus. In addition to protein, the virus contains about 27% lipid and 0.8% RNA, which is equivalent to a molecular weight of about 3.7×10^6 daltons of RNA (Lyons and Moore, 1965).

The presence of the virus and estrogenic hormones are necessary for tumors to develop (Moore, 1967). Female mice deprived of estrogens by removal of the ovaries have a decreased incidence of mammary tumors. Male mice have a low incidence of tumors despite the presence of virus, but if estrogens are administered to them, the males have a greatly increased incidence of tumors (Hall and Moore, 1966).

One of the most striking of the new properties acquired by viral-transformed cells is their loss of contact inhibition (Rubin, 1961; Stoker and Rubin, 1967). This deregulation is probably the essence of the oncogenic process.

(ii) Tumor viruses that contain DNA

The DNA-containing viruses that are known to induce tumors are in the pox, polyoma-papilloma, adenovirus and herpesvirus groups. All the known DNA tumor viruses cause solid tumors, usually sarcomas. None has been found that induces leukemia or other blood malignancies.

(a) Pox viruses. Borrell in 1903 postulated that cancer was viral induced. He observed that cancer is unregulated cell growth, that viruses propagate in cells and

usually kill them, but that certain pox viruses induce a temporary stage of cellular proliferation before the cells die.

Viruses of the pox group other than those observed by Borrell produce a cellular proliferation that more clearly qualifies as a tumor. One of those is the Yaba virus (Andrewes et al., 1959; Niven et al., 1961), isolated from tumors that appeared in a colony of rhesus monkeys in Yaba, Nigeria (Bearcroft and Jamieson, 1958). The tumors are usually about 2 to 5 cm in diameter (Sproul et al., 1963). They occur on the head, face, or limbs of monkeys, and they regress spontaneously in 1 to 3 months. The monkey develops permanent immunity to reinfection.

The DNA virus that causes molluscum contagiosum is also considered to be a tumor virus. The tumors, occurring only in human skin, are small nodules (about 2 mm in diameter) that may persist for several months before regressing spontaneously. Virions are present in the tissue and can be seen by electron microscopy in thin sections (Hasegawa, 1964; Middelkamp and Munger, 1964). The virus appears to induce interferon synthesis in chick embryo cell cultures (Friedman-Kien and Vilcek, 1967).

Shope (1932a, 1932b) described a virus that causes fibromas in rabbits. The virus is closely related to the myxoma virus that infects rabbits. Although information is limited, the viruses seem to be different in certain

biological characteristics. In tissue culture, the myxoma virus produces plaques and the fibroma virus produces foci of proliferating cells (Padgett et al., 1962).

The pox viruses do not produce malignant tumors, but do cause self-limited cellular proliferation.

(b) Herpes viruses. Two viruses of the herpes group may induce tumors.

One is a virus that might be responsible for causing a renal carcinoma of certain leopard frogs. This is called the Lucke's virus because of Lucke's thorough investigation of the virus. The intranuclear inclusions that form in kidney cells and in cultured cells (Maes et al., 1967), the base composition of the viral DNA (Maes and Granoff, 1967; Morris et al., 1966), and electron microscopic morphology (Darlington et al., 1966) all suggest that the virus is of the herpes group.

A second candidate virus is often found in cultures derived from Burkitt's tumors. Cells from many of these tumors have been cultured (Epstein et al., 1965). On examining the cultures by electron microscopy, a high percentage of them are found to contain particles that are morphologically identical to viruses of the herpes group (Toplin and Schidlovsky, 1966; Yamaguchi et al., 1967; Hinuma et al., 1967; Hummeler et al., 1966). It has been possible to demonstrate the presence of virus by its interference with the replication of other viruses (Henle and

Henle, 1965) and by the presence of an antigen in the cells that reacts with antibody in the serum of patients with the tumor (Old et al., 1966).

Serologic tests have shown that people in all parts of the world have a high incidence of antibody to the virus in cultured cells (Gerber and Birch, 1967). There is also strong serologic evidence that having infectious mononucleosis causes the development of antibodies against the virus in the cultured cells (Henle et al., 1968).

Stewart et al. (1968) has successfully infected animals with this virus. Virus from freshly established cell lines from Burkitt tumors was inoculated intracerebrally in hamsters and could be passed serially from hamster to hamster. Inoculated hamsters develop central nervous system disease resembling that of other herpes viruses.

(c) Polyoma-papilloma viruses. All known viruses in this group except the K virus produce tumors (Kilham and Murphy, 1953). The papilloma viruses cause warts of the skin or mucous membranes in human, rabbit, cow, pig, dog and others (Olson, 1963; Rowson and Mahy, 1967). Morphologically these viruses are closely similar and they all seem to have a circular DNA molecule. They are, however, not closely related serologically (Le Bouvier et al., 1966).

The virus may be present in high concentration in the wart (Barrera-Oro et al., 1962). The rabbit papilloma, caused by the Shope papilloma virus, seems to be the only wart that regularly becomes malignant. After a rabbit

papilloma becomes malignant, the virus can no longer be isolated from the tumor. However, DNA extracted from malignant warts will cause tumors in rabbits (Ito, 1960, 1961; Ito and Evans, 1961, 1965).

Polyoma virus was discovered during experiments in which a Gross' leukemia agent was being passed in mice. In addition to the leukemia that occurred in some mice, parotid tumors also developed. After further cell-free passage of the unknown virus, other tumors also occurred (Stewart et al., 1957; Stewart, 1960), accounting for the name, polyoma, that was given to this virus that can cause many kinds of malignancies — sometimes several in a single animal.

Polyoma virus is widespread among the wild mouse population (Rowe, 1961), in which it causes little or no apparent disease. Due to its widespread nature and its ability to cause tumors only rarely under natural conditions, and because the tumors are of many kinds, polyoma virus has brought about renewed interest in the possibility that human malignancy is viral.

The vacuolating agent, SV 40, was isolated from cultures of rhesus monkey kidneys. SV 40 does not produce extensive cytopathic effect. However, in kidney cultures from African green monkeys, SV 40 grows actively and produces an extensive cytopathic effect consisting of vacuolations of the cells.

SV 40 causes malignant tumors in hamsters (Eddy et al.

1961, 1962; Girardi et al., 1962, 1963; Hsiung and Gaylord, 1961), but unlike polyoma-induced tumors, SV 40-induced tumors are slow to appear. In tissue culture, SV 40 causes transformation of cells from many species including man. Yet SV 40 has not been shown to induce tumors in its natural host species, the rhesus monkey.

(d) Adenoviruses. Trentin and his co-workers (Trentin et al., 1962; Yabe et al., 1962, 1963, 1964) were able to produce tumors in baby hamsters by the injection of human adenovirus type 12. Other workers, using the same approach, soon showed that human adenovirus type 18 (Huebner et al., 1962; McLeod and Ham, 1963), type 7 (Girardi et al., 1964), and type 31 (Pereira et al., 1965) also were oncogenic. Once a tumor has been induced, the virus can no longer be isolated from it.

Adenoviruses of other species also induce tumors, either in animals or in cultured cells of the monkey adenoviruses, six of 18 have demonstrated oncogenicity, and one of the three serotypes of bovine adenoviruses is oncogenic (Darbyshire, 1966). One of the two chicken adenoviruses is oncogenic (Sarma et al., 1965), and the virus of infectious canine hepatitis is oncogenic (Sarma et al., 1967).

From the foregoing discussion on tumor viruses, it appears that the tumor cell does not necessarily contain detectable infectious virus particles. Once transformed, the cell may contain only a part of the viral genome. This

property may explain why it has been so difficult to isolate viruses from human cancers. Another possibility is that human cancers may not be virus-induced. However, with the large number of cancers of lower animals that are known to be viral induced, and with the belief that uniformity exists among organisms, it would be surprising if at least some human tumors were not viral induced. Nevertheless, irrespective of the causes of human tumors, tumor associated antigens have been detected using serological as well as transplantation methods. This subject of tumor antigens has been reviewed in detail in the past (Gorer, 1961; Habel, 1969; Harris and Sinkovics, 1960; Klein, 1966; Klein, 1968; Klein, 1961; Law, 1970; and Old and Boyse, 1965).

III. Virological Aspects of Human Leukemia

Since the nature of the etiological agent of human leukemia has not been elucidated, it is necessary to examine critically all the published data on the particulate in addition to the non-particulate matter in a significant association with human leukemia.

In order to be etiologically related to human leukemia, an agent must be present in a recognizable form in a great majority of lymphoid-tissue specimens obtained from proven cases of leukemia. It must possess a unique antigenic specificity, possibly different from all other viruses, except the viruses associated with leukemia, and different from normal human antigens. It must also react specifically

with antibodies present in patients with leukemia (if such antibodies are present), but not with the sera from individuals with other illnesses and from normal, non-leukemic people.

Three general categories of virus-like particles possessing a strong affinity for human lymphoid tissues have been demonstrated repeatedly with the aid of the electron microscope:

(a) Virus-like particles resembling avian and murine leukemia viruses having diameter of 90 nm and consisting of an inner zone surrounded by one or two membranes, found in lymph nodes and plasma pellets (Dmochowski, 1957, 1959, 1968; Ito, 1958; Beard, 1958).

(b) The murine leukemia type C particles, with a diameter of 100 nm, showing an electron-dense nucleoid and a unit membrane (Almeida et al., 1963; Murphy and Furtado, 1963; Dalton et al., 1964; Negroni, 1964).

(c) Herpes-type virus possessing an icosahedral capsid consisting of 162 capsomeres.

Virus-like bodies were first discovered in ultrathin sections of lymph nodes from patients with various forms of leukemia. These particles were 90 nm in diameter and consisted of an inner zone surrounded by one or two membranes (Dmochowski et al., 1959).

Murine leukemia type C particles were detected by a direct electron-microscopic examination of human blood plasma and lymph nodes from patients with acute lymphocytic leukemia. When specimens from patients with different types of leukemia were examined, the type C particles were observed in 27 of 164 samples, such as lymph nodes, blood plasma and bone marrow (Seman, 1968).

The virus-like particles were discovered in intercellular spaces, intracytoplasmic vacuoles and occasionally as budding from plasma membranes. Similar particles, resembling the immature forms of murine leukemia viruses, were detected in the cytoplasm of the blast cells obtained from the buffy coat of 30% of patients with acute leukemia (Seman, 1968). Herpes-type virus particles were not observed in any of the cultures.

Virus-like particles resembling the virus of avian myeloblastosis were detected with a similar frequency (Burger et al., 1964; Porter et al., 1964). Since those particles were also found in children with infectious mononucleosis, it has been suggested that those virus particles may be biologically related.

Levine et al. (1967) correlated the presence of C-type particles in plasma pellets with their clinical manifestations. More C-type particles were found in the acute than in the chronic form of leukemia and no C-type particles were detected in remissions.

RNA viruses of the C-particle type have not been isolated from human leukemic specimens by means of tissue cultures. But it has been reported (Epstein et al., 1964; O'Connor et al., 1968; Griffin et al., 1966; Iwakata and Grace, 1965; Grace, 1967; Henle, 1967; Stewart, 1968) that Herpes-like viruses have been found in tissue culture lines from cases of Burkitt's lymphoma and from myeloid leukemia. The virus particles possessed icosahedral capsids consisting of 162 capsomeres. When examined by a cross-immunofluorescence technique, those bodies appeared as antigenically different from, and not related to the common herpes viruses, i.e., Herpes Simplex, varicella, pseudorabies, reoviruses, and human cytomegalovirus. These findings were confirmed by Moore et al. (1966) and Zeve et al. (1966).

Similar, if not identical, virus particles were observed by electron microscope in concentrated, partially purified extracts from leukemic organs (especially the liver and spleen) (Lee and Kwapinski, 1972). These particles partly purified by continuous particle electrophoresis and isopycnic centrifugation, reacted with the immunoglobulins of antisera produced in rabbits injected with the purified, particulate antigen preparations, using the immunoferritin test and an electron-microscopic precipitation technique. In contrast, the antisera, absorbed with pooled, normal human tissue antigens, did not react with the extracts from normal human organs. Cross-reactions were not found on

examinations of the non-disrupted and disintegrated virus particles against antisera to murine viruses and to feline leukemia viruses.

Contrary to the reports above, some researchers were unable to detect any virus particles associated with human leukemia. The materials examined were blood plasma, cerebrospinal fluid, bone marrow and spleen from leukemic individuals (Bernard and Lephis, 1964; Arnault and Hognenau, 1966; Murphy and Zarafonetis, 1968). Virus particles were not found in the cell lines established from the blood cells of patients with acute leukemia and from marrow cells (Clarkson et al., 1967; Murphy and Zarafonetis, 1968; Rechar et al., 1969).

IV. General Considerations on Tumor Associated Antigens

Tumor associated antigens are usually classified according to the causative agent of the tumor, e.g., tumors induced by chemicals and by viruses. One of the roles of circulating tumor-specific antigens in the tumor-host has been elucidated recently by Alexander and Cumè (1973). They found that the sera of patients with melanoma, hypernephroma, and bladder carcinoma contain factors which specifically inhibit the cytotoxic effects of the patient's circulating lymphocytes directed against cultured tumor cells. The inhibitor does not combine with the target cells and cannot be considered to be a blocking factor. A number of different tests indicate that the active material is

circulating tumor-specific antigen which in the *in vitro* assay competes with the tumor cell for the cytotoxic lymphocytes. In rats bearing sarcomas, the presence of circulating tumor-specific transplantation-type antigens was demonstrated directly by neutralizing of antisera directed against the tumor-specific transplantation-type antigens.

In both the human and animal tumors studied (Alexander and Cumè, 1973), circulating antigen disappears after removal of the tumor. The significance of circulating antigens in the tumor-host relationship is still not clear and it cannot be predicted that removal of circulating antigen will necessarily have a favorable influence on prognosis because tumors recur following treatment of the primary that has resulted in the elimination of circulating antigens. Nevertheless, the release of circulating antigen may explain the reason treatment of tumor-bearing animals with irradiated tumor cells sometimes facilitates the growth of metastases, while under other conditions it is therapeutic.

In both man and animals, it has been found that in the presence of clinically detectable tumors, active immunization stimulates humoral and cell-mediated immunity. The resultant increase in antibody production presumably results in the removal of antigen from the circulation. Conversely, in animals in which only minimal numbers of tumor cells remain and in which cell-mediated immunity can

be detected in distant nodes, autoimmunization with irradiated tumor cells results in the appearance of antigen in the circulation and in some cases the growth of metastatic lesions is promoted. The data of Alexander and Cumè (1973) are consistent with their hypothesis that the outcome of treatment with irradiated tumor cells is determined by the balance between the beneficial effects of stimulation of lymphoid tissue distant to the tumor and the harmful effects of antigen over-load.

From the findings of Alexander and Cumè (1973), it appears that soluble tumor associated antigens may be important in the outcome of the tumor stricken host. It is in this perspective that the following review of tumor-associated antigens is included.

(i) RNA-virus induced tumor antigens

When animal cells are infected by RNA oncogenic viruses, the cells usually release virus particles. Therefore, virion antigens may be detected inside the cells, on the cell membrane and in the serum of the tumor bearing animals. The antigens are divided into two major groups: the structural virus antigens which form part of the virion and the non-viral antigens.

In the infection of animal cells by murine leukemia viruses (an RNA virus), the following main components of murine leukemia associated antigens have been identified.

(a) A group specific (gs), internal virus antigen (Huebner et al., 1964; Huebner, 1967). The gs antigen of Gross' virus has an extimated molecular weight of 35,000 to 40,000 and a sedimentation coefficient of 2.7S. Two immunologically identical macromolecules, with α and β-globulin mobilities in immunoelectrophoresis, may be separated. This antigen can be fractionated into two molecular species by iso-electric focusing and into three by polyacrylamide gel electrophoresis.

The Gross, Friend, Moloney and Rauscher murine leukemia viruses share gs antigens (Harris et al., 1970). This antigen is found in the plasma of mice infected at birth and in artificially infected cells (Hartley et al., 1971; Parr, 1970). The gs antigen is common to all members of the avian leukosis and sarcoma virus group. Gs antigen is also found in RSV-induced tumors in hamsters, guinea pigs, and chickens. Infectious virus is not usually found in significant quantities in RSV-induced mammalian tumors. Although only small amounts of virus are found in primary tumors, gs antigen is present in detectable quantities. It is found in the nucleus, cytoplasm and cell surface of infected cells. A large proportion of chickens that are free of avian leukosis virus contain an antigen that is identical to the gs antigen (Dougherty, 1968).

Gs 1 and gs 2 antigens of the murine leukemia viruses are absent from the cat, hamster and rat viruses. The gs 3

antigen or murine leukemia viruses was also found in cat and hamster leukemia viruses as detected by Ouchterlony test. There is no serological cross-reaction between mammalian and avian leukemia viruses (Geering et al., 1970).

In the publication by Maruyama and Dmochowski (1969), it was reported that when cell cultures were infected in one of the following oncogenic RNA virus strains: Rauscher, Moloney, Friend leukemia virus of mice, murine sarcoma virus (Moloney), feline lymphoma virus, or reticulum cell sarcoma virus of SJL/J strain of mice, all the cultures gave a positive mixed hemadsorption reaction with anti-Rauscher leukemia virus serum, showing titers of 1:128 or higher. The sera from six cats strickened with leukemia or lymphoma reduced the titer between feline or murine cultures and the anti-Rauscher leukemia virus serum by two-fold. Similar effects were observed using sera of mice with Friend leukemia and of a hamster with sarcoma induced by Moloney sarcoma virus. Control sera from normal mice or hamsters had no effect. Type C virus particles and budding were demonstrated in all cell lines by electron microscopy. These results suggest that common antigen(s) existed in the cultures containing type C virus particles derived from animals of different species. However, species-specific antigenic determinants also exist in the gs antigen of mouse, hamster, cat and rat C-types viruses.

Gilden et al. (1971) observed that a human cell line,

ESP-1 lymphoma, which contained C-type virus, also possessed an antigen with determinants identical to those detected on the group-specific antigen of murine C-type viruses. This result appears to suggest the viral etiology of at least some human tumors.

Charney and Moore (1971) incubated purified mammary tumor virus of mice with sera of women with breast cancer and of normal individuals, then injected into mice that are susceptible to mammary tumor virus. The mean incidence of mammary tumor virus infection was significantly lower when sera of tumor bearing patients was used, i.e., 87% versus 69% ($P = 0.02$). Therefore, it was concluded that these results suggest a viral etiology for the disease and that human and murine viruses are closely related.

In a related study, Dmochowski (1970) discovered that autologous and homologous serum-cell systems from patients with different types of leukemia, lymphoma, osteogenic sarcoma and breast carcinoma gave positive reactions in a number of cases. Sera from some of the patients and their relatives gave positive results with cultures from leukemia, reticulum cell sarcoma and bone tumors that contain type C particles. These were derived from mice, rats, hamsters and cats. The same sera were negative with embryo cell cultures derived from animals of different species in which C particles were not found.

The murine and feline leukemia viruses share an

antigen. Sarma et al. (1971) detected the common antigen by complement fixation using broadly reactive rat anti-murine leukemia virus sera.

In the mice, cats and virtually all the chickens studied, antibody was not produced to the group-specific antigens characteristic of the RNA tumor viruses of their own species (Huebner et al., 1970). However, Noronha et al. (1972) reported that antibody against feline gs antigen could be stimulated in the cat.

(b) Type specific (ts) antigens. The ts antigens are components of the viral protein envelope (Hartley, 1971). These antigens are specific for the various strains of oncogenic RNA viruses although some cross-reactivity may be present. The serological differences between the Graffi, Moloney, Friend, Rauscher and Rich strains of murine leukemia viruses can be demonstrated with anti-ts sera, by the techniques of virus neutralization, immunodiffusion, and fluorescent antibody tests.

Cells that contain the ts antigens of murine leukemia viruses react with cytotoxic antibodies and then lyses in the presence of complement. However, the cytotoxic test reveals no significant differences between leukemic cells induced by the Moloney, Rauscher or Gross viruses.

The ts antigens of murine leukemia viruses have been grouped into the G-type (Gross) or FMR-type which are the cross-reacting antigens of Friend, Moloney and Rauscher

viruses (Harris and Sinkovics, 1970; Hartley et al., 1971; Pasternak et al., 1970).

