ISOLATION AND IDENTIFICATION OF MELANOMA ANTIGENS FROM HUMAN MELANOMA TUMOR CELL MEMBRANES

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ABSTRACT

ISOLATION AND IDENTIFICATION OF MELANOMA ANTIGENS FROM HUMAN MELANOMA TUMOR CELL MEMBRANES

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This study describes the isolation and purification of plasma membrane antigens specific for human malignant melanoma. Plasma membranes were isolated from melanoma tissue by differential and discontinious sucrose density gradient centrifugation. These membranes were solubilized and chromatographed on sepharose beads coupled to the patient's autologous melanoma specific IgG. The presence of tumor specific antibody against melanoma cell surface antigens in preabsorbed patients' sera was investigated by Direct and Indirect membrane immunofluorescence technique. Of sixteen (16) patients studied ten (10) showed positive activity for plasma membrane fluorescence. The melanoma specific IgG fractions were then isolated from sera, showing relatively strong reactivity, by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose ion exchanger. The molecular weight species of the purified plasma membrane antigens, from five(5) different melanomas, were found to be in the range of 50,000 to 185,000 by SDS-polyacrylamide gel electrophoresis. On counter current

immunoelectrophoresis, melanotic sera from the five(5) patients precipitated the autologous as well as the allogeneic antigens, whereas there was no reactivity against eight(8) normal controls.

Although the presence of autologous antigens could not be ruled out, these proteins appear to be melanoma specific allogeneic antigens.

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PART A

INTRODUCTION

I. HISTORICAL BACKGROUND AND THE GENERAL DISCRIPTION OF HUMAN MALIGNANAT MELANOMA

Human malignant melanoma has stimulated the concern of a large group of investigators since the onset of recorded history in the fifth century B.C. (Urteaga and Pack, 1966). In 1806 the condition was brought before the medical profession for the first time by Laennec in a paper read before the faculty of medicine in Paris; Laennec was also the first to use the term "La Melanose" to describe the color of the lesions. In 1833 the lesion was recognized as a malignant tumor under the term "Melanoma" by Robert Carswell.

Pemberton in 1858 was the first one who explained accurately the appearance of the primary tumor in the skin and other areas of the body. In 1907, the lymphatic spread and penetration by melanotic cells was observed by Sampson Handley and in 1908 Pringle, with respect to the Handley observation, suggested the surgical dissection and excision of the primary tumor, the "inbetween" lymphatics and the regional nodes for the treatment of melanomas.

The name melanoma was subsequently designated to a class of skin growth of melanocyte cell population which appears on exposed area of the body and represents two types of closely related tumor cells.

One of the benign nevi, and moles that remain localized at the site of origin throughout life, were known as beauty marks during the eighteen century; the other, composed of highly malignant and transplantable tumor cells with the capacity to invade and metastasize through any organ in the body, leaves behind mostly pigmented lesions. This is the reason why the tumor had been known as black cancer or black death (Urteaga and Pack, 1966; Ackerman and Regato, 1962).

II. VARIOUS TYPES OF MALIGNANT MELANOMA

From the standpoint of pathology and histogenics there exist a few dominant types of melanomas. Extensive studies by Clark et al (1969, 1976) had distinguished four (4) distinctive forms of melanomas, namely, lentigo malignant melanoma, superficial spreading melanoma, nodular melanoma and arcle lentiginous melanoma, which are different on the basis of their histological appearances and the biological behaviour patterns of the primary tumor.

III. THE ORIGIN OF MELANOMA

Although the relationship between malignant melanomas and moles was reported by Laennec (1806), the histogenetic correlation between them has been controversial and a reason for disagreement.

Allen and Spitz (1953) believed that almost all melanomas of the skin and mucous membrane are derived from

pre-existing hevi with junctional activity, but Becker (1948) indicated that pre-existing nevi are responsible for less than 25% of melanomas, and the rest originate from the melanocytes of the normal skin that are located at the epidermal-dermal junction.

Several other observers such as Russel and Reyes (1959) and Trozak et al (1975) reported a high incidence of malignant melanoma to be aroused from congenital nevi.

With respect to the occurrence of melanomas in the colored races, Pack (1948) stated that moles and melanomas are not common in the pigmented races. In contrast to this Hewer (1935) reported the presence of nevi in 95% of 200 Sudanese Africans who were chosen at random. However, collected evidence seems to suggest that melanoma is not a rare disease in the pigmented races and indicates the occurrence to be much greater on the soles of the feet than other areas of the body (Lewis, 1967a).

Studies by Lewis (1967a) in Uganda Africans clearly demonstrated the relationship in the incident of melanoma and what he called "a district pigmented spot" with junctional activity on the sole of the foot.

