THE ISOLATION AND PARTIAL PURIFICATION OF TUMOR-SPECIFIC ANTIGENS ASSOCIATED WITH THE NUCLEOLI OF HUMAN MALIGNANT MELANOMA

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A Thesis

in

The Department

of

Biological Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of Masters of Science at Concordia University, Montreal, Québec, Canada

May 1979

ABSTRACT

THE ISOLATION AND PARTIAL PURIFICATION OF TUMOR-SPECIFIC ANTIGENS ASSOCIATED WITH THE NUCLEOLI OF HUMAN MALIGNANT MELANOMA.

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Tumor-specific antigens were found to be associated with nucleoli of human melanoma tumor cells by indirect and direct cytoplasmic immunofluorescence techniques employing autologous sera that had been shown to be specific for antigens in the patients' tumor cells. Nuclei were isolated from melanoma tumor cells by differential and discontinuous sucrose density centrifugation. Nucleoli were obtained by careful sonication of the nuclei and purified by gradient centrifugation. Soluble antigenic material was extracted from these nucleoli by 3M KCl. Antigens were obtained from the KCl extracts by chromatographing on affinity columns made up with the patients' purified IgG coupled to Sepharose 4B. The molecular weight of proteins obtained from affinity columns done on KCl extracts of nucleoli from tumors of five (5) malignant melanoma patients were found to be in the range of 66,000 to 170,000 daltons by SDS acrylamide gel electrophoresis. Immunological analysis of these proteins by counter-current immunoelectrophoresis indicated that some or all of them were antigenic. Tests for specificity suggest that these antigens may be melanoma-specific allogeneic.

This thesis

is

dedicated to Marie José Gélinas

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the following people:

1. My director, Dr. E. Preddie, for his guidance and understanding throughout the duration of this project. I would like to thank him also for doing the serum specificity studies with ^{125}I labelled IgG and for running the experiments on purity of the different IgG fractions.
2. Dr. M.G. Lewis, for telling us about the nucleolar antigens, for making all the facilities of the McGill Cancer Research Unit available to me and for his continued interest in the work.
3. Dr. T.M. Phillips, for many helpful discussions.
4. Ms. Gail Deutch, for doing the electronmicroscope examinations.
5. Dr. Dan Hartmann and the entire staff of the Cancer Research Unit for their interest, encouragement and constant cooperation.
6. Mr. Marcel Dupré and fellow workers at Cartier Sugar Refinery with whom I had the pleasure of working for several summers.
7. My classmates, Serge Persad, Mohammed Khosravi, Rudolph Tubbeh, and Ron Parisella for their moral support and encouragement during many difficult and frustrating times.
8. Dr. Sylvia Ruby, for her unbending faith in me.

Finally, I am deeply endebted to Marie José Gélinas without whose continual support and encouragement this project could not have been successfully completed.

FOREWORD

Cancer has been known to man since antiquity, yet it remains the least understood group of diseases, and one of the costliest in terms of human suffering. The fundamental problem in cancer research today is the cancer cell, which possesses the ability of division without being subjected to the normal hemeostatic control mechanism of the host. The concept that malignant transformation (of normal cells to cancerous cells) is associated with the acquisition or reexpression of antigens specific for the cancer cell (neoplastic cells) has lead to great interest in the isolation and purification of these antigens, which is a required first step in order to define the role they must play in modulating response or lack of response in the host. However, the ultimate aim of this area of research is the eventual use of these antigens in (i) early diagnosis, (ii) in monitoring the clinical course of the disease, and (iii) in developing an immunological method of treating cancer.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

INTRODUCTION

HISTORICAL REVIEW OF MALIGNANT MELANOMA

Melanin pigmentation of the human skin is produced by the pigment synthesizing cells called melanoblasts which are located in and proximal to the portion of the epidermis. Malignant melanoma represents a group of tumors derived from those pigment-forming cells. Despite references to the fact that it was known to early physicians, it was the French physician Laennic in 1806 who first coined the term "la melanose" to describe this group of neoplasms (Pemberton 1964). Later, Robert Carswell in 1838 used the term melanoma to describe these pigmented malignant tumors (Carswell 1974). The term melanoma is now used to designate two types of closely related tumors originating in the pigment-synthesizing cells: a) benign nevi and mole, b) malignant nevi. It is generally believed that it is the latter group which posess the inherent capacity for lymphatic and blood-borne metastasis (Nicolson 1979). Malignant melanoma is generally found in all races and in many mammals as well as lower animals.

According to many investigators (Elwood and Lee 1974, Lee 1973, Magnus 1973) there has been a tremendous increase in both the incidence and mortality of malignant melanoma in individuals born since 1923. Despite this, malignant melanoma is still a relatively rare disease, constituting approximately 20% of all malignant tumors

of the skin (Cole 1974) and 2-3% of all malignancies in the U.S.A. (Golub 1975). However, in spite of its rarity, malignant melanoma has received a disproportionate share of interest from tumor immunologists. The reasons for this focus include: a) its unpredictable natural history (Everson and Cole 1966, Baab and McBride 1975), b) the abnormally high rate of spontaneous regression of the primary tumor (Smith and Stehlin 1965, Everson and Cole 1966) and its high rate of metastasis (Bodurtha et al 1976, Everson 1964, Cole 1974, and Nicolson 1979). These factors strongly suggest that malignant melanoma was ideal as a human tumor in which the host immune system appeared to be involved in modulating the growth and development of the tumor.

Subsequently, during the last 30 years, malignant melanoma has been used as a model by researchers investigating the immunological parameters of host-tumor relationships in human tumors (Mavligit et al 1974a,b, Clark 1977, Golub 1975). These and many other investigators have attempted to show that: a) malignant melanoma tumor cells possess tumor-specific antigens, b) that the host immunological system recognizes and reacts with these antigens, and c) this reaction correlated with/or has some impact on the course of the disease (Lewis et al 1971, Lewis 1974, Zeigler et al 1969, Fass et al 1970, Hellstrom and Hellstrom 1973).

TYPES OF MELANOMA

According to Clark et al (1969), there are three main

biological variants of malignant melanoma: 1) Lentigo maligna, 2) superficially spreading, and 3) nodular melanoma. These three groups represent 85% of all melanoma generally found, the remaining 11.5% consist primarily of a rare group called acral lentiginous melanoma. More recently, Everall and Dowd (1977) studied the various types of melanoma, and concluded that the three main types of melanoma differed in: a) site of origin, b) pattern of growth, and c) occurence.

ORIGIN OF MALIGNANT MELANOMA

The origin of malignant melanoma remains very controversial ever since Laennic in 1806 postulated that malignant melanoma was derived from pre-existing moles. With respect to the role of the nevus, there are essentially three schools of thought: 1) some (Allen 1949, Allen and Spitz 1953) believe that the junctional nevus is the formal histogenic precursor of all malignant melanoma; (2) others, most notably Mishima 1960, Mishima 1967, have suggested that all malignant melanoma except those of the lentigo maligna type, arise from nevi, and (3) Clark *et al* 1969, 1975, believe that only some malignant melanoma arise from pre-existing nevi since they represent a concentration of the cellular targets (Melanocytes). In support of the latter view was the recent study of Rippey *et al* 1977. These authors reported that nevi cells were found in 22% of 54 primary cutaneous malignant melanoma studied. However, nevi cells were found in 64% of the melanomas of the trunk. It appears therefore that some nevi, especially those of the junctional and compound types, predominantly found on the trunk, may have a higher malignant

potential for neoplastic transformation, and may well be the precursor of some malignant melanoma.

CAUSES OF MALIGNANT MELANOMA

The exact cause of malignant melanoma remains unknown. However, evidence to date strongly suggests that malignant melanoma results from a complex interaction of several factors. These factors can include some exogeneous physical or chemical agent and susceptible cellular characteristics of a host (i.e. melanocytic moles) which permit the inductive agent to act in a transforming manner. The inductive agents more often cited in the literature are trauma, viruses, (Balda et al 1975) and sunlight (Elwood and Lee 1974, 1975, Lee 1973, Magnus 1973). As stated earlier, recently there has been a tremendous increase in the incidence of melanoma; moreover, this increase seems to be related to melanoma occuring at certain specific sites of the body, e.g. lower limbs and back above the waist of both men and women. These facts prompted Elwood and Lee (1975) to suggest that changes in the kind of clothing and modes of dress were responsible for the incidence of melanoma. However, since the increased incidence of melanoma did not occur in the most exposed parts of the body (i.e. face and back of hands), and was most prominent in professional and managerial workers, it is suspected that sunlight acting in an indirect unknown manner, in conjunction with other factors, i.e. susceptible nevi, are involved in the increased incidence of melanoma.

THE HOST IMMUNOLOGICAL RESPONSE TO MALIGNANT MELANOMA

a) Role of Tumor Antigens in Human Neoplasia

It is now well accepted that tumor cells whether induced by (a) viruses, (b) chemical, (c) carcinogen, (d) physical agents, or from tumors of unknown etiology, synthesize macromolecules which normal adult cells do not (Alexander 1972, 1974). The presence of these macromolecules (generally referred to as "new antigens") on the tumor cell surface is detected indirectly through the immune response which they usually but not always evoke in the host.

The existence of host immunological resistance directed specifically against these molecular markers in patients with malignant tumors is well established (Hamilton-Fairley 1969, Hamilton-Fairley et al 1971, Nagel et al, Alexander and Hamilton-Fairley 1967, Hellstrom et al 1971a,b,c, Lewis 1967, Lewis et al 1969, Old 1977). This concept was first postulated in 1909 by Erhlich who demonstrated the existence of tumor-specific antigens in experimentally induced animal tumors (Erhlich 1957). In the 1950's, Foley (1953) and later Prehn and Main (1957) provided further evidence for the existence of tumor-specific antigens in mice sarcomas tumors induced by the chemical agent methylcholanthrene.

With respect to human tumors in general and malignant melanoma in particular, the amount of available data concerning the existence of tumor antigens is not so great. This partly reflects

the recent development of sereological methods (those using antibody as the analytical probe to replace the transplantation techniques used in inbred mice animal studies). In spite of this, the available evidence does indicate that human malignant tumor cells in general (Old and Boyse 1966, Haddow 1965) and malignant melanoma in particular also possess similar tumor-related antigens. Moreover, the evidence suggests that some of these antigens (in human tumor cells, depending on their location in the cells) significantly influence the growth and development of the tumor by partly determining both the nature and magnitude of the immunological response they elicit in the host.

b) Mechanism of the Host Immune Response

The host immunological response to tumor antigens can be divided into two components: a) cellular mediated response involving thymus-derived lymphocytes and macrophages, and b) humoral mediated response involving bone-marrow derived lymphocytes and plasma cells. However, it must be remembered that the two components frequently interact, and furthermore this interaction can significantly affect the growth and deveopment of the tumor, e.g.: (i) T-lymphocytes are known to act as "helper" cells for B-lymphocyte antibody production; (ii) humoral antibodies can suppress the cellular mediated response by reacting with the tumor-specific antigens on the tumor cell surface (Hellstrom et al 1971, Hellstrom et al 1973).

The existence of host immunological response, i.e.

cellular and/or humoral to tumor-specific antigens in human malignant tumors in general (Hellstrom et al 1968, Currie and Bagshawe 1967) and malignant melanoma in particular is now well established (Hamilton-Fairley 1969, Nagel et al 1971, Alexander and Hamilton-Fairley 1967, Bodenham 1968). It was always felt that the unusual biological behavior of malignant melanoma, that is, its unpredictable and variable natural history and its high rate of spontaneous regression, indicated the host immunological response was influencing the growth and development of the tumor. Convincing evidence for the existence of both cellular and humoral immunity in human malignant melanoma has been derived from several studies, e.g. Samson-Handley (1907). He was the first investigator to demonstrate a correlation between regression of the malignant tumor and the degree of infiltration of the tumor mass by mononuclear leucocytes. More recently, Thompson (1972) also showed that degree of lymphocyte infiltration correlated with the deveopment of the tumor.

With respect to humoral immunity, many investigators (most notably Lewis 1967, Lewis et al 1969, Ikonopisov et al 1970) have demonstrated the presence of specific serum immunoreactivity in patients with malignant melanoma. Despite the tremendous number of studies done concerning the host-tumor relationship in malignant melanoma, the importance of the role of each component of the immune system and the relevence of the entire host-immune system in the modulationof tumor growth and deveopment remains very controversial. With respect to the former, it was originally believed that host immunological response to tumor cells was almost exclusively a

function of the cellular mediated response. In addition, it was known that humoral antibodies in general had limited access to solid tumors "in vivo", thus they were not expected to play a significant role in the host immune response to localized tumor cells. Nevertheless, it is now believed that humoral antibodies may play a role when tumor cells are readily accessible as with blood-borne metastasis. Strong support for humoral mediated response in affecting the growth and development of the tumor has been provided by the findings of several investigators (Gold 1967, Lewis 1967, Lewis et al 1969, Morton 1970). Their evidence strongly suggests that humoral antibodies play a crucial role in delaying dissemination of the primary tumor.

The question as to whether or not the host immunological response is responsible for the unusual behavior of malignant melanoma remains unresolved. In this regard, there are essentially two schools of thought. Some investigators (Lewis et al 1969, Lewis 1971b, 1972a, Morton et al 1970, Hamilton-Fairley 1969, Hamilton-Fairley et al 1971) believe that there exists some correlation between the host immune response (humoral) and the growth and development of the tumor. On the other hand, other investigators (Nairn et al 1972) believe that there is no correlation between the host immune response (humoral and cellular) and the growth and development of the tumor. These different conclusions as to the role of the host immune response in part reflects:

(i) different techniques used to assess the patient's immunological competence.

(ii) variation of the patient's immunological competence at different stages of the disease.

(iii) specificity of the different techniques used.

c) Methods of Detecting Host-immunological Response to Malignant Melanoma-associated Antigens

The existence of both cellular mediated and humoral mediated host response to melanoma-associated antigens in melanoma target cells has been shown by a number of "in vivo" and "in vitro" immunological tehcniques. Cellular immunity has been investigated using:

(a) Delayed Cutaneous Hypersensitivity (DCHP) to tumor extracts (e.g. 3M KCl) by Roth et al 1976a,b, Mavligit et al 1973a, and Holmes et al 1975.

(b) Lymphocyte Mediated Cytotoxicity; Hellstrom et al 1971, DeVries et al 1972, and Nairn et al 1972, Hamilton-Fairley 1971.

(c) Leucocyte Migration Inhibition; Cochran et al 1972, Segal et al 1972.

However, the most widely used technique, and the one believed to best reflect the actual ("in vivo") relationship between the host and the melanoma tumor cell is lymphocyte cytotoxicity. Hellstrom et al (1971) were the first investigators to demonstrate that lymphocytes from most patients with malignant melanoma are cytotoxic to melanoma target cells "in vitro". The available

evidence strongly suggests that lymphocytes from patients with malignant melanoma were (i) specifically cytotoxic to melanoma target cells, whether the cells were autologous or allogeneic, and (ii) more cytotoxic in patients with less advanced disease (localized tumors). Although there remains some unanswered questions concerning both the specificity of this assay (Heppner et al 1973, Oldham et al 1975) and the relationship between the degree of lymphocyte cytotoxicity and the extent of the disease (Roenigk et al 1975), it appears certain that the host cellular mediated response to melanoma-associated antigens does exist and has some impact on the growth and development of the tumor.

Host Humoral Mediated Response

Lewis (1967) was the first investigator to demonstrate that serum from patients with localized tumors contained anti-melanoma antibodies which were cytotoxic to their own autologous tumor cells "in vitro". Three methods have been used extensively to study both the existence of host humoral response to melanoma-associated antigens and to assess the impact of this response on the growth and development of the tumors.

(a) Immunofluorescence (Oettgen et al 1968, Morton et al 1968, Romsdahl and Cox 1970, Fossati et al 1971, Lewis et al 1969, Phillips and Lewis 1970).

(b) Immune Adherence (IA) (Corain et al 1973, Irie et al 1974)

(c) Complement-dependent Cytotoxicity (Lewis 1967, Lewis et al 1969, Nairn et al 1972, Bodurtha et al 1975).

In summary, the bulk of available data concerning host-tumor relationship in malignant melanoma indicates that (a) melanoma cells carry tumor-specific antigens against which the host immunological system has been shown to react, and (b) that the host immunological responses humoral (cytotoxic antibody) and cellular (lymphocyte cytotoxicity) elicited by these antigens have some impact on the growth and development of the tumor.

RELATIONSHIP BETWEEN HOST TUMOR IMMUNITY AND GROWTH AND DEVELOPMENT OF TUMORS

"Metastatic melanoma is one of the cancers least susceptible to treatment in man largely because of the frequency of lymphatic and blood-borne metastases" (Nicolson 1979). A number of theories have been postulated to explain why the primary tumor can persist localized for indefinite periods of time then suddenly acquire the capacity for rapid profileration and metastasis in face of a strong and intact immune system. Evidnece to date suggests that the capacity of malignant melanoma tumor cells to metastasize is related primarily to the absence of tumor-specific immunity mediated by humoral antibodies and/or T-lymphocytes. This does not rule out the possibility that secondary factors, e.g. absence of complement, might greatly affect the immune response as a whole.

Zeigler et al (1969) and Fass et al (1970) demonstrated that the major difference between the immunological response of patients with localized forms of melanoma and those with advanced stages of metastases was the absence of tumor-specific antibodies against their tumor cell in the latter. That patients with advanced stages of malignant melanoma were not non-specifically immunologically abnormal has subsequently been confirmed by several other investigators (Morton et al 1970, Lewis 1974, 1972). Consequently, four main hypotheses have been postulated to explain the absence of tumor-specific antibodies in patients with disseminated malignant melanoma.

I) Sneaking through hypothesis: This suggests that initially the individual tumor cells are not specifically immunogenic; consequently, by the time the immune system recognizes the presence of the tumor-specific antigens on these cells- the tumor is well established and too large for the immune system to effectively deal with it.

(II) Antigenic modulation: This suggests that the tumor cells involved in metastasis possess the ability to mask or even lose their antigenic determinants in face of immunological attack (Old and Boyse 1968, Alexander, 1972,1974).

(III) Antigenic blindfolding: This suggests that the growing tumor may shed excessive amounts of tumor surface antigens which then bind to the tumor-specific antibodies or to specific receptors on

lymphocytes and thus prevent them from recognizing and destroying the tumor.

(IV) Blocking hypothesis: (May also involve antigenic blindfolding) - This suggests the existence of several blocking substances which effectively neutralize the tumor-specific antibodies produced by the host (Romsdahl 1974, Levy 1973, Hellstrom et al 1971c, 1973a, Lewis et al 1976, Hartmann et al 1974, Hartmann and Lewis 1974, Preddie et al 1979).