The avian leukosis and sarcoma viruses have also shown complex antigenic relationships by the technique of neutralization. Three major sub-groups may be recognized: A, B and C (Dougherty, 1968). Within each sub-group the viruses share common ts antigens, exhibit common patterns of interference and have the same host range in chicken cells.

(c) Surface (S) antigens of RNA virus induced tumors. The S antigens are non-viral in nature. These antigens are detectable in non-virus-producing tumors induced by defective viruses, and were able to induce transplantation immunity even though virion antigens were not present. Harris and Sinkovics (1970) reported that the Moloney virus that induced XM-1 hemangiosarcoma in mice, did not release virus but induced transplantation immunity. This phenomenon was also demonstrated by the murine sarcoma virus Harvey (MSV-H) in inbred hamsters. This tumor had a faster rate of growth in X-rayed than in unirradiated hosts and killed 14/14 and 5/12 challenged hamsters, respectively. The hamsters that were immunized with irradiated nonproducer tumor cells resisted challenge with viable cells; 13/15 normal controls and 0/11 preimmunized hamsters developed tumors. The results (McCoy et al., 1972) showed that no MSV-H neutralizing antibodies were detected in sera of

hamsters immunized and resisting challenge with the non-producer line, but were seen in the sera of hamsters immunized with the producer line or MSV-H. The results suggest that the non-producing MSV-H induced tumor possess a new tumor antigen(s) which is not an MSV-H virion antigen but can be induced by MSV-H. On the other hand, Stephenson and Aaronson (1972) demonstrated that the nonproducer BALB/3T3 cell line that was transformed *in vitro* by Rauscher leukemia virus, was unable to induce any detectable transplantation immunity in mice.

In addition to the gs and ts antigens, the Gross virus induces another "Gross cell surface antigen" (GCSA) and a soluble antigen that is present in infected animals which is a non-virion antigen. The Gross virus induced another cell surface antigen, the G_{IX} (G = Gross, IX = the determining gene that is located in linkage group IX). As shown by Stockert et al. (1971) this antigen exhibits Mendelian inheritance and also appears *de novo* in cells that have become productively infected with Gross leukemia virus. GCSA was found to be associated with virus infection, but G_{IX} sometimes occurs independently of productive infection. The structural gene coding for G_{IX} is common to all mice, like the T_{Ia}. However, it is still not certain whether the two segregation genes, G_{IX} and T_{Ia}, belong to the murine leukemia virus genome rather than to the cellular genome.

Vaage (1970) discovered that pretreatment of mice that are free of mammary tumor virus with mammary carcinomas results in immunity to the tumor. This suggests that the tumors are cross-reactive and the principal tumor antigens recognized are probably those of the inducing virus and virus associated products.

(ii) DNA virus induced tumor antigens

(a) Papova and Adenoviruses are able to induce tumor (T) antigens localized in the nucleus of transformed cells (Pope and Rowe, 1964). The tumor antigens are specific for the virus that caused their formation and not for the cell type or species of the host cells. Adenoviruses had been found to cross-react with their T antigens; however, the T antigens are not identical with the components of the virion or the enzymes of DNA metabolism (Huebner, 1967). However, all the present evidence points to their being coded by the viral genome incorporated in the transformed cell (Habel, 1966). As with the cell-surface antigens, the specificity is determined by the virus not the cell; for example, the same T antigen can be found in hamster, mouse and human cells transformed by SV 40 virus (Habel, 1965).

Rapp et al. (1965) showed that the production of T antigens in a lytic infection system can be prevented by inhibitors of RNA and protein synthesis, but not by DNA inhibitors, indicating that input virus genome is sufficient

to initiate it. In addition, the whole biologically active virus genome is not required to initiate T antigen production since this capacity is more resistant to UV irradiation than is virus infectivity (Carp and Gilden, 1965).

In the lytic system T antigen synthesis can be partially segregated from infectious virus production by incubating cultures at a reduced temperature (Kitahara and Melnick, 1965), whereas T antigen production as well as transformation of 3T3 cells on exposure to SV 40 virus may be prevented by interferon. This viral inhibitor has no such effect on cells already transformed (Oxman et al., 1966; Oxman et al., 1967; and Todaro et al., 1965). In view of the present explanation for the mechanism of action of interferon, this would suggest that once viral genome is integrated into cell genome the messenger RNA for T antigen synthesis acts the same as normal cell messenger RNA.

In the nucleus of the tumor cell the antigen seems to be associated with RNA (Gilden et al., 1965), but when extracted the CF activity of the T antigen is not destroyed by RNase. By ferritin-labeled antibody staining and electron microscopy, it is shown to be present in structures resembling groups of fibers (Kalnins et al., 1966). The extracted T antigen is heat-labile destroyed by trypsin and in the case of adenovirus 12 has an average sedimentation coefficient of 2.40S and an iso-electric point of 5.0 (Gilead and Ginsberg, 1968). A summary of the biological properties of

the virus-induced tumor antigens is given in Table II, and the major antigens of the murine leukemia virus group in Table III.

An oncogenic hybrid of adenovirus type 7 and SV 40 has been described by Harris (1970). In the transformed cells T antigen of the SV 40 virus was present; however, the hybrid virus could not be neutralized by SV 40 antiserum. In addition, non-oncogenic adenovirus type 2 was observed to acquire oncogenic potency by association with the SV 40 virus genome.

One of the most important uses of the discovery of T antigen has been as a marker in basic biochemical investigations with the DNA tumor viruses. Since this type of antigen is produced in both complete lytic and incomplete abortive or transforming infections, and is one of the earliest virus-coded proteins made in the infectious cycle, it is a convenient fingerprint demonstrating the presence of at least a partially functioning viral genome. The extensive work on adenovirus hybrids with SV 40 virus could not have been possible without T antigen monitoring, since this is the only evidence for the SV 40 virus element (Huebner et al., 1964; Melnick et al., 1964; Rowe, 1965).

The group-specific CF antigens in avian (Armstrong et al., 1964) and murine (Hartley et al., 1965) leukemia viruses have provided a relatively simple means of quantitating infectivity of these agents in tissue culture systems.

TABLE II

SUMMARY OF SOME BIOLOGICAL PROPERTIES
OF VIRUS-INDUCED TUMOR ANTIGENS

Type of Antigen[a]	Surface		Internal	
Type of Virus	DNA	RNA	DNA	RNA
In tumor cells	+	+	+	+
In *in vitro* transformed cells	+	+	+	+
In lytic infected cells	+	+[b]	+	+
Specificity: Type	+	+	+	-
Group	-	+	+[c]	+
Structural part of virus	-	+	-	+
Cross-reacting between species	+	+	+	+
Host reaction - cell mediated	+	+	-	-
- serum antibody	+	+	+	+
- produced by virus	+	+	+	-
- produced by tumor	+	+	+	+

a - No distinction is made here between structural and non-structural virus antigens.

b - Cells producing mature virus usually survive.

c - Cross-reactions between T antigens of certain types of adenoviruses.

(From Habel, 1969)

TABLE III

MAJOR ANTIGENS OF THE MURINE LEUKEMIA VIRUS GROUP

	Source	Designation	Specificity	Method of Detection	Presumed Origin in Virus
	Tumor and lymphoid cells	Cell surface (G, FMR)	Type	Transplant rejection Cytotoxicity and absorption of cytotox	Envelope protein
Infected cells				Immunofluorescence	
	Plasma and tissue extracts	Soluble (GSA, FMR sol.)	Type	Absorption on indicator cells - cytotox (FMR) - immunofluor.(G)	Envelope protein
	Plasma and tissue extracts, tissue culture harvests	Soluble (gs)	Group	Immunodiffusion Complement fixation Immunofluorescence	Internal protein
Virion		Virion surface	Group and Type	Neutralization (Immunofluorescence)	Envelope protein
		Soluble (gs)	Group	Immunodiffusion Complement fixation Passive HA	Internal protein

(From Hartley et al., 1970)

One of the significant uses of these tumor associated antigens has been in establishing that a given tumor or *in vitro* transformed cell was induced by the specific virus inoculated. In the case of surface antigens in the leukemias and the T antigen in DNA virus tumors, a possible approach to specific diagnosis is the demonstration of specific antibody in the serum of the animal developing a tumor. The implications of these procedures for establishing the viral etiology of human tumors are obvious. However, thus far no definitely positive results have been obtained in human tumors with the possible exception of the Burkitt lymphoma (Klein et al., 1966) where the virus responsible for the specific antigen and antibody appears to be the causative agent of infectious mononucleosis (Henle et al., 1968), but has yet to be proven of etiological relationship to the tumor. For convenience, this agent has been designated as the EB (Epstein-Barr) virus after the EB-1 line of Burkitt's cells in which it was first observed. Similar particles have, however, also been observed in cell cultures from non-malignant patients, such as mononucleosis (Chessin et al., 1968) as well as from peripheral leukocytes of healthy patients (Moore et al., 1967).

It is, therefore, uncertain whether EB virus is the etiologic agent in Burkitt's tumor, a passenger within the tumor tissue, or merely a contaminant of long-term cell culture.

However, Klein et al. (1968) and Klein et al. (1968) demonstrated that the plasma membrane of the Burkitt's cell may contain tumor-specific antigens capable of inducing a corresponding antibody response on the part of the host. In addition, sera from a large number of patients demonstrate cross-reactions with lines of Burkitt's cells other than their own, suggesting the presence of the same tumor-specific antigen(s) in tumors of different individuals. These observations are therefore in agreement to those which have been made with virus-induced tumors. Nevertheless, the results have not been conclusive and at present, cautious inferences are necessary.

(iii) Antigens of chemically induced tumors

Tumors induced by chemical carcinogens are characterized by the induction of neoantigens that show an extreme, individual antigenic specificity. The new tumor antigens are specific for each individual tumor, even though they are induced by the same agent in identical animals of an inbred strain (Klein, 1968; McKhann, and Haywood, 1971).

Studies by McKhann (1971) have raised the possibility that some methylcholanthrene-induced sarcomas may share common antigens. By the use of indirect isotope-labeled antiglobulin technique, it was found that antisera recovered after amputation of a methylcholanthrene sarcoma was strongly positive for that sarcoma. It failed to show any absorption to cells of two other similar sarcomas, but reacted strongly

with a fourth tumor.

(iv) Onco-fetal antigens

In 1906, G. Schone reported that mice which had been injected with fetal tissue acquired the capacity to reject transplants of tumor tissue which otherwise grew and killed. Adult tissues did not evoke this response. It was therefore suspected that fetal and neoplastic tissues have some common immunological properties. However, convincing evidence supporting this observation has been provided only recently that various tumors and fetal tissues share common antigens. This knowledge provides valuable leads for basic cancer research concerned with the nature of the malignant transformation and perhaps in the near future, assist in the estimation of certain fetal antigens in tissue fluids thereby providing a tool for diagnosis and for monitoring the treatment of some forms of cancer. This has been recently realized by the use of radioimmunoassays where the truly remarkable sensitivity - nanogram measurements are routine and at picogram levels possibly feasible in the near future (Thomson et al., 1969; Addison and Hales, 1971). With a better understanding of Schöne's observations, it is not unlikely that eventually a new approach to the treatment of malignant disease by immunological means may be evolved.

(a) Carcino-embryonic antigen. Gold and Freedman (1965) first introduced the term carcinoembryonic antigen, to designate a constituent found in all adenocarcinomas of

the human digestive system, but which is normally present only in embryonic and fetal digestive tissues in the first two trimesters of gestation.

The antisera to the tumor were prepared in rabbits and rendered tumor-specific by absorption with an excess of corresponding normal tissue extracts and by utilizing the phenomenon of acquired immunologic tolerance.

The results of those investigations revealed that all of the colonic adenocarcinomas examined contained an identical, qualitatively tumor-specific antigen which was absent from the corresponding, autologous normal colonic tissue (Gold and Freedman, 1965b ; von Kleist and Burton, 1969; Kronman, 1970).

Using this discovery, it was then demonstrated that all human adenocarcinomas arising from the entodermally-derived digestive system epithelium (esophagus, small bowel, liver, pancreas, rectum, colon and stomach) contained the same tumor-specific constituent.

By using agglutination studies with tissue cultured cells of colonic cancer origin, Gold et al. (1968) found that the CEA was a constituent of the tumor cell surface. This was confirmed by immunofluorescent studies employing frozen sections of digestive system tumors and fetal intestines and viable cells explanted from freshly resected colon cancers (von Kleist and Burton, 1969; Denk et al., 1972; Goldenberg et al., 1972). By using the ferritin-anti-

CEA technique, Gold et al. (1970) found that a portion of the CEA is situated in the glycocalyx of the tumor cell immediately adjacent to the surface membrane.

Physicochemical properties of the CEA. The carcinoembryonic antigen is a large, water soluble glycoprotein with an approximate carbohydrate to protein ratio of 2 or 3 to 1, a sedimentation coefficient of 7-8S, and a mobility in the beta-globulin range upon immunoelectrophoresis in agar gel. The antigen has been purified by extraction in perchloric acid and subsequent fractionation by preparative gel chromatography and gel block electrophoresis (Krupey et al., 1968; Krupey et al., 1972).

The data obtained by acid degradation of CEA suggest that N-acetyl-D-glucosamine and D-mannose may be important constituents of the tumor-specific site(s) of the CEA molecule. In addition, D-galactose, L-fucose, D-glucose and sialic acid were also found in CEA (Banjo, 1972).

Immunologic reactivity of CEA in human. Cell-mediated immunity. Delayed type hypersensitivity of the skin were observed in 17 or 19 patients with carcinomas of the colon and rectum when they were challenged intradermally with soluble membrane fractions obtained from the autochthonous tumor cells (Hollinshead, 1970). Normal tissue fractions gave negative results. The skin-reactive antigen was also found in the digestive tract cells of both first and second

trimester fetuses.

Humoral immunity. Gold (1967) examined the sera of 212 individuals for the presence of circulating antibodies against the CEA by the bis-diazotized benzidine hemagglutination technique. He found that a specific IgM humoral anti-CEA antibody response was detected in 30 of 43 patients with non-metastatic digestive system cancer and 28 of 46 pregnant women in all trimesters of pregnancy and in the immediate post-partum period.

Thomson et al. (1969) described a radioimmunoassay for the detection of circulating CEA in the sera of patients bearing digestive system cancers. These observations have been confirmed in other laboratories (Lo Gerfo et al., 1971; Snyder, 1972; Kleinman et al., 1971; Kupchick and Zamcheck, 1972; Kleinman and Turner, 1972; and Chu et al., 1972). In addition, the above mentioned investigators employing the modified radioimmunoassay techniques and different preparations of CEA and anti-CEA antisera have reported the detection of "CEA-like" substances, termed "tumor associated antigen(s)", in the sera of patients suffering from non-digestive system cancers, certain inflammatory bowel diseases, and other pathologic entities.

Studies made by Mach and Pusztaszeri (1972), von Kleist et al. (1972) and Ørjasaeter et al. (1972) suggest that the tumor associated antigens may be similar to a glycoprotein extractable from normal lung, spleen, liver,

breast, gut and colonic cancer tissues. This material has a mobility in the beta-globulin region upon immunoelectrophoresis. The molecular weight of this material is less than that of CEA, sedimenting at 3-4S in the analytical ultracentrifuge. These investigators have designated this material as normal glycoprotein, non-specific cross-reacting antigen and Beta External.

Rabbits that have been immunized with 0.6M perchloric acid colonic cancer tissue extracts appear to result in antibodies directed against the immunodominant regions of this macromolecule, as well as a comparable site on the CEA molecule which is not tumor specific. The anti-normal antibodies may be removed by absorption with normal colonic mucosa extracted in 0.6M perchloric acid. Ørjasaeter et al. (1972) claimed that this treatment does not diminish the CEA-anti-CEA reaction in any way.

These data suggest that the CEA molecule has a tumor-specific antigenic determinant and one or more antigenic sites present in some normal, adult tissues. Apart from the gastrointestinal epithelium tumors, other tumors may also possess the normal glycoprotein present in colonic tumor. These tumors may also contain other antigenic determinants present on both CEA and TAA. Therefore, the immunobiological nature of the CEA and TAA is still not fully understood.

(b) <u>α-foeto protein</u>. Abelev (1963) demonstrated that one of the abnormal proteins synthesized by a chemically

induced hepatoma in mice was antigenically identical to an α-globulin present in embryonic and neonatal mouse serum but absent from the adult mouse. Shortly following a similar material had been found by Tatarinov (1964) in the serum of patients with primary hepatoma. Recently, Ruoslahti (1972) developed a radioimmunoassay for α-foeto protein and, using this method, very low levels ($\sim$ 5 to 10 ng ml^{-1}) which are quite undetectable by gel diffusion have also been found in the serum from normal subjects. The level found in hepatoma patients is 1 to 100 mg ml^{-1}.

In some areas of Africa 80% of all patients with hepatomas give a positive test, whereas in western countries rates as low as 30% have been reported. Whether a hepatoma produces α-foeto protein may depend on its mode of causation. For example, 100% of hepatomas induced in rats with a carcinogenic azo dye gave a positive serum whereas none did so with hepatoma caused by aflatoxin B (Stanislanski-Birenewajg, 1967).

(c) $\underline{\text{Alpha}_2 \text{ ferroprotein}}$. It is produced by the liver in fetal and post-natal life up to two years of age (Alexander, 1972; Gold, 1971; Gold, 1971b). It appears in the sera of 81% of young children with a large spectrum of tumors not confined to those of embryonal origin (Buffe et al., 1970). It is found in the sera of children suffering from a variety of cancerous conditions, e.g., nephroblastoma, lymphosarcoma, hepatoma, teratoma, neuroblastoma, reticulum

cell sarcoma and cerebral tumors.

(d) Gamma fetal protein - 2. Edynak et al. (1971) demonstrated the existence of a fetal antigen with electrophoretic mobility of α-globulins, in saline extracts of human tumors, including carcinomas of the breast, colon, ovary, stomach, leukemias and sarcomas. Antibody was found in the sera of a minority of cancer patients but not in normal sera. Edynak therefore suggests that this antigen may be the product of a cell with malignant or near malignant physiology.

(e) Fetal sulphoglyco protein. Hakkinen (1969) found a sulphoglyco protein with blood group substance A activity in the gastric juice of nearly all patients with carcinoma of the stomach (75-80%), but it is also found occasionally (14/148) in the absence of cancer both in the gastric juices as well as the mucosa, and more frequently in older patients (Gold, 1971 and Gold, 1971b).

(f) Regan iso-enzyme. The foetal alkaline phosphatase is characterized by high heat stability and was isolated from a bronchiocarcinoma of a man called Regan; it is found in the placenta and serum of pregnant women but not in any normal adult tissues. In surveys by Fishman (1968) and Stolbach et al. (1969), the Regan iso-enzyme was found to be present in the sera of some 4% of patients with a wide variety of different carcinomas. This enzyme has provided a reliable guide for either progression or regression of the

tumor following treatment.

(g) Cross-reaction of leukemia associated antigens with fetal sera. Harris et al. (1971) prepared a rabbit antiserum against a partially purified human leukemia antigen. After suitable absorption with normal antigens, it reacted in the Ouchterlony test with a circulating antigen present in one third of the leukemia patients. Five of six fetal sera also gave a positive reaction. Twenty-two sera from healthy volunteers, solubilized extracts of normal spleens, buffy coat, or hemolysed group A red cells gave negative results.

In spite of the large volumes of literature on the oncofetal antigens, the immunobiological functions of the antigens are still unclear.

(v) Antigens of human leukemia

The evidence for the existence of human leukemia associated antigens has been provided by a number of researchers using different immunological techniques (De Carvalho, 1960; Messineo, 1961; Garb et al., 1962; Fink et al., 1964; Holeckova, 1962; Kamiya, 1969; Cohen et al., 1970; Viza et al., 1970 and Sahasrabudhe et al., 1971) (See Table IV).

De Carvalho (1960) segregated the antigens from human leukemic and tumoral cells by fluorocarbon extraction.

Leukemic blood and tumor suspensions were mixed with the fluorocarbon trifluorotrichloroethane at speeds of 45,000 to 70,000 r.p.m. at 2°C in air turbine homogenizers.