Although the cytogenetic origin of malignant melanomas is still a debatable issue, it appears most people believe that pre-existing junctional nevi and moles are the precursor of melanomas.

IV. HISTORICAL BACKGROUND OF HOST IMMUNE RESPONSE TO MALIGNANT DISEASE

For any immunological reactive cells, there must exist antigens distinct from those of adult host cells. These antigens could be virus specific antigens, fetal antigens, or of unknown origin.

The first scientist that suggested the possible presence of host immune response was Ehrlich in 1906, who stated that the immunity is not directed only against microbial and parasitic infection but also against malignant cells.

It was Sampson Handley in 1907 who demonstrated the presence of lymphocyte infiltration in malignant melanoma and stated that this was responsible for the tumor rejection. In 1908, Wade explained the infiltration by lymphocytes to be the factor responsible for the rejection of transplanted sarcoma in dogs. Attempts were made by Ehrlich in 1909 and Bashford and Russel in 1910 to study the phenomenon of tumor transplantation. The result indicated that the rejection of transplanted tumor in most cases were due to immunological reactions directed against the normal allogeneic histocompatibility antigens, because in those experiments, tumors were grafted between genetically different animals.

However, the original demonstration of tumor specific immunity is attributed to Gross (1943) and Foley (1953) who studied tumor transplantation of a carcinogen-induced sarcoma in genetically identical mice. Following this observation, the existence of tumor specific transplantation antigen was clearly demonstrated by Prehn and Main in 1957.

Due to the above observation, rejection of transplantation antigens between inbred animals has been used in the study of animal tumors. This type of experimental approach can not be used in humans because of ethical reasons and the lack of a synogeneic donor-host relationship, except in the case of identical twins. Therefore, indirect evidence for the immunity in man has been collected by using a variety of "in vitro" and "in vivo" experiments to determine the serological reaction of the host humoral and cellular responses against the tumor-associated antigens.

V. MALIGNANT MELANOMA AND IMMUNITY IN MAN

One of the early indications regarding the possibility of the influence of host defense mechanism in malignant melanoma came from the differences in biological behavior of the disease, such as its unpredictable and variable natural history, and the spontaneous regression of the tumor.

1. With Respect to the Natural History , several investigators including Coley and Hoguet in 1936 noticed the

variability of such a phenomena in malignant melanoma and in some cases, the ability of the primary tumor to stay localized for a long period of time during its development (Bodenham, 1968).

In 1968 Lewis and Kiryabwire, studying the incidence of melanomas in Uganda, were able to distinguish three different type of patients. The first group was characterized by a large primary tumor localized on the sole of the foot for a long period of time without any sign of metastases. In the second group, the tumor was rapidly disseminated in a few weeks or months, and the third group was distinguished by secondary deposits in lymph nodes with complete regression of the primary tumor.

The influence of such factors of sex, age, size of the tumor and degree of pigmentation failed to explain the above differences in the biological behavior. Consequently, the possible involvement of the host-defense mechanism was considered to be responsible for the delayed metastatic spread.

Using complement-dependent cytotoxicity assay, Lewis (1967b) and his co-workers (Lewis et al 1969) demonstrated that the differences in the natural history of the disease is closely related to the presence or absence of anti-melanoma antibodies in the sera of the patients.

2. Spontaneous Regression of Malignant Melanoma.

Spontaneous regression of malignant melanoma is a common event in the natural history of the disease and accounts for about 11% to 15% of all cases of spontaneous regression in human tumor (Everson and Cole, 1966; Kopf, 1971).

The incident of such a phenomena was further documented by Cole (1974) who reported 17 cases of spontaneous regression of human melanomas.

Recently, in a reported case by Bodurtha et al (1976), a considerable increase in lymphocyte cytotoxicity values was observed during the regression period of the tumor. This suggests that the host control mechanism may be immunologic.

There are a large number of histological studies indicating that the host immune response is mediated in part through lymphocyte infiltration in melanomas (Handley, 1907). Lymphocyte and monocyte infiltration of the tumor was further reported by Couperus and Rucker in 1954. Little (1972), studying the relationship between lymphocyte infiltration and subsequent development of tumor, noticed the inverse relationship between prognosis of the disease and the degree of lymphotic infiltration.

Recently, Roubin et al (1975) reported 25 cases of histological appearance of lymphotic infiltration in melanomas and indicated that the progression of the tumor

corresponded to the decrease in the number of lymphatic cells. Added to this is the finding of Bodurtha et al (1976) that the presence of lymphocytic infiltration, melanophages, and degenerated melanocytes proceeded the spontaneous regression of the tumor.