Blocking factors may include: (i) circulating soluble antigen (ii) circulating antigen-antibody complexes, (iii) idiotypic type of auto-antibodies, or (iv) other specific substances produced in individual patients.

In summary, it is reasonably certain that melanoma tumor-specific antigens play a crucial role in both the elicitation of tumor-specific immunity in the host in the metastases of the tumor. Therefore, it is obvious that the isolation, purification and characterization of these antigens is a necessary prerequisite for the eventual understanding of the role of these antigens in the regulation of the host's immune response to the invading tumor cells.

MELANOMA SPECIFIC ANTIGENS

As a working hypothesis, tumor antigens can be divided into two types: (a) tumor-associated antigens, and (b) tumor-specific

antigens. Antigens such as carcino-embryonic antigens (CEA), α-fetal proteins (AFP) and antigens related to infection with Eptein-Barr viruses are examples of tumor-associated antigens. These antigens which are secreted by the tumor cells as well as normal cells such as the embryonic cells of the corresponding organ are not believed to be directly related to the neoplastic transformation process. On the other hand, the tumor-specific antigens for which there are no corresponding molecules on normal cells are believed to be involved in the host immunological response to the tumor (Foley 1953, Klein 1968, Haddow 1965).

Tumor-specific antigens have been extensively studied in animal systems where it was initially shown by Foley (1953), and later by Prehn and Main (1957), that all chemically induced tumors had distinct and unique tumor-specific antigens, whereas virus induced tumors had antigens in common within each etiological group. Tumor-specific antigens in human tumors have not been as well studied mainly due to technical problems involved in their isolation, identification and characterization. Recently, several authors have convincingly demonstrated the presence of both tumor-associated (Federman and Lewis 1974, Carrel and Theikaes 1973, Wood and Barth 1974) and tumor-specific antigens (Van Allstyne 1977, Thompson et al 1976, Preddie et al 1976, 1977, 1978a, 1978b, Persad 1979, Khosravi 1978) in the tumor cells of human malignant melanoma.

There is good evidence for two groups of tumor-specific antigens in malignant melanoma cells. These are: (1)

tumor cell membrane associated antigens, and (2) antigens associated with the cytoplasmic contents of the tumor cell. It is highly likely that the membrane-associated tumor-specific antigens are involved in the recognition and selective destruction of the invading tumor cells by the patient's immune system. Consequently, this group of antigens is presently being intensively researched by several workers, most notably Preddie et al 1978 and Khosravi 1979. The cytoplasmic antigens have been receiving a little less attention, partly due to (i) a lack of ideas about a possible role for them in the immune response, and (ii) problems involved in identifying their exact cytoplasmic location without prior fractionation of the cytoplasmic organelles. One group of these antigens has been shown to be associated with the nucleoli in melanoma tumor cells (McBride et al 1972, Lewis et al 1973, and Bowen et al 1976) and Persad (1979) has demonstrated that some of these antigens are associated with rough microsomes in tumor cells.

Tumor-specific nucleolar antigens have been studied by several investigators using different techniques. The results obtained by the variety of methods used are not in total agreement as to the nature of these antigens. Because our group has been successful in isolating tumor-specific antigens from purified plasma membranes (Khosravi 1978, Preddie et al 1977, Preddie et al 1978a,b) and from subfractions of microsomes prepared from malignant melanoma tumor cells (Persad 1979, Preddie et al 1979) it was decided to apply some of these experimental approaches to try to demonstrate defin-

itely the presence and the nature of melanoma tumor-specific antigens which were shown by immunological methods to be associated with tumor-cell nucleoli. The technique most widely used to date to localize and characterize human tumor-associated antigens is immunofluorescence. Originally developed by Coons and Kaplan (1950) and subsequently modified to its present form by Lewis and Phillips (1972) and Phillips and Lewis (1970), this technique has recently been used by investigators to study cytoplasmic antigens associated with human malignant melanoma. Clank et al (1977) suggested that the specific cytoplasmic fluorescence observed by many workers may be directed against an antigen common to many malignant cells, possibly a fetal antigen. In support of this finding is the work of Whitehouse (1973) who demonstrated cross-reactivity between melanoma sera and human fetal target cells. These results may in part reflect the fact that there exist both melanoma-specific and non-specific antigens associated with the cytoplasm of malignant melanoma cells. Lewis et al (1973) suggested that there are at least six types of antigens present on melanoma cells which they classffied as follows: (a) surface membrane autologous, allogeneic, membrane and foetal, (b) cytoplasmic autologous, allogeneic, and (c) nucleolar allogeneic. They further suggested that these antigens were expressed at different stages of the disease as the tumor progressed from the localized nevus stage to the widely disseminated state, and they demonstrated that the anti-nucleolar antibodies were found in the patient's blood only in the latter stages of the disease (i.e: when wide-spread dissemination was taking place). These findings were confirmed by McBride et al 1972, and Bowen et al 1976. However, all of these workers used indirect immunological and morphological methods to

demonstrate these antigens. An understanding of the nature, and the role of the nucleolar antigens as well as the interrelationship between these and the other groups of tumor-specific antigens requires that these antigens be isolated, characterized, and studied in a homogenous molecular form.

MELANOMA ANTIGENS ASSOCIATED WITH THE NUCLEOLUS OF MALIGNANT MELANOMA CELLS

THE NUCLEOLUS

The nucleolus is an intranuclear organelle whose primary function is the production of most (80-90%) of the cellular RNA, mainly ribosomal (rRNA). In addition, it is believed that the nucleolus also serves as the site of organization of the ribonucleoproteins which are processed through a number of stages from one percursor to another until they eventually leave the nucleolus and are transferred through the nucleoplasm and into the cytoplasm. Recently, a number of investigators have attempted to ellucidate both the organization and function of this interesting organelle. In terms of structure, the nucleolus consists of at least three distinct regions: (a) pars amorpha, (b) the nucleolema, and (c) perinucleolar area. With respect to its function, much remains to be known; for example, little is known about the factors that control the rate of nucleolar activity and the number of nucleoli in a given cell. The role of the nucleolus in neoplasia has been a subject of considerable interest to many investigators ever since MacCarty et al 1936, MacCarty 1937, MacCarty and Haumeder 1934, presented evidence of structural irregularities in the nucleoli of cancer cells.

NUCLEOLI OF CANCER CELLS

It was originally suggested that the only constant

feature of neoplasia was nucleolar aberration in morphology and function (Busch et al 1963, Busch and Smetana 1970, Long and Taylor 1955, Cowdry 1951). Therefore, many investigators have studied differences in nucleolar size, number & morphology between normal and neoplastic cells in an attempt to use these differences as an indication of neoplasia and to gain insight into the structure and function of this organelle. No constant and/or specific morphological differences have been found between cancer and non-cancerous cells. That is, although the shape of the nucleoli of tumor cells is pleomorphic, this characteristic is also found in non-cancerous growing cells. In addition, the nucleoli of cancer cells are strikingly very large and variable in number, but these characteristics have also been shown in other non-cancerous cells, e.g. hepatic cells and cells undergoing some hyperactive function. On the other hand, the atypical characteristics of nucleoli found in neoplastic cells are more intense and occur with a greater frequency in malignant cells as compared to normal cells. Long and Taylor (1955) found that with increased malignancy, the corresponding nucleolar variations included: (1) multiple nucleoli, (2) larger size of single nucleoli, (3) variations in size of multiple nucleoli, and (4) irregularly shaped and vacuated nucleoli. With the advent of methods for isolation of purified nucleoli from neoplastic and non-neoplastic cells, biochemical analysis of nucleolar constituents has strongly suggested that the major differences between nucleoli of these cells may reside at the gene level. In other words, it has been postulated that perhaps the one feature that may distinguish the nucleoli of normal and malignant cells may involve the nucleolar-chromosomal complex in which disassociation

(resulting from neoplastic transformation) between the nucleolus and its chromosome may lead to unregulated production of both nucleolar material and chromatin (Kopac and Mateyko 1958).

In view of the fact that the nucleolar chromosomal complex may be a fundamental regulatory mechanism in the cell, it is possible that disassociation of the nucleolar chromosomal complex may result in the nucleoli of cancer cells producing abnormal products e.g. ribonucleoprotein which could affect the responsiveness of the cancer cells to both internal and external control systems.

This is important in light of the preliminary findings of McBride et al (1972), i.e. that the nucleolar antigen associated with malignant melanoma was an RNA-bound protein, and that this antigen was melanoma-specific since it was not found in non-malignant tissue from melanoma patients. Further evidence for the above concept was provided by the work of Farhad-Marashi et al 1979. They showed that the antigens associated with the nucleoli of Novikoff hepatoma differed from those of normal liver nucleoli. Subsequent isolation and partial characterization of this antigen showed that it was an extranucleolar chromatin-type molecule closely associated with the nucleolar DNA.

PURPOSE AND DESIGN OF PROJECT

In view of the fact that techniques for the isolation of purified nucleoli in adequate amounts necessary for biochemical analysis are now available, the isolation and partial purification of melanoma tumor-specific antigens from nucleoli purified from tumor tissue was undertaken. The experimental approach was as follows:

(a) Immunofluorescence using fluorescent autologous tumor-specific serum was used to show that tumor-specific antigenic activity was associated with the nucleolus.

(b) Nucleoli were isolated from tumor cell nuclei and shown by morphological, chemical and enzymatic analysis to be pure as defined by published criteria for purity of nucleoli.

(c) Tumor-specific antigens were extracted from purified nucleoli, and purified by affinity chromatography employing columns made up of purified IgG's from melanoma patients covalently bound to Sepharose 4B.

(d) Isolated antigens were partially characterized.

The results of these studies should (a) provide some insight into the nature of the nucleolar antigen/s, (b) help us to understand the immunological relationship between this and other melanoma tumor-specific antigens, and (c) provide the impetus for a study of this antigen as a possible candidate for a diagnostic agent in determining the clinical status of malignant melanoma.

MATERIALS AND METHODS

i. Source of Chemicals

All chemicals used were of reagent grade. Indole, ammonium sulfate (grade III), sucrose (crystalline, grade 1), polyethylene glycol, maleic acid repurified, DL-dithiotheoiotol (Sigma grade), ethylenediaminetetracetic acid (disodium), DEAE cellulose (medium mesh) and p-nitrophenyl phosphate (Sigma grade) were obtained from Sigma Chemical Company, St-Louis, U.S.A. Sepharose 4B, Sephadex G-200, RNA and proteins used for molecular weight markers were from Pharmacia, Dorval, Quebec. Trichloracetic acid (TCA), Triton X-100 were obtained from Fischer Scientific Company, Montreal, Quebec. Trypsin (grade A), ribonuclease (grade A), orcinal (grade A) were obtained from Calbiochem, as were AMP and G-6-P. Polyvinyl Sulphate was from Estman. TC199 was a gift from Dr. T. Phillips.

ii. Concentration of Protein Fractions

Three methods were used to concentrate the various fractions, depending on their volumes and on the molecular weights of the protein constituents involved.

(a) For a very small quantities (i.e. less than 1.0 ml), the method involving the use of dry calcium chloride provided the best results in terms of time and recovery of the material. The fractions were concentrated by placing the frozen samples in a desicator containing dry calcium chloride at $4^{o}C$ and evacuating with a vacuum pump.

(b) For larger quantities (1-10 ml), the fractions were placed in dialysis tubing of appropriate molecular weight cut-off, and concentrated by immersing the tubing bag, in a beaker and surrounding it with finely crushed polyethylene glycol.

(c) For concentrating very large volumes (10-25 ml) with high molecular weight constituents, e.g. IgG isolation, an Amicon concentrator, model 75 P.S1, was used. The power source was a nitrogen tank and a membrane filter with a molecular weight cut-off of 50,000 was employed.

iii. Dialysis

Dialysis tubing of different widths and different molecular weight cut-off was used extensively throughout this project for a variety of purposes. The dialysis tubing, which was obatined from Spectrum Medical Industries, Los Angeles, California, was prepared for use by heating for 30 minutes at 80°C in distilled water containing sodium bicarbonate 0.1M ($NaHCO_3$). After heating, the tubing was washed extensively by rinsing in distilled water. Dialysis was done at 4°C (in a cold room) for varying lengths of time (12-48 hours) with changes of buffer every eight (8) hours.

iv. Sources of Tumor

Human malignant melanoma tissue and patients' sera used in this project were obtained from the Cancer Research Unit, McGill University, Montreal, Quebec.

(a) Tissue specimens - tumors which were obtained immediately after surgical excision were placed in clean petri dishes containing small amounts of TC 199, sealed, and subsequently stored in a walk-in deep freezer at -136°C (Phillips and Lewis 1970).

(b) Sera - Serum specimens from twelve (12) patients with malignant melanoma were collected and stored at -20°C until used for cytoplasmic immunofluorescence. In addition, sera were obtained from three other types of non-melanotic tumors. Normal sera obtained from the Hematology Department of the Royal Victoria Hospital were used as control.

v. Absorption of Test Sera and Determination of Specificity

To increase the specificity of the results (Cytoplasmic IF), absorption of positive sera was done according to the method of Minden et al (1974). Positive sera were abosrbed with (a) normal human spleen cells, and (b) tumor cells of non-melanotic tumors using the following procedure: approximately 4.5 X 10^7 normal human spleen cells, and 2 X 10^7 tumor cells of the other tumors were washed in sterile PBS (0.01 potassium phosphate, 0.15M NaCl pH 7.3) and then mixed with 1 ml of the serum in a tube which had been brought to 37°C. This absorption was performed at 37°C for one (1) hour and continued overnight with constant stirring of suspension. After absoprtion, the samples were centrifuged at 2000 X g for 30 minutes and the supernatant was removed and kept at -20°C until used for cytoplasmic immunofluorescence. Aliquots of the absorbed sera were tested for specificity. Specificity of the sera was determined by

studying the effect of unlabelled sera on the interaction between ^{125}I labelled serum and autologous tumor-specific antigens obtained from 3M KCl extracts of the tumors. These studies were performed by Preddie according to the method described by Preddie et al 1978a).

vi. Cytoplasmic Immunofluorescence

Direct and indirect immunofluorescence was performed by the method of Phillips and Lewis (1970) and Lewis and Phillips (1972): a cell suspension was prepared from fresh tumor material for snap-freezing: the tumor specimen was finely minced in a petri dish containing a small amount of TC 199 medium. The suspension was filtered through sterile gauze, the cell number was determined and subsequently adjusted to 1×10^6 per ml in the same medium. Snap-freezing was performed primarily to open the cell membrane to the entry of immunoglobulins, but also to facilitate both adhesion and flattening of the cells. Approximately 30,000 cells/smear (3 smears per microscopic slide) were added by pipetting one (1) drop of the 1×10^6 cells/ml suspension onto a clean microscopic slide and then allowing the slides to dry at room temperature for 30-60 minutes. After drying, the slides were fixed by freezing in a mixture of isopentane and liquid nitrogen for one (1) minute at -160^oC and then stored at -180^oC until used. To perform cytoplasmic immuno-fluorescence analysis, the slides were removed and allowed to thaw at room temperature for thirty (30) minutes. The smears were numbered and then the slides were washed with PBS to remove any residual isopentane. During the washing procedure the sera were prepared by

diluting 1:4 with the phosphate buffer. After washing, the excess water was removed and a Pasteur pipette was used to apply an equal amount of the diluted serum to each smear. The slides were covered with large lids and allowed to stand at room temperature for thirty (30) minutes in order for the serum reaction to occur. After this time, the slides were carefully rinsed twice with PBS then placed in a bath containing buffer for thirty (30) minutes to remove all unreactive serum proteins.

During the washing procedure, the conjugate fluorescein isothiocyanate isomer I (FITC) obtained from the Behring Institute (Germany) was prepared. The anti-gammaglobulins that it is conjugated to are IgG, IgA, and IgM, raised in goat. Subsequently, one drop of the conjugate (prepared by dissolving one ampoule content in 7 ml of 0.01 M PBS buffer pH 7.3 and 1 ml of distilled water) was added to each smear. The slides were then incubated for 30 minutes at room temperature in a moist chamber to permit reaction between the conjugated anti-gammaglobulins and the gammaglobulins bound to cytoplasmic antigenic components.

After incubation, the slides were washed three times for twenty minutes each time, with PBS solution. Before mounting, the excess buffer was carefully removed by wiping the area around the smears. Then one drop of 1:1 (glycerol:0.01M potassium phosphate pH 7.6) was applied to each smear which was then covered with a coverslip making sure no bubbles were trapped beneath. The slides were viewed under a microscope illuminated by UV light. Note that the

basic procedure used for direct (plate 1a) and indirect (plate 1b) cytoplasmic immunofluoresence is the same, except in the former the patient serum is omitted.

Microscopy and Scoring

All slides were number coded, ramdomized and subsequently examined and scored.

ISOLATION AND PURIFICATION OF NUCLEI

Solutions used in the preparation and purification of nuclei and nucleoli:

TKM Buffer

- 0.05M Tris
- 0.005M $MgCl_2$
- 0.025M KCl
- pH adjusted to 7.5 with HCl

a) 0.25M sucrose in TKM buffer

b) 2.3M sucrose in TKM buffer

c) Phosphate buffered saline

- 0.01M phosphate buffer containing 0.15M NaCl pH 7.4

d) S1 = 0.34M sucrose in TKM

e) S2 = 0.88M sucrose in TKM medium

f) Staining Solution

- 0.1% azure C, made up in 0.25M sucrose or in distilled water.

The sucrose-calcium procedure, which was originally developed by Hogeboom et al (1952) and subsequently modified by Chauveau et al (1956), has become the most widely used procedure for the isolation of nuclei from liver and other tissues. Since, for the purpose of isolation of nucleoli, it is essential that a divalent ion be present in the medium during the isolation of the nuclei, a modification of this procedure was employed. Therefore, the method used to isolate and purify the nuclear preparations was essentially that of Blodel and Potter (1966), which is a significant modification of the procedure of Chauveau et al (1956).