After centrifugation the homogenate separates in three layers: a supernatant fluid to be re-extracted, a middle gel layer of protein precipitate which is collected and an undernatant of excess of fluorocarbon which is discarded. The extraction of the supernatant fluid continues until it does not yield middle protein layer any more. The protein layers from the several extractions are mixed and stirred vigorously at 2°C. The fluorocarbon is expressed out of its semisolid emulsion with the proteins and can be decanted freely. To the protein is then added chilled 0.15M phosphate buffer at pH 7.2.

After fluorocarbon homogenization of human leukemic and tumoral cells, two main fractions were separated, one a ribonucleic acid-rich and the other a protein-rich fraction, but antigen analysis of the protein-rich moiety was attempted. When tested by a gel diffusion method against both rabbit antisera prepared with proteins from malignant cells and against antisera prepared with proteins from normal tissues, the tumoral proteins showed antigens common to both normal and malignant fractions and antigens specific for the malignant fractions. The normal antigens, but not the malignant ones, could be removed from the whole malignant protein extract by absorption of the latter with rabbit anti-normal gammaglobulin. The specific antigens found in solid tumors were different from the ones found in leukemias and, within the leukemias, there were different antigens in acute stem

TABLE IV

LITERATURE ON SOLUBLE HUMAN LEUKEMIA ASSOCIATED ANTIGEN

SOURCE OF HUMAN LEUKEMIA SPECIFIC ANTIGENS	FORM OF ANTIGENS	METHOD OF DETECTION	CHARACTERISTICS OF THE ANTIGENS	AUTHORS
Human leukemic leukocytes extracted with glycine to obtain deoxyribonucleo-protein.	Crude.	Immunodiffusion in gel.	Cross reactions were not observed between normal and leukemic DNPs except for one instance out of 18.	L. Messineo, 1961
Human leukemic leukocytes.	Crude.	Use of immune tolerant sheep and cytotoxic tests.	Immune tolerant sheep produced cytotoxic antibodies specific for leukemic cells.	Holeckova, 1962
Leukemic blood.	Crude.	Immune tolerant rabbits and immunodiffusion in gel.	Immune tolerant rabbits produced a minimum of 2 precipitant lines with leukemic leukocytes.	S. Garb, 1962
Protein rich preparations from bone marrow of acute leukemias and other tumors.	Crude.	Immune tolerant rabbits and immunodiffusion in gel.	Several precipitation lines produced.	S.DeCarvalho & H.J. Rand, 1963
Concentrated plasma from leukemic individuals containing virus-like particles.	Crude.	Absorption of rabbit antisera against leukemic plasma, with normal human antigens and labelling with fluorescein isothiocyanate immunofluorescent technique.	Absorbed leukemia sera reacted specifically with cellular elements of bone marrow and blood from patients with leukemia. Antisera against Rauscher leukemia virus cross-reacted with the human leukemia material.	M.A. Fink et al., 1964

Cont'd.

SOURCE OF HUMAN LEUKEMIA SPECIFIC ANTIGENS	FORM OF ANTIGENS	METHOD OF DETECTION	CHARACTERISTICS OF THE ANTIGENS	AUTHORS
Saline extract of leukemic leukocyte and brain tissue.	Crude.	Immunodiffusion and use of immune tolerant rabbits.	Leukemia specific precipitant line observed in immunodiffusion.	H.Kamiya, 1969
Leukemic plasma.	Crude.	Immunodiffusion	Antigen cross reacts with the avian leukosis antigens. Antigen detected in only 1 of 47 leukemia plasma tested.	S.Cohen et al., 1970
Membrane material from peripheral white blood cells eluted and solubilized by the addition of papain.	Soluble antigens partially purified through Sephadex. Four fractions used as antigens to produce antisera. DEAE-cellulose ion exchange used on 1 of the crude fractions.	Successive absorption of the leukemic sera with normal human serum, soluble normal HL-A antigen pooled from spleen and papain. Immunodiffusion.	Absorbed antisera gave positive reactions with the five soluble crude leukemic preparations, each produced a different precipitant pattern. Leukemia specific antigen was eluted where transplantation and mouse leukemia TL antigens eluted.	A.Viza et al., 1970
Leukemic white blood cells mixed with 1-fluoro--2,4-dinitrobenzene.	Crude.	(a) WBC-FDNB injected into a horse. Serum absorbed with pooled A, B and O human RBC's.	Using immunodiffusion absorbed anti-leukemic serum reacted with the soluble extracts of leukemic WBC but no reaction with normal WBC's.	Sahasrabudh et al., 1971
		(b) WBC-FDNB injected into a human. No absorption was mentioned.	Human serum revealed one precipitant line with leukemic cells. No mention of normal control.	Sahasrabudh et al., 1971

cell and in chronic lymphatic leukemias.

De Carvalho claimed that by the use of absorption of fluorocarbon extracts from human tumoral and leukemic cells with anti-normal rabbit serum prepared with fluorocarbon extracts of human amnion had resulted in a purified tumoral and leukemic antigen preparation. He justified the conclusions by the fact that the antisera in rabbits against this purified antigen contained no demonstrable antibodies against normal tissue antigens.

However, he did not fractionate the crude antigen extract and test for the homogeneity of the purified antigens. In addition, the normal control antigens used for the preparation of anti-normal sera were extracted from primary cultures of human amnion cells, and were not the homologous normal counterparts of the tumor tissues that were used. Therefore the normal antigenic components of the leukemic and tumor tissues may have been different from that of the amnion cells. Therefore, his conclusions may not be justifiable.

Shortly following De Carvalho's work, Messineo (1961) demonstrated immunological differences in deoxyribonucleoproteins from white blood cells of normal and leukemic human beings. The extraction of deoxyribonucleoproteins was performed with 0.01M glycine overnight after washing leucocytes with 0.01M sodium citrate and 0.01M glycine solution. Purification of the extract was achieved by centrifugation

at 24,000 g for 25 minutes. Antibodies were prepared by injecting intravenously a total amount of 25-30 mgm of deoxyribonucleoproteins into rabbits three times at weekly intervals. Both normal and leukemic deoxyribonucleoproteins were found to induce formation of high-titre antibodies in rabbits. They always gave a single precipitin line when fresh preparations were employed. Sera of rabbits immunized with normal deoxyribonucleoproteins did not cross-react with leukemic deoxyribonucleoproteins and vice versa. However, in one instance a preparation of deoxyribonucleoproteins from a person with chronic lymphatic leukemia gave a positive reaction with rabbit sera immunized with normal deoxyribonucleoproteins and a negative one with rabbit sera immunized with leukemic deoxyribonucleoproteins. In eighteen studies of leukemia versus normal serum combinations, only one gave a positive reaction. With these results, Messineo concluded that the antibodies produced in rabbits are specific for the normal and leukemic deoxyribonucleoproteins. This specificity argues for the nativity of these preparations; and it implies a genetic differentiation of the nuclear deoxyribonucleoproteins in the growth of white blood cells.

Along similar lines of research, Garb et al. (1962) studied the white blood cells of human leukemia but using the technique of immune tolerance in rabbits. He demonstrated leukemia specific reactions with human acute myelogenous leukemia.

In the attempt to induce tolerance, blood from several donors with different blood types was injected into each rabbit. With the exception of 2 animals, each rabbit received human AB, O, Rh positive and Rh negative blood. The two exceptions received Ab, Rh positive blood.

The injection of normal human blood were begun in some rabbits on the day of birth, and in others at the age of two days. The injections were continued through the age of 14 days. After the rabbits had reached the age of nine weeks, immunization to myelogenous leukemia was begun. The animals of one group received 2 ml of whole, fresh leukemic blood intramuscularly every 7 days for three injections. The other two groups received injections of a buffy coat mixture, prepared by centrifuging the leukemic blood at 2,500 r.p.m. for 30 minutes. No attempt was made to obtain pure leukemic cells. The materials injected into the rabbit contained erythrocytes, leukocytes, platelets and plasma. The second group of animals received 0.5 ml of leukemic buffy mixture subcutaneously every 7 days for four injections. The third group of animals received the leukemic buffy mixture mixed with equal parts of Freund's adjuvant given intradermally in four separate sites, 0.1 ml per site, and repeated in 10 days. Ten or more days after the last immunizing injection, the rabbits were bled.

Garb's results show that in the rabbits which received less than 1 ml of human blood in the neonatal

period, there was no evidence of production of immune tolerance. In the rabbits which received between 1 and 2.4 ml of human blood in the neonatal period, the incidence of adequate immune tolerance was about 70%.

Of the 14 animals whose serum showed definite positive responses to acute myelogenous leukemic buffy coat, 9 reacted to the leukemic material even when diluted to 1:25, in agar immunodiffusion. All 14 showed at least two separate precipitation lines at a 1:25 dilution. The sera of rabbits which responded positively to buffy coat of a patient with acute myelogenous leukemia also responded positively to buffy coat of other patients with acute myelogenous leukemia. However, attempts to demonstrate reactions between the rabbit antiserum and buffy coat mixture from patients with chronic myelogenous leukemia were unsuccessful, even in cases in which the reactions to acute myelogenous buffy coat were decidedly positive.

In general, the results of the skin tests on the rabbits paralleled those of the Ouchterlony plates.

Garb, therefore, concluded that it is possible to produce in rabbits antibodies which react to one or more antigens in the blood of patients with acute myelogenous leukemia, but which do not react to normal human blood constituents, and that his evidence strongly suggests that the antigen is related to leukemia. With the controls he ruled out the possibility that the antigens specific for

leukemia are isoantigens.

By the application of immunofluorescence to the study of human leukemia, Fink et al. (1964) demonstrated leukemia specific antigens in the plasma of leukemic patients. Pooled plasma, from 5 patients with acute leukemia, were used as an antigen in the production of an antiserum in rabbits. These plasmas were chosen because of the presence of virus-like particles of possible etiologic significance, demonstrated by electron microscopic examination. After absorption with normal human antigens and labeling with fluorescein isothiocyanate, this serum reacted specifically with various cellular elements of the bone marrow and peripheral blood of a significant number of patients with leukemia (13/23 blood smears and 49/72 marrows) and of none of the 25 marrows from normal persons. It also reacted with tissue-cultured cells from a Burkitt's lymphoma, but not with HeLa cells infected with either Herpes viruses. A similarly prepared antiserum to Rauscher leukemia virus (murine) cross-reacted with the bone marrow and peripheral blood cells from some humans with leukemia and especially with the cell from a patient with erythroleukemia. These findings are interpreted as supporting the hypothesis of a viral etiology of human leukemia, and as suggesting a strong probability of antigenic similarities among strains of leukemia virus infecting various species.

The presence of tissue-antigens specific for human

leukemia was demonstrated by Greenspan et al. (1963). The technics used to demonstrate the presence of antibodies included passive cutaneous anaphylaxis (PCA), immunodiffusion, microprecipitin, and immunofluorescent tests.

The antigens of normal brain, leukemic brain, normal human lymph node and Hodgkin's disease lymph node were prepared in a 10% homogenate with sterile saline solution. The preparations were passed through a Seitz sterilizing filter and either frozen or lyophilized for future use. Freon 113 (Fluorocarbon) extractions of normal and leukemic human tissues were also prepared by the method of Stone and Moore (1959).

Human volunteers were used to prepare the antisera against the various extracts. They were divided into three groups: one group received human leukemic cell-free brain extract; one group a cell-free extract made from a Hodgkin's disease node and the third group, human non-leukemic cell-free brain extract.

Five per cent pooled homogenates of frozen tissue extracts were prepared in buffered physiologic saline solution by grinding the tissues in a mortar with sand. The resulting homogenates were passed through a No. 6 Seitz sterilizing filter. Blood samples were collected from all volunteers for control studies and at the time of each inoculation. Nine men were given leukemic brain filtrate, two Hodgkin's disease node filtrate, and three non-leukemic brain filtrate. 0.1 cc

was given intracutaneously and 0.9 cc intramuscularly. After three and six weeks, the inoculations were repeated. One month later each man received an intramuscular injection of one ml adjuvant combined with antigen. The subjects were bled three weeks later. Both the normal human brain and leukemic brain antisera were absorbed with normal human brain or liver before testing.

The results with passive cutaneous anaphylaxis was positive when the serum from an immunized subject was injected intraperitoneally into the guinea pig, and the challenging antigen was obtained from either human or mouse leukemic tissue. When leukemic tissue antigens were used to challenge, a positive result was obtained. The specificity of the reaction was retained after absorption with normal human or mouse brain but was lost by the absorption with leukemic human or mouse brain. Extracted gammaglobulin from positive sera gave a strong positive reaction. The sera of the nine men injected with leukemic brain extract reacted positively with leukemic human brain, leukemic mouse brain, and fluorocarbon-extracted leukemic brain. The sera from these men collected before immunization did not react with the same antigens. The serum from the two men injected with Hodgkin's disease node extract gave positive reactions with Hodgkin's disease node extract, and a less intense reaction with leukemic tissue extracts.

Further evidence of the specificity has demonstrated

in six persons working with the leukemic mice and leukemic human tissues. Their sera were negative initially; however, after six months of working with experimental leukemia, their reactions became positive to the leukemic extracts. Sera from 24 normal adults and 6 children were negative to both leukemic and normal brain extracts.

These immunologic studies demonstrate specific antigenic differences between normal and leukemic tissue extracts. From the results of the experimental findings, the authors postulated that the difference between normal and leukemic extracts is the consequence of the presence of viruses or the alterations caused by them.

The existence of the specific human leukemia antigens was also demonstrated by Izawa et al. (1966) by the techniques of immunofluorescence, immunodiffusion and leuko-agglutination tests. They obtained anti-human serum against leukemic bone marrow aspirates, injecting them to the human volunteers and demonstrated the presence of circulating antibodies against leukemic cells, using the fluorescenced and I^{131} radio-iodinated serum. They concluded that man was capable of responding to the leukemic antigens, and that the specific antigen was present in human leukemic blood. They did not, however, attempt at isolating the leukemic antigen from the blood.

Kamiya (1969) made a thorough immunological study on leukemia with special reference to the circulating anti-

bodies against the human leukemic antigens in human sera. He demonstrated the presence of the leukemia specific antigens by passive cutaneous anaphylaxis and immune adherence hemagglutination.

In order to demonstrate the specific antigen in leukemic leukocytes and leukemic brain tissue, antiserum was prepared by injecting the leukemic leukocytes antigen to the rabbit, immunotoleranced to normal leukocytes. The specific antigenicity was demonstrated by the techniques of passive cutaneous anaphylaxis and immune adherence test between the anti-rabbit serum and leukemic leukocyte antigen.

Antibodies to the leukemic antigen were tested on the sera of 205 persons, including 54 patients with leukemia, 25 patients with leukemia analogous disease and 101 persons of their families.

The results show that by passive cutaneous anaphylaxis, no positive reaction was elicited with the sera of leukemic patients, but a relatively higher percent of positive reaction was observed with sera of patients with leukemia analogous disease. Positive reactions were found in 2.6% with sera of leukemic families and in 25% with sera of families of leukemia analogous disease.

It was found that by immune adherence test, the rate of positive reaction was significantly low with sera of leukemic families. Thus from this paper, the author could not predict the presence of transmissible antigenic agent in

human leukemia. However, it was noted that circulating antibodies to leukemic antigens were encountered more often with sera of patients with leukemia analogous disease, mainly lymphosarcoma.

To account for the absence of detectable circulating antibodies in leukemic patients, Kamiya explained that it may be possibly due to failure of their production by one of the following mechanisms: antigen excess, immuno-selection, immunotolerance or immunological surveillance of the test.

The presence of leukemia-specific antigens was recognized by its ability to induce specific antibodies in a xenogeneic system (Davies, 1966). A more direct evidence has been obtained from the study of partially purified antigens (Viza et al., 1970). These preparations were obtained by the solubilization of white blood cell membranes with papain. They partially purified the solubilized antigens by using Sephadex G-75, G-200 and DEAE-cellulose ion exchange columns. The antisera obtained against the four partially purified fractions were then absorbed with normal human serum and soluble extracts of spleen from normal tissues. The absorbed sera gave positive reactions with five soluble leukemic preparations, but each produced a different pattern of precipitation lines with the other preparation in immunodiffusion. The activity specific of leukemia was found in fractions eluted where transplantation

antigens elute. It is significant that in the appropriate mouse leukemic preparations, the TL antigen appears in the same fractions (Viza et al., 1970).

Leukemic cells are antigenically indistinguishable from their normal counterparts, but their net surface charges are different (Ambrose, 1965). This charge on the cell surface regulates the ability of the antibodies to approach the target cells. In order to exploit the differences in the net charge on the cell surface of leukemic and normal cells, antibodies specific to leukemic cells may be prepared as described by Sahaszabudhe et al. (1971). The net surface charge on the white blood cells of normal O group people was altered by tagging the cells with chemicals such as 1-fluoro-2,4-dinitrobenzene (FDNB). These cells were then used as antigens for producing antibodies against human leukemic cells in rabbits and horses. The antisera were then heat inactivated and absorbed with pooled A, B and O human erythrocytes; globulins were separated by ammonium sulfate and isoelectric precipitation.

The antisera were used for the detection of a leukemic-specific agglutinogen in leukemic cells. For the agglutination test, antiserum dilutions were mixed with an equal volume of a suspension of leukemic or non-leukemic cells containing 10^5 cells in siliconized microprecipitin tubes. After incubating at 37°C for 1 hour, the tubes were centrifuges at 600-800 rpm for one minute and the degree of

agglutination was read.

By means of this immunological procedure, it was found that the white blood cells from all 11 patients with myeloid leukemia and from all 5 lymphocytic leukemic cases reacted with the antisera diluted as high as 1:2048 to 1:8192, whereas no agglutination was given by any of the 15 non-leukemic white blood cells of antiserum dilutions of 1:4 or stronger. These results were confirmed by immuno-diffusion studies for which extracts of the leukemic cells were used. No reaction was observed in a control immuno-diffusion test in which plasma proteins from normal and leukemic persons were crossed. These observations indicate that the anti-leukemic serum can be used as an immunological specific detector of leukemic cells.

The evidence for the existence of human cancer-specific antigens, that is, antigens which are present in cancer and not present in the normal tissues of the same person is reviewed with special emphasis on leukemia. The major technical problems that have been encountered by most of the researchers were the difficulty of detecting and distinguishing the individual components of a complex mixture of antigens and possibly is the major cause of error in the search for cancer-specific antigens.

It may be concluded that some of the studies which purport to show cancer-specific antigens have probably shown only differences in the tissue antigens of different people.

However, some of the more recent and stringently controlled studies have so greatly reduced this source of error that it seems quite probable that these observed antigenic differences are actually leukemia-specific. Further investigations with appropriate comparison of normal and leukemic tissues seem necessary. These studies also show the lack of information as to the immunochemical nature of these elusive but very important leukemia associated antigens. However, in order to elucidate the immunochemical nature of the leukemia associated antigens, these antigen(s) must first of all be isolated and purified. My research project is, therefore, designed to provide some data on the immunochemical nature of the leukemia associated antigen(s), and hopefully to bring us a step closer to the truth in tumor immunology.

B. ORIGINAL RESEARCH

In my research work, I have tried to isolate soluble leukemia associated antigen(s) (LAA) from suitable tissues of human origin, purify them by physical methods and characterize them immunochemically.

In order to achieve these goals, first crude extracts were prepared from the various organs obtained from human leukemic and non-leukemic autopsy cases. Crude soluble constituents of the enzyme disrupted cells were then resolved by a consecutive series of fractionation procedures in order to separate the LAA from other cell constituents.

In the initial stages, spleen, liver, kidney and bone marrow were collected from 8 leukemic and 10 normal autopsied humans. Each tissue was extracted in the manner described in Materials and Methods. The final soluble extracts were injected individually into rabbits for the production of antisera. The antisera were absorbed with the homologous normal tissue extracts (pooled from 10 humans). Then, immunodiffusion tests were set up with unabsorbed and absorbed antisera, against leukemic and normal soluble extracts. With all the unabsorbed antisera, there were many crowded precipitation lines formed against both the leukemic and normal extracts. However, with the absorbed antisera, only one, that produced against 69B371 leukemic <u>liver</u> extract, formed a line against its

homologous liver extract, and no lines against normal liver extracts. All the other absorbed antisera did not produce any precipitation lines after absorption with normal extracts.

Because of the above findings, I decided to continue using only livers for the following project. Thereafter, the antiserum against the 69B371 was used as a tool to identify LAA in the various stages of fractionation.