The phenomenon of halo naevus is additional evidence that suggests the influence of host-immune mechanism in melanoma, and is believed to be related to the spontaneous regression of the tumor (Lewis and Copeman, 1972). The process of the rejection was recently found to be mediated through cellular immunity by lymphocyte and monocytes and finally phagocytosis of the tumor by macrophages (Rowden and Lewis, 1975).

Although the mechanism of tumor regression is not known yet, it has provided another line of evidence in favor of host-response immunity against melanomas in man.

VI. EVIDENCE FOR HUMORAL IMMUNITY

The existence of circulating anti-melanoma antibodies have been demonstrated by several types of "in vitro" techniques.

1. Immunofluorescence.

Using an indirect immunofluorescence technique Morton et al (1968) demonstrated the presence of cross-reactive anti-melanoma antibody in the sera of seven (7) different

patients when tested against cytoplasmic and cell surface components.

The same technique was also employed by Romsdahl et al (1970) and Potra et al (1971). These investigators also reported that sera from patients contained cross-reactive anti-melanoma antibodies directed against both cytoplasmic and surface membrane determinants.

The presence of a common anti-melanoma cell surface components antibody was recently confirmed by Leong et al (1977a, 1977b) in the patients' post auto-immune anti-melanoma sera.

There is however, a great deal of controversy with respect to the cross-reactivity of the anti-cell surface antibodies. Lewis et al (1969), studying the humoral immunity in 103 melanoma patients, demonstrated the presence of two types of antibodies, one autologous plasma membrane antibodies which reacted only against the patients own viable cells with no or very little cross-reactivity, and the other, cross-reactive cytoplasmic antibodies. Additional evidence came from the work of Phillips and Lewis (1970) who reported that the cell surface antibodies demonstrated by the membrane immunofluorescence technique are individually distinct for each melanoma. Whereas the antibodies against cytoplasmic antigens are shared by most of all melanomas patients.

More recent work by Lewis and co-workers, (Lewis and Phillips, 1972; Lewis et al 1975) has supported their previous observation indicating that all surface antibodies are patient-specific with no cross-reactivity.

2. Complement Dependent Cytotoxicity

This technique have been used to demonstrate the cytotoxicity effect of patients' sera against the surface of melanoma cells.

The first attempt in this respect was reported by Lewis (1967b) who studied the effect of sera from 16 patients on the tissue culture of tumors from these patients. The results indicated the cytotoxicity effect of autologous sera in 15 of 16 cases in patients with localized tumor. No reactivity was found in those with disseminated melanomas (Lewis et al 1969).

The above finding was recently confirmed by Bodurtha et al (1975) who reported cytotoxicity in sera of 90% of his patients with localized disease and in less than 1% of the patients with advanced metastases when examined in an autologous system.

There are a number of reports which suggest the presence of common cell surface and cytoplasmic antibodies (Fritze et al 1976; Stuhimiller and Seigler, 1977). These groups of investigators have used xenoantisera (i.e. antisera raised

against cell surface antigens in animals) in order to increase the titer of reactive antibodies, and after absorption studies, demonstrated the exsitence of shared membrane antibodies when tested against cultured melanoma cells.

3. Immune Adherence Assay(IA)

The assay is a specific immunological reaction which is used for the study of antibody to surface membrane melanoma cell antigens. Melanoma cells are incubated with serum plus complement and, if the serum contains antibody against surface membrane antigens, the antibody (pluse complement) will bind to the antigen . This complex is then demonstrated by the subsequent adherence of indicator cells (normal human erythrocytes).

Using IA assay, Hiroshi et al (1976) studied the presence of antibodies against surface antigens of cultured melanoma cells as targets. They detected two classes of antibodies with positive reaction, one autologous which could not be removed by absorption tests on 12 allogeneic melanoma cell lines, and the other represented a class of common melanoma antibodies against cell surface determinants.

Recently Seibert et al (1977) found a high reactivity of sera with allogeneic melanoma cells, which is in agreement with the above finding and disagree with those of Lewis et

al (1969), Lewis and Phillips (1972), and Bodurtha et al (1975), who found positive reactivity only in autologous systems.

Although the above findings indicate the presence of melanoma-specific antibodies in patients' sera, the inconsistency of the reported results could be due to the use of different serological techniques and differences in the source of sera and target cells.

4. Evidence for "In Vivo" Binding of Anti-melanoma antibody

One of the most reasonable lines of evidence for the presence of melanoma specific antibodies and involvement of humoral immunity in melanoma could be indicated by the elution of tumor specific antibodies from melanoma tumor cells.

Romsdahl and Cox (1973) studying the reactivity of melanoma sera against a soluble extract of the tumor by immunodiffusion, noticed the presence of immunoglobulins, mainly of IgG types, in the tumor extracts. These immunoglobulins were subsequently isolated from the cell surface and were found to block effectively sensitized lymphocyte cytotoxicity in an allogeneic system.