The procedure was as follows: approximately 5-7 g. of tumor tissue was thawed rapidly in a 40°C water bath and finely minced in 2 volumes of ice-cold 0.25M sucrose in TKM buffer. The product was homogenized manually with 10-15 complete strokes using a glass homogenizer, and then filtered through four layers of cheese-cloth to remove fibrous tissue. The suspension was then centrifuged for 10 minutes at 700-800 X g in a Sorvall Superspeed RC refrigerated centrifuge. The crude nuclear pellet obtained was redissolved in 7 ml of 0.25 sucrose in TKM buffer by gentle homogenization and then pipetted into a 30 ml capacity nitrocellulose tube. Then 14 ml of 2.3M sucrose in TKM buffer was added by means of a large syringe and thouroughly mixed by inversion with the 0.25M sucrose-TKM homogenate. The sucrose concentration of the homogenate (mixture) was thereby raised to approximately 1.62M sucrose, the density necessary to float mitochondria and rough endoplasmic reticulum, thus preventing them from co-sedimenting with the nuclear pellet. The mixture was then

underlaid with 7 ml of 2.3M sucrose in TKM. Centrifugation was in a Spinco SW 27L rotor at 25.5 rpm at 0-4°C for 45 minutes. To further purify the nuclear pellet, the nuclear pellet was redissolved in 5 ml of TKM buffer pH 7.4 containing 1% Triton X-100. The nuclei were washed by centrifugation (0-4°C) at 700-800 X g for ten (10) minutes in a Sorvall refrigerated centrifuge. The purified nuclei were then taken up in phosphate buffered saline and dialyzed against the same buffer overnight.

1.1 DETERMINATION OF THE PURITY OF THE NUCLEAR PREPARATIONS

1.1.1 Morphological Analysis

Electronmicroscopy

Electronmicroscopy provides one of the most valuable morphological evaluations of nuclear purity by examination of the nuclear membrane and the nucleolus. Consequently, this method was used mainly to asses to degree of purity of the nuclear preparation during the various stages of the isolation procedure. Samples were prepared for electronmicroscopy in the following way: pellets were washed in distilled water by centrifugation and fixed by the addition of 2.0 ml of 4% gluteraldehyde in 0.2M sodium cacodylate, pH 7.2, and allowed to stand at 0°C for approximately 1 hour. The nuclei were then pelleted down by centrifugation (1600 X g) for thirty (30) minutes and fixed again at 0°C in the same buffer for two (2) hours. All subsequent preparations and microscopic examinations were done by

Gail Detuch, at the Cancer Research Unit, McGill University.

Light Microscopy

The morphological integrity of the nucleoli can be used as a criteria of nuclear purity: therefore, samples of nuclear preparations from various stages of the isolation procedure were routinely stained and examined by light microscopy. In addition, samples of nuclear preparations were also examined to determine the degree of contamination by cytoplasmic material.

1.1.2 Enzymatic Analysis

Those nuclear preparations which were of acceptable purity, as determined by electronmicroscopy, were further analyzed by enzymatic and chemical techniques. Enzymatic analysis involved the determination of commonly used plasma membrane and cytoplasmic marker enzymes. For example, the degree of contamination of the final nuclear preparations by fragments of plasma membrane was assessed by assaying for 5' nucleotidase, while the degree of cytoplasmic contamination was assessed by assaying for glucose-6-phosphatase and acid phosphatase. All enzyme assays were conducted within three (3) months of isolation of nuclear preparations using fractions which had been stored at -20°C and thawed just before use. The basic principle involved in both assays was similar: the incubation of the specific substrate with each enzyme sample and subsequent determination of the amount of liberated inorganic phosphate.

5' Nucleotidase

The method employed to assay for 5' nucleotidase activity was a modification by Schimmel et al (1973) of the original procedure of Schachter et al (1970) . The incubation mixtures for the assay of 5' nucleotidase contained (in millimoles): adenosine 5' monophosphate (AMP), 7.5; glycine-HCl (buffer pH 8.5) 125; $HgCl_2$, 12.5; EDTA, o.2; and enzymes (50 ug protein) in a final volume of 0.3 ml. The reaction tubes were incubated at 37°C for 60 minutes, and then the reaction was terminated by the addition of 0.3 ml of cold 10% TCA (trichloracetic acid). The tubes were then centrifuged to remove the precipitated protein, and 0.2 ml of the clear supernatant was removed for determination of enzymatically released inorganic phosphate.

Inorganic Phosphate Release Assay,

To determine the amount of inorganic phosphate released by 5' nucleotidase and D-glucose-phosphatase, the method of Lowry and Lopez (1946) was employed: 2 ml of 2% ascorbic acid (freshly prepared), and 2 ml of 0.5% ammonium molybdate made up in 2N H_2SO_4 were added to 0.2 ml aliquots taken from the supernatant of the reaction mixtures. The reaction tubes were shaken and then incubated at 45°C for 20 minutes. The reaction was terminated by immediately cooling, and the absorbance at 820 mu was determined using a Beckman Spectrophotometer. A solution, 1 mM KH_2PO_4, was used as a standard. A unit of activity was expressed as micromoles of inorganic phosphate released per 60

minutes, and specific activity was expressed as units of activity per mg of protein.

Acid Phosphatase Assay

The enzyme was assayed by measuring the release of p-nitrophenyl from p-nitrophenylphosphate. The incubation medium contained 0.5 ml buffer substrate, 0.5 ml enzyme/H_2O and 0.05 ml of 5% Triton X-100.

(a) Citrate buffer (50 mM)

50mM citrate buffer pH 4.8 was prepared by dissolving 0.5253 g citric acid in approximately 45 ml of distilled water. The pH was adjusted to 4.8 with NaOH, and the final volume was brought to 50 ml.

(b) Buffer substrate (6.8×10^{-3}M)

The buffer substrate solution was prepared by dissolving 0.1015 g Na-p-nitrophenylphosphate in 50 ml citrate buffer.

(c) P-nitrophenyl (5.5×10^{-3}M)

5.5×10^{-3}M p-nitrophenyl solution was prepared by dissolving 6.96 mg of p-nitrophenyl in 10 ml of 0.02M NaOH.

Procedure: The assay tubes were agitated by shaking vigorously and then incubated for 60 minutes at 22°C. After the incubation, the reaction was terminated by the addition of 1.0 ml of 0.1N NaOH. The release of p-nitrophenyl was determined by measuring absorbance at 405mu in a Beckman Spectrophotometer. 5.5×10^{-3}M p-nitrophenyl solution was used as standard, and absorbance versus u moles p-nitrophenyl was plotted.

D-Glucose-6-phosphatase Assay

D-glucose-6-phosphatase assay was performed according to the method of Swanson (1965) using the following solutions:

(a) Glucose-6-phosphate solution (0.1M)

0.1M glucose-6-phosphate solution was prepared by suspending 260 mg of barium salt in 2 ml of distilled water, and dissolving in a minimum amount of0.1 NHCL. Then 72 mg of anhydrous Na_4SO_4 was added, and the resulting precipitate was removed by centrifugation. The supernatant was subsequently tested for complete precipitation by the addition of a small quantity of Na_4SO_4. The pH of the supernatant was adjusted to 6.5 with 10% NaOH and the volume was brought to 10 ml.

(b) Maleic acide-NaOH buffer (0.1M)

Maleic acid buffer was prepared by dissolving 116 mg of maleic acid in approximately 8 ml of distilled water. The pH was then adjusted to 6.5with 10% NaOH and the volume was brought to 10 ml.

The incubation mixture for the assay of D-glucose-6-phosphatase contained 0.1 ml of 0.1M glucose-6-phosphate solution, 0.3 ml of maleic acid NaOH buffer pH 6.5 and approximately 50 ug protein in a final volume of 0.5 ml. The reaction tubes were incubated at 37°C for 60 minutes, and the reaction was terminated by addition of 0.5 ml of 10% TCA. The reaction mixture was then diluted to 2.5 ml with distilled water, then centrifuged to remove the precipitated proteins. Then 0.2 ml of the supernatant was used for the determination of inorganic phosphate released as outlined earlier.

1.1.3 Chemical Analysis

Chemical analysis of the purity of the nuclear preparations involved the determination of the major constituents of nuclei, i.e. RNA, DNA, and proteins.

RNA Determination

The RNA content of the nuclear preparations was done according to the Orcinol procedure as outlined below. Assay mixtures containing 2 ml of enzyme/water, and 2 ml of Orcinol reagent (i.e. 0.2 g Orcinol in 20 ml of concentrated HCl containing 0.1 g $FeCl_3$ freshly prepared) were agitated and then placed in a boiling water bath for 20 minutes. The reaction tubes were then cooled and absorbance at 660 mu was read in a Beckman Spectrophotometer. 1.0 mg/ml RNA solution was used as a standard. It should be noted that since sucrose in high concentration interferes with both the Orcinol test (Schactner et al 1970) and Lowry (Bonitati et al 1969) all fractions from the discontinuous sucrose gradient were first dialyzed against 0.01M PBS pH 7.4 overnight with three (3) changes of buffer.

Protein Determination

Protein assay was done according to the method of Lowry et al (1951) with the following modifications: the final volume was decreased to 3.8 ml, and BSA (1 mg/ml) was used as the standard.

Deoxyribonucleic Acid (DNA) Determination

DNA was determined by Ceriotti's modification of the diphenylamine procedure, except that 2.5N concentrated HCl was used and smaples were heated for 20 minutes instead of 10 minutes as suggested by Blodel and Potter (1966). Calf thymus DNA (Sigma) concentration 2.5-15 ug/ml was used as a standard. Assay mixtures contained 2 ml of protein/H_2O, 1 ml of Indole (as prepared below), and 1 ml of 2.5N HCl. The mixtures were shaken well, and then the tubes were covered with aliminium caps and placed in a boiling water bath for 20 minutes. The tubes were then cooled under running water, extracted 3 times with 4 ml of chloroform each time, and centrifuged to give a completely clear interphase. The intensity of the yellow color which developed was read in a Beckman Spectrophotometer at 490 mu. The indole solution was prepared by dissolving the indole (4 mg) in 8 ml of warm distilled water, cooled under running water and diluted to volume.

1.2 ISOLATION AND PURIFICATION OF NUCLEOLI

1.2.1 Sonication of Nuclei

Those nuclear preparations which were of adequate yield and purity, as determined by electronmicroscopy and enzymatic analysis, were sonicated essentially according to the method of Bush (1967). Purified nuclei were suspended in a volume of 20 ml of 0.34 sucrose-TKM medium containing an RNA polymerase inhibitor (polyvinyl sulphate) in a final concentration of 1%. For release of the nucleoli from the nuclear preparations, a sonic 300 dismembrator (obtained from Fischer Scientific Company) was used. To minimize nucleolar breakdown due to increased temperature, the sample container was immersed in an ice bath throughout the sonication process. The nuclear preparations were sonicated for 20 second periods. At each time interval, a drop of the sonicate was used to determine the extent of the destruction of the nuclei. Sonic oscillation was discontinued when most of the nuclei were virtually destroyed. Under these conditions (i.e. temperature and volume which were optimum for sonication) the maximum destruction of the nuclei was achieved with minimum destruction of the nucleoli. This did not usually require sonic oscillation periods longer than 60-100 seconds.

1.2.2 Identification of Nucleoli

Since excessive sonic oscillations result in increased destruction of the nucleoli, it was necessary to carefully follow

the various stages of destruction of the nuclei. At each step of the sonication procedure the aliquots of the sonicate were mixed with a few drops of 1% Azure C made up in 0.25M sucrose or distilled water. The mixture was placed on a clean microscopic slide, covered with a coverslip, and examined using a light microscope. This stain permitted the rapid and easy visualization of the nucleoli as well as ribonucleoprotein components of the cytoplasm.

1.2.3 Purification of the Nucleoli from Sonicate

The procedure used for purification of the nucleoli was basically that of Ro et al (1964) . The whole sonicate in a volume of 20 ml of 0.34M sucrose-TKM is layered over 20 ml of 0.88M sucrose in TKM, and then centrifuged at 2000 X g in a refrigerated centrifuge for 20 minutes at $0^{o}C$. The sedimented pellet containing purified nucleoli was furtherpurified by redissolving in 10 volumes of 0.88M sucrose-TKM and centrifuging once more to remove any co-precipitated nucleoprotein. To ensure maximum recovery of nucleoli from the sonicate, aliquots of sonicate and supernatants from the first and second centrifugations were routinely checked for loss of small nucleoli by staining with Azure C as outlined above. In addition. since the number of nuclei was not determined, it was impossible to calculate the recovery of nucleoli from the sonicated nuclear preparation. Consequently, to further maximize the recovery of nucleoli from the sonicate, the following fractions were routinely assessed by chemical (RNA determination) and enzymatic (acid phosphatase) analysis. The fractions used for analysis were (a) intact purified

nuclei suspended in phosphate buffered saline, (b) the sonicate, (c) upper layer of the gradient designated NuS_1, (d) the lower layer of the gradient designated NuS_2, and (e) purified nucleoli.

1.3 DETERMINATION OF THE PURITY OF THE NUCLEOLAR PREPARATIONS

Analysis

The criteria used to determine the purity and yield of nucleoli from normal as well as tumor tissue generally includes morphological, chemical and enzymatic analysis.

1.3.1 Morphological Analysis

Since light microscopy provides a ready and reliable method for direct visualization of nucleoli in cell fractions, especially when used in conjunction with cytochemical techniques, e.g. staining with Azure C, this method was used primarily to assess the purity of the nucleolar preparations (see section entitled "Identification of Nucleoli").

1.3.2 Enzymatic Analysis

Although there are no specific nucleolar enzymes which are readily and easily distinguishable from nuclear enzymes as a whole, it would be expected that the purified nucleolar preparation would contain a much lower level of the specific activities of both plasma membrane

and cytoplasmic marker enzymes than the purified nuclei from which they were isolated. Consequently, enzymatic analysis of nucleolar purity was done using the same procedures, and under the same conditions as was done for nuclear analysis.

1.3.3 Chemical Analysis

The three major constituents of nucleoli are RNA, DNA and proteins - as is the case for nuclei. Thus, these were assayed by the same methods used for determination of nuclear purity.

ISOLATION AND PURIFICATION OF KCl-EXTRACTED NUCLEOLAR ANTIGENS

2.1 THE ISOLATION AND PURIFICATION OF IgG's

Gammaglobulins were isolated from preabsorbed sera showing high tumor specificity. The gammaglobulin fractions were isolated by ammonium sulfate fractionation according to the following procedure: with constant stirring, 0.666 ml of cold saturated ammonium sulfate solution was added to 1 ml of serum, thus effecting a 40% saturation. The mixture was further stirred for another ten (10) minutes and then allowed to stand at 4°C for 30 minutes. It was then centrifuged at 20,000 X g for ten (10) minutes. The supernatant was discarded and the pellet containing the precipitated proteins, primarily gammaglobulins and some traces of other globulins and albumin, was redissolved in enough phosphate buffered saline to restore the volume of the solution to that of the original serum

sample (Sapin et al. 1975). The gammaglobulin fraction was then dialyzed at 4°C against 0.01M PBS pH 7.5 for 24 hours with several changes of buffer to remove the ammonium sulfate. After dialysis, the protein concentration was determined, and then approximately 10-15 mg of gammaglobulin was chromatographed on DEAE cellulose in order to isolate the IgG fraction.

2.2 DEAE CELLULOSE CHROMATOGRAPHY

The gammaglobulin fractions which were isolated by salt precipitation were subjected to chromatography on DEAE cellulose.

Preparation of Absorbant: DEAE cellulose was washed in alkali and acid to remove impurities and fine particles using the following procedure: approximately 10 g of DEAE cellulose was suspended in 900-1000 ml of 0.2M NaOH. The suspension was gently stirred and then allowed to stand at room temperature for 30 minutes to allow the heavier particles to settle. The fine particles were then removed by aspiration. This procedure was repeated twice, followed by several washings with the same volume of 0.2M HCl. The slurries were then washed with several volumes (900-1000) of distilled water until the pH was approximately 5.0, then neutralized with concentrated K_2HPO_4. The slurries were finally washed 3 times in phosphate buffered saline (0.01M, containing 0.15M NaCl, pH 7.4) and resuspended in 500 ml of the same buffer until used.

2.2.1 Preparation and Elution

A column (20 X 1.5 cm, Pharmacia Fine Chemicals) was filled to 1/3 its height with starting buffer. Then the gel slurry in the same buffer was added, and the column was packed to the required height using a flow rate induced by gravity. To further equilibrate the column prior to application of the sample, approximately 500 ml of the starting buffer was used to continuously wash the column for 10-12 hours. During this equilibration procedure, the ammonium precipitated gammaglobulin fractions were dialyzed for 10-12 hours against the starting buffer. The equilibrated fraction, in a volume of 1.0 ml, was absorbed onto the column by washing in with two (2) volumes of the same buffer. The column was then filled with buffer and the elution gradient was attached. The eluting salt gradient, consisting of 0.005M and 0.5M NaCl made up in 0.01M phosphate buffer, was set up by siphoning 200 ml of the lower (0.005M) buffer, keeping constant the volume of the mixing chambers. The flow rate was adjusted to 3 ml per 20 minutes and 3 ml fractions were collected using a Minirac 1700 fraction collector from LKB Biocal Instruments.

2.2.2 Analysis of Fractions

The fractions were then dialyzed against 0.01M phosphate buffer overnight, and the protein concentration was subsequently determined by U.V. absorption (optical density 280 mu). Fractions which were found to contain very high concentrations of Immunoglobulin G as determined by immunological techniques (see section 3.2, Immunological analysis), were pooled and concentrated by ultrafiltration to a final concentration of 10-15 mg/ml. In addition, the purity of the

IgG-rich fraction was assessed by double diffusion (Outerlony) and cross-over immunoelectrophoresis.

2.3 KCl EXTRACTION OF PUTATIVE ANTIGEN(S)

The purified nucleolar preparations were tested for antigenic activity by using the three immunological techniques described below. Those nucleolar preparations which were of high antigenic activity were used to extract the putative antigen(s). Soluble antigenic material was extracted from purified nucleolar preparations with hypertonic (3M) potassium chloride, essentially according to the method of Mavligit et al (1973). An aliquot of the nucleolar preparation was suspended in 5 ml of 3M KCl made up in 0.1M phosphate buffered saline pH 7.2. The mixture was stirred for one hour at $4^{o}C$ and then transferred to a dialysis bag, and then dialyzed against 0.01M PBS pH 7.2 for 24 hours with three changes of the buffer. The mixture was then centrifuged in the micro-eppendorf centrifuge for thirty (30) minutes at $4^{o}C$. The supernatant, containing the extracted nucleolar proteins, was transferred to another dialysis bag and dialyzed against 0.01M PBS pH 7.4 for eight (8) hours. After dialysis, the mixture was removed and concentrated using polyethylene glycol. The protein concentration was then estimated by measurement of optical density at 280 mu. An aliquot of the KCl extract was retested for antigenic activity, and the remainder was chromatographed on Sepharose 4B-IgG columns.

2.4 AFFINITY CHROMATOGRAPHY OF 3M KCl EXTRACTS

Partially purified IgG's were coupled to Sepharose 4B using the cyanogen bromide procedure of Cutrecasas (1970)and affinity chromatography was carried out as described by Thomson et al (1976).