I. Materials and Methods

(i) Source of Material

The main materials for the research work were the livers obtained from autopsies of humans who died of leukemia and those who died from cases other than leukemia. Full autopsy reports were obtained. Eight leukemic liver specimens were obtained with the following clinical diagnosis: one acute myeloid leukemia, one chronic myeloid leukemia, one acute myeloblastic leukemia, one acute monomyelocytic leukemia, three acute lymphocytic leukemias and one congenital leukemia (Table V). Their ages ranged from 9 days to 69 years with six males and two females.

The control specimens of livers were obtained from 10 non-leukemic, non-infectious cases such as heart failures, multiple traumatic injuries, coronary thromboses and drug overdose (Table VI), and as far as possible, the latter specimens were matched with the leukemic group in respect of the age, sex and blood type of the deceased. The specimens were frozen at -20°C immediately after the autopsies until the time of extraction.

(ii) Extraction of Soluble Antigens

Liver specimens were cut into small pieces with scissors, and washed with 10 changes of 0.85% sodium chloride solution to remove the red blood cells and the extracellular fluids. The washed and minced tissues were then processed as outlines in Table VII.

The specimen that was washed with the physiological saline was resuspended in Hanks' buffered saline solution (HBSS) at pH 7.2 to make a 15% suspension (w/v) and homogenized for 10 minutes in a Sorvall electric omnimixer at 4°C. An ice bath was used to cool the homogenizer cup. The pH of the homogenate was readjusted to 7.2 using 0.17% sodium bicarbonate solution.

Hyaluronidase (250 I.U./ml)(ovine lyophilized salt free, Mann Res. Lab) was added to a final concentration of 250 I.U. per ml of tissue. The enzyme tissue mixture was stirred slowly for one hour at room temperature. The digested tissue was centrifuged in an International Refrigerated Centrifuge at 6000 g for 15 minutes at a temperature of 4°C.

The supernatant was discarded and the sediment was washed three times with 10 X the volume of tissue in 0.85% saline. To facilitate the washing of the tissues, a stirring rod was used to disperse the tissues. Between each washing, the suspension of tissue was centrifuged at 6000 g for 15 minutes. The washings were discarded.

After the third washing, the sediment was resuspended in 3 volumes of 1M KCl and homogenized for 5 minutes at 4000

TABLE V

THE SOURCE OF LEUKEMIC LIVER SPECIMENS

Autopsy No.	Age at Death/Sex	Clinical Diagnosis	Treatment
69B371	23/F	Chronic myeloid leukemia	Pentamidine Penicillin
71B47	24/M	Acute lymphoblastic leukemia	L-Asparaginase Prednisone Cyclophospha-mide
A70/45	4/M	Acute myeloblastic leukemia	Prednisone Vincristine Methotrexate Cytosine Arabinoside Rubidomycin
70B272	69/M	Acute monomyelocytic leukemia	Micostatin Cloxacillin Gentamycin Allopurinol
86243	14/M	Acute lymphocytic leukemia	Phenergan Tarwin Morphine Cytosine Arabinoside
71B51	35/M	Acute lymphocytic leukemia	Intrathecal methodrexate Prednisone Antibiotics (Penicillin, doxacillin, kanamycin, vancomycin)
71B69	47/M	Acute myeloid leukemia	Aquamophyton Demerol Tetracycline
A70/29	9 days/F	Congenital leukemia (undetermined)	Not treated

TABLE VI

THE SOURCE OF NON-LEUKEMIC LIVER SPECIMENS

Autopsy No.	Age at Death/Sex	Clinical Diagnosis
25335	59/M	Congestive heart failure
70B129	62/F	Congestive heart failure
A25643	58/M	Multiple traumatic injuries
A25597	46/F	Congestive heart failure
25802	5/F	Multiple traumatic injuries
A25905	35/M	Coronary thrombosis
25943	28/M	Drug overdose (Barbiturate)
25962	56/M	Coronary thrombosis
25980	42/M	Coronary thrombosis
26002	23/F	Car accident

TABLE VII

EXTRACTION PROCEDURE FOR SOLUBLE TISSUE ANTIGENS

Suspend the washed tissue in Hanks' buffered saline solution (HBSS), pH 7.2, to make a 15% suspension (w/v). Homogenize for 10 minutes in a high-speed Sorvall Omnimixer.

↓

Tissue Homogenate

↓

Digest with hyaluronidase (250 I.U./100 ml) at room temperature for 1 hour with stirring. Centrifuge at 6,000 g for 15 minutes

↓ Sediment

↓ Supernatant (Discard)

↓

Wash in 0.85% saline 3 times. Resuspend cells in 3 volumes of 1M KCl and homogenize for 5 minutes. Add 3 volumes of distilled water and homogenize for 5 minutes. Centrifuge at 20,000 g for 20 minutes.

↓ Sediment (Discard)

↓ Interphase

↓ Upper layer (Discard)

↓

Formalization (12 hrs at room temp.)
(0.2% formalin concentration)

↓

Dialyzed against .001M PBS, pH 7.2

↓

Dialysate
(Centrifuge at 20,000 g for 20 minutes)

↓ Supernatant

↓ Sediment (Discard)

↓

Concentrate by lyophilization

↓

SOLUBLE-ANTIGENS

rpm in the Sorvall omnimixer. The mixture was allowed to stand at room temperature for 1 hour. Then, three volumes of distilled water was added and the suspension homogenized again for 5 minutes. The sudden change in osmotic pressure caused the cells to disrupt as evidenced by observing through the light microscope at 1000 X magnification, samples of the cells before and after adding the 3 volumes of distilled water.

The suspension was then centrifuged at 20,000 g for 20 minutes in the International Refrigerated Centrifuge at 4°C. Three layers resulted from the foregoing centrifugation. The sediment and upper layer were discarded. The interphase consisting of soluble materials was collected and formalin was added to a final concentration of 0.2% and incubated at room temperature for 12 hours.

The formalinized soluble extract was centrifuged again at 20,000 g for 20 minutes to remove the precipitate that was formed during the formalization period. The sediment was discarded. The supernatant, which contain the soluble part of the extract, was dialyzed (Visking dialysis tubings) against 100 times its volume of 0.001M PBS at pH 7.2 for 48 hours at 4°C. This was intended to remove excess formalin and KCl ions. The dialysate was centrifuged at 20,000 g for 20 minutes in the IEC centrifuge at 4°C to remove any precipitate that was formed during dialysis. The sediment was discarded.

The supernatant was concentrated by lyophilization in 100 ml portions using the Virtis apparatus. The soluble fraction was first frozen along the sides of the lyophilization

flasks in a freezing bath of acetone and solid carbon dioxide before applying to the Virtis lyophilization machine. The lyophilization usually took about 12 hours to complete.

The freeze-dried materials were stored in small aliquots in small glass bottles and kept at -20°C until used. Before use, the lyophilized materials were dissolved in 0.001M PBS at pH 7.2

(iii) Purification of Soluble Antigens

The constituents of the extracts obtained as described in the preceding section were first fractionated by means of Vesterberg's et al. (1966) method of isoelectric focusing.

For this procedure, a 20 mg sample of the lyophilized extract was first dissolved in 5 ml of 0.001M phosphate-buffered saline (PBS), pH 7.2, and dialyzed (Visking dialysis tubings) overnight against 5 litres of the saline buffer, at 4°C and stirred with a magnetic stirrer.

A sample equivalent to approximately 10 to 20 mg of the lyophilized material was placed on an isoelectric focusing column (LKB 8101 with 110 ml capacity, see Fig. 2) consisting of 1% ampholine in a sucrose gradient in the initial range of pH 3-10. A current of 4 mA, 500 volts, was applied for 24 hours. During the experiment, the current dropped to a steady 2 mA from the initial 4 mA.

After completion of the focusing, a peristaltic pump (Buchler) was used to withdraw the material from the column into an automated refrigerated (Buchler) fraction collector (see Fig. 5) at 4°C. 3 ml portions of the eluate were collected. The

Figure 2. The isoelectric focusing equipment.

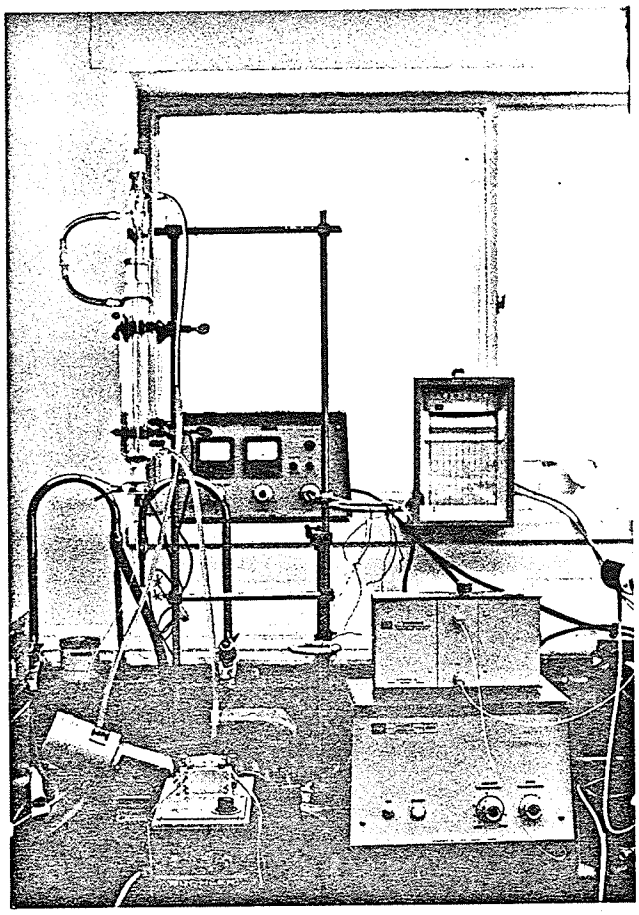


Figure 2

collected fractions were dialyzed individually against 3 changes of 5 litres 0.001M PBS at pH 7.2 in the cold room at 4°C for 48 hours.

The optical absorbance of the components present in the solution were measured at wavelengths of 210, 260 and 280 nm in a Unicam SP1800 Spectrophotometer.

The various peaks from the column were pooled and concentrated to the original applied volume by lyophilization. These concentrated samples were then screened for the presence of leukemia associated antigen(s) in immunodiffusion using cellulose acetate membranes and the antiserum against 69B371 leukemia extract but absorbed extensively with normal tissue antigens to remove all the antibody activities against normal tissue components. This absorbed antiserum reacted only with leukemia extracts and not with normal tissue antigens.

The fractions that indicated the presence of human leukemia associated antigens were purified further by isoelectric focusing at pH range of 4-6, because the antigen has an isoelectric point of between pH 4 and pH 6.

The procedure for pooling and examining these fractions are identical to that described for pH range of 3-10. The peak area(s) that contain the leukemia associated antigens were then subjected to the agarose-gel electrophoresis in the following manner.

Glass slides (2.5 cm wide, 7.5 cm long and 1 mm thick) were first coated with a 2-3 mm thick layer of 1.0% agarose (Mann Research Laboratory) in 0.002M Tris buffer, pH 7.2

The wells (1 mm diameter) cut in the middle of the gel (Fig.13) were then filled with the partially purified antigen preparation and the material was subjected for 5 hours to the electrophoresis in an 0.1M pH 8.6 barbital buffer using a current of 5 mA per slide. Whatman 3M filter paper was cut to the dimensions of 2.5 cm x 7.5 cm and used as wicks for the electrophoresis. It was firstly soaked in the barbital buffer and then placed on each end of the glass slide with 5 mm touching the gel, the other end dipping in the electrode solution of barbital buffer. The LKB D.C. Power Supply 3371E was used to deliver the current for the electrophoresis.

In order to identify the position of the leukemia associated antigen, a trough (2 mm x 5 cm) was cut out in the gel between the two wells after the electrophoresis. Rabbit antiserum produced against the partially purified leukemia associated antigenic fraction of pI 4.85 and absorbed with normal human tissue extracts to remove all visible detectable anti-normal antibodies, was applied to the trough. This absorbed antiserum visibly reacted in immunodiffusion, with only the leukemia antigens and not with the normal tissue antigens. The glass slide was then incubated for 72 hours in a moist chamber at 23°C (that is, room temperature). The leukemia associated antigen was identified in the β_1-globulin position as shown in Fig. 14.

In accordance with the position of a leukemia associated antigen in the agarose gel, a 3 mm length (5 mm

wide) area immediately adjacent to the cathode side of the wells was cut out, the gel from this area was collected and the antigen was eluted in 50 ml 0.001M PBS, pH 7.4 by gentle rocking for 72 hours at 4°C. The eluate was then concentrated by lyophilization to 10 X its original concentration.

(iv) Chemical analysis of the antigens

(a) Enzyme digestion. In order to determine the gross chemical nature of the leukemia associated antigens, 2 ml samples of the soluble antigen digested by the following enzymes at the concentrations show below: 0.03 mg/ml of DNase (Bovine Pancreas Sigma Chemical Co.), 0.01 mg/ml RNase A (Worthington Biochemical Corp.), 0.1 mg/ml Trypsin and 0.1 mg/ml Pronase (B grade, Calbiochem., Los Angeles).

Each enzyme was added to a 1 ml sample solution of the leukemia associated antigen to a final enzyme concentration as shown above. The mixture for the DNase digestion also contained 0.05M $MgSO_4$ in 0.01M PBS at pH 7.2. Each enzyme-substrate mixture was incubated at 37°C for 1 hour with gentle shaking in a mechanical shaker.

The mixtures were then dialyzed against 5 liters of 0.01M PBS at pH 7.2 for 24 hours at 4°C. Control samples of the leukemia associated antigen was treated in a similar way; but instead of adding the enzyme solution, 0.01M PBS at pH 7.2 was added. Each of these samples were then tested in immunodiffusion (cellulose acetate) against the antiserum to the leukemia associated antigen. This antiserum was absorbed with normal antigen to remove any detectable anti-

normal reactions.

The purified antigen preparation was analyzed as to the composition of saccharides and amino acids in the following manner.

The chromatographic analysis of saccharides was conducted on MM300 cellulose pre-coated plates (20 cm x 20 cm). In order to liberate the saccharides, the purified leukemia associated antigen was first suspended in 2 ml of 1.5N sulfuric acid and was heated in an oven for 5 hours at 100°C. The hydrolyzate was adjusted to approximately pH 4.5, using barium hydroxide. Test samples were placed on a pH indicator paper to determine the pH of the hydrolyzate. The resulting sediment was discarded by centrifugation at 20,000 g at 4°C for 30 minutes. The clear supernatant fluid was collected, recentrifuged and 0.1 ml of the solution was spotted on the glass plate coated with MM300 cellulose. 0.1% sugar standards were also placed in duplicate on the glass plates. A hot air dryer was used to dry the spots in order to minimize the spreading of the solution.

The plates were then placed in Kwapinski's (1972) solvent, consisting of a 10:5:2:1 mixture of n-butanol, ethyl alcohol, acetic acid and distilled water for 4 hours at the temperature of 23°C. Afterwards, the plates were dried in the air at room temperature (23°C). The plates were then sprayed with a 1:1 mixture of 0.1% aniline in ethanol and 2.5% oxalic acid at 100°C.

After drying the chromatograms for 10 to 15 minutes at 100°C, colors and locations of monosaccharide spots are recorded and compared with a map of standards. Color of hexose is brown, of pentose pink, and of rhamnose yellow. Pentose gives cerise color; aldohexose brown-yellow; hexulose yellow; 6-oleoxyaldohexoses yellow or yellow-pink.

The position, color and Rf_R values of each sugar spot thus detected were determined. Saccharides were identified by comparing the Rf_R values with those of the appropriate sugar standards. The value of Rf_R can be calculated from the following formula:

$$Rf_R = \frac{\text{Rf of examined saccharide}}{\text{Rf of rhamnose}} \times 100$$

(b) Amino acid analysis. The amino acids content of the purified leukemia associated antigen was determined by using the Beckman Automatic-Analyzer Model 120C.
Principle: Amino acids by definition are organic compounds containing at least one carboxyl (acid) group and one amino (basic) group. They are the basic constituents of all proteins and either neutral, acidic, or basic depending on their structure. The standards that were used for the experiment were the neutral amino acids: the monoaminomonocarboxylic acids (i.e., glycine, adenine, valine, ieo-leveine and leucine), the hydroxyamino acids (i.e., serine and threonine), the sulphur-containing amino acids (i.e., phenylalanine and tyrosine), the pyrrolidyl amino acids (proline).

The acidic amino acids are aspartic acid and glutamic acid, and the basic amino acids are histidine, arginine, tryptophan and lysine.

The Beckman amino acid analyzer effects the separation of the amino acids in a sample by chromatography on ion-exchange resins. When an amino acid is placed on a column of the sodium salt of a polysulfonic resin, ion-exchange takes place.

The amount of a given quantity of an amino acid which is bound to the ion-exchange resin relative to that remaining in solution at equilibrium and under a given set of conditions is usually expressed as a distribution coefficient, K, and the magnitude of this coefficient depends on the structure of the individual amino acid.

Before the assay, the purified leukemia associated antigen (approximately 1-3 mg protein) was hydrolyzed in 5 ml of 5N HCl for 18 hours in a sealed vial at 110°C in an oven. The hydrolyzates were decolorized with charcoal, centrifuged and evaporated in vacuo in the presence of P_2O_5.

The amino acid content of the purified leukemia associated antigen was assayed in the Beckman Model 120C Autoanalyzer using the method of Dus et al. (1966). The times for elution of the various amino acids from the leukemic sample were compared directly against the amino acid standards and consequently the entire amino acid content of the leukemic sample was identified.

(v) Examination of physical properties of the leukemia associated antigen

The leukemia associated antigen dissolved in 0.01M PBS at pH 7.2 was subjected to the action of heat. Two ml samples of the antigen was placed in three water baths at temperatures of 37°C, 56°C, and 76°C for half an hour and one hour periods. After the incubation period, the heat treated samples and control antigen were tested for leukemia specific activities in immunodiffusion against an antiserum (suitably absorbed) that produced detectable reactions in immunodiffusion only with leukemia associated antigens and not with normal antigens.

(vi) Production of antisera

The various antigen preparations obtained after the last step of the extraction procedure and each major step of purification thereafter were adjusted to a concentration corresponding to 0.3 absorbance at 280 nm using the Beckman SP1800 spectrophotometer.

One albino rabbit per antigen was injected at 3 to 4 day intervals according to the following schedule: subcutaneous injections of 0.1, 0.2 and 0.2 ml of the antigen preparation emulsified in an equal volume of incomplete Freund's adjuvant followed by two subcutaneous injections of 0.4 ml of the antigen above, then 0.4 ml of the antigen injected intravenously and finally 0.6 ml of the antigen injected subcutaneously. The rabbits were bled from the

ear 5 days after the last injection. The collected antisera were kept frozen at -20°C until needed.

(a) Partial purification of antibodies. The immunoglobulins were extracted from the rabbit antisera by ammonium sulfate precipitation. For this purpose, half the volume of saturated ammonium sulfate, adjusted to pH 7.8 with 2N NaOH, was added to every volume of serum. This was done in a dropwise fashion with constant stirring at 4°C. The suspension was left standing at 4°C for 3 hours after which it was centrifuged at 1000 g for 30 minutes at 4°C in the IEC refrigerated centrifuge. The precipitate was dissolved in 0.01M PBS at pH 7.2 to half the original volume of serum. This solution of immunoglobulins was dialyzed at the PBS for 72 hours at 4°C with 3 changes of 5 liters of PBS. The prepared antisera were kept frozen at -20°C without preservatives until used.

(vii) Absorption of antisera

Absorption of the antisera was carried out by adding two parts of a crude soluble antigen preparation, adjusted to the O.D. of 0.3 using 0.01M PBS at pH 7.2, from non-leukemic cases, to three parts of the antisera. The mixture was incubated for 3 hours at 37°C with gentle shaking on a mechanical shaker. The absorbed serum was then centrifuged at 20,000 g for 20 minutes at 4°C in the IEC centrifuge. The resulting clear supernatant was subjected to a second absorption and centrifuged under the same conditions.

Finally, it was concentrated to half the original volume of antiserum by adding lyphogel (Gelman Instrument Company).

(viii) The immunological assay

The antigen preparations were assayed with the antisera prepared as above by means of immunodiffusion using two different techniques, namely, the cellulose acetate and agarose gel techniques.

(a) Principles of the immunodiffusion reaction.