The report of Gupta and Morton (1975) has provided more evidence for the presence and specificity of these antibodies. In this study immunoglobulins were eluted from the surface of five(5) different melanomas and found to be

reactive against both sonically treated autologous and allogeneic homogenates of melanomas but not against normal and other types of tumor extracts. Also they found about 32-fold increase in the antigenic activity following elution as compared to that against autologous sera.

The above findings could possibly provide a reason for the failure of cell-mediated immunity in malignant melanoma.

VII. EVIDENCE FOR CELLULAR IMMUNITY.

A number of "in vivo" and "in vitro" techniques have been employed in order to demostrate cellular immunity against melanoma.

1. Lymphocyte Cytotoxicity

This technique has been widely used to test lymphocytes from melanoma patients for their ability to destroy melanoma target cells "in vitro".

De Vries et al (1971) studied lymphocyte cytotoxicity against melanoma cultured cells and reported positive reaction in 13 of 23 cases against autologous tumor cells and in 26 of 48 cases in the allogeneic situation. No reaction was detected against normal skin fibroblast. Similar findings were also reported by Potra et al (1971) and confirmed by Narin et al (1972) who reported a high degree of cross-reactivity in 5 of 18 cases.

Searching for lymphocyte cytotoxicity, Hellstrom and Hellstrom (1973) reported lymphocyte cytotoxicity to be more effective in patients with localized tumor. A recent report by Bodurtha et al (1976) is in agreement with that of Hellstrom. This group of investigators measured lymphocyte cytotoxicity, that was obtained from two different established melanoma cultured cells. They reported a significant increase in cytotoxicity against the two allogeneic melanoma target cells during the regression period of the tumor which appear to be a good evidence for tumor destruction through cellular immune mechanism.

2. Delayed Cutaneous Hypersensitivity Reaction

Cellular immune reaction in melanomas has been also demonstrated by skin testing against melanoma extracts.

Stewart (1969) was one of the early investigators who reported skin reactivity in 25% of his patients; it was also reported to be more common in patients with localized disease (Fass et al 1970).

Hollinshead et al (1974) studied skin reactivity to autologous and allogeneic soluble components of the tumor cell membranes in a number of melanoma patients. Positive reactions were produced in 17 of 22 patients with early melanoma, in 7 of 19 patients in the late stages of the disease, and in only 1 of 22 controls. The reactivity was not shown in non-melanotic patients.

The allogeneic skin reactivity of salt extracted cell surface antigens was recently reported by Roth et al (1976a ,1976b) and supported by the finding of Grimm et al (1976) who reported the release of melanoma-associated antigens into the tissue culture medium of a single cell line that produced delayed cutaneous hypersensitivity reaction in 4 of 5 melanoma patients.

3. Leucocyte Migration Inhibition

This technique has been also used to demonstrate cellular immunity in melanoma patients.

Cochran et al (1972) by using antigens from 10 melanomas demonstrated leucocyte migration inhibition in 80% of 46 patients, and in only 5 of 31 normal controls. Also, he found a significant decrease in the reactivity following surgical operation. The cross-reactivity of the antigen was further reported by Segall et al (1972).

The above finding is in agreement with those of Flak et al (1973) who reported reactivity by using a preparation of membrane antigens from three (3) melanoma patients. Positive reaction were found in 16 patients with progressing melanoma and in none of the normal controls or patients with non-melanotic tumors.

VIII. MELANOMA ANTIGENS

Recognition of melanoma-associated and specific antigens has been the subject of intensive research and has generated a great deal of controversy with respect to the cross-reactivity of the tumor cell surface antigens, (Lewis et al 1975).

There are two major methods by which the presence of these antigens has been identified. One method is to demonstrate the reactivity of anti-melanoma antibodies in the sera of the patients or in xenoantisera, after appropriate absorption, against tumor antigens or cells. The other method is to show the ability of the cellular defense mechanism of melanoma patients to recognize these antigens "in vivo" or "in vitro".

Since most of the experimental evidence in this respect have been presented in the previous sections, only the main findings and some other reports regarding cell surface antigens will be discussed here.

Immunofluorescence staining of viable cell surface membranes by several investigators (Morton et al 1968 ; Romsdahl and Cox, 1970 ; Potra et al 1971 ; Leong et al 1977a, 1977b) has revealed the existence of melanoma surface antigens which appear to be highly cross-reactive. Romsdahl and Cox (1970) reported the complete elimination of membrane

immunofluorescence after absorption studies of sera with allogeneic melanoma cells.