2.4.1 Coupling of Ligand to Sepharose

Approximately 15 ml of Sepharose 4B was washed three (3) times with distilled water by centrifugation at 7000 rpm for five (5) minutes at $0^{o}C$. The packed Sepharose volume was determined, and then mixed with an equal amount of cold distilled water. Approximately 4.0 g (1g/5 ml of suspension) of finely divided cyanogen bromide was added at once to the stirred suspension. The pH of the suspension was immediately raised to and maintained between 10.5 and 11.1 by the addition of 10% NaOH. At the same time, the temperature was maintained at approximately $20^{o}C$ by the addition of pieces of ice as needed. The reaction was completed in about 10 minutes; this was shown by the cessation of proton release and concommitant stabilization of the pH. As soon as the reaction was completed, a large amount of ice was added to the suspension, which was then quickly transferred to a Buchner funnel and immediately washed rapidly under suction with 200 ml of cold citrate buffer (0.2M, pH 6.5). IgG's in the same volume of citrate buffer as the packed Sepharose 4B were added to the washed,

activated Sepharose, and the suspension was mixed gently in a beaker using a glass stirring rod. The suspension was stirred gently for four (4) hours in a small beaker, and then allowed to stand at $4^{o}C$ overnight to ensure complete loss of reactive groups. Since 'activated' Sepharose is very unstable, extreme caution was taken to ensure that the pH of the suspension during the activation process did not rise about pH 11.1. Also, care was taken to make sure that the entire procedure, i.e.from the activation of Sepharose through to the coupling stage, did not last longer than 10-12 minutes.

2.4.2 Preparation of Column

Using a column (1 X 5 cm), approximately 4 ml of coupled Sepharose 4B-IgG slurry was gently added. The column was packed using a flow rate induced by gravity. To remove all the unreactive ligand, the column was washed continually for ten (10) hours with 50 ml of starting buffer, i.e.0.01M tris-HCl pH 7.4 containing 0.15M NaCl. During the washing procedure, the fractions collected were monitored for protein content by determination of absorbance at 280 mu. When approximately five (5) consecutive fractions had abosrbance 280 readings of zero, it was assumed that the ligand (IgG) was no longer being removed. At this point, a known amount of KCl extract (in the starting buffer) was applied onto the column. The sample was absorbed onto the column by washing in with two volumes of the same buffer. The flow rate was then stopped for one hour to permit maximum reaction between the ligand and the KCl extracted proteins. Collection of fractions was resumed and the column was again washed continously with tris-HCl buffer

until the absorbance returned to zero. At this point, the column was further washed until approximately ten (10) more fractions of zero absorbance were collected. This was done to ensure that the KCl extracted proteins were no longer being eluted from the column. The column was then filled with buffer, and connected to a buffer well containing 3M KCNS. The column was eluted and 0.7 ml fractions were collected by hand. Fractions were read at 280 nM in a spectrophotometer. When the abosrbance readings of the fractions reached the level at which KCNS read, ten (10) more fractions were collected. The fractions were dialyzed for forty eight (48) hours against three (3) changes of 0.001M tris-HCl buffer containing 0.015M NaCl pH 7.4. After dialysis, protein concentration was determined on aliquots of fractions by the method of Lowry et al (1951) and the fractions were concentrated using the calcium chloride procedure as outlined earlier. Samples were pooled and analyzed by SDSPAGE, and by cross-over immunoelectrophoresis.

BIOCHEMICAL AND IMMUNOLOGICAL ANALYSIS

3.1 SDS-ACRYLAMIDE GEL ELECTROPHORESIS

Samples of (a) purified nucleoli, (b) KCl extracts before and after affinity chromatography were analyzed by SDS polyacrylamide gel electrophoresis according to the method of Fairbanks et al (1971) with several modifications: In order to make three gels, the concentrated stock solutions were mixed in the order and proportions given in table (3). The mixture was deaerated and then the ammonium persulfate

TABLE 3

Solutions used for preparation of 5.6% SDS acrylamide gels.

A) Stock solutions

- acrylamide 40 gm
- methylene-bis-acrylamide 1.5 gm
- H_2O to 100 ml

- 20 gm SDS in H_2O to 100 ml (w/v) (20%)
- 15 mg $(NH_4)_2S_2SO_4$ in 1 ml water (1.5%)
- 5 ul TEMED in 1 ml water (0.5%)

Buffer 10 X concentrated pH 7.4

- 1.0M tris (40 ml)-hydroxymethylaminomethane
- 2.0M sodium acetate (10 ml)
- 0.2M EDTA (10 ml)-ethylenediamine tetra acetic acid
- H_2O to 100 ml

B) Electrophoresis buffer (per liter)

- buffer 10 X concentrated 100 ml
- 20% SDS 50 ml
- H_2O 850 ml

C) Gels (per 10 ml of solution)

- concentrated acrylamide solution 1.4 ml
- buffer 10 X concentrated 1.0 ml
- 20% SDS 0.5 ml
- 1.5% $(NH_4)_2S_2SO_4$ 1 ml
- 0.5% TEMED 0.5 ml
- H_2O 5.6 ml

Staining and destaining solutions (D,E,F)

D) - 25% isopropyl alcohol
- 10% acetic acid
- 0.05% Coomasie brilliant blue

E) - 10% isopropyl alcohol
- 10% acetic acid
- 0.005% Coomasie brilliant blue

F) - 10% acetic acid
- 0.002% Coomasie brilliant blue

and Temed were added, stirred, and quickly transferred to 13 cm glass tubes (13 cm X 8 mm diameter) that had been previously cleaned by soaking in chromic sulfuric solution for ten (10) hours, followed by extensive rinsing in distilled water. Each column (height 11 cm) was overlaid gently with a solution consisting of 0.1% SDS, 0.15% ammonium persulfate and 0.05% Temed. The gels were left to polymerize (approximately 30-45 minutes at room temperature), and then the tops of the gels were rinsed with distilled water and overlaid with about 0.5 ml of the electrophoresis buffer solution B, table (3). The gels were covered with parafin paper and allowed to stand at room temperature for 10-12 hours to ensure minimum band curvature.

3.1.1 Preparation of Samples

Approximately 100 ug protein samples of 1% SDS solubilized nucleoli preparations were prepared for electrophoresis by first dialyzing against 10 mM tris-HCl pH 8.0 containing 1 mM EDTA for 10-12 hours at $4^{o}C$, followed by the addition of the following to final concentrations: 1% SDS, 1mM EDTA (pH 8.0), 10 mM tris-HCl (pH 8.0) and 40 mM DTT. In addition, samples containing salts (i.e. KCl extracts) were first dialyzed against the same buffer plus 1% SDS. The samples were then incubated at $37^{o}C$ for 30 minutes to ensure full reduction of disulfide bonds by DTT. After incubation, the samples were made 5% in sucrose, and then 5-10 ul of 0.05% bromophenol brilliant blue was added. Each sample was then taken up in a disposable Pasteur pipette and discharged gently beneath the electrophoretic buffer onto

the top of the gel. A mixture of proteins of known molecular weight, which was used as molecular weight markers was treated in the same manner as samples with the exception that they were incubated at $45^{o}C$ for 20 minutes.

3.1.2 Running of the Gels

Electrophoresis was performed using a disc-electrophoresis apparatus connected to a power supply purchased from Instrumentation Specialities Company. The current was approximately 8 mA per tube. When the tracking dye had travelled approximately 3/4 of the gel length (normally 3-4 hours), electrophoresis was terminated. After electrophoresis, the gels were removed from the tubes by gently squirting distilled water between the walls of the glass tube and the gel, using a 5 ml syringe fitted with a 2-inch blunted needle. The gel length and the position of the tracking dye was recorded, and then the gels were stained and destained .

3.1.3 Staining and Destaining

The gels were placed in culture test tubes and stained for protein with Coomasie brilliant blue using the following procedure: gels were fixed in solution (D) for 8-10 hours, followed by staining in solution (E) for approximately 6 hours. To further intensify the protein pattern, the gels were then stained overnight in solution (F). Afterwards,the gels were destained with several changes of 10% acetic

acid until the background allowed the bands to be seen clearly.

3.1.4 Analysis of Protein Patterns

The molecular weights of the various molecular weight species involved were calculated by use of a standard curve constructed from the mobilities of the marker proteins as a function of their known weights on semilogarithmic coordinates. Since gels expand to different degrees during the staining procedure, the relative mobilities were calculated using the following equation:

$$\text{Mobility} = \frac{(\text{distance of protein migration})(\text{gel length before staining})}{(\text{distance of dye migration})(\text{gel length after staining})}$$

Molecular Weight Markers

Ribonuclease A (13,700), chymotrypsinogen A (25,000), aldolase (40,000), ovalbumin (45,000), bovine serum albumin - BSA - (68,000), and β-galactosidase (135,000).

3.2 IMMUNOLOGICAL ANALYSIS

The following immunological techniques were used to detect antigens: (a) immunoprecipitation, (b) double diffusion and (c) counter current immunoelectrophoresis. Because of the ease and rapidity with which the above analyses can be performed, the first two methods were routinely used for (1) checking the retention of antigenic activity

during the various stages of cell fractionation, and (2) localization and subsequent analysis of the purity of the IgG fraction during ion exchange chromatography.

It should be noted that since opalescence interferes with the visualization and subsequent interpretation of precipitin bands, the serum was first clarified by centrifugation in a micro-eppendorf 3200 centrifuge for ten (10) minutes. After centrifugation, the clairified serum was removed and used for analysis. Prior to analysis, all fractions to be tested were dialyzed for 8-16 hours at $4^{o}C$ against 0.15M NaCl.

3.2.1 Immunoprecipitation

Approximately 20 ul of fraction to be tested was placed on a glass coverslip coated with wax and then gently inserted into a 50 ul capliiary tube. To prevent mixing, the tube was slanted slightly, and an equal volume (20 ul) of test serum was carefully added by introducing the clairified serum by a Hamilton syringe along the walls of the tube near the surface of the bottom solution. The tubes were allowed to stand for one hour then viewed for formation of a precipitin line. A precipitin ring, which is indicative of a positive reaction. occurred as a result of the diffusion of the antigen and antibody until their concentration ratio was optimum for precipitation.As controls, 20 ul of 0.01M tris-HCl buffer pH 7.4 containing 0.15M NaCl and 20 ul of IgG, and 20 ul of goat anti-human IgG were used.

3.2.2 Double Diffusion (Ouchterlony)

Double diffusion in two dimensions, a procedure developed by Ouchterlony (1958), involves placing an antigen and an antibody solution in separate wells cut in agar, and allowing the reagents to diffuse freely and form precipitin bands at a point where they meet at equivalent proportions. The procedure of double diffusion is outlined below:

(a) Preparation of Agar Plates

1. 1% Noble agar, prepared by dissolving 1 g in 90 ml of 0.1% saline, was melted and cooled to approximately 50-60^{0}C, then 10 ml of 10^{-5}M merthiolate solution was added, and mixed well.
2. Immediately, approximately 2.5 ml of hot agar was pipetted onto a clean microscopic slide and allowed to solidify to an even coat.
3. The agar slides were covered and further cooled for fifteen (15) minutes at room temperature.
4. Agar slides were placed over the geometric pattern required, and either a rounded metal cutter or special gel puncher was used to cut wells into the agar according to the pattern.
5. Agar from the wells was removed by using a Pasteur pipette attached to a vacuum line.
6. Then the wells were filled with approximately 15 ul of samples to be tested using a 50 ul Hamilton syringe.
7. As controls, 15 ul of human IgG, anti-human IgG or buffer were included.
8. Slides were marked to facilitate easy identification during the

subsequent staining and destaining procedure.

9. Slides were placed in a special gel chamber on top of pieces of lintless paper dampened with distilled water, and were examined periodically for development of precipitin lines.

10. When the development of precipitin lines seemed optimum (10-12 hours), they were graded 1 - (slight) to 3 - (very intense), or further dried and stained (see below).

(b) Washing and Drying Plates

After visual analysis of the precipitin lines, the unreacted proteins were removed by immersing the slides in a bath of 0.01M phosphate buffer, pH 7.5 containing 0.015M NaCl. The slides were removed, placed between large pieces of filter paper, and subjected to a steady stream of warm air. As the filter paper dries, it removes water and salts from the agar gel; consequently, the agar dries to a thin and even surface.

(c) Staining and Destaining of Plates

The slides were stained according to the procedure of Uriel (1971). The dried plates were carefully placed in a staining solution consisting of 0.1% Ponceau red or 0.1% amido black, 0.425M acetic acid, and 0.0425M sodium acetate for two (2) hours. Excess stain was removed by transferring slides to 2% acetic acid. The slides were further destained for approximately 30-45 minutes with three changes of the destaining solution. When the gel bands could be seen

clearly, the slides were removed and air-dried in a vertical position.

3.2.3 Counter Current Immunoelectrophoresis

Counter current immunoelectrophoresis was performed by the method of Dr. D. Hartmann (Meerovitch et al 1977) of the Cancer Research Unit, McGill University.

Preparation of Plates: Approximately 0.5 g of Sigma type II agarose medium EEO was dissolved in 50 ml of Veronal acetate-HCl buffer pH 8.2 and heated. As soon as the solution started to boil, it was removed from the heat and poured into a clean plate and allowed to solidify slowly at room temperature to an even surface. A specially made template containing cylindrical openings was placed over the plate and secured by use of two side hooks. The well pattern was first marked by use of a rounded steel cutter and the gel was subsequently cut using a square metal cutter. Finally, the agar was removed by a Pasteur pipette under negative pressure.

Sample Preparation: Samples to be tested, i.e. sera and antigenic fractions, were removed from the freezer, thawed rapidly in a 40°C water bath, and allowed to stand at room temperature for thirty (30) minutes. After this time, the test serum was acidified by a 1:8 dilution with 0.02M glycine-HCl buffer pH 2.2. In addition, the controls (human IgG and anti-human IgG), were diluted (1:8) with Venonal-acetate HCl, and glycine buffer respectively. After thirty (30) minutes of further incubation, approximately 15 ul of either acidified

sera and the anti-IgG control was carefully placed in the wells located on the anode side, while equal amounts of antigenic fractions and buffered IgG's were placed in the cathode side of the plate. Any excess reactant was quickly removed by a Hamilton syringe and then electrophoresis was done.

Electrophoresis: The electrophoretic plates, cells and power supply used were purchased from Hyland, California, U.S.A. An electrophoretic cell filled with Veronal acetate buffer was mounted above the power supply. A connective metal electrode was placed in each chamber and covered with conductive sponge which had been soaked in the same buffer. The filled plate was carefully inverted and fitted over the electrophoretic cells so that the serum-containing wells were positioned near the anode side of the power supply. Electrophoresis was carried out at 30 mA for sixty (60) minutes at room temperature. After electrophoresis, the plates were removed, washed, and analyzed using the same method as used for immunodiffusion.

SPECIFICITY OF NUCLEOLAR ANTIGEN(S)

To demonstrate the specificity of nucleolar antigen(s), the following experiments were done. Normal serum was diluted 1:5 with PBS (0.01M pH 7.4) and a known amount of purified nucleoli was added. The mixture was incubated at 37°C for five (5) minutes and cooled, and then overnight at 4°C. Nucleoli were collected and washed twice with three (3) volumes of the same buffer by centrifugation in a micro-eppendorf 3200 centrifuge at 4°C for ten (10) minutes. The nucleoli

were recollected by centrifugation. Following centrifugation, the supernatant and the pellet were collected. The pellet was suspended in 3M KCl and extracted as described previously. After extraction, the suspension was dialyzed against phosphate-buffered saline pH 7.4. The dialyzed suspension was centrifuged, and the supernatant was collected and concentrated. The fractions were then analyzed for antigenic activity by cross-over immunoelectrophoresis. The experiments were repeated with autologous tumor serum and sera from other non-melanotic cancer patients substituted for normal serum.

RESULTS

CYTOPLASMIC IMMUNOFLUORESCENCE

The fluorescent antibody technique has been used successfully in the identification and localization of a wide variety of melanoma tumor-specific antigens. Many authors, Muna et al 1969, Lewis et al 1971, Lewis and Phillips 1972, McBride et al 1973, have observed specific immunofluorescence on the surface of living cells or in the cytoplasm and nucleoli of snap-frozen cell preparations. However, unlike the surface membrane reaction, the cytoplasmic reaction exhibited a wide degree of cross reactivity in that positive sera from a number of malignant melanoma patients reacted strongly against many cell preparations from other melanoma patients. Therefore, to determine which melanotic sera and tissue were to be used for the isolation of the immunoglobulin components and subsequent isolation of putative nucleolar antigens against which the components reacted, indirect cytoplasmic immunofluorescence was performed using the procedures described by Phillips and Lewis (1972) (Lewis and Phillips 1970).

To establish specificity of the cytoplasmic reaction, sera were first examined for the existence of autologous tumor-specific antibodies by inhibition studies with KCl extracts of the tumors and for titer of these antibodies to cytoplasmic antigens by reacting them with autologous cells. Positive sera were absorbed with normal spleen cells and with pooled non-melanotic tumor cells and re-tested

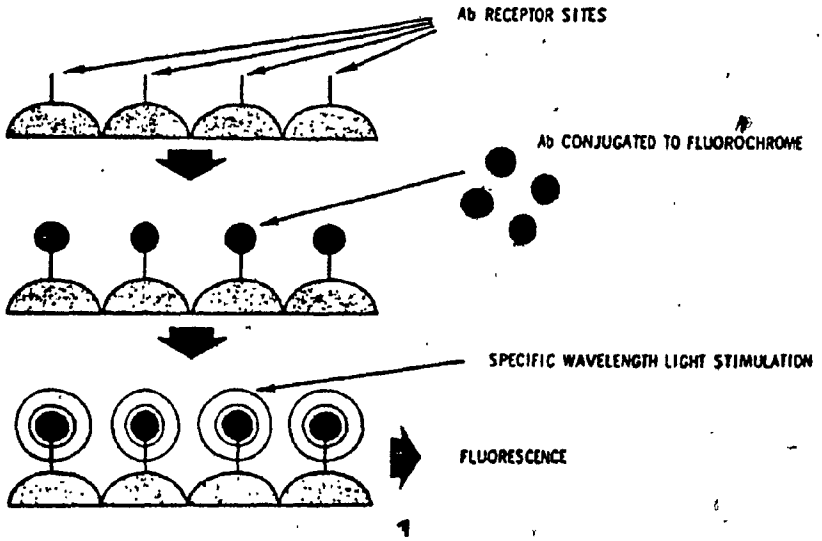
PLATES 1 and 2

Schematic representation of cytoplasmic immunofluorescence.
1) direct fluorescence
2) indirect fluorescence.