The immunodiffusion precipitation test is a method of immunologic analysis of complex soluble materials by optically revealing complexes of antigens and antibodies, formed in gel or on the cellulose-acetate membrane. The visible complexes are formed after the diffusion of antigen and antibody molecules towards each other and concentrated in an area of optimal proportion. The diffusion of antigen and antibody through semisolid media consists in the migration of a zone of high concentration to areas of lower concentration. Individual antigens have a different electric charge, molecular structure and specific weight and therefore, possess various diffusion coefficient. The components of a complex soluble material therefore separate in the gel as they diffuse and may react with the corresponding antibodies in different sites of the gel medium, forming visible precipitate bands. An immunoprecipitate formed in gel or in cellulose acetate membrane allows immunologically unrelated molecules to pass through the zone of the immunoprecipitate, but antigens and

antibodies immunologically similar to those which have formed the precipitate cannot penetrate the barrier. It is due to the immunoprecipitate acting as a selective barrier that makes it possible to discriminate between and compare different antibodies and antigens. Consequently, different antigen-antibody complexes form distinctly different immunoprecipitant lines.

The equipment for the test consists of a suitable plastic template, selected from 20 different templates designed by Kwapinski for macro- and microprocedures (Fig. 3). The template rests on a plastic plate (Immunocell, National Instrument Labs, Rockville, Md.), equipped with a rubber base, on which a cellulose acetate membrane is placed. The membrane is previously soaked in a 0.01M PBS pH 7.2. After the plastic chamber has been reassembled, 0.16 ml of the antigen solutions are placed in wells (4 mm diameter in macro-plates) and 0.3 ml of antiserum is pipetted in the center well. If microtemplates are employed, the amounts of antigen and antiserum are reduced to 0.03 and 0.1 ml, respectively. The well diameter is then 2 mm.

The plates are incubated at 37°C in a moist chamber for 3 days, and then the membranes are removed, washed in 0.85% saline and stained with 0.2% Porceau S (Allied Chemicals) in 3% acetic acid, and then decolorized with 6% acetic acid. The membranes are examined for the presence of red-colored precipitation bands in a transmitted light viewer, equipped

Figure 3. Micro- and macro-plastic templates and a base equipped with a rubber base and a cellulose-acetate membrane.

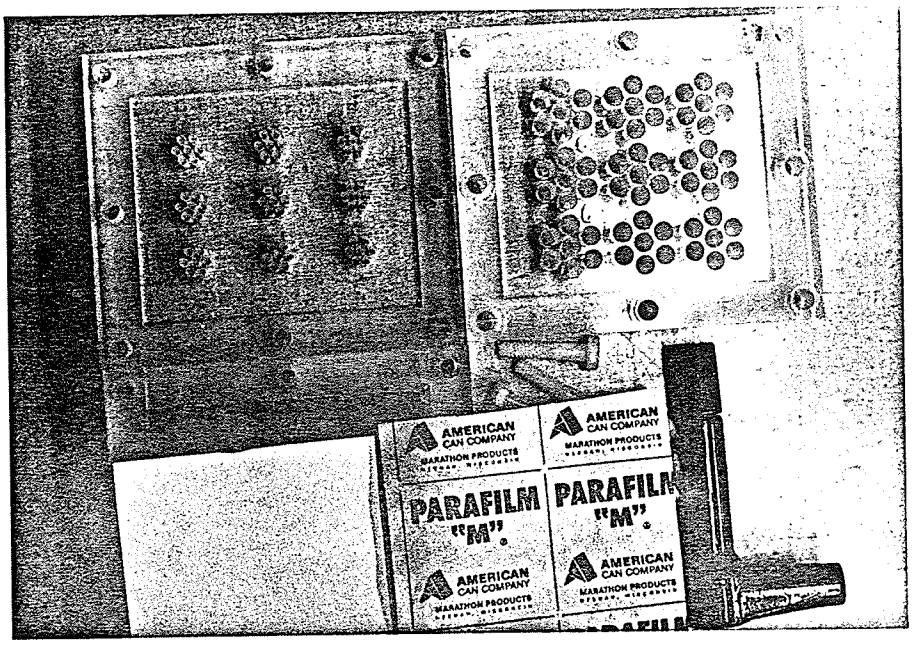

AMERICAN
CAN COMPANY
MARATHON PRODUCTS
AMERICAN
CAN COMPANY
MARATHON PRODUCTS
PARAFILM
"M"
PARAFILM
"M"
AMERICAN
CAN COMPANY
MARATHON PRODUCTS
AMERICAN
CAN COMPANY
MARATHON PRODUCTS

Figure 3

with a 10 X magnifying glass.

The membranes may be cleared in liquid paraffin or in a 1:10 mixture of glacial acetic acid and ethyl alcohol. In the latter case, the membranes must immediately be placed on a glass plate since they shrink. Caution must be applied at the clearing of membranes since faint precipitation bands sometimes are not visible after the clearing of membrane.

Each test was set in duplicate and was repeated twice. The number of precipitation bands and the relation and position of adjacent or fused bands was noted.

For the agarose-immunodiffusion test, Scheidegger's (1955) method was used. Thus, the agarose was prepared in 0.85% sodium chloride with the addition of a few drops of 1:10,000 merthiolate to make an 0.9% final concentration of agarose. Wells were cut with a Feinberg agar-gel cutter. The diameters of the center well and the outer wells were 14 mm and the distance between the center and the outer wells was 10 mm apart. Approximately 0.5 ml of the antigen (O.D. 0.3 at wavelength of 280 nm) was applied to the outside wells and the center well received the same volume of the antiserum.

The plates were incubated in a moist chamber at 37°C for 48 hours. Precipitant bands were recorded as lines of identity, non-identity and partial identity of the different soluble antigens with the antiserum.

For the micro-immunoelectrophoresis test, the method

described by Prince (1968) was used.

An 0.9% melted agarose was prepared by dissolving in 0.002M Tris buffer of pH 7.2, by autoclaving at 121°C for half an hour. One ml of an 0.1M sodium azide was added to 100 ml of the melted agarose. Microscopic slides (2.5 cm x 7.5 cm) were first precoated with a thin film of 0.5% agarose for a better adherence of the agarose layer proper. The precoated glass slides were placed on a level surface and 2.5 ml of the molten agarose preparation was pipetted onto each slide. A cover was placed over the slides to allow the agarose to solidify at 23°C without drying out in the process. The agarose slides were stored in a moist chamber at 4°C for at least 5 hours before the wells were cut.

The trough and wells were cut in the agarose slide using a gel cutter (Shandon). By using suction, the agarose from the wells only was removed.

The electrophoresis apparatus (Colab Canada) was filled with 0.1M Veronal buffer, at pH 8.6. The wells were filled with the appropriate antigens. Whatman 3M filter paper wicks were used. A power source (Vokam) was used to provide a current of 5 mA per agarose slide. The electrophoresis was continued for 1 1/2 hours or 5 hours as needed.

After the electrophoresis, the agarose in the antibody troughs (2 x 40 mm) were removed by the use of a specially shaped spacer which had the width of the trough.

The appropriate antibody or serum was then placed in a level, humid chamber for 24-72 hours, at 23°C. The slides were examined for the presence of precipitant lines with oblique illumination against a black background.

For preservation, the slides were washed at 4°C with 1 liter of phosphate buffered saline (0.01M,pH 7.2) for 48 hours with one change of saline. After the washing, the slides were placed between layers of dry filter paper and left for a minimum of 8 hours in a 37°C incubator. The agarose was dried to a thin film in this process. The slides were then stained with an .002% nigrosine (The British Drug House, B.D.H. Eng.) in 2% acetic acid solution for 5 minutes at 23°C. The slides were then destained by washing in 6% acetic acid for 2 minutes, and then air-dried. The slides were examined for immunoprecipitant lines using a strong light, and photographed.

II. RESULTS

When the liver specimens were cut to small pieces, it was found necessary to wash the cut tissues with 5 to 6 changes of physiological saline before the tissues were free of hemolysed red blood cells. On adding three volumes of distilled water to the tissue suspension that was in 1M KCl, the sudden change in osmotic pressure caused the cells to disrupt as evidenced by observing through the light microscope at 1000 X magnification, the samples of cells before and after adding the 3 volumes of distilled water. After centrifuging this solution, three layers of materials could be seen, the upper layer consisted of a thin membranous material, the middle layer was the clear liquid containing the soluble antigens; and the third layer was the tissue sediment. The middle soluble layer was collected and on formalization, additional precipitates were formed. These precipitates were removed by centrifugation as described earlier. The resulting soluble extract on dialyzing against 0.001M PBS at pH 7.2, resulted in more precipitates coming out of solution. After removal of these precipitates by centrifugation the resulting solution contained only soluble material. The solution was colorless.

An antiserum that was prepared against the soluble leukemia antigen (69B371) reacted in immunodiffusion using cellulose-acetate membranes, with both leukemic and normal

liver extracts producing approximately 6 or more precipitant lines (Fig. 4). However, after absorption of the antiserum with pooled normal liver antigens, the resultant antiserum reacted in immunodiffusion with only the myelocytic leukemic liver extracts (Fig. 5 and Table VIII) but not with any of the normal liver extracts or the lymphocytic leukemia liver extracts. This was the only one of 9 antisera which behaved in this manner. This absorbed antiserum was used in the assay for leukemia associated antigen(s) in the fractionation experiments, and shall be referred to in the following description as the "leukemia specific antiserum".

The soluble liver extract of specimen 70B272, fractionated by the isoelectric focusing method with pH 3-10 ampholine, yielded 3 major peaks appearing at pI 4.0, pI 4.4, pI 6.1, and two smaller peaks at pI 7.5 and pI 8.8 for specimen 70B272 (acute monomyelocytic leukemia) (Fig. 6).

The absorbed leukemic antiserum which did not react with normal liver antigens on immunodiffusion in cellulose acetate, produced one immunoprecipitant band with the fraction corresponding to the peak of pI 4.4 (shaded area in Fig. 4) and not with any of the other 4 peak areas.

Similarly, specimen A70/45 (acute myeloblastic leukemic) was fractionated and the peaked areas processed as described in Materials and Methods. The peak corresponding tp pI of 4.4 reacted in immunodiffusion with the leukemia specific antiserum. All the other peak areas did not react (Fig. 7).

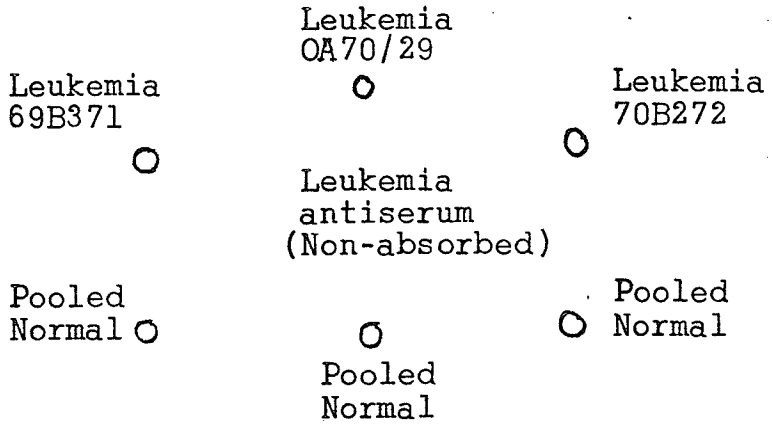


Figure 4. The immunodiffusion assay on cellulose-acetate membrane of leukemia antigens and normal tissue antigens versus leukemia antiserum (non-absorbed).

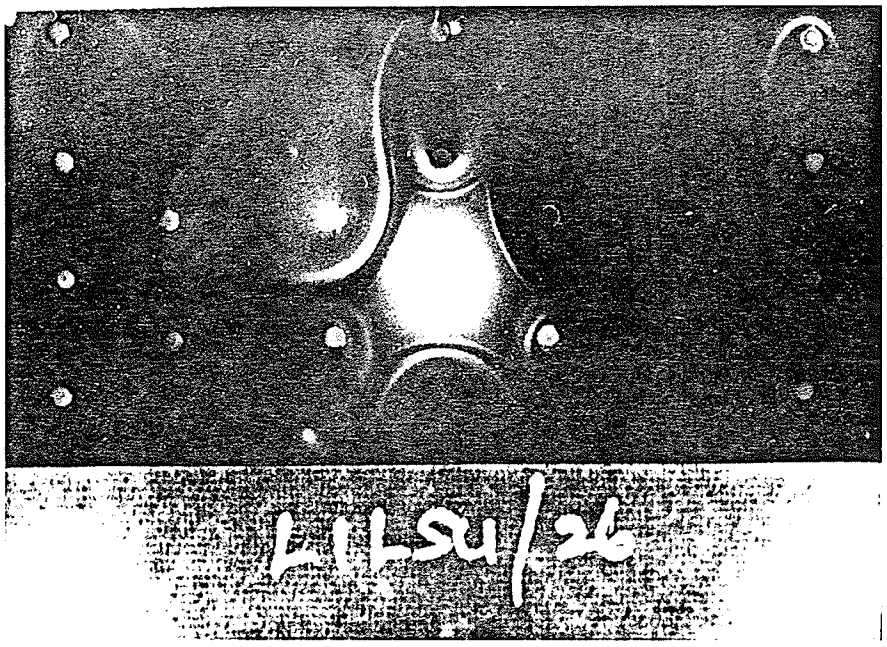


Figure 4

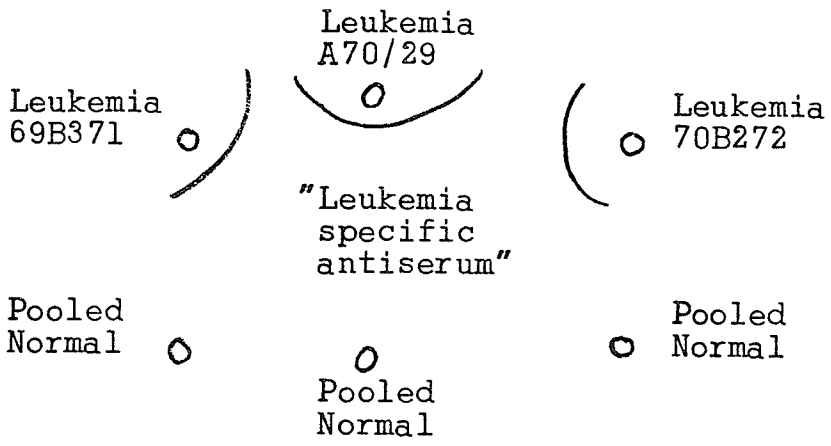


Figure 5. Immunodiffusion assay on cellulose-acetate membrane of leukemia antigens and normal tissue antigens versus absorped leukemia antiserum.

Figure 5

TABLE VIII

REACTIONS IN IMMUNODIFFUSION OF LEUKEMIA SPECIFIC ANTISERUM WITH SOLUBLE HUMAN LIVER EXTRACTS

Specimen Number	Leukemia Specific Antiserum to 69B371 Absorbed with Pooled Normal Antigens
A70/29 (Congenital leukemia)	+ (one immunoprecipitant line)
69B371 (Acute myeloid leukemia)	+ (one immunoprecipitant line)
A70/45 (Acute myeloblastic leukemia)	+ (one immunoprecipitant line)
70B272 (Acute monomyelocytic leukemia)	+ (one immunoprecipitant line)
71B69 (Acute myeloid leukemia)	+ (one immunoprecipitant line)
71B47 (Acute lymphoblastic leukemia)	-
86243 (Promyelocytic leukemia)	-
71B51 (Acute lymphocytic leukemia)	-
Non-leukemics	
25335	-
70B129	-
A25643	-
A25597	-
25802	-
A25905	-
25943	-
25962	-
25980	-
26002	-

Figure 6. An example of the separation of constituents of the 70B272 specimen extracted by isoelectric focusing using ampholine of pH 3-10. The leukemia-reacting antigen was contained in the pI 4.4 area.

Figure 6

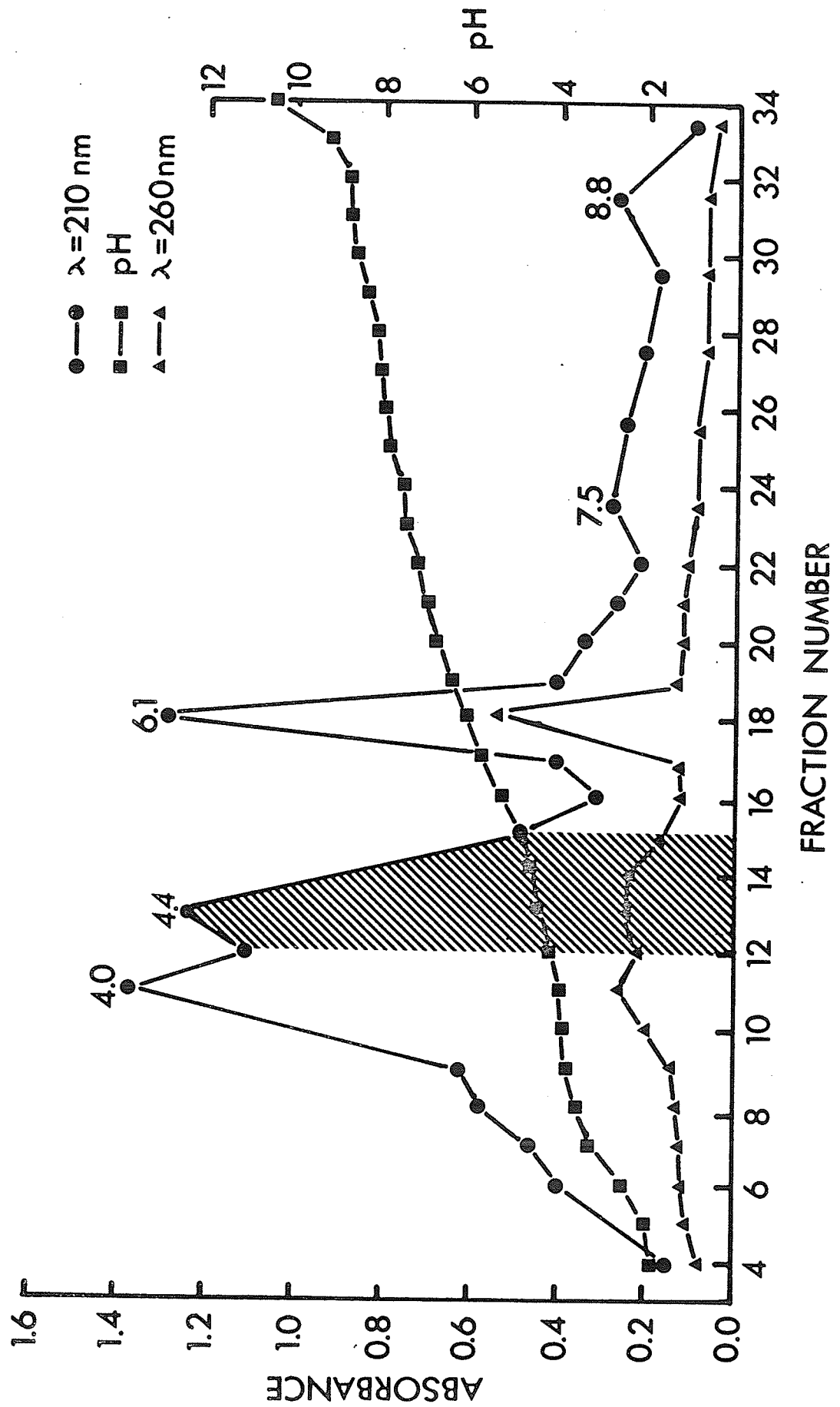
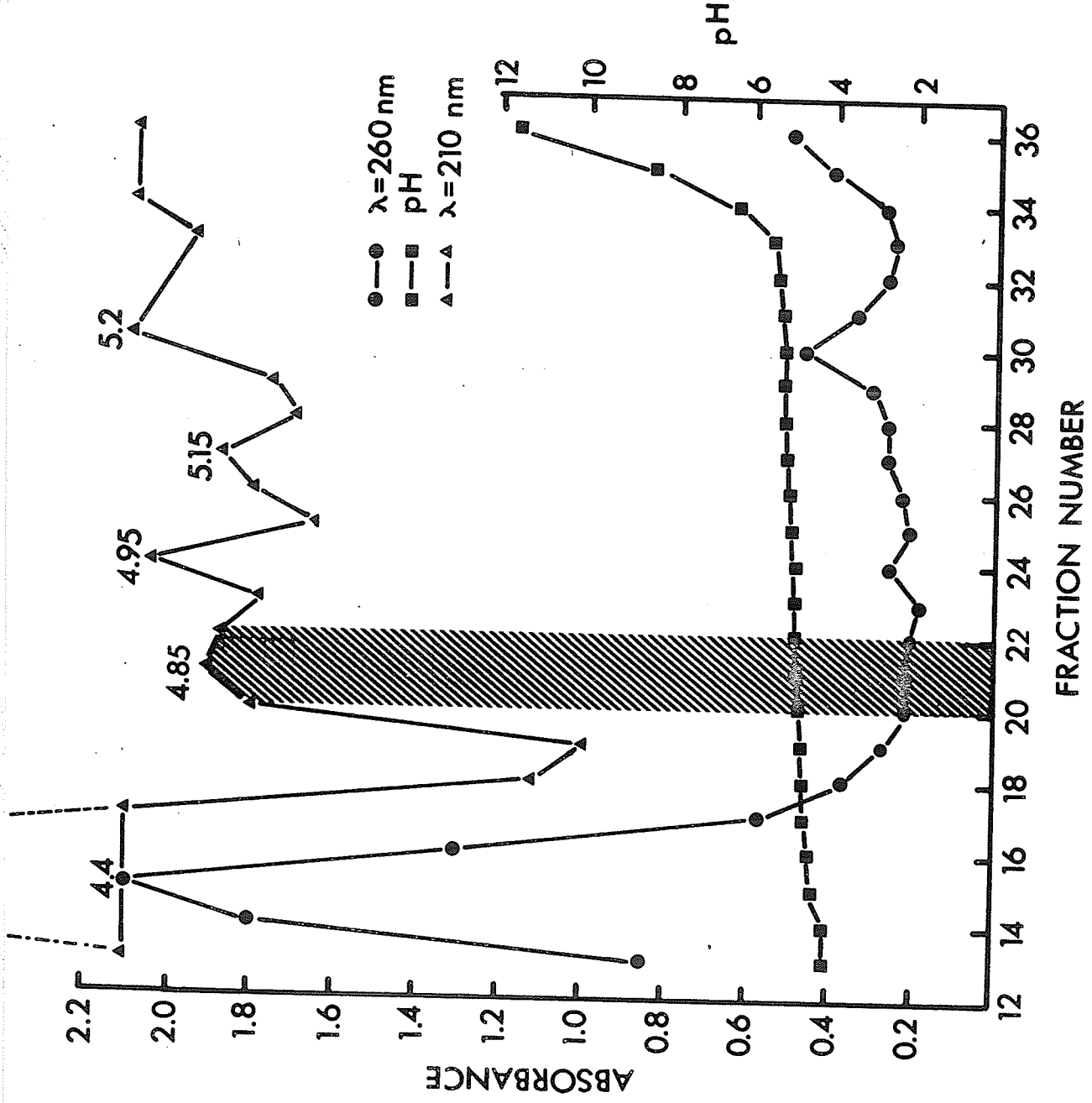