The above findings disagree with those of Lewis and co-workers (Lewis et al 1969 ; Phillips and Lewis,1970 ; Lewis and Phillips, 1972 ; Lewis et al 1975) which have provided evidence for the presence of autologous tumor-specific antigens on the surface of melanomas, and showed that absorption of the sera with only autologous cells eliminated immunofluorescence staining.

The individuality of surface antigens was further demonstrated by Lewis (1976b), Lewis et al(1969), and Bodurtha et al (1975) using a complement dependent cytotoxicity technique.

Several other investigators (Fritze et al 1976 ; Stuhimiller and Seigler, 1977 ; Hakim, 1977) have recently localized common cell surface antigen(s) on cultured melanoma cells using xenoantisera against tumor cells after appropriate absorption. Although these findings are in agreement with the previous reports in which patients sera were used, the exact correlation between them requires more understanding in respect to the specificity of xenoantisera.

Hiroshi et al (1976), using the technique of immuno-adherence assay, demonstrated the presence of two classes of antigens localized on the tumor cell plasma membranes : one autologous and the other shared melanoma surface antigens.

Seibert et al (1977) by using the same technique also found cross-reactive membrane antigens on cultured melanoma cells which appeared to be similar to antigens on fetal cell membranes. The presence of fetal antigens on a number of tumor cells have also been reported by Lewis et al (1973a), Fritze et al (1976), and Irie et al (1977).

Hollinshead et al (1974) has isolated two types of skin reactive antigens from primary melanomas as well as large metastatic deposites (Hollinshead, 1975) by using 3M KCL extraction. One antigen, a glycoprotein, in "Sephadex fraction II", appeared to be melanoma-specific and produced positive reaction in 17 of 22 patients with skin or ocular melanoma, and gave negative reaction in 21 of22 patients with other types of cancer. The second antigen, in "Sephadex fraction III" appeared to be more widely distributed, producing positive reaction in 9 of 21 patients with early melanoma, in 13 of 18 patients with advanced melanoma and in 5 of 6 patients with breast cancer, and also appeared to be similar to some of the proteins isolated from fetal skin. The molecular weights of these partially purified antigens were reported to be between 10,000 to 40,000 daltons.

The presence of a more broadly reactive group of melanoma-associated antigen in common with neoplasms other than melanoma has been found in tissue culture medium of a single cell line of human melanoma which , after partial

purification by chromatography, was found to be a rather large glycoprotein, slightly negatively charged, with a molecular weight greater than 200,000 daltons, Grimm et al (1976).

Roth et al (1976a, 1976b) used 3M KCL to isolate melanoma-associated antigens from a fresh surgical specimen. Positive skin reactions to these antigens were noted in 25 of 39 melanoma patients and 7 of 30 patients with other neoplasmic disease. Only 4 of 28 patients reacted to autologous muscle extract. Partial purification of the soluble antigen fraction, by the same technique as Hollinshead et al (1974) indicated a large molecular weight species with the similar size as that found by Grimm et al (1976).

Bystryn and Smalley (1977) employed lactoperoxidase-iodinated cultured melanoma cells and xenoantisera raised in rabbit to identify cell surface melanoma-associated antigens. After solubilization of the cell in non-ionic detergent, labelled melanoma antigens were identified by a quantitative double antibody-antigen binding assay.

Appropriate absorption studies suggested the possibility that melanoma cells carry both melanoma-specific and fetal antigens on their surface. After partial purification of these antigens by chromatography, their molecular weights were reported to be greater than 160,000 daltons.

Although progress is being made in order to isolate melanoma cell surface antigens , there seem to be numerous inconsistancies in the literature, the major problem being the tissue specificity of the tumor antigens.

However, it seems clear that there is more than one group of antigens present on the surface of melanoma cells. One group being individually specific antigens , and the other, tumor specific and associated antigens. Inconsistancies in the results could be due to the different sources of tumor materials, and differences in the isolation and solubilization techniques that have been used. Standardization of the techniques may be the answer to these contradictory results.

IX. THE RELATIONSHIP BETWEEN HOST IMMUNE RESPONSE AND THE VARIOUS STAGES OF THE DISEASE

Although the accumulated evidences indicates that melanoma patients respond to their tumor by developing humoral and cellular immunity, critical evidence must exist to explain the significance of this relationship by demonstrating that the presence of host immune response is related in some way to the natural history of the tumor.

Several investigators have attempted to correlate the presence of circulating antibody in patients' sera with the clinical stages of the disease and demonstrated that

circulating anti-melanoma antibodies are important in preventing or delaying the metastases of the tumor.

Morton (1971), by using membrane immunofluorescence assay, reported the presence of a high titer of antibody in patients with localized disease. Lewis et al (1973a) has also reported that surface antibodies are present mostly in patients with localized disease and disappear as dissemination occurs. These antibodies were not found to be related to the total mass or volume of the tumor present, but to the degree of localization.