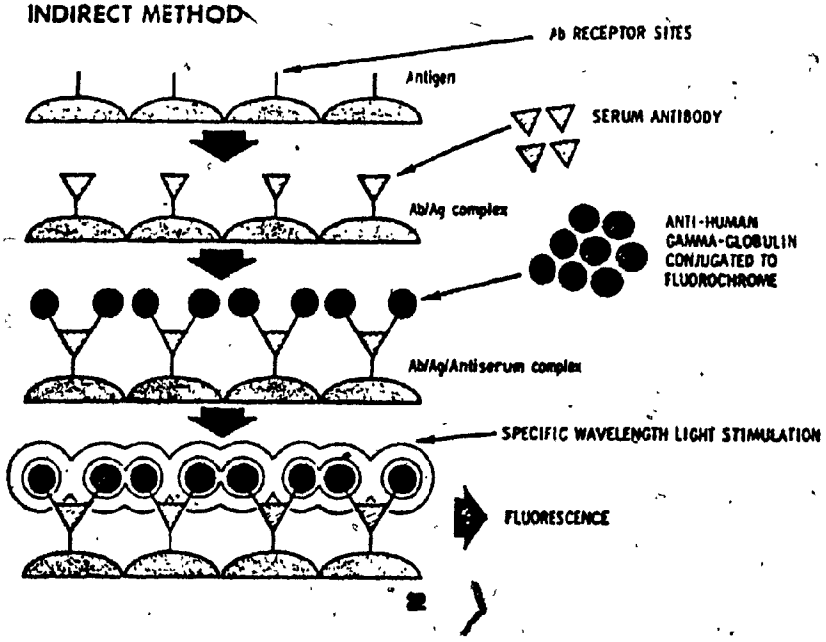
(from Lewis 1974)

PLATES 1 and 2

DIRECT FLUORESCENT METHOD



INDIRECT METHOD



for reactivity. Tumor tissue which was found to have high reactivity was used to make cell suspensions and smears of these suspensions were snap-frozen in a mixture of isopentane and liquid nitrogen. For analysis, smears were thawed, washed with phosphate buffered saline (PBS) and incubated with autologous positive sera (1:4 diluted with PBS) for a period of time sufficient to allow the reaction between the serum immunoglobulin molecules and any tumor-specific antigenic molecule present to occur. To further ensure the specificity of the cytoplasmic reaction, direct immunofluorescence in which the smears were directly treated with the conjugate, was also performed. In addition, to check the quality of the commercially prepared conjugate in terms of free fluorochrome and non-gammaglobulin protein content, control smears were treated with PBS and analyzed for non-specific fluorescence.

Under the above conditions, positive fluorescence was recorded when the cytoplasm (nucleolar region) stained a deep grass green in contrast with the light reddish green surroundings. The results obtained with smearsof tumor cells from several malignant melanoma patients are shown in tables (1,2). It can be seen that there was a wide range of cross reactivity between highly positive sera from any one of the patients and snap-frozen cell preparations of most of the melanoma tumors tested. In terms of degree of positivity, the results in table (1) indicate that five (5) patients (Ros , Ven, Duc, McK, Dal) were strongly positive, while the sera of other patients showed a lesser degree of positivity. Since the degree of positivity observed reflects the amount of antigenic molecules present in the cell preparations and also the titer of the immunoglobulins present, sera and tissue material

TABLE 1

CYTOPLASMIC IMMUNOFLUORESCENCE

Patient's Name	Immunofluorescence Pattern
Ros	+
Duc	+
McK	+
Fei	$\pm$
Per	$\pm$
Shu	$\pm$
Dal	+
Ven	+
Tre	+
Control Roi	-
Control Cha	-
Normal Sera	-

Determination of melanoma specific positive antibodies: (+) indicates high titer, ($\pm$) indicates low titer, and (-) indicates absence of detectable melanoma specific antibodies.

TABLE 2

CYTOPLASMIC IMMUNOFLUORESCENCE

Determination of titer of positive antibody against autologous and allogeneic smears.

Patient's serum	Corresponding Patient's Smear				
	Ros	Duc	McK	Dal	Ven
Ros	1/128	1/64	1/128	1/64	1/32
Duc	1/32	1/128	1/32	1/64	1/32
McK	1/64	1/32	1/128	1/64	1/64
Dal	1/128	1/128	1/64	1/128	1/64
Ven	1/32	1/64	1/64	1/32	1/64

All sera were diluted (1/4, 1/8, 1/16, 1/32, 1/64, 1/128) with PBS (0.01M potassium phosphate containing 0.15M NaCl) pH 7.3. The sera with the highest titer of positive antibodies were used for further work.

were selected from those sera and cell preparations which exhibited high reactivity. However, in some experiments other positive sera, normal sera and non-melanotic tumor tissue were used.

ISOLATION AND PURIFICATION OF IMMUNOGLOBULIN G.

It is known that the two main types of immunoglobulin molecules involved in the specific reaction against tumor-specific antigens are IgG and IgM. (Romsdahl and Cox 1973, Seibert et al 1977, and Lewis et al 1977). Therefore, sera (Ros , Ven, McK, Dal, Duc) which were strongly positive by cytoplasmic immunofluorescence (i.e. appeared to have a high titer of tumor-specific immunoglobulin, tables (1,2)) were used for the isolation and purification of IgG's. The immunoglobulin component of the positive sera was precipitated by the addition of ammonium sulfate to 40% saturation. As ion exchange chromatography requires that the sample be applied at low ionic strength, immunoglobulin pellets were dialyzed extensively for 10-12 hours at 4°C against three changes of phosphate buffer (0.01M PBS pH 7.4). DEAE cellulose was washed to remove impurities and fines, and then pre-cycled to maximize the availability of charged reactive groups. A column was then packed to the required height with gel slurries and equilibrated by continuous washing with starting buffer for 8-10 hours. After equilibration, 10-15 mg of the 40% ammonium sulphate precipitate, previously dialyzed against the same buffer, was absorbed onto the column. The column was then eluted with a linear salt gradient consisting of 0.005M NaCl and 0.5M NaCl made up in the phosphate buffer (0.01M PB, pH 7.4). To maximize the resolution, the flow rate of

the column was adjusted to 3 ml/20 minutes and 3 ml fractions were collected and checked for protein on a Beckman spectrophotometer at 280 nm.

The protein elution profiles from DEAE cellulose chromatography of 40% ammonium sulphate precipitate of sera for five (5) melanoma patients are shown in figures (1,2). The profiles are very similar. It can be seen in figure (1),which shows the complete profiles from patients Ros and Ven, that there are three main regions of protein-containing material: (a) a large unretarded peak which is eluted at very low ionic strength. This peak was presumed to be the IgG-rich fraction on the basis that most IgG molecules carry a slightly net positive charge, and therefore would bind very loosely to the positively charged DEAE cellulose; (b) a well defined peak of intermediate height occuring between fraction 24 and fraction 30 presumed to be IgM; and, (c) a series of poorly defined, small peaks presumably other type immunoglobulin molecules.

In order to confirm which fraction contained the IgG's, fractions from the three main peaks were pooled, concentrated and analyzed by the following immunological techniques: approximately 10-15 ul aliquots ofthe pooled fractions were analyzed by micro-precipitation and double diffusion using commercially prepared goat anti-human IgG and anti-human serum proteins. The results of these analyses are presented in tables (4,5) & plate (3). Plate (3) shows that fractions 11 to 16, in the first peak, contained IgG's. Analysis of the IgG fractions by the method described by Preddie et al 1978a,

FIGURES

1. DEAE cellulose chromatography of 40% ammonium sulphate precipitate from patients Ros - ■ - ■ - ■ - and Ven -●-●-●-.

2. DEAE cellulose chromatography of 40% ammonium sulphate precipitate from patients McK - ▲ - ▲ - ▲ -, Duc -●-●-●- and Dal - ■ - ■ - ■ -.

FIGURE 1

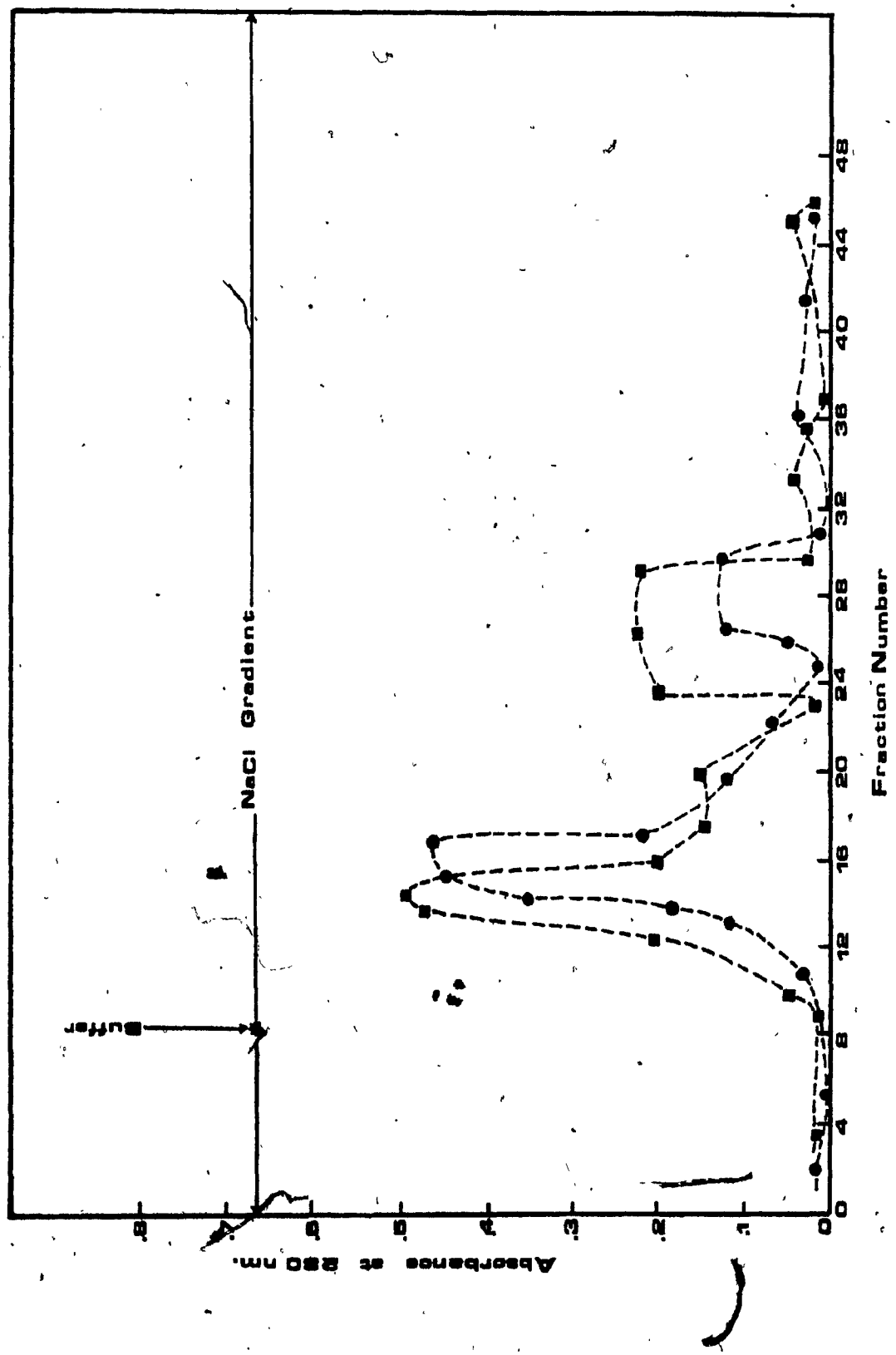


FIGURE 1

FIGURE 2

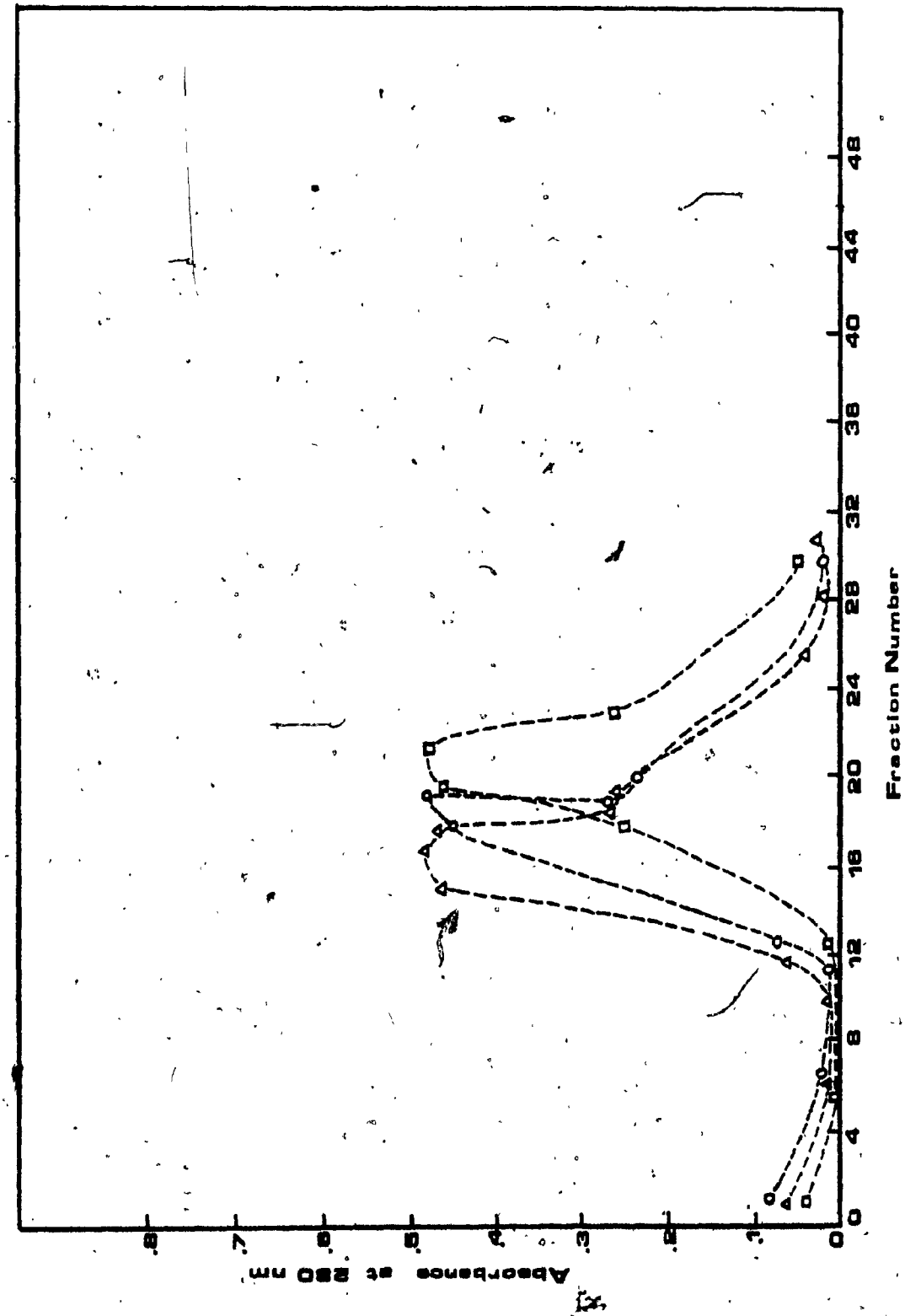


FIGURE 2

TABLE 4

Determination of purity of IgG-rich fraction isolated by DEAE cellulose chromatography.

The following fractions were tested against commercially prepared goat anti-human IgG using the following two immunological techniques (a) microprecipitation, (b) double diffusion.

Table (4) Use of Ros serum	Strength of precipitin line	
Fraction number	Microprecipitation	Double Diffusion
Controls: 1) Buffer (0.01M PBS pH 7.4)	-	-
2) IgG	+++	+++
10	-	-
11	+ -	+ -
12	++	++
13	++	++
14	++	++
15	+++	+++
16	++	++
17	-	-
18	+	+
19	+ -	+ -
20	++	+
21	+ -	+ -
22-30	-	-
43-50	-	-

TABLE 5

Determination of purity of the IgG-rich fraction isolated by DEAE cellulose chromatography.

Use of Dal serum.

Fraction number	Degree of Positivity	
	Microprecipitation	Double Diffusion
Controls (1) Buffer	-	-
(2) IgG	+++	+++
1	++	++
5	+	+
13	+	+
15	++	++
16	++	++
17	++	++
18	+	++
19	+	+
20	+ -	+ -
21	+ -	+ -
26-29	-	-

PLATE 3

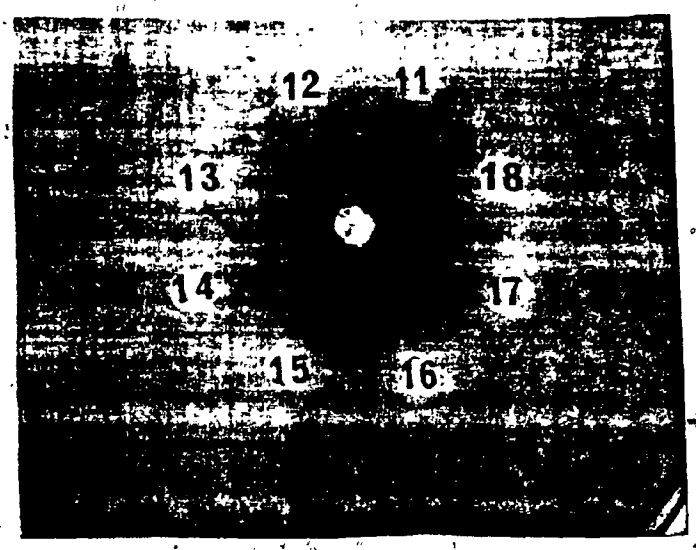
Localization of IgG in Fractions Following DEAE Cellulose Chromatography

Double diffusion precipitin reaction.