λ=210 nm
pH
λ=260nm
ABSORBANCE
pH
FRACTION NUMBER
4.0
4.4
6.1
7.5
8.8

Figure 7. An example of the separation of constituents of the 70B272 specimen extracted by isoelectric focusing using ampholine of pH 4-6. The leukemia-reacting antigen was contained in the pI 4.85 area.

Figure 7


ABSORBANCE
FRACTION NUMBER
pH
λ=260 nm
pH
λ=210 nm
4.4
4.85
4.95
5.15
5.2

On the other hand, when specimen 71B51 (acute lymphocytic leukemia) was similarly fractionated, none of the peak areas reacted with the leukemia specific antiserum. The fractionation patterns are shown in Fig. 8. The peak areas fractionated from specimen NPA (Normal pooled antigens) as shown in Fig. 13 also did not react in immunodiffusion with the leukemia specific antiserum.

Since the leukemia reactive antigen was identified in fractions of pI 4.4, a narrower pH gradient of ampholine having a range of pH 4-6 was used in order to achieve finer separations of the macromolecules.

The soluble antigen preparations of the five specimens that reacted in immunodiffusion with the leukemia specific antiserum (i.e., specimens A70/29, 69B371, A70/45, 70B/272, and 71B69) were fractionated using the LKB 8101 isoelectric focusing column with ampholine of pH 4-6 range.

When the various fractions were examined by immunodiffusion procedures in agarose gel and cellulose-acetate membranes against the "leukemia specific antiserum", only the fractions corresponding to the pI of 4.85 (#A70/29, Fig. 10), pI 4.82 (#69B371, Fig. 11), pI 4.8 (#A70/45, Figs. 12 and 13), pI 4.85 (#70B272, Fig. 6) and pI 4.8 (#71B69, Fig. 14) reacted, producing a single precipitant band each. All the other peak areas did not react with this "leukemia specific antiserum".

Figure 8. Fractionation of leukemic specimen #71B51 by isoelectrofocusing with pH gradient of 3-10.

Figure 8

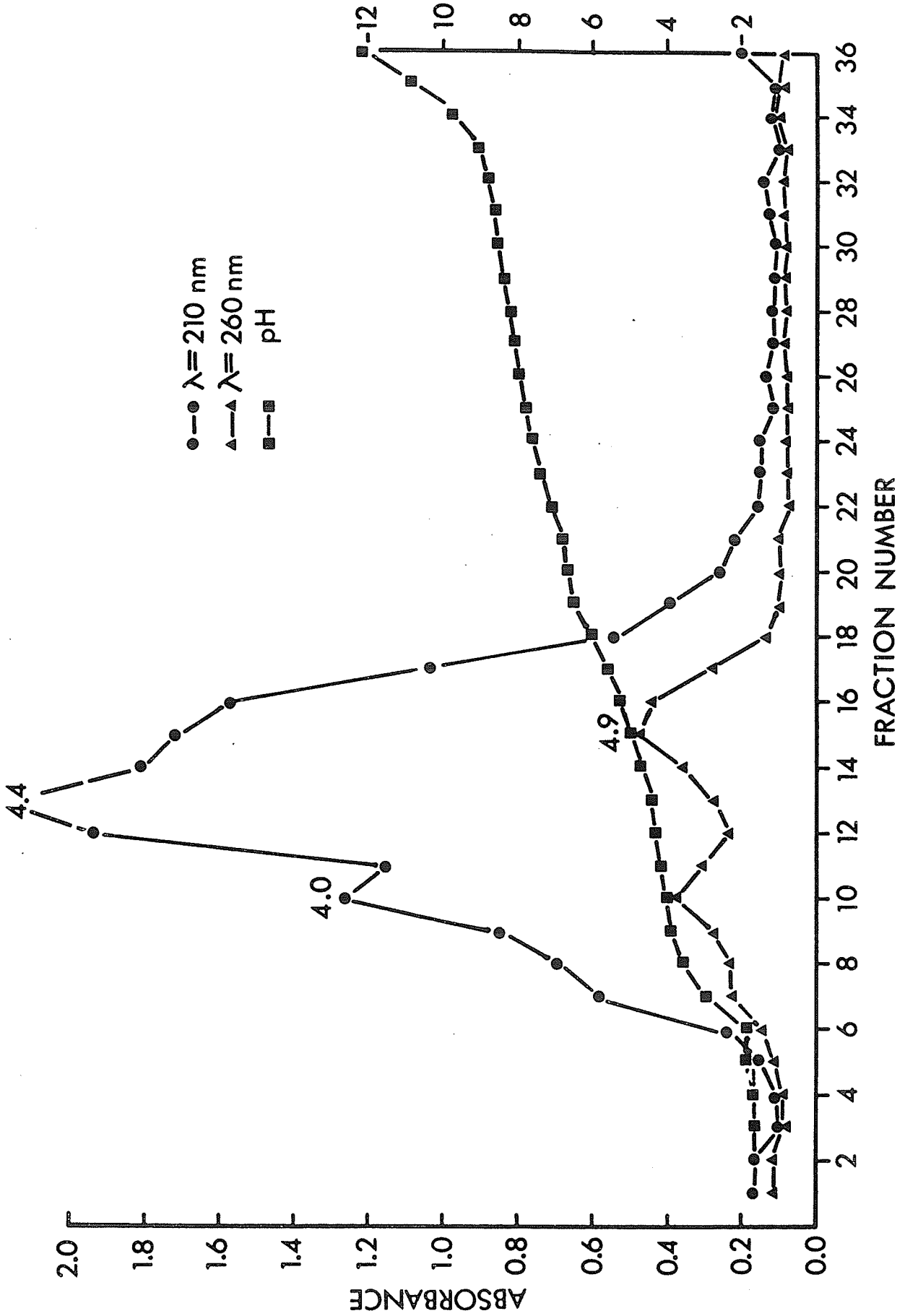


Figure 9. Fractionation of normal pooled antigen by isoelectrofocusing with pH gradient of 3-10.

Figure 9

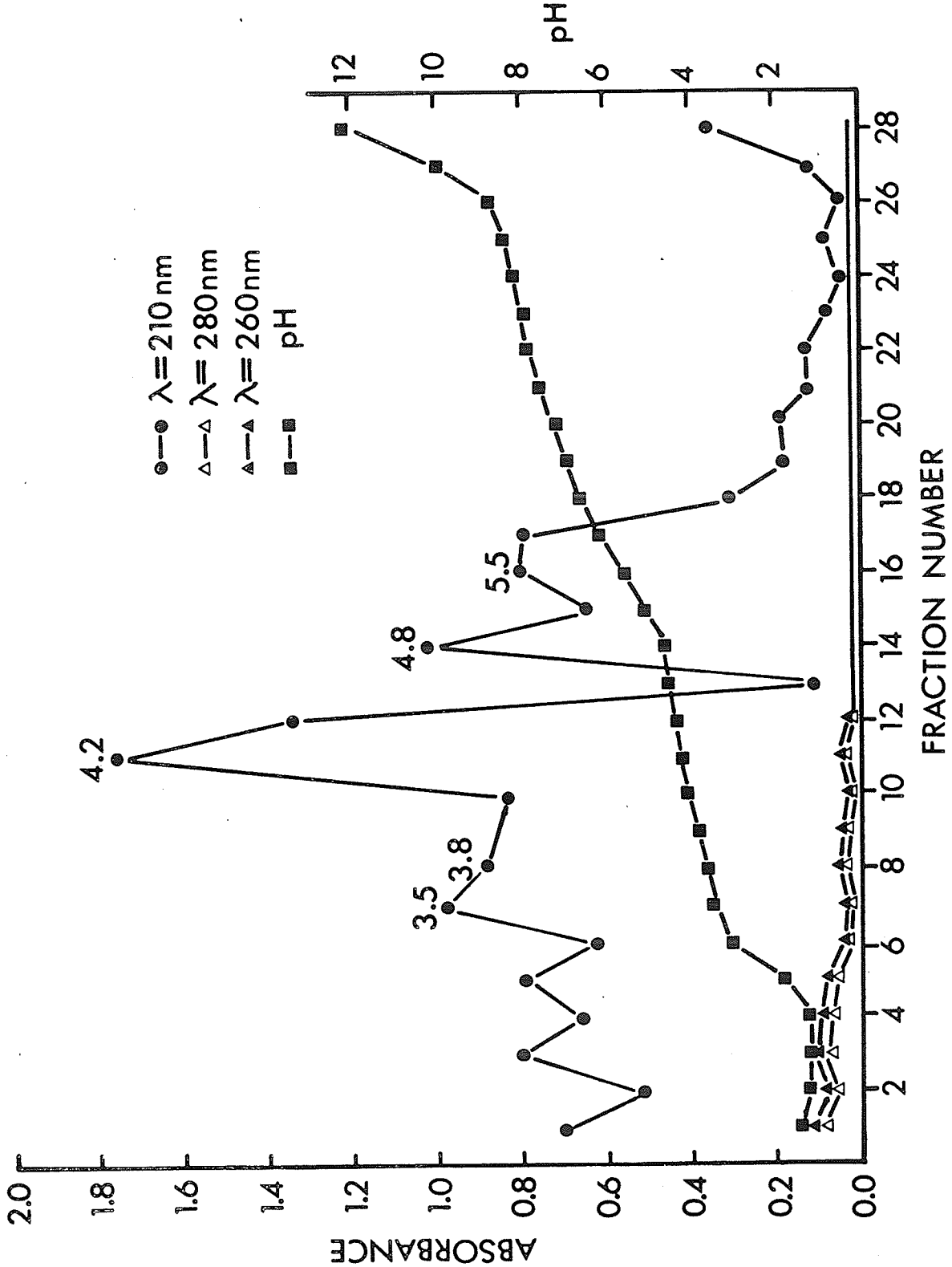

λ=210nm
λ=280nm
λ=260nm
pH
ABSORBANCE
FRACTION NUMBER
4.2
3.8
3.5
4.8
5.5

Figure 10. Fractionation of leukemic specimen #A70/29 by isoelectrofocusing with pH gradient of 4-6.

Antigen in shaded area reacts with leukemia specific antiserum.

Figure 10

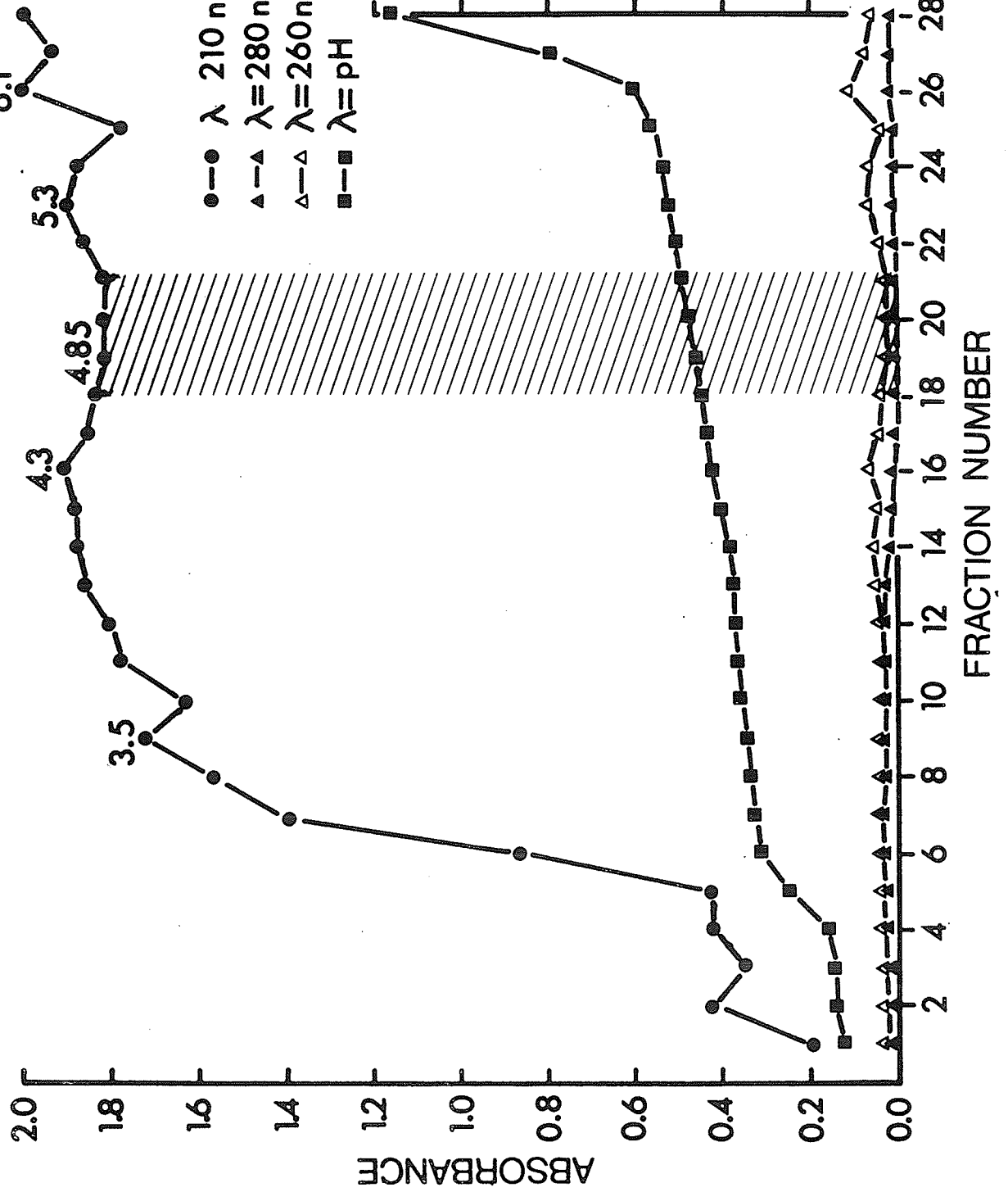

ABSORBANCE
FRACTION NUMBER
pH
λ 210 nm
λ=280 nm
λ=260 nm
λ= pH
3.5
4.3
4.85
5.3
6.1

Figure 11. Fractionation of leukemic specimen #69B371 by isoelectrofocusing with pH gradient of 4-6.

Antigen in shaded area reacts with leukemia specific antiserum.

Figure 11

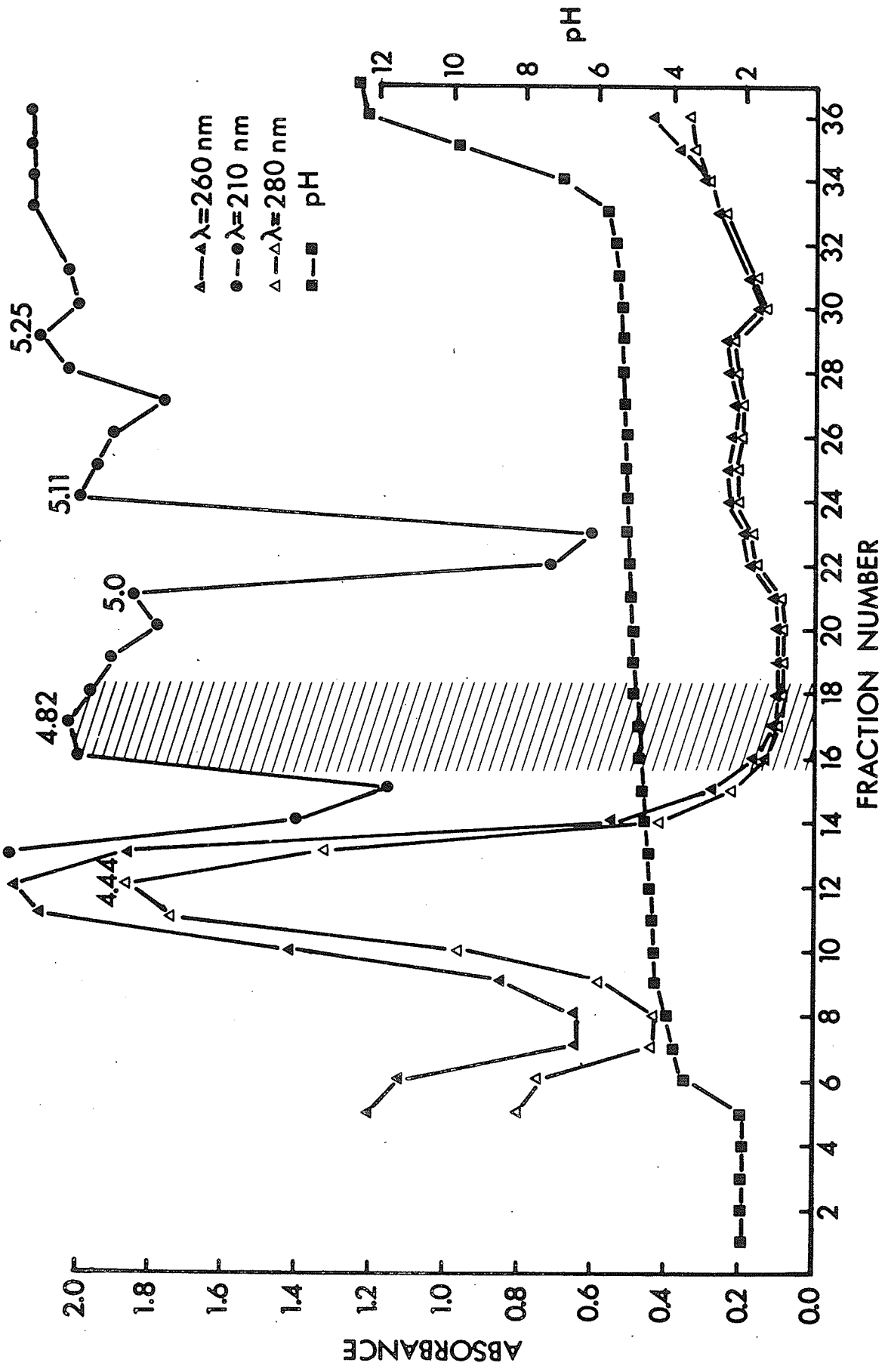

λ=260 nm
λ=210 nm
λ=280 nm
pH
4.82
4.44
5.0
5.11
5.25
ABSORBANCE
FRACTION NUMBER
pH

Figure 12. Fractionation of leukemic specimen #A70/45 by isoelectrofocusing with pH gradient of 4-6.