These workers provide additional evidence for a number of different tumor-associated antigens and proposed the appearence of antibodies to these various antigens at different times in the course of the disease (Lewis et al 1973b).

The relationship between the circulating antibodies and the natural history of the tumor is also documented by the demonstration of complement-dependent cytotoxic antibodies against the surface determinants (Lewis et al 1969 ; Bodurtha et al 1975). These investigators reported that the antibodies are present in patients with early stages of melanoma (Stages I and II), and do not occur in highly metastesized tumors (Stage IV). In these examples, dissemination of the tumor with metastases was associated with a lack of detectable antibody.

With respect to the cytoplasmic antibodies, the situation is not as clear. Wood and Barth (1974) reported that patients with local, regional, and disseminated melanoma showed cytoplasmic antibodies with equal frequency; but they also mentioned that patients with disseminated disease have a higher antibody titer. The anti-cytoplasmic antibodies have been reported to have less stage relationship and occur in more advanced stages of melanoma (Lewis et al 1973b).

The relationship between cellular immunity and the natural history of melanoma is more confusing.

Fass et al (1970) reported skin reactivity against autologous saline extract of tumor in 100% of his tests and only in patients with Stage I melanoma. Hollinshead et al (1974) found autologous and allogeneic skin reactivity against his two partially purified surface antigens in patients with stage II and stage III of the disease.

Hellstrom and Hellstrom (1973) stated that lymphocyte from patients with advanced melanoma are less reactive than those from patients with a small tumor load, by using "in vitro" lymphocyte cytotoxicity assay.

The above findings and many more suggest that cellular and humoral immunity are more reactive in patients with early stage of the disease and tend to decrease with progression of the tumor, except for antibodies against

cytoplasmic components.

Lewis et al (1971) clearly demonstrated that the presence of antibody in the circulation has a strong influence on the duration of localization, and the drop in detectable antibodies often proceeds the appearance of metastatic spread within weeks or months. Therefore, the question to be answered is that if circulating antibodies and cellular immunity are important in preventing dissemination, why does the mechanism eventually become ineffective?

X. REASON FOR THE FAILURE OF IMMUNE MECHANISM

Cell surface antigens are believed to be responsible for rejection phenomena in malignant tumor because of their susceptibility to attack by humoral and cellular immune systems. There are a number of mechanisms described which may be responsible for the failure of host immune mechanism in malignancy.

Old and Boyse (1968) postulated that tumors develop, despite their antigenecity, because their growth exceeds the capacity of immune response. They also suggested that metastatic tumor cells are different in antigenicity from those in the primary tumors, or, they may lose their antigenic determinants completely (i.e. Antigenic modulation).

The second mechanism implies that in some patients with progressive tumor the immune system becomes incompetent and can no longer recognize and react against tumor antigens.

Using autologous sera from melanoma patients in early stages of the tumor development, it was shown that these sera are able to react against autologous primary tumor cells as well as the metastatic cells obtained at various times during the course of the disease. However, the sera obtained at the late stage failed to react against either autologous primary cell or cells from metastatic tissue. These results clearly ruled out the involvement of the first possibility and led to the suggestion that some kind of change in the patient's serum was responsible for the above differences (Lewis, 1972).

To study the second mechanism, Ikonopisov et al (1970) showed that in patients with non-detectable anti-melanoma antibody, auto-immunization with autologous irradiated metastatic tumor cells could stimulate the immune response and raised tumor-specific antibodies which could react with both autologous primary and metastatic tumor cells, and lasted for up to 14 days in the circulation.

The third possibility suggested that tumor cells, in the metastatic phase, act as a sponge and absorb specific antibodies from the blood stream. Elution of these anti-tumor antibodies, which have been stated to be bound to the

cell surface membrane, has been previously reported and was found to effectively block cell-mediated cytotoxicity "in vitro" (Romsdahl and Cox, 1973 ; Gupta and Morton, 1975).

Other investigators (Hellström, 1967 ; Hellstrom et al 1971) reported the presence of blocking factors (especially in the sera of patients with progressive disease) which also appeared to inhibit cell-mediated responses and were found to be immunoglobulin. These workers suggested that tumor-specific antibodies were masking the antigenic determinants and therefore, protecting the tumor cell from being recognized and destroyed by cellular immune mechanism.

The recent report of Lewis et al (1976a), however, is not in agreement with the above findings and demonstrated that antibody could not be detected on the surface of tumor cells and that the soaking of the anti-melanoma antibodies can not be related to the tumor load.