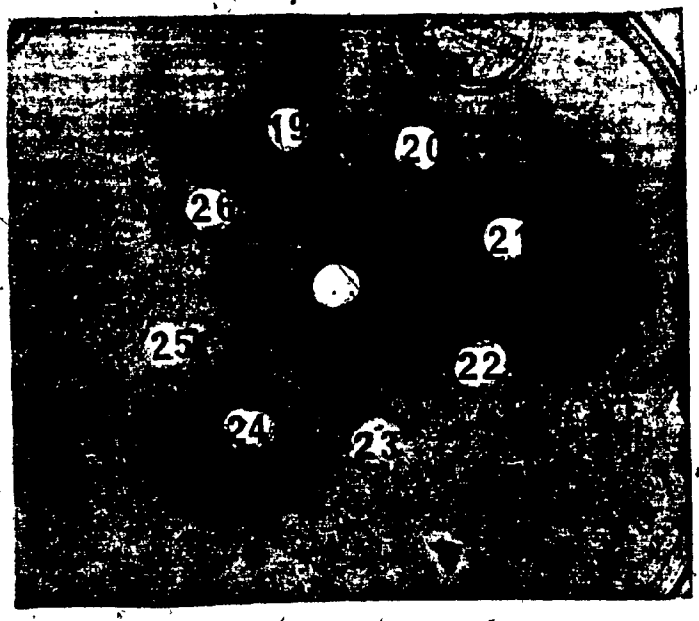
A) Anti-human IgG was placed in the center well, and fractions from the column were placed in wells 10 to 16 and well 18; wells 15 and 17 contained commercially prepared IgG and tris-HCl (0.01M pH 7.4) respectively.

B) Anti-human IgG was placed in the center well, and column fractions were placed in wells 19 to 23. Wells 24,25 contained commercially prepared IgG, and well 26 contained buffer.

PLATE 3



A



B

show that these were highly purified molecules. Therefore, only this peak was routinely used as the IgG source for all further experiments. Note that with sera from patients McK, Duc and Dal elution was stopped after the first peak emerged.

LOCALIZATION OF THE ANTIGENIC ACTIVITY ASSOCIATED WITH NUCLEOLI OF MALIGNANT MELANOMA CELLS

To do this, it was first necessary to show that the nuclear pellets from which the nucleolar preparations were to be derived were of acceptable purity in terms of morphological integrity and enzymatic activity. Therefore, nuclei isolated by the sucrose-calcium procedure were analyzed by: (a) electronmicroscopy - the results from electronmicrographs of nuclei isolated from patients Ros and Ven (these are representative of nuclei obtained from tumors of the other three patients in this study) are shown in plate (4)and indicate that the method used provides nuclei of high morphological integrity. One can also observe that there is no plasma membrane or cytoplasmic contamination visible at this high level of magnification (33,000); (b) that the nuclei were highly purified can be determined from the results in tables (6,7)on the chemical analyses of the nuclear preparations. It can be seen that the percent recovery of nucleic acid (i.e. DNA) in the nuclear pellet relative to that in the crude homogenate averages 71%, and the RNA:DNA ratio of the nuclear pellet ranged from 0.22 to 0.37 , an average value of 0.31.

Having determined that the nuclear pellets were of acceptable morphological and chemical purity, the next step was to show

PLATE 4

Electronmicrographs of purified nuclei.

A) Nuclei isolated from Ros tumors

B) Nuclei isolated from Ven tumors

The magnification was X 33,000. The nuclei were purified as described in the Methods section.

Chemical Analysis of Purity of Nuclear Preparations. TABLE 6

Patient No	Protein in Hom mg/ml	Protein in Nuclear pellet mg/ml	Protein in Nuclear pellet %	DNA in Hom mg/ml	DNA in Nuclear pellet mg/ml	DNA in Nuclear pellet %	RNA in Hom mg/ml	RNA in Nuclear pellet mg/ml	RNA in Nuclear pellet %	Ratio of Nuclear RNA/DNA	Ratio of Nuclear Protein DNA
Ros	30.2	4.23	14.1	5.25	4.20	78.72	15.01	1.38	9.2	0.33	1.01
Ire	27.31	5.05	18.51	4.78	3.30	70.53	11.73	0.76	6.5	0.23	1.53
McK	18.94	4.06	21.3	3.25	2.27	69.83	9.87	0.81	8.2	0.35	1.79
Duc	21.87	3.48	15.90	3.87	2.96	76.5	14.3	0.99	6.9	0.33	1.17
Ven	34.72	4.79	13.81	7.59	4.72	62.7	22.0	1.72	7.8	0.37	1.02
Dal	23.41	3.06	15.42	4.70	3.16	67.3	13.22	0.94	7.3	0.31	1.14

The values are the average of two (2) experiments. Each experiment was done in duplicate. Approximately 5 gm of tissue was used for each isolation. The volumes of Homogenate and nuclear pellet were adjusted to 0.5 ml with Saline (0.15M NaCl).

Chemical Analysis of Purity of Nucleolar Preparations

TABLE 7

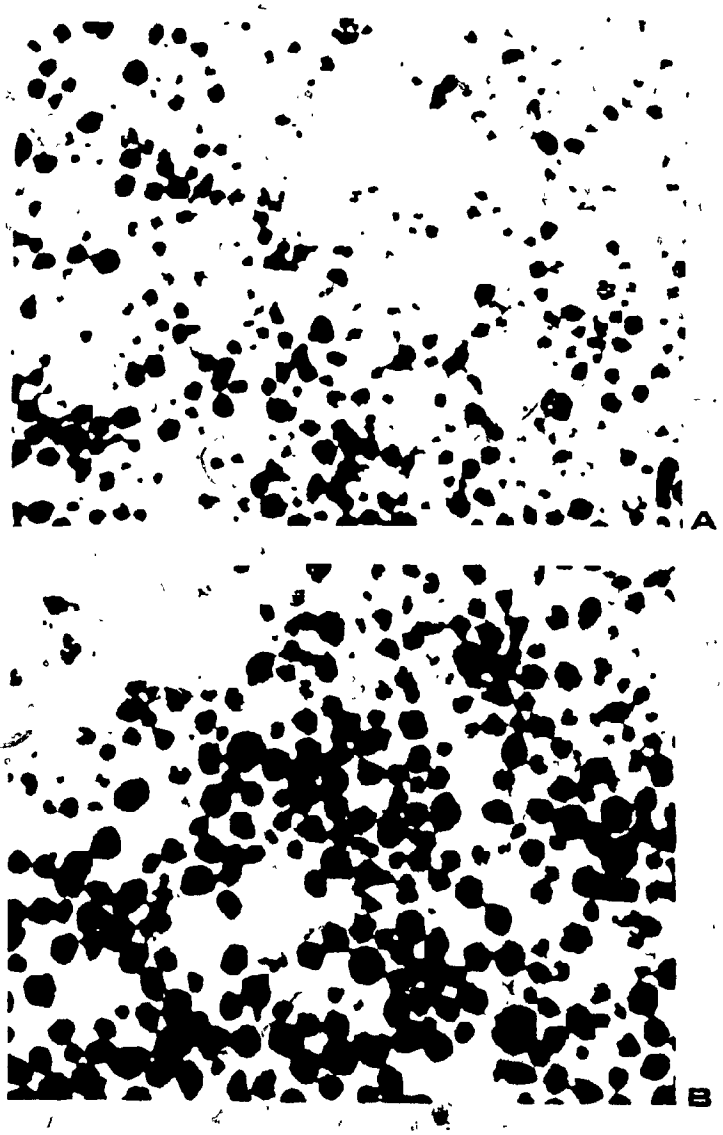
Patient number	RNA mg/ml	DNA mg/ml	Protein mg/ml	Ratio of RNA:DNA	Ratio of RNA:protein
Ros	0.36	0.26	2.25	1.38	0.16
Tre	0.26	0.14	2.75	1.86	0.09
McK	0.24	0.12	3.19	2.03	0.07
Duc	0.32	0.26	2.89	1.20	0.11
Dal	0.30	0.24	2.19	1.25	0.14
Ven	0.46	0.29	3.04	1.54	0.15

The values are the averages of two (2) experiments. Each experiment was run in duplicate. The average RNA:DNA and RNA:Protein values were 1.54 and 0.120 respectively. The volumes of all nucleolar preparations were adjusted to 0.5 ml with Saline (0.15M NaCl).

that nucleoli isolated from these purified nuclei were also of high purity. The electronmicrographs of purified nucleoli are shown in plates (5a,b). The nucleoli appear to be morphologically intact and free of visible comtaminants. These nucleoli purified from nuclei isolated from patients Ros and Ven are representative of the other three patients which are a part of this study. The nucleolar pellets were further analyzed for purity by enzymatic and chemical methods. Results of enzymatic analyses (table 8,9,10) appear to confirm that in all five tumors the nucleolar pellets were free of (a) cytoplasmic contamination as determined by glucose-6-phosphatase an enzyme marker commonly used for smooth endoplasmic reticulum, (b) lysosomes as determined by acid phosphatase, and (c) plasma membrane as determined by 5' nucleotidase activity. Since the specific activities of the marker enzymes in the nucleolar pellets represented only a fraction of the activity found in the nuclei from which they were derived, it is reasonable to conclude that the nucleoli are free from contamination by pieces of nuclei. On the other hand, the yield of nucleoli which was obtained indirectly by measuring the recovery of glucose-6-phosphatase activity and ribonucleic acid appeared to be very good in all five tumors. The results in tables (11,12) (from three patients) appear to indicate that the method used to isolate nuclei resulted in nucleolar pellets of high yield and low degree of contamination. Thus, those preparations which were previously found to be morphologically pure and enzymatically and chemically satisfactory were used to extract the putative antigens. Soluble antigenically active material was extracted from purified nucleolar preparations with hypertonic 3M potassium chloride essentially according to the

PLATE 5

Electronmicrographs of purified nucleoli isolated from melanoma cells.



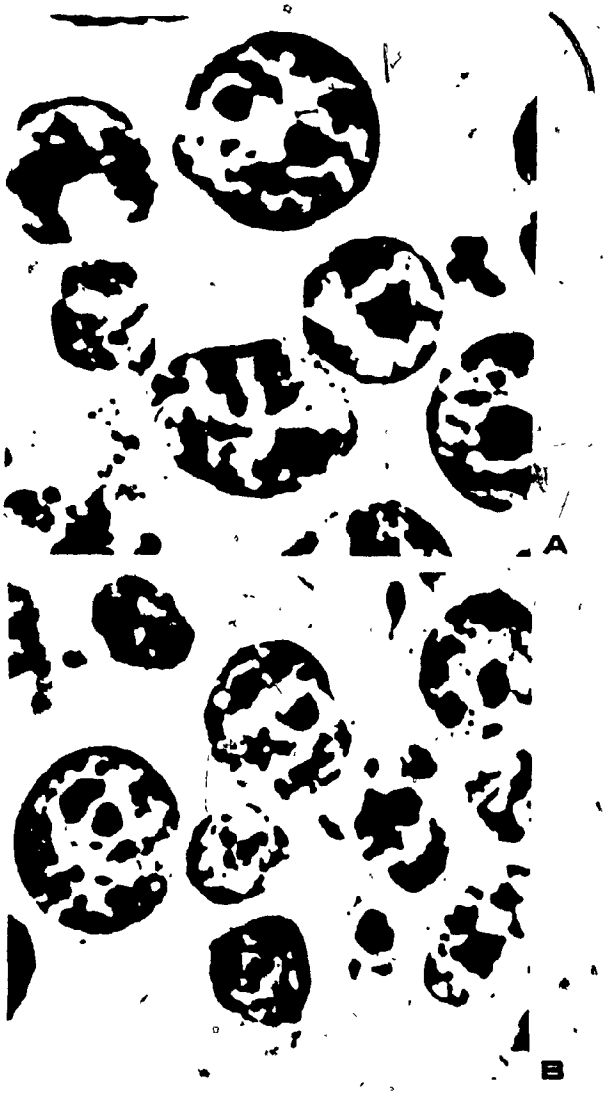
A) Nucleoli from Ros tumor cells. Magnification approx. X 20,000

B) Nucleoli from Ven tumor cells. Magnification approx. X 33,000

Nucleoli were purified as described in the Methods section.

PLATE 6

Electronmicrographs of isolated nuclei stained with Azure C.



A) Nuclei isolated from Duc tumor cells.

B) Nuclei isolated from McK tumor cells.

Magnification was approx. 33,000. Isolation and staining of nuclei was done as described in the Methods section.

TABLE 8

Nucleotidase, Enzymatic Analysis of Nucleolar Purity.

Patient	Fraction	O.D. 820nm	µm Pi/hr/50ug protein	Specific Activity	Rel. Spec. Activity
Ros	Homogenate	0.042	0.105	0.035	1
	Nuclei	0.037	0.228	0.076	2.17
	Nucleoli	0.002	0.023	0.026	0.74
Tre	Homogenate	0.014	0.126	0.042	1
	Nuclei	0.021	0.108	0.039	0.857
	Nucleoli	0.006	0.045	0.015	0.357
McK	Homogenate	0.008	0.084	0.028	1
	Nuclei	0.025	0.165	0.055	1.945
	Nucleoli	0.002	0.027	0.009	0.321
Duc	Homogenate	0.012	0.105	0.035	1
	Nuclei	0.034	0.219	0.073	2.085
	Nucleoli	0.003	0.032	0.017	0.486
Dal	Homogenate	0.008	0.085	0.029	1
	Nuclei	0.052	0.288	0.096	3.31
	Nucleoli	0.006	0.047	0.016	0.552
Ven	Homogenate	0.007	0.081	0.027	1
	Nuclei	0.030	0.189	0.063	2.33
	Nucleoli	0.007	0.060	0.024	0.88

The values presented in table 8 are the average of two (2) experiments. Each determination was done in duplicate. Specific activity was calculated as micromoles Pi/min./mg protein. Relative specific activity (Rel. Spec. Activity) was calculated with respect to the homogenate.

TABLE 9

Enzymatic Analysis of Purity of Nucleolar Preparations.

Glucose-6-phosphatase

Patient	Fraction	O.D. 820nm	µm Pi/hr/50ug protein	Specific Activity	Rel. Spec. Activity
Ros	Homogenate	0.035	3.49	1.17	1
	Nuclei	0.066	44.33	14.77	12.67
	Nucleoli	0.012	8.67	2.88	2.47
Tre	Homogenate	0.038	4.121	1.37	1
	Nuclei	0.034	20.42	6.81	4.95
	Nucleoli	0.017	10.36	3.46	2.52
McK	Homogenate	0.024	3.45	1.15	1
	Nuclei	0.029	20.38	6.78	5.95
	Nucleoli	0.005	2.351	0.78	0.68
Duc	Homogenate	0.027	3.43	1.14	1
	Nuclei	0.039	31.82	10.61	9.27
	Nucleoli	0.013	7.27	2.42	2.12
Dal	Homogenate	0.032	3.45	1.15	1
	Nuclei	0.033	24.16	8.05	7.23
	Nucleoli	0.015	11.98	3.99	3.48
Ven	Homogenate	0.049	4.11	1.37	1
	Nuclei	0.062	36.40	12.13	8.86
	Nucleoli	0.018	9.62	3.20	2.34

The values presented in table 9 are the average of two (2) experiments. Each determination was done in duplicate. Specific activity was calculated as micromoles Pi/min./mg protein. Relative specific activity (Rel. Spec. Activity) was calculated with respect to the homogenate.

TABLE 10

Enzyme Analysis of Nucleolar Purity

Acid Phosphatase

Patient	Fraction	O.D. 405 nm	µm Pi/hr/50ug protein	Specific Activity	Rel.Spec. Activity
Ros	Homogenate	0.042	0.070	0.023	1
	Nuclei	0.065	0.112	0.037	1.611
	Nucleoli	0.003	0.006	0.002	0.087
Tre	Homogenate	0.039	0.063	0.021	1
	Nuclei	0.068	0.113	0.038	1.809
	Nucleoli	0.001	0.002	0.001	0.048
McK	Homogenate	0.032	0.054	0.018	1
	Nuclei	0.054	0.090	0.030	1.667
	Nucleoli	0.002	0.005	0.002	0.111
Duc	Homogenate	0.046	0.073	0.024	1
	Nuclei	0.051	0.080	0.027	1.125
	Nucleoli	0.004	0.009	0.003	0.125
Dal	Homogenate	0.037	0.061	0.020	1
	Nuclei	0.059	0.103	0.034	1.700
	Nucleoli	0.002	0.005	0.002	0.100
Ven	Homogenate	0.047	0.075	0.025	1
	Nuclei	0.068	0.113	0.038	1.520
	Nucleoli	0.003	0.006	0.002	0.080

The values presented in table 10 are the average of two (2) experiments. Each determination was done in duplicate. Specific activity was calculated as micromoles Pi/min./mg protein. Relative specific activity (Rel. Spec. Activity) was calculated with respect to the homogenate.

TABLE 11

Determination of Recovery of Nucleoli

Intranuclear Distribution of Glucose-6-phosphatase

Patient	Fraction number	Recovery activity µM Pi/min/mg protein
Ros	Nuclei	14.38
	Sonicate	17.12
	NuS_1	10.03
	NuS_2	2.66
	Pur. nucleoli	1.48
Tre	Nuclei	6.93
	Sonicate	8.15
	NuS_1	4.93
	NuS_2	1.51
	Pur. nucleoli	1.23
Dal	Nuclei	7.95
	Sonicate	8.66
	NuS_1	4.53
	NuS_2	2.36
	Pur. nucleoli	1.26

Recovery of nucleoli was determined by comparing the recovered activity of glucose-6-phosphatase in the various fractions of the gradient relative to that of the nuclei. The data for these enzyme assays are given for three (3) patients only and is representative of all the other patients studied.

TABLE 12

Intranuclear Distribution of Ribonucleic Acid (RNA)

Patient	Fraction	RNA mg/ml
Ros	Nuclei	1.42
	Sonicate	1.56
	NuS_1	0.11
	NuS_2	0.32
	Pur. nucleoli	0.93
Tre	Nuclei	1.15
	Sonicate	1.21
	NuS_1	0.05
	NuS_2	0.08
	Pur. nucleoli	0.87
Dal	Nuclei	1.68
	Sonicate	1.76
	NuS_1	0.25
	NuS_2	0.49
	Pur. nucleoli	1.05

Recovery of nucleoli was determined by comparing the recovery of RNA in the various fractions of the gradient relative to the activity in whole nuclei. The volumes of the various fractions used to isolate and purify the nucleoli were adjusted to 0.5 ml with Saline (0.15M NaCl).

method of Mavligit et al (1973). After extensive dialysis to remove KCl, aliquots of the KCl extracts were tested for antigenic activity by immunodiffusion and cross-over immunoelectrophoresis. The remainder of the KCl extracts were subjected to affinity chromatography on Sepharose 4B-IgG columns.

AFFINITY CHROMATOGRAPHY

Purified IgG's from sera of five patients were coupled to Sepharose 4B and affinity chromatography was done as described in the Methods section of the text. Aliquots, 5.5 to 8.5 mg of KCl extracts, were absorbed onto the columns. The columns were washed with the same buffer and then eluted with 3M KCNS. Fractions (0.7 ml) werecollected and protein concentration was determined by absorption at 280nm. The protein elution profiles of KCl extracts of the five tumors are shown in figures (3,4,5). The elution profiles are similar, whether the affinity column was prepared with autologous or with allogeneic tumor-specific IgG. Three distinct areas are observed in the elution profiles: (a) peak 1 represents unbound ligand eluted during the first washing with starting buffer; (b) peak 2 represents material for which there is no corresponding IgG bound to the Sepharose 4B, and (c) peak 3 represents bound antigenic material that is eluted by 3M KCNS. The two curves represent the absorbance at 280 nm before and after dialysis to remove KCNS which affects the absorbance at 280 nm.