Antigen in shaded area reacts with leukemia specific antiserum.

Figure 12

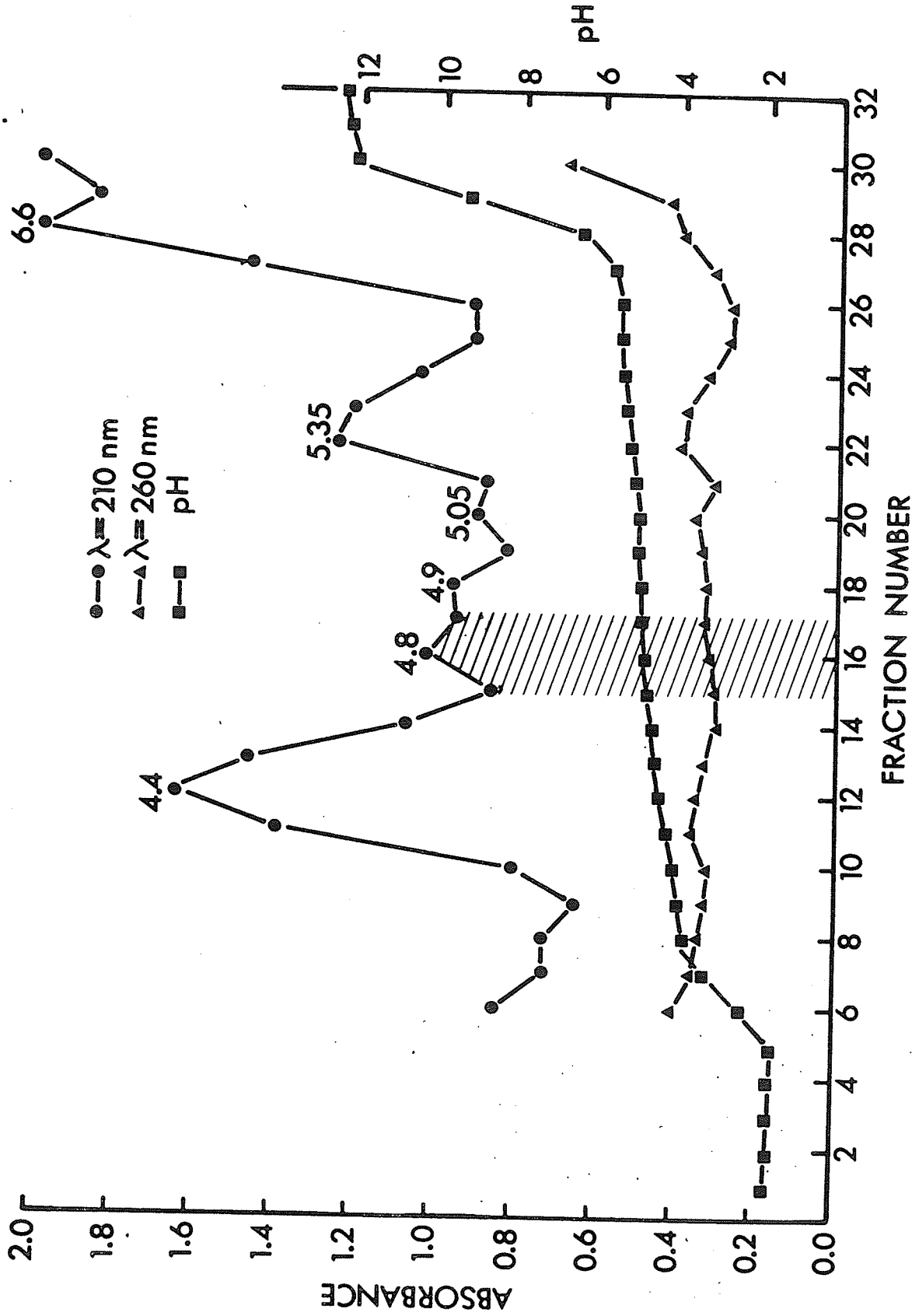

λ= 210 nm
λ= 260 nm
pH
6.6
5.35
5.05
4.9
4.8
4.4
ABSORBANCE
FRACTION NUMBER
pH

Figure 13. Fractionation of leukemic specimen #A70/45 by isoelectrofocusing with pH gradient of 3-10.

Fraction in shaded area reacts with leukemia specific antiserum.

Figure 13

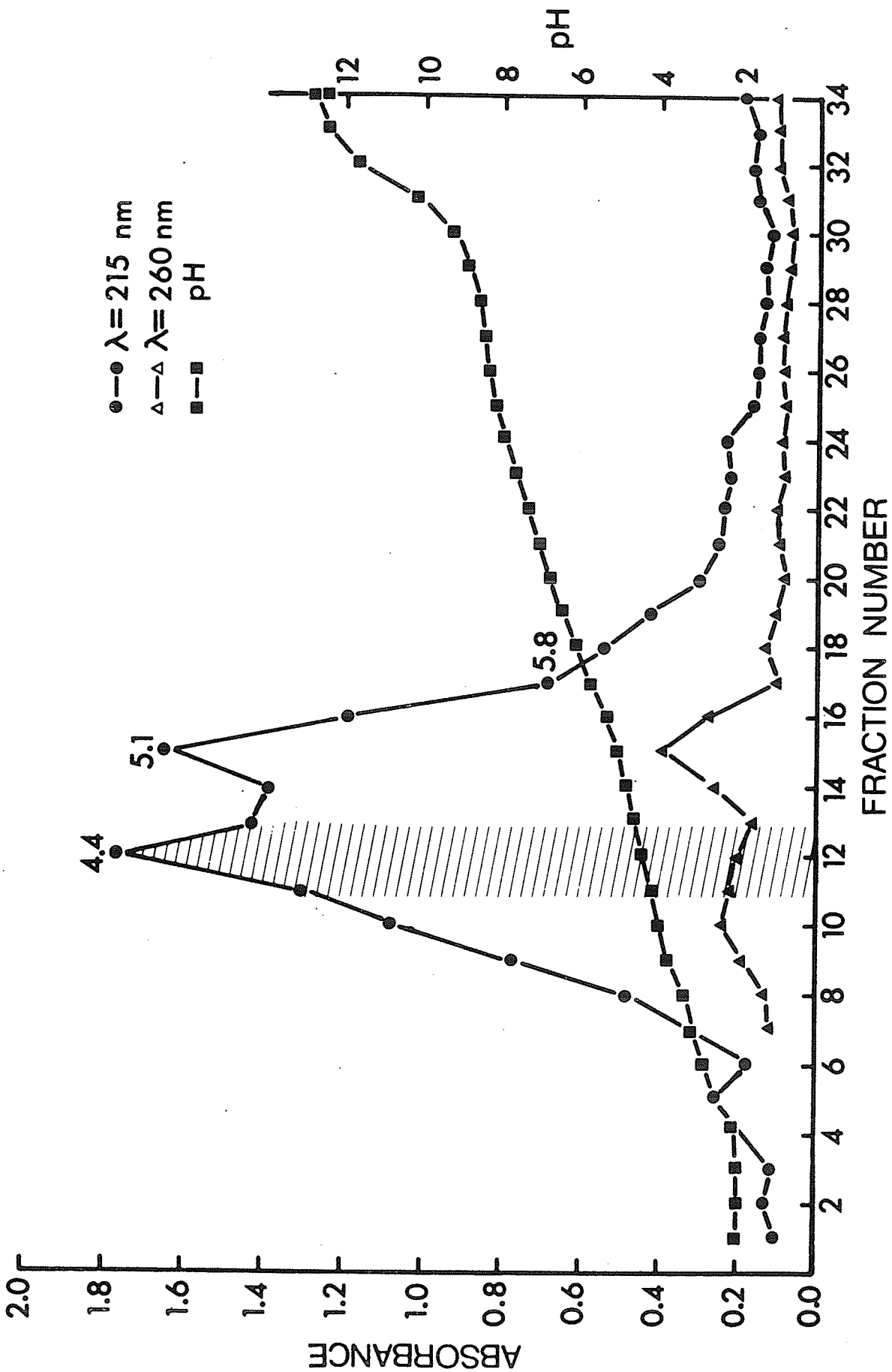
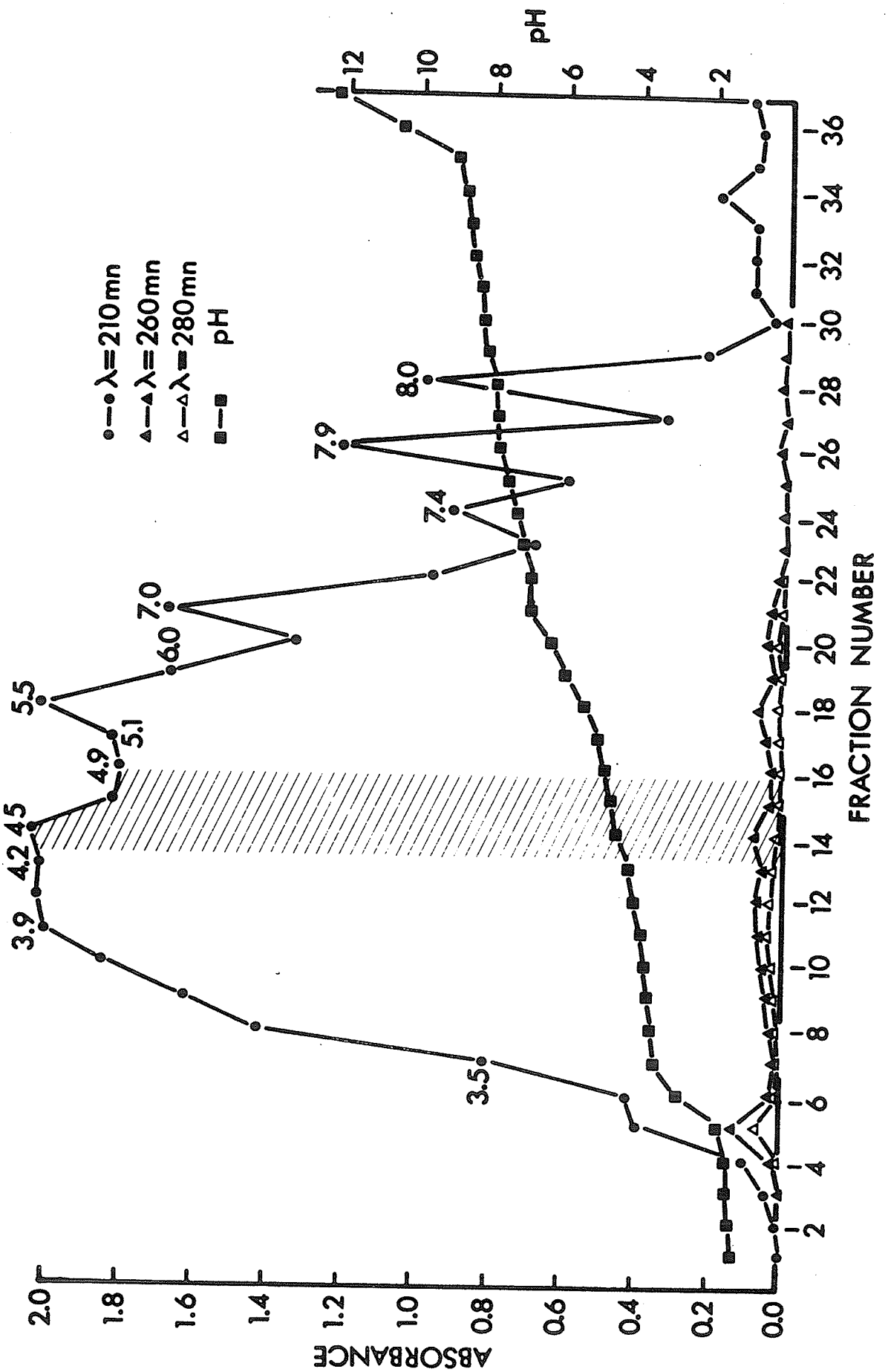


Figure 14. Fractionation of leukemic specimen #71B69 by isoelectrofocusing with pH gradient 3-10.

Fraction in shaded area reacts with leukemia specific antiserum.

Figure 14


λ=210mn
λ=260mn
λ=280mn
pH
ABSORBANCE
FRACTION NUMBER
pH
3.9
4.2
4.5
4.9
5.1
5.5
6.0
7.0
3.5
7.4
7.9
8.0

Having identified the pI 4.85 fraction of 70B272 as containing LAA, an antiserum was made against it by the method used previously. This was found on immunodiffusion to react with 6 out of 9 preparations of soluble leukemic preparations (Table IX) three of which were derived from myelocytic and one from congenital leukemia. The lymphocytic leukemia extracts did not react with the absorbed leukemic serum. The unique antigen associated with the myeloid leukemias will be referred to as the leukemia associated antigen in this thesis.

Using an unabsorbed antiserum to the pI 4.85, it was revealed that 3 precipitant bands were formed on the immunoelectrophoresis of the leukemic pI 4.85 fraction (Fig. 15) and only two bands with pooled normal liver extracts. On absorption of the leukemic antiserum, only one precipitant band was formed with the leukemic pI 4.85 fraction, and no reaction with the normal antigens. This leukemia associated antigen migrated to the β_1 region as shown in Fig. 16.

However, it was observed that on prolonged electrophoresis (5 hours instead of 1 1/2 hours) the "normal" antigenic fractions migrated away from the β_1 position leaving the leukemia associated antigen isolated at the β_1 position (Fig. 17).

Taking advantage of this finding the leukemia associated antigen was further purified by prolonged electrophoresis on agarose gel. The agarose gel corresponding

TABLE IX

THE NUMBER OF PRECIPITATION BANDS FORMED IN IMMUNOELECTROPHORESIS IN AGAROSE-GEL

Antiserum against:	Soluble antigens extracted from the liver of autopsy No.:									
	69B371	A70/29	A70/45	71B69	70B272	pI 4.85 70B272	86243	71B47	71B51	Normal (Pooled) extracts
	Acute Myeloid Leukemia	Congenital Leukemia	Acute Myeloblastic Leukemia	Acute Myeloid Leukemia	Acute Mono-Myelocytic Leukemia	Acute Mono-Myelocytic Leukemia	Promyelocytic Leukemia	Acute Lymphoblastic Leukemia	Acute Lymphocytic Leukemia	
pI 4.85 fraction from 70B272	3	3	3	3	3	2	2	2	2	2
pI 4.85 fraction from 70B272 (absorbed with pooled normal tissue-antigens)	1	1	1	1	1	1	0	0	0	0

Figure 15. Precipitant bands formed on immunoelectrophoresis

Upper well: Leukemia antigen No. 70B72.
Lower well: Pooled normal antigen.
Trough: Non-absorbed antiserum against partly purified antigen No. 70B72.
Length of electrophoresis: 1 1/2 hours.

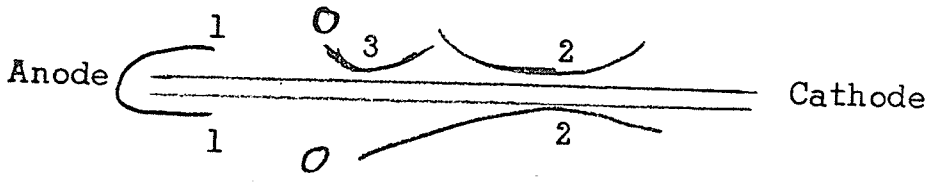


Figure 15

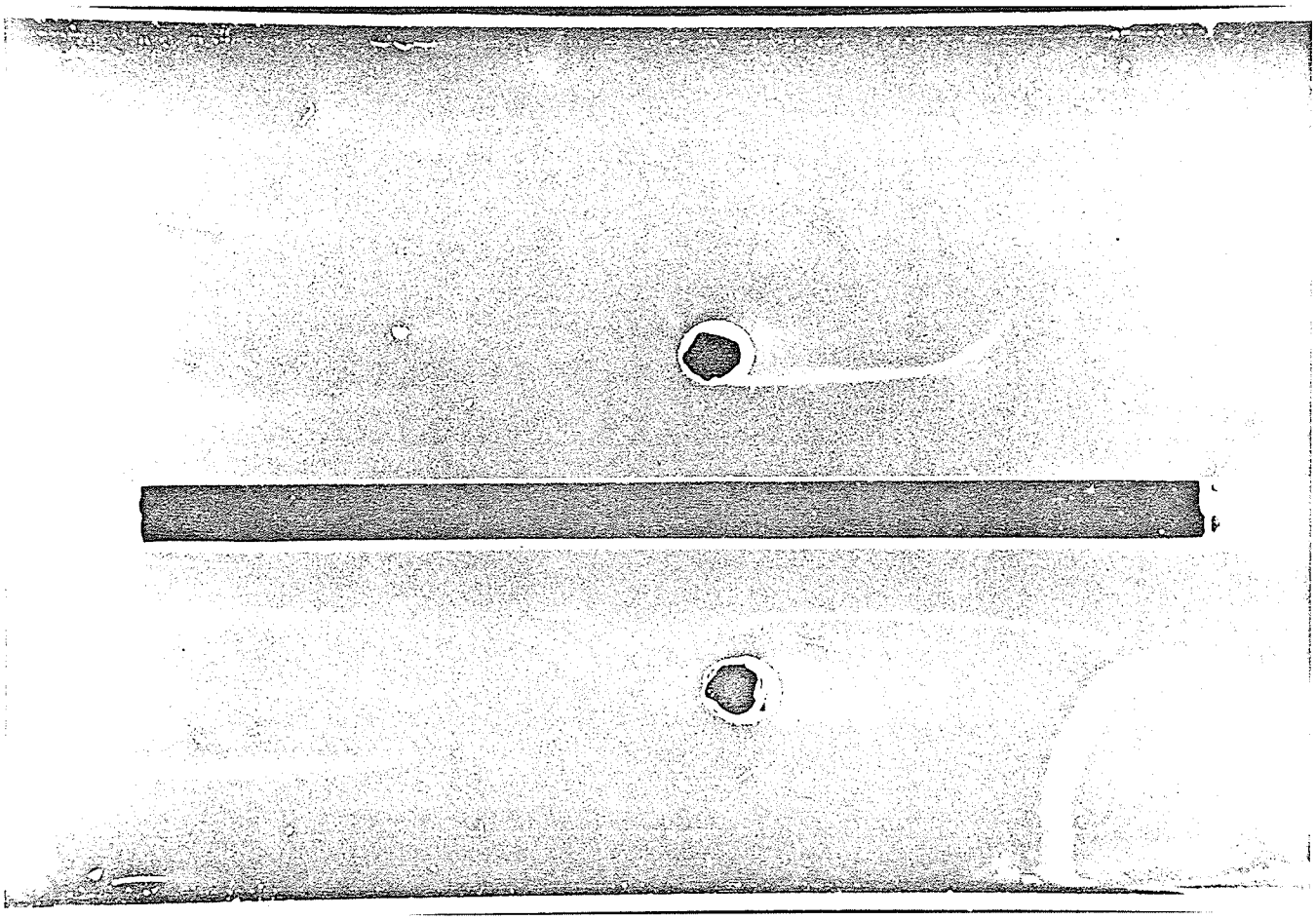


Figure 16. Precipitant band formed on immunoelectrophoresis (ii).