The disappearence of anti-tumor antibodies has also been reported to be due to the shedding of surface antigens which bind to the specific antibodies and therefore, form complexes that block lymphocyte cytotoxicity (Alexander, 1974). For such a mechanism to be operative, a rapid membrane antigen turnover and release from tumor cell into the body fluid would be a prerequisite. Evidence for "in vitro" shedding of melanoma antigens that also reported by several investigators, suggests similar "in vivo" phenomena

of tumor protection against host defense mechanism by either reducing the number of recognizable antigens on tumor cells, or by forming immune complexes with free antibodies that react with cytotoxic T cells before they reach their targets. (Grimm et al 1976 ; Stuhimiller et al 1977 ; Leong et al 1977a).

Another mechanism which effects the host-tumor relationship has been described by Lewis et al (1971). These investigators reported that the development of an anti-antibody is responsible for inactivation of the tumor-specific antibodies in melanoma. They demonstrated, by membrane immunofluorescence technique against the patients' tumor cells, the presence of an IgG-type molecule in the negative sera of patients with disseminated melanoma which could specifically inhibit the activity of the autologous positive sera taken from the same patients after auto-immunization.

Additional evidence in favor of the above mechanism was also reported by Hartmann and Lewis (1974) and more recently by Lewis et al (1976b).

From the above studies it seems clear that the potential for "in vivo" blocking of the host anti-tumor immune responses does exist and may therefore permit the growth of immunogenic tumors.

XI. IMPORTANCE OF CELL SURFACE ANTIGENS

There is now evidence for the spontaneous regression of malignant melanoma due to the influence of humoral and cellular immune responses.

Anti-cell surface antibodies have been shown to have a close stage relationship with the disease and appear mostly in the early stages or localized stage of melanoma and disappear prior to dissemination of the tumor. It is believed that they may be responsible for delayed dissemination.

There may be several different blocking factors present in patients' sera that protect cancer cells from lymphocytes or are responsible for the decrease in the level of anti-membrane antibodies during the progression of the disease.

However, if one considers the relationship between host immune mechanism and the phenomena of spontaneous regression in some patients, then the recognition of the tumor in its early stages and maintaining the level of circulating anti-melanoma antibodies may well be helpful in the control of the tumor.

There is evidence which seems to explain the importance of early diagnosis in malignant melanoma, regarding the prognosis of the disease (Bodenham, 1968).

The existence of tumor-specific antigens has been established by the demonstration of such antigens in melanoma cell extracts and by the presence in patients' sera, of antibody that is specifically directed against melanoma cell surface determinants.

Since the cell surface is more accessible to immunological reactions, the antigens thereon are more likely to elicit cellular or humoral responses capable of inhibiting tumor growth. Clearly, isolation, purification, and characterization of such antigens could be an essential first step in their eventual possible use in immunotherapy, especially in the early stages of the disease. This could help in understanding the nature of blocking factors which seems to somehow be responsible for tumor growth. At the very least, the availability of purified antigens are potentially useful as immnuodiagnostic agents, and could be used to develop more sensitive diagnostic serological tests. They could also be useful, in a more limited way, in increasing host immune responses against the tumor and the production of highly specific antisera.

XII. BIOLOGICAL MEMBRANE AND FUNCTION OF HISTOCOMPATIBILITY ANTIGENS (HLA)

It is now known that cell membrane structure and function are intimately related. The cell surface components are thought to be related to specific molecular

events occuring in differentiation, malignant transformation, and in both normal and tumor immune responses (Bretscher and Raff, 1975).

It has been well documented that there are differences between the cell surfaces of normal and malignant cells.

The major histocompatibility antigens represent a group of cell surface glycoproteins and are present on almost all types of cells, like most structural proteins; hence they can interact directly with materials, such as serum antibody and surface substances of other cells, in the extracellular environment. The function of HLA antigens have been well recognized with relation to organ transplantation. There are allogeneic systems on the lymphocytes of man analogous to the Ia antigens of mice. The genetic control of the serum complement components has been also attributed to the function of HLA complex, (Cunningham, 1977 ; Gill et al 1978). In addition, HLA antigens have been associated with susceptibility to a variety of immunologically mediated disease including malignant melanoma, (Clark et al 1973 ; Singal et al 1974 ; Bergholiz et al 1977). Moreover, melanoma antigens have been suggested to be altered histocompatibility antigens, (Thomson et al 1976).

According to the theory of immunological surveillance, harmful mutations, such as those that cause cancer, are constantly causing changes in the cell surface structure

which are succeedingly identified by the immune system, especially a class of lymphocytes that constantly checks the body's own cells and tissues. These lymphocytes will then transfer the signal back to the lymph nodes and alter a class of lymphocytes known as Killer - T cells to attack and destroy the defective cells. The Killer- T cells function in much the same way as they kill foreign cells during graft rejection. In graft rejection, the Killer- T cells attack the foreign histocompatibility antigens; whereas in the case of the body's own abnormal cells, the histocompatibility antigens on the T-cells and the target cell are identical. Therefore, T-cells must recognize some signals such as a viral antigen or tumor-specific antigens that allow them to distinguish the altered cells from normal ones.