The fractions from the three major peaks were pooled

FIGURE 3

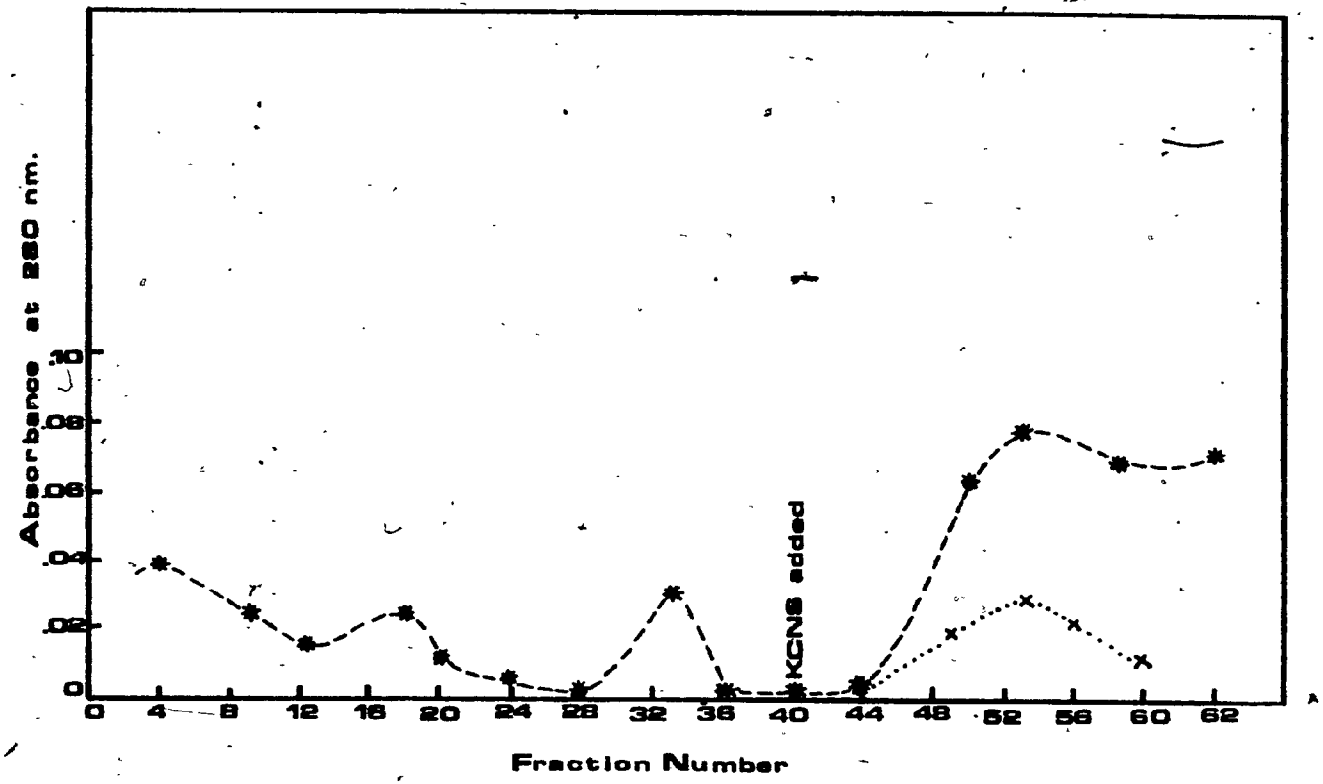


FIGURE 3

3. Elution profile of 3M KCl extracts on affinity columns -(Ros).
....X....X....X after removal of KCNS by dialysis.

FIGURE 4

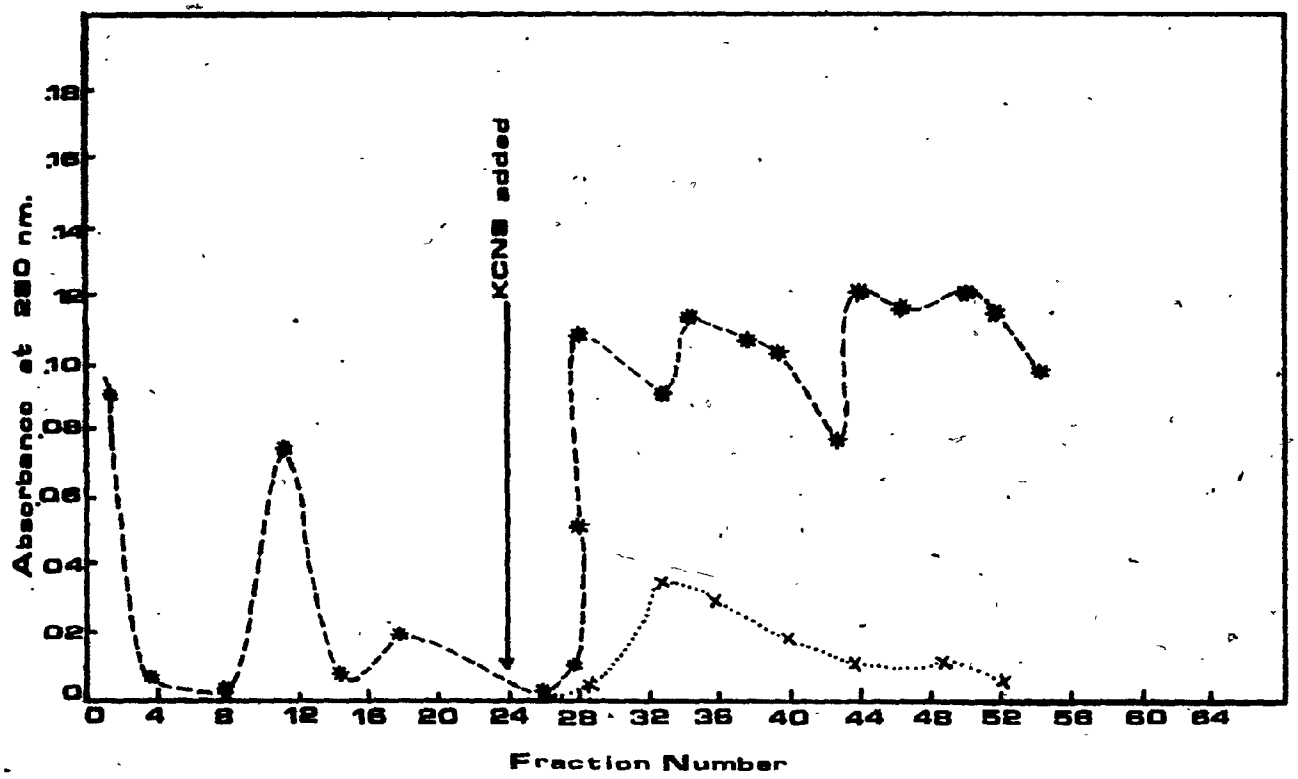
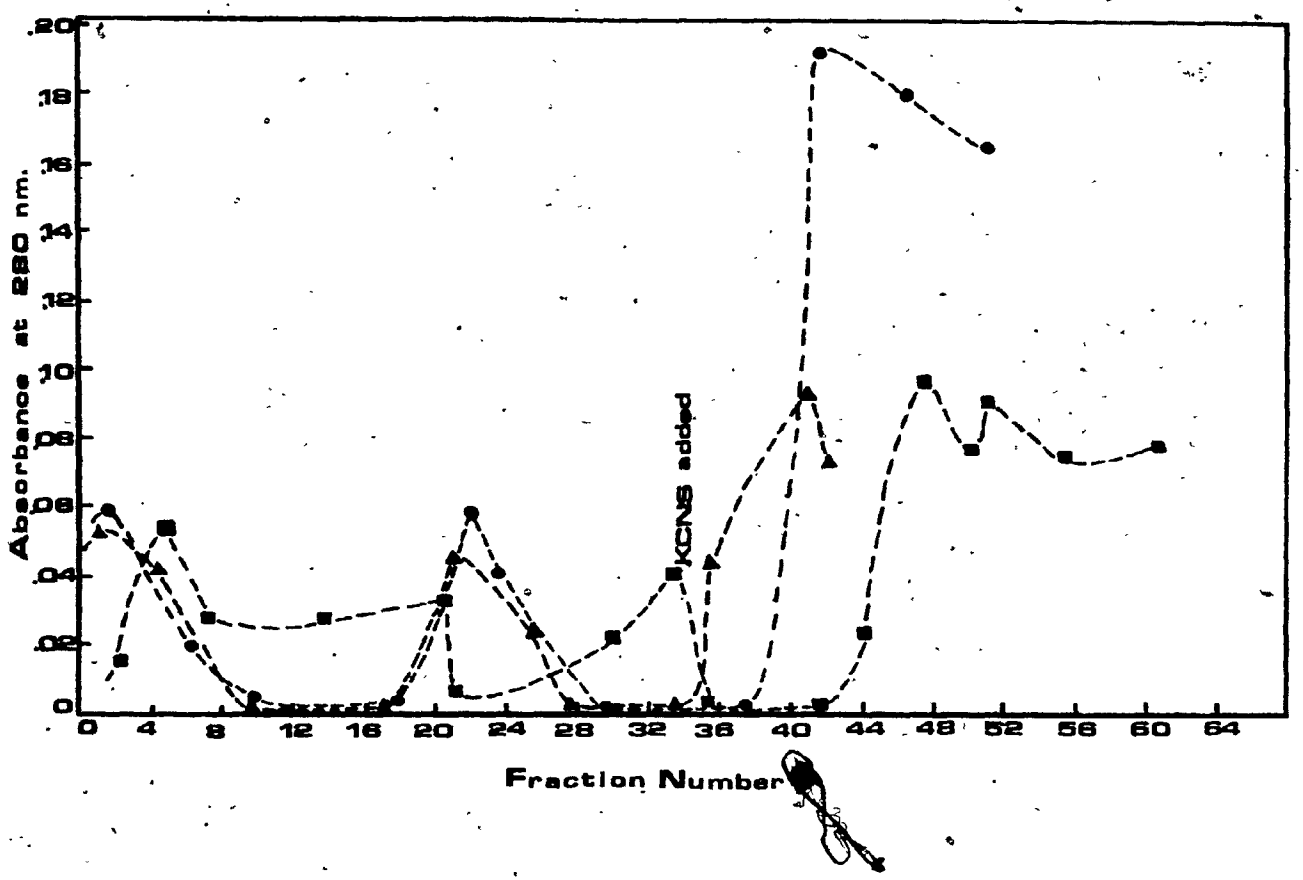


FIGURE 4

4. Elution profile of 3M KCl extracts on affinity columns -(Dal).
....X....X....X after removal of KCNS by dialysis.

FIGURE 5



5. Elutions profiles of 3M KCl extracts from affinity columns.
   McK -- ■ -- ■ -- ■ --;
   Duc -- ● -- ● -- ● --;
   Ven -- ▲ -- ▲ -- ▲ --.

FIGURE 5

and dialyzed for forty eight (48) hours against three changes of buffer (0.001M tris-HCl pH 7.4 containing 0.15M NaCl). After dialysis, the fractions were re-checked for protein, pooled and concentrated 10-fold by the calcium chloride method. Aliquots of concentrated fractions were tested for antigenic activity and electrophoresed on SDSPAGE.

The results of immunological analysis of the fractions eluted from Sepharose 4B-IgG columns are shown in table (13) and plates (8a,8b). Due to very low concentration of protein in the 3M KCl extracts from two patients, these were pooled. This was considered very reasonable because the material from these two patients cross-reacted with each other ;in other words, they both contained allogeneic tumor-specific antigens. However, the presence of undetected autologous tumor-specific antigens in either or both of these tumor fractions cannot be ruled out. The KCNS fraction eluted from each of the five patients contained melanoma tumor-specific antigenic material.

Cross-over immunoelectrophoresis was used to monitor antigenic activity during tumor cell fractionation. The results in table (14) show that in all cases except for Tre nuclei (this preparation of nuclei suffered some spurious breakage) the nuclei were negative, whereas homogenates, nucleoli and KCl extracts of nucleoli were positive. The results also show that pre-absorption of the nucleoli with normal serum and/or serum from other non-melanoma cancer patients had no effect on the antigenic activity recovered in KCl extracts, but on the other hand, pre-absorption with autologous tumor-specific serum abolished all antigenic activity.

TABLE 13

Identification of the Antigenic Activity by Cross-over Immunoelectrophoresis of Fractions Eluted by 3M KCNS in Affinity Chromatography.

Affinity Chromatography system	Fraction number	Cross-over immuno-electrophoresis
Ros and Dal KCl extracts on Ros IgG	31-32	-
	33-34	-
	35-36	+
	37-38	+
	39-40	+
	41-42	-
	43-44	-
McK KCl extracts on Ros IgG	44-45	-
	46-48	+
	49-50	+
	51-52	+
	53-55	-
Duc KCl extracts on Duc IgG	57-58	-
	59-61	+
	62-63	+
	64-65	+
	66-67	-
Fei KCl extracts on Ven IgG	40-41	-
	42-43	+
	44-45	+
Tre KCl extracts on McK IgG	37-38	+
	39-40	+
	41-42	+

KCNS elution profiles of these experiments, except for McK KCl extracts on Ros IgG, are not shown in figures 3, 4 or 5.

PLATE 8

Cross-over immunoelectrophoresis.

Immunoelectrophoresis was done as described in the Methods section of the text.

A) 1. KCNS eluate of KCl extracts of Ros nucleoli which was eluted from an affinity column.
2. KCNS eluate of KCl extracts of Ven nucleoli which was eluted from an affinity column.
3. KCNS eluate of KCl extracts of Ros nucleoli which were absorbed with normal serum before extraction with KCl.
4. KCNS eluate of KCl extracts of Ven nucleoli which were absorbed with normal serum before extraction with KCl.
5. same as (3) but the nucleoli were absorbed with serum from a patient with colon cancer before extraction with KCl.

B) 1. same as A) 4. but the nucleoli were absorbed with serum from a patient with breast cancer before extraction with KCl.
2. Normal human IgG versus anti-human IgG.
3. KCl extract of nucleoli from Ros tumor cells which were absorbed with Ros autologous melanoma tumor-specific serum before extraction with KCl.
4. Normal human IgG versus anti-human IgG.
5. KCl extract of nucleoli from Ven tumor cells which were absorbed with Ven autologous melanoma tumor-specific serum before extraction with KCl.

PLATE 8 (a)

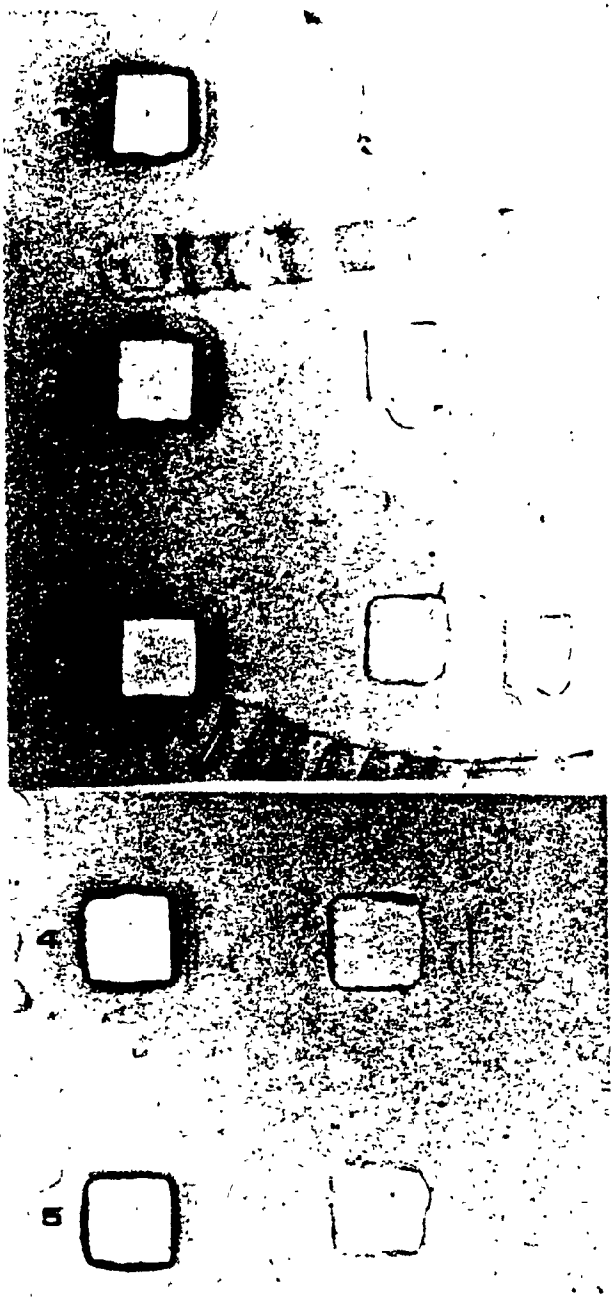


PLATE 8 (b)

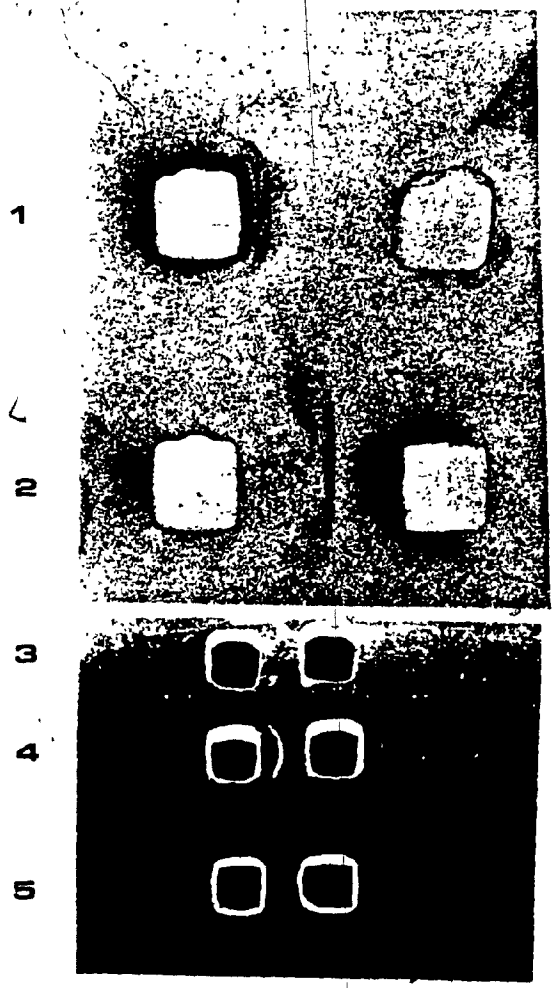


TABLE 14

Determination of Antigenic Activity during Cell Fractionation by Cross-over Immunoelectrophoresis (CIE).