Upper well: Leukemia antigen A70/45.
Lower well: Pooled normal antigen.
Trough: Leukemic antiserum absorbed with pooled normal human antigens.
Time of electrophoresis: 1 1/2 hours.

Figure 16

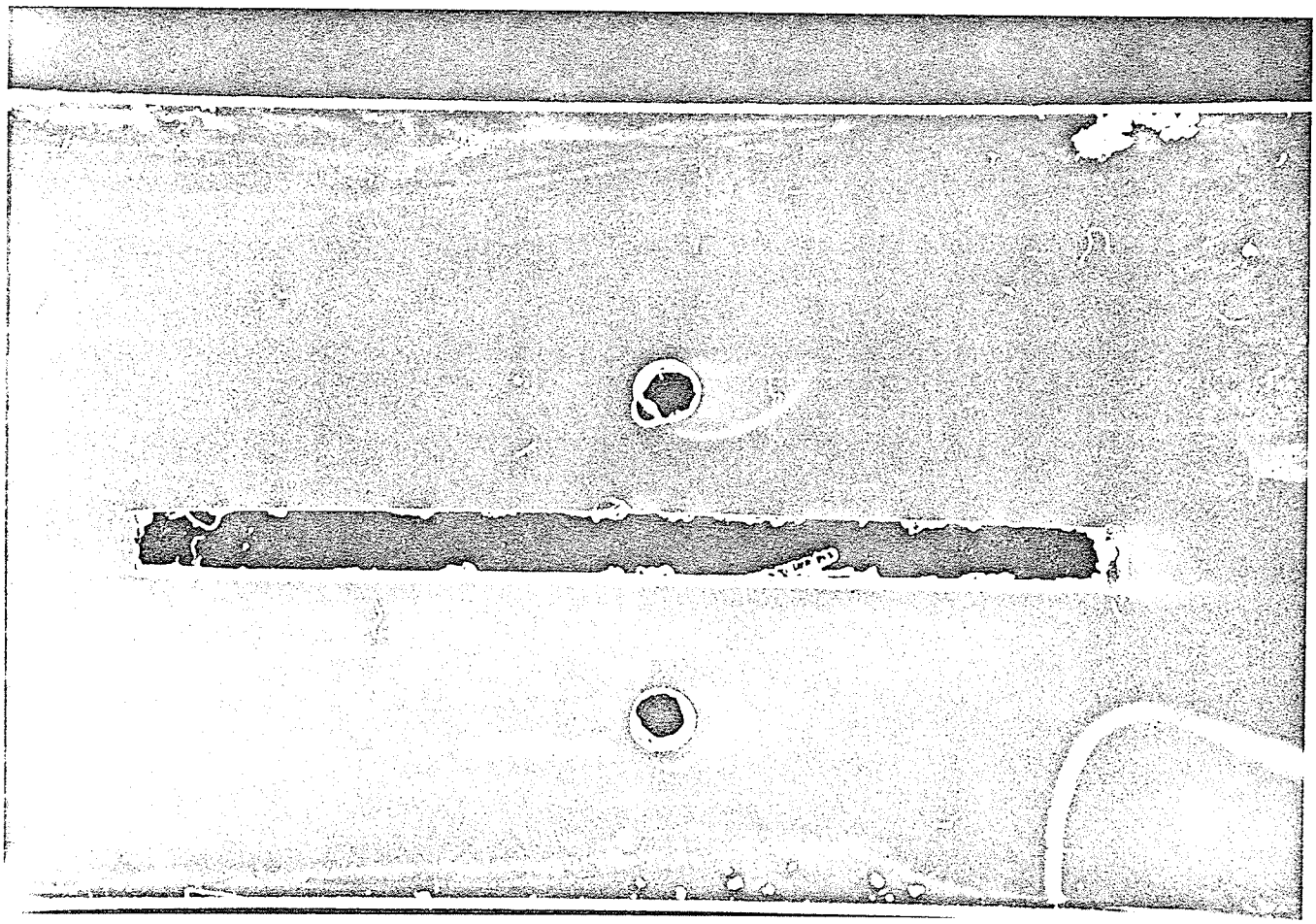


Figure 17. Precipitant bands formed on immunoelectrophoresis (iii).

Well: Leukemia antigen.
Trough: Non-absorbed antiserum against the partly purified leukemia antigen.
Time of electrophoresis: 5 hours.

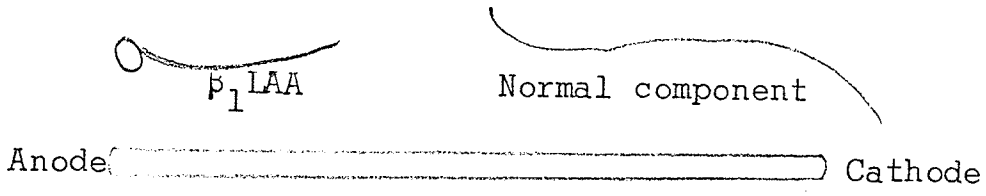
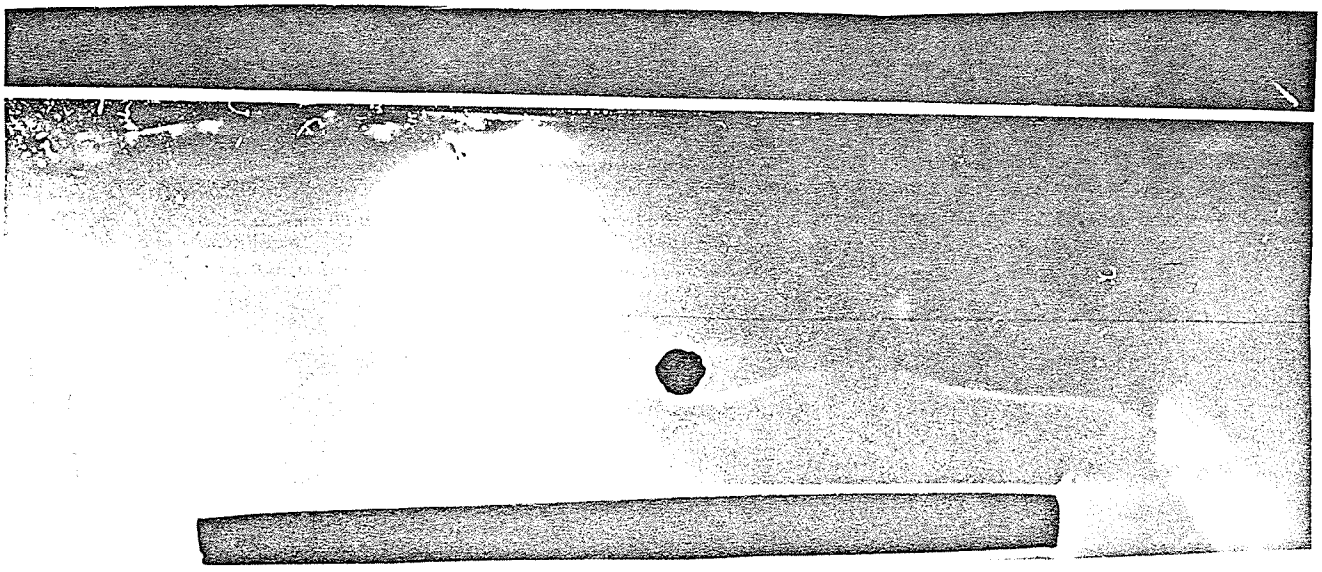


Figure 17



to the β_1 position, i.e., a 3 mm area immediately adjacent to the cathode side of the wells was cut out and the antigen eluted in 0.001M PBS at pH 7.2. This purified antigen reacted with unabsorbed leukemia antiserum producing one precipitant line and did not react with antiserum to normal tissue antigens.

The pure leukemia associated antigen that was eluted from agarose was then subjected to physico chemical analysis. The thin layer chromatography analysis of the purified antigen preparation has revealed the presence of galactose.

The Amino Acid Autoanalyzer has revealed the presence of thirteen different amino acids. The amino acids were identified as lysine, arginine, glycine, aspartic acid, threonine, serine, glutamic acid, proline, alanine, valine, isoleucine, leucine, and phenylalanine. The molecular quantities are calculated from the area under each elution peak as shown in Table X and Fig. 18.

The enzyme digestion studies showed that the serological activity of the leukemia associated antigen was not destroyed by the action of deoxyribonuclease, ribonuclease, or trypsin, but it was completely destroyed by the action of Pronase. The serological activities of the two normal components were destroyed by boty trypsin and pronase.

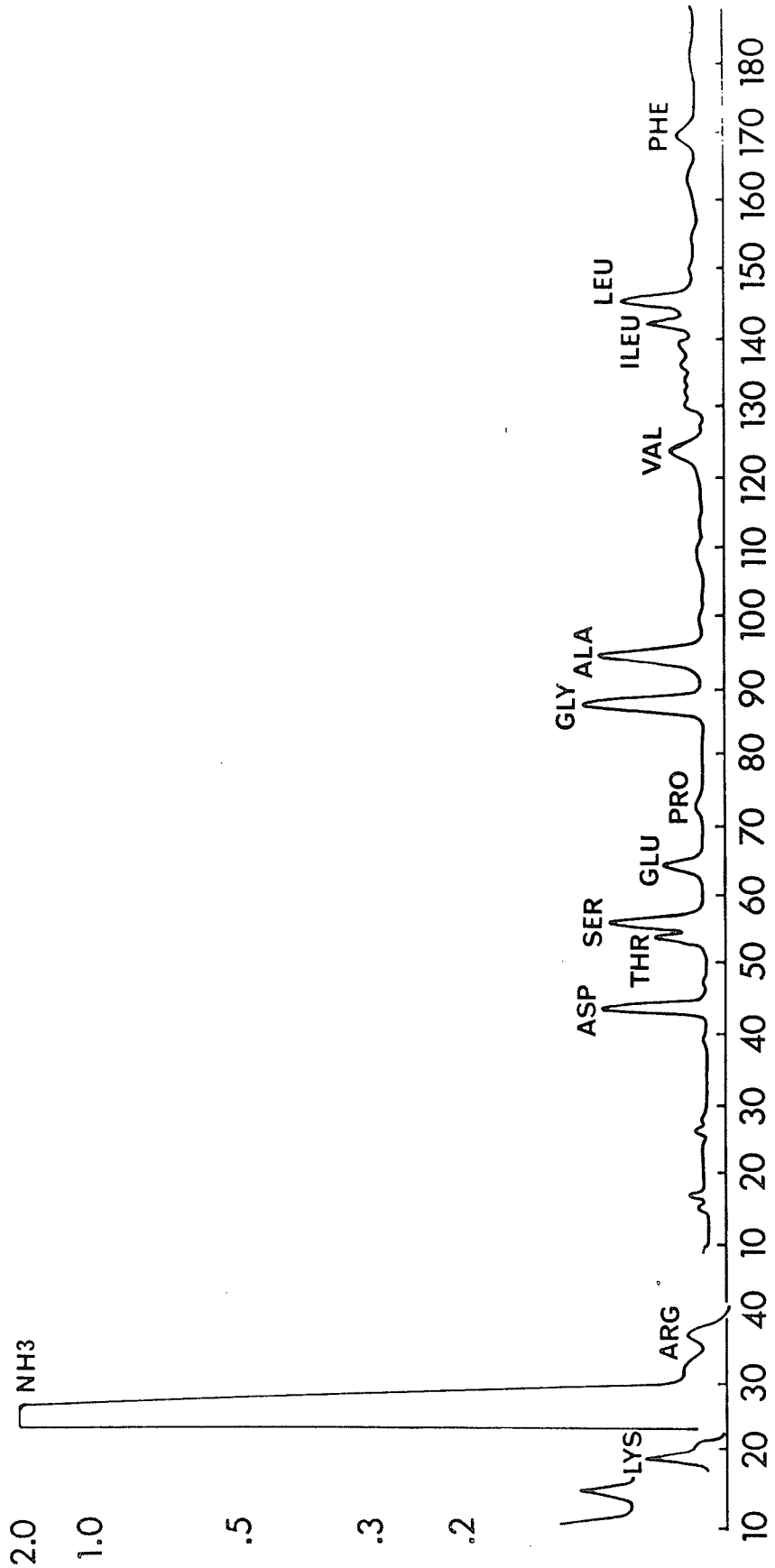
The leukemia associated antigen was found to be relatively heat stable. The action of heat at 37°C, 56°C at 1/2 and 1 hour did not destroy the serologic activity of the antigen. However, incubation of the antigen at 76°C

TABLE X

AMINO ACID CONSTITUENTS OF LEUKEMIA ASSOCIATED ANTIGEN
(Beckman Model 120C Amino Acid Analyzer)

Amino Acid	Base Line	Ht.	Half Ht.	H Net Ht.	W Width (Dots)	H x W	Micro Moles $\frac{H \times W}{C}$
Lysine	0.006	0.052	0.029	0.046	13.5	0.621	0.0092
Arginine							<0.005
Aspartic Acid	0.010	0.081	0.046	0.071	12.3	0.8733	0.0156
Threonine	0.011	0.043	0.027	0.032	15.1	0.4832	0.0082
Serine	0.011	0.078	0.045	0.067	14.4	0.9648	0.0158
Glutamic Acid	0.011	0.041	0.026	0.030	17.1	0.513	0.0086
Proline							<0.005
Glycine	0.012	0.096	0.054	0.084	17.5	1.47	0.0253
Alanine	0.012	0.085	0.049	0.073	20.2	1.475	0.0250
Valine	0.012	0.034	0.023	0.022	28.0	0.616	0.0107
Isoleucine	0.020	0.050	0.035	0.030	14.2	0.425	0.0069
Leucine	0.020	0.069	0.045	0.049	15.0	0.735	0.0121
Phenyl-alanine	0.018	0.030	0.024	0.012	24.0	0.288	0.0048

Figure 18. Chromatogram of the leukemia associated antigen from Beckman Model 120C AminoAcid Analyzer.


O.D. 570nm
2.0
1.0
.5
.3
.2
NH3
LYS
ARG
ASP
SER
THR
GLU
PRO
GLY
ALA
VAL
ILEU
LEU
PHE
10 20 30 40 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
TIME (min)

for half an hour destroyed the serologic activity of the antigen.

DISCUSSION

The findings to be discussed are:

1) One among liver extracts (69B371) from 8 autopsies of patients dying of leukemia (4 myelocytic, 3 lymphocytic and one undetermined) induced in rabbits an antiserum which when absorbed with normal liver extracts had the following properties. In cellulose acetate immunodiffusion preparations, one precipitation line was produced using extracts of each of the four myelocytic and one undetermined leukemic livers and no lines were produced using extracts of normal livers or lymphocytic leukemia livers. The active liver extract was obtained from one of the myelocytic leukemias.

2) The characteristics of the antigen which reacts with this antiserum were found to be

a) a galactopeptide with 13 amino acids.

b) Heat stable at 37°C and 56°C for 1 hour.

c) It migrates in the β_1 globulin region and can be separated from other materials in the pI 4.85 fraction by using immuno-electrophoresis.

d) It is not dialysable and therefore its molecular weight is more than 12,000.

3) Although the original extract 70B272 from a myelocytic leukemic liver did not induce such an antiserum as described in 1) above, nevertheless, material obtained from the original 70B272 extract by further purification

(isoelectrofocusing) did induce the production of such an antiserum.

The first conclusion we can suggest from the above is that there is an antigen present in myelocytic leukemia livers which is not present in lymphocytic or normal livers. This statement assumes that the congenital leukemia is of a myelocytic nature.

It has been suggested that we might have encountered an antigen that is present in relatively small quantities in normal myelocytic tissue.

However, this assumption cannot be concurred with for the following reasons:

1) The suggestion that normal liver contains little or no myelocytic antigens whereas the livers from myelocytic leukemia contain a greater quantity is not valid, because normal liver tissues contain normal myelocytic cells and all kinds of soluble and insoluble human tissue antigens. Furthermore, the autopsy reports in fact indicate that there were very little normal myelocytes in the myelocytic leukemia liver tissues, but contained large quantities of leukemic cells.

2) The normal liver extracts used for absorbing the leukemic antiserum were obtained from large quantities of 10 normal livers (more than one kilogram) and then concentrated at least twenty times after extraction, before they

were used for absorption and immunological studies. The leukemia antiserum, even after extensive absorption with the concentrated normal liver extracts, consistently reacted with the myelocytic liver extracts, producing one prominent precipitant line in immunodiffusion. The same absorbed antiserum did not produce a precipitant line with any of the ten normal liver extracts.

It is interesting to know that there is only one of the five original extracts of myelocytic leukemia livers induced a specific antiserum. This suggests that the concentration of the LAA antigen in the liver 69B371 is higher than that of the others, or that there may have been interference by normal materials such as antibodies. This indicates that it is much more advantageous to employ purified LAA for immunological studies including the production of leukemia-specific antiserum, than depend on non-purified materials and absorption procedures.

The discovery of the LAA may offer an immunological diagnostic tool for the detection of the myelocytic leukemia. Cancer of the colon is diagnosed by the detection of carcinoembryonic antigen in the blood stream and therefore, possibly the findings of this thesis may be used in a similar way.

Other workers (Cohen et al., 1970; DeCarvalho and Rand, 1963; Garb et al., 1962; Kamiya, 1969; Sahasrabudhe et al., 1971; Viza et al., 1970) who have reported on

soluble leukemia antigens have not reported its chemistry in enough detail for a comparison of this finding.

It is desirable that the above research work which has revealed the immunochemical nature of a human leukemia associated antigen, be followed by further research, leading to the utilization of the technique for diagnosis and for prevention of leukemia. The immunochemical relationship of the LAA to other animal leukemic antigens should be examined in future research work.

SUMMARY

The aim of this research work was to isolate and immunochemically characterize the soluble leukemia associated antigen in human leukemic liver tissues.

Soluble antigens were obtained from the liver of myelocytic and congenital human leukemia cases by hyaluronidase treatment of liver tissues extraction with 1M KCl and purification by isoelectric focusing and prolonged gel electrophoresis.

By use of isoelectric focusing with pH range 3-10, the LAA was identified in the fraction corresponding to the pI of 4.4. This fraction was further purified by isoelectric focusing at pH range 4-6. The LAA was then identified in the fraction corresponding to the pI 4.85. This fraction was injected into a rabbit and antiserum was obtained that reacted with the pI 4.85 fraction producing 3 immunoprecipitant lines on immunoelectrophoresis. The same antiserum on absorption with pooled extracts of normal liver was found on immunodiffusion to react with 6 out of 9 preparations, four of which were derived from myeloid and one from congenital leukemia. In contrast, the absorbed antiserum did not react with lymphoid leukemia extracts or normal extracts.

The pI 4.85 fraction containing the LAA was purified a further step using a prolonged technique of electrophoresis in agarose-gel. The position of the LAA was identified by applying rabbit antiserum against the pI 4.85 fraction,

absorbed with normal human tissues, to a trough cut in the gel between the two wells and leaving the slide for 72 hours in a moist chamber, at room temperature.

The LAA was located in the β_1 position, i.e., in the area close to the well toward the cathode side, whereas the two normal fractions migrated away from the β_1 position, completely separated from the LAA. Consequently, a 3 mm area immediately adjacent to the cathode side of the wells was cut out, and the antigen eluted in 0.01M PBS, pH 7.4 by gentle rocking for 72 hours at 4°C. The eluate was then concentrated by lyophilization.

A physico-chemical analysis of the purified antigen preparation by chromatography and by means of an Amino Acid Autoanalyzer has revealed the presence of galactose and of thirteen different amino acids. The amino acids were identified as lysine, arginine, glycine, aspartic acid, threonine, serine, glutamic acid, proline, alanine, valine, isoleucine, leucine, and phenylalanine; and their relative molecular quantities approximately were as follows: 9:4:25:6:8:16:9:4:25:11:7:12:5.

The results, therefore, have demonstrated the presence of a unique water soluble antigen closely associated with human leukemia in liver extracts obtained from four cases of myelocytic leukemia and one congenital leukemia. The leukemia associated antigen migrates immunoelectrophoretically in the β_1 globulin region. The leukemia specific antiserum

appears to react specifically with the antigens from myeloid type of leukemia and not with antigens from normal tissue extracts. This technique may possibly be suitable for use in the diagnostic immunological tests for the myelocytic type of leukemia. It is hoped that further research be done as a follow-up to this initial finding which may hopefully lead to the rapid diagnosis and possibly prevention of leukemia.

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