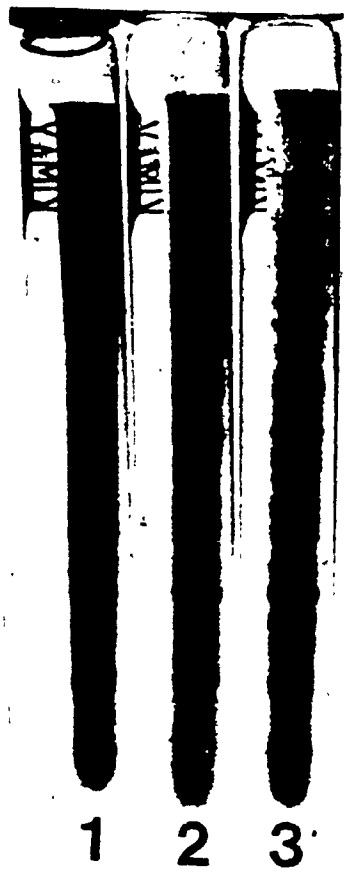
Sera Source	Tumor Source	Tumor Fraction	CIE results	CIE results (a)	CIE results (b)
Ros	Duc	Homogenate	+		
Ros	Duc	Nuclei	-		
Ros	Duc	Nucleoli	+		
Ros	Duc	3M KCl ext.	++	++	-
Ros	Dal	Homogenate	+		
Ros	Dal	Nuclei	-		
Ros	Dal	Nucleoli	+		
Ros	Dal	3M KCl ext.	++	++	-
Ros	Tre	Homogenate	+		
Ros	Tre	Nuclei	+		
Ros	Tre	Nucleoli	+		
Ros	Tre	3M KCl ext.	++	++	-
Ros	Ros	Homogenate	+		
Ros	Ros	Nuclei	-		
Ros	Ros	Nucleoli	+		
Ros	Ros	3M KCl ext.	++	++	-
Ros	Fei	Homogenate	+		
Ros	Fei	Nuclei	-		
Ros	Fei	Nucleoli	+		
Ros	Fei	3M KCl ext.	++		
Fei	McK	Homogenate	+		
Fei	McK	Nuclei	-		
Fei	McK	Nucleoli	+		
Fei	McK	3M KCl ext.	++	++	-

CIE results (a) - the nucleoli were absorbed either with normal serum or with sera from non-melanotic cancer patients before they were extracted with 3M KCl. CIE results (b) - the nucleoli were absorbed with autologous tumor-specific serum before they were extracted with 3M KCl.

Aliquots of 3M KCl extracts and 3M KCNS eluted fractions were electrophoresed on SDS PAGE as described in the Methods section of the text. The results of electrophoresis shown in table (15) and plates (7, 9) lead to the following conclusions: (a) purified nucleoli from each of the five patients contained between 20 and 25 different molecular weight species; and (b) only four molecular weight species wereobserved in the eluate from the affinity columns suggesting that at least four protein-containing species among the species present in the nucleoli are melanoma tumor-specific antigenic molecules. SDS PAGE of KCNS affinity columns eluates of KCl extracts of nucleoli (Ros and Ven) which were pre-absorbed with autologous tumor-specific IgG, showed no protein-containing bands, indicating that pre-absorption with autologous sera neutralizes these antigenic molecules.

PLATE 7

SDS acrylamide gel electrophoresis.



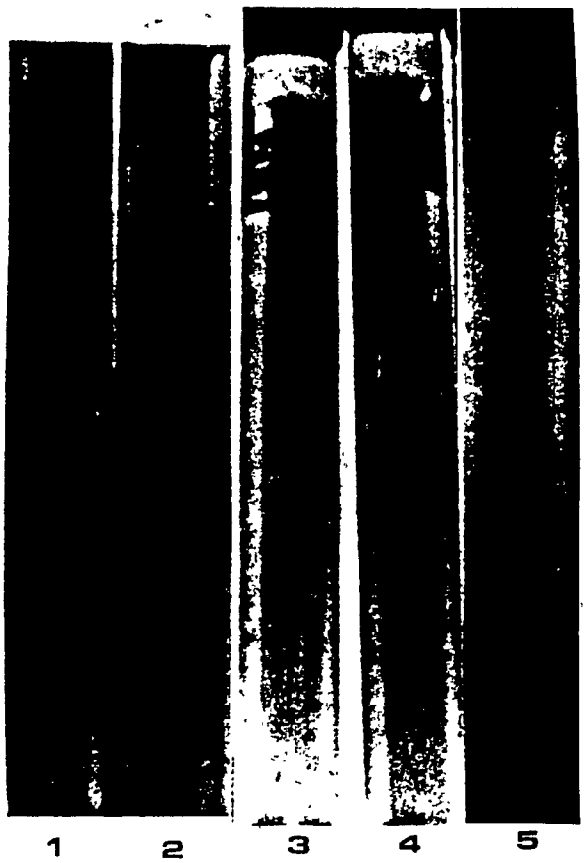
1) KCl extract of nucleoli isolated from Ros tumor cells.

2) KCl extract of nucleoli isolated from Ven tumor cells.

3) KCl extract of nucleoli isolated from Dal tumor cells.

PLATE 9

SDS acrylamide gel electrophoresis of KCNS eluates of 3M KCl extracts, from affinity columns.



1. Ros
2. Dal
3. McK
4. Duc
5. Ven

TABLE 15

SDS acrylamide gel electrophoresis of KCNS fractions from the affinity columns: molecular weights of Coomasie brilliant blue stained species.

Patient	Mobility	Molecular weight X 10^{-3}
Ros	0.255	165
	0.427	93
	0.492	75
	0.526	69
Tre	0.270	165
	0.413	95
	0.524	69
McK	0.265	163
	0.430	93
	0.506	74
	0.521	67
Duc	0.232	170
	0.420	95
	0.493	73
	0.523	69
Dal	0.253	165
	0.432	93
	0.493	73
	0.530	66

DISCUSSION

The importance of studies on the structure and function of the nucleoli in cancer cells has been apparent since the early 20th century when several investigators suggested that nucleolar abberation was a constant, and possibly a diagnostic indication of malignancy (MacCarty and Haumeder 1934, MacCarty et al 1936, MacCarty 1937, and Busch et al 1963). However, until recently such studies have been limited primarily to cytological and morphological examination partly due to problems involved in the isolation and purification of nucleoli from mammalian tissue in general and human tumor tissue in particular. These studies appeared to indicate that although nucleolar size, number and morphology were more frequently abberrant in neoplastic as compared to the corresponding normal cells, these differences seemed neither specific not constant.

With the advent of more powerful techniques (e.g. histochemical and autoradiography) coupled with the development of methods for isolation of purified nucleoli and biochemical analysis of purified material from these nucleoli, it has been possible to suggest that perhaps the major differences between the nucleoli of normal cells and cancer cells may reside at the level of the nucleolar chromosomal-complex. It has been postulated that neoplastic transformation causing dissociation of the nucleolar chromosomal complex may result in the nucleoli of cancer cells synthesizing abnormal products (i.e. ribonucleoproteins) which could affect the responsiveness of the cancer cells to both internal and external control systems. In support of

this postulate is the finding of several investigators that 'antigens' of normal cells differ from those of cancer cells of the corresponding organ (Farhad-Marashi et al 1979). However, little is known concerning the immunological characteristics of the nucleoli. Several investigators have reported that antibodies to nucleoli in auto-immune diseases (Nelson 1977) and in malignant melanoma (McBride et al 1972, Lewis et al 1973, and Bowen et al 1976). With respect to malignant melanoma, all three groups of investigators found that there exists in the sera of patients with advanced malignant melanoma, antibodies to antigens associated with their nucleoli. However, neither of these groups sought to isolate the nucleoli and show definitively that this was the organelle against which the tumor-specific antibodies were reacting. Moreover, no attempt was made to isolate and purify the antigenic determinants of the nucleoli involved in the tumor-specific immunity reaction. Nevertheless, there appears to be broad agreement that the antibodies directed against the antigenic determinants of the nucleoli were primarily present in the latter stages of the disease, and that their presence (i.e. frequency and titer) appeared to have some correlation with the clinical stage of the disease. Since the antigen(s) was detected and analyzed indirectly in all cases, little information was available concerning:

(i) the number of antigenic species involved- i.e. the number of molecular species and their molecular weights-

(ii) the immunological relationship between these antigenic species and those of other tumor-specific cytoplasmic and membrane-associated antigens (Preddie et al 1978a, 1976b, Khosravi 1978, Persad 1978, Persad 1979), and

(iii) the chemical nature of the nucleolar associated antigen(s).

The results presented in this thesis provide the following:

(a) a reliable method for the isolation and purification of the nucleoli of malignant melanoma cells, and the isolation of tumor-specific antigen(s) from these cells. These methods may be adaptable to other types of malignant cells.

(b) some preliminary information on the immunological specificity of these antigens.

The information on the relationship between the tumor-specific nucleolar antigens and other tumor-specific antigens associated with malignant melanoma has already been provided in part by Preddie et al 1978 and is not included in this thesis.

LOCALIZATION OF THE ANTIGENIC ACTIVITY ASSOCIATED WITH THE NUCLEOLI OF MALIGNANT MELANOMA CELLS

For this investigation, twelve (12) patients were selected, 10 with malignant melanoma and 2 with non-melanotic malignancies. Since auto-antibodies against nucleolar determinants were found to be highly stage-specific, i.e. they appeared in the patient's serum only during the latter stages (stages 3 and 4) of the disease (Lewis et al 1973a,b, McBride et al 1972, Bowen et al 1976), indirect cytoplasmic immunofluorescence was used to select those patients possessing high titres of positive antibody against cytoplasmic antigenic determinants of melanoma cells. The widespread use of this technique is due

to its sensitivity and specificity. It has been employed to show specific positive immunofluorescence on the surface of viable cells and in the cytoplasm and nucleoli of snap-frozen cell preparations. Although there remains some questions concerning its specificity (i.e. the reaction between normal sera and melanoma target cells - Lewis et al 1969, Whitehead 1973), the bulk of the available data appears to indicate that immunofluorescence provides an excellent serological tool for analyzing the presence and the distribution of tumor-specific antigens in tumor cells.

In order to establish specificity, sera containing tumor-specific antibodies were absorbed with normal human spleen cells as well as with a variety of non-melanotic tumor cells. This absorption procedure ensured specificity of the reaction when tissue was being tested against sera from other melanoma patients as well as normal controls. In addition, the specificity of the cytoplasmic fluorescence observed was further confirmed by the use of the direct immunofluorescence test in which the addition of the patients' own sera was omitted (plate 1,2).

The immunofluorescence studies (table 1) show that 70% of the patients selected had strong positive immunofluorescence for the nucleoli of snap-frozen smears, when tested with the patients' autologous sera. On the other hand, there was cross-reactivity between the highly positive sera and nucleolar preparations obtained from other malignant melanoma patients. Keeping in mind that in all cases studied, this reaction was found to be less intense than the autologous reaction.

That the reaction observed was specific can be inferred from: (a) the lack of positive reaction between the normal sera and all nucleolar preparations of malignant melanoma cells, and (b) a lack of positive reactions between the highly positive sera from patients with other types of malignancies and nucleolar preparations of malignant melanoma.

The 70% frequency of positive reaction, although high, was found to be in the region reported by other investigators. Moreover, it reflects the bias selection of tissue and sera used, in that tissue and sera were selected mainly from patients whose disease was primarily in the latter stages. McBride et al (1972) reported 15% of positive cells tested with autologous sera were positive, as seen by the presence of single large brightly stained intranuclear body. Moreover, sera with high antibody titer, when tested with tumor cell preparation of other melanoma patients, 39% were positive for the presence of the nuclear antigen(s). Overall, the nucleolar antigen was found to be present in 45% if the 140 patients tested, of which 57% and 34% were in stages 3 and 4 respectively. Bowen et al (1976) reported that 19% (33 of 175 patients) of autologous serum cell combinations were positive for the nucleolar antigen, while there was cross-reactivity between positive sera and 45% of patients tested (63 of 175). About 91% of the positive patients were in clinical stages 3 (57%) and 4 (34%).

ISOLATION AND PURIFICATION OF THE NUCLEOLI

Because the isolation of the nuclei was a necessary

prerequisite for the isolation of nucleoli, the method selected for isolation of nucleoli had to meet the following criteria: (i) isolated nuclei had to retain their morphological structure, (ii) the nuclei had to be isolated in a highly purified form, and (iii) the isolated nuclei had to show retention of high enzymatic activity. Blodel and Potter's (1966) modification of the sucrose calcium procedure proposed by Chauveau et al (1956) was the method of choice since it resulted in isolated nuclei which met the criteria stated above. Of crucial importance to the isolation of acceptable nuclei was the degree of homogenization. Too much homogenization resulted in excessive damage to the nuclei and thus decrease in whole nuclei recovered in the nuclear pellet; on the other hand, insufficient homogenization resulted in an increased contamination of the nuclear pellet by cytoplasmic contents. Therefore, the conditions selected for homogenization represented a compromise to achieve maximum recovery of the nuclei with minimum contamination by cytoplasmic and/or membrane components.

Electronmicrographs of sample nuclear preparation showed that the nuclei were of high purity and were not contaminated to any significant degree by other non-nuclear material (plate 4). In addition, evidence derived from chemical and enzymatic analysis (tables 6, 8, 9, 10), and the fact that the yield of nucleic acid in the nuclear pellet averaged 71%, suggested strongly that the isolated nuclei were quite acceptable for use to obtain nucleoli. In this study, the method of Ro and Busch (1964), using sonication to disrupt the nuclei, resulted in nucleoli of high purity, as indicated by electronmicrographs of the preparations (plates 5, 6, 7).

The purified nucleoli contained only a fraction of the specific activities of the enzyme markers found in the nuclear pellets from which they were derived (0.56, 2,263, and 0.091 respectively). Assuming that the nuclei fromwhich the nucleolar preparations were made were relatively free from major cytoplasmic contamination, then these nucleoli were considered to be of acceptable purity for the isolation of antigens.

ISOLATION OF TUMOR-SPECIFIC ANTIGENS ASSOCIATED WITH NUCLEOLI OF MALIGNANT MELANOMA CELLS

Having shown that the nucleoli were of acceptable purity, the next step was to extract and characterize the melanoma specific antigens from these nucleoli. Antigens were extracted with 3M potassium chloride and purified by affinity chromatography on columns of tumor-specific IgG coupled to Sepharose 4B. Extraction with 3M KCl has been usedby other investigators (Roth et al 1976, Mavligit et al 1972, Mavligit 1973) to extract antigens from solid tumors.

The appropriate fractions were tested for antigenic activity using purified tumor-specific IgG and anti-human IgG. The results (table 13 and plates 8, 9) show that all of the antigenic activity was eluted with 3M KCNS. Fractions which showed antigenic activity and were in adequate amounts were used to re-establish the specificity of the nucleolar antigens and were analyzed by SDS-acrylamide gel electrophoresis.

PROBLEMS INVOLVED IN DETECTION OF ANTIGENIC ACTIVITY

Three immunological techniques of varying sensitivities were used throughout this project, to monitor antigenic activity during the various isolations, concentrations, and analytical procedures. Therefore, it is likely that any antigenic molecules present were detected. In particular, table (14) shows that cross-over immunoelectrophoresis provided a reliable means of monitoring antigenic activity during tumor cell fractionation. However, since the amount of antigenically active KCL-extracted material was always in very limited quantity, it is highly possible that dilution of these fractions prior to and during affinity chromatography could have resulted in the loss of some antigenically active molecules.

CHARACTERIZATION OF THE NUCLEOLAR ANTIGENS

The identification of the antigens associated with the nucleolus of malignant melanoma tumor cells was done primarily by cross-over immunoelectrophoresis, a technique widely used for the detection of both antigens and antibodies. The major advantages of this method are: (a) its sensitivity (i.e. it can detect less than 10 ug protein), (b) it is highly reproducible and (c) it is very rapid.

By this method, the fractions (KCNS eluted) from 5 affinity columns were re-tested for antigenic activity against purified allogeneic and autologous sera. In all cases, except for column No.2, where the material recovered was in very low concentration, the fractions showed good antigenic activity. To determine the number of moleuclar weight species present in the antigenic fractions isolated by affinity chromatography, aliquots of fractions were analyzed by SDSPAGE.

The data in tables (15) and plate (7) indicates the following:

(I) There appears to be about 4 molecular species varying in molecular weight from 65,000 - 165,000 in KCl extracts of nucleoli obtained from melanoma tumor of five patients.

(II) The molecular weight species found in fractions isolated by affinity chromatography were also found in the KCl extract and in the solubilized nucleoli.

The variation in number of molecular weight species obtained for the different patients may be due to one or all of the following poss-- bilities:

(a) the nucleolar pellets were prepared from different metastatic tissue.

(b) the loss of protein (antigenic molecules) during the various isolation and concentration procedures.

THE SPECIFICITY OF THE NUCLEOLAR ANTIGENS

In order to confirm that the antigens isolated from nucleoli were indeed melanoma-specific antigens, purified nucleoli were absorbed with normal sera , with pooled sera from patients with other neoplastic diseases, and with autologous tumor-specific sera. In the cases of absorbance with normal sera and sera from non-melanotic cancer patients, it was possible to extract the total amount of antigens from these nucleoli after absorption. On the other

hand, in the case of the autologous tumor-specific serum, it was not possible to obtain antigens after absorption. This confirmed the antigens were indeed melanoma tumor-specific.

CONCLUSIONS

The preceeding discussion describes a specific approach to the localization and isolation of antigens associated with the nucleolus of malignant melanoma cells. These antigens were extracted with 3M KCl from purified nucleoli that were isolated by differential and sucrose density centrifugation and then purified by affinity chromatography on Sepharose 4B-IgG bound columns. The identification of these antigens was made by employing the technique of cross-over immunoelectrophoresis. The antigens isolated appeared to be (a) allogeneic, i.e. they were present in all patients and freely cross-reacted, and (b) specific, i.e. they did not react with sera from normal patients or with sera of patients with other forms of cancer, and (c) specifically located in the nucleolus. The chemical nature of these antigens will be the subject of additional investigation.

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