Evidence to support the above phenomena came from the finding that Killer-T cells could not destroy cells infected with viruses unless the killer cells and the target cells had at least one H-2 antigen in common. The same was also found to be true for the killing of cancer cells by Killer-T cells. Further evidence indicating that specific antibody blocking of the shared H-2 antigen on the target cells inhibits the lymphocyte cytotoxicity effect.

Based on the above findings, two general mechanisms have been proposed to explain the role of H-2 antigens in the destruction of abnormal cells by Killer-T cells. One hypothesis suggested that the killer lymphocytes have two

types of receptor molecules on their surface: one that binds to the H-2 antigens, and the other that binds to the viral or tumor specific antigen. According to this hypothesis, the binding of the two receptors is necessary for the elimination of the abnormal cell. The second hypothesis suggests that the killer lymphocyte has only one receptor which selectively binds to a hybrid antigen consisting of an H-2 antigen bound to an abnormal antigen.

Although the exact function of histocompatibility antigens in the destruction of abnormal cells by Killer-T cells is not fully understood, the reported evidence by Cunningham appears to be in favor of the second hypothesis and indicated that H-2 and foreign antigens can interact with each other to form a hybrid molecule recognizabale by the Killer-T cells, (Cunningham, 1977).

XIII. PURPOSE AND OBJECTIVES OF THE PROJECT

From the above results it can be inferred that tumor-associated and specific antigens are present on the surface of melanoma cells and can be identified by several "in vitro" and "in vivo" serological techniques measuring humoral and cellular immunity. Although the presence of common melanoma cell surface antigens have been controversial, there are several reports describing the cross-reactivity and/or individuality of these antigens using totally different approaches.

With respect to the importance and possible usefulness of these antigens as a diagnostic tool and from the immunotherapical point of view described previously, studies were initiated to identify and isolate melanoma cell surface antigens from metastatic specimens in a highly purified form.

In line with the objectives outlined above, the results reported in this thesis give evidence to some initial success achieved in our laboratory by the experimental approach we have taken in an attempt to identify and isolate melanoma specific surface antigens. In this part of the study three points have been considered.

1. Absorption studies of the sera to establish specificity of any positive or negative reaction.

2. Membrane isolation and purification to eliminate cytoplasmic contamination.

and, 3. Autologous reaction to eliminate contribution of antibodies to histocompatibility antigens.

This study, however, is part of the effort underway in our laboratory to identify and isolate melanoma-specific antigens from various fractions of human malignant melanoma tumor cells.

PART B

MATERIALS AND METHODS

I. CHEMICALS

All chemicals were reagent grade. Acrylamide, AMP (Type II), ATP (disodium salt), Coomassie brilliant blue, DEAE-cellulose (medium mesh), DTT, EDTE (disodium salt), EGTA, G-6-phosphate (Barium salt), Maleic acid and SDS were purchased from Sigma Chemical Company, St. Louis. Mo. U.S.A.

Cyanogen bromide , TCA, Triton X-100 were obtained form Fisher Scientific Company; Sephadex G-200, Sepharose 4B, from Pharmcia, Dorval, Quebec.

Glass distilled water was used throughout.

II. SONICATION

The sonic 300 dismembrator used was a product of Fisher Scientific Company. For suspension of total volume of about 2 ml a 1/8 inch probe was used. The power was normally set to a reading between 33-35 of a possible maximum of 100. The sample container was always immersed in an ice bucket during sonication. Sonication was performed in four 15-second periods interspersed with one minute cooling intervals during which the sample was stirred. The probe was never allowed to touch the sides of the container as this was found to produce excessive heating. If these precautions are not taken the temperature of the suspension can rise as high as 40°C . even when the sample is surrounded by

crushed ice.

III. CENTRIFUGATION

Centrifugation at 10,000 g or below was performed in a Sorvall superspeed RC-2B refrigerated centrifuge at a temperature of 0°C . to 4°C .

Centrifugation at higher g forces was performed in a L2-50 Model Beckman ultracentrifuge using swinging bucket 65 titanium rotor and polyallomer centrifuge tubes.

IV. TEMPERATURE CONTROL

Unless otherwise stated, all operations were performed at 0°C . to 4°C . to ensure protein stability. All large scale operations such as column chromatography, dialysis, etc. were performed in a 4°C . cold room.

V. DIALYSIS

Dialysis was carried out overnight with several buffer changes using spectrapor membrane tubings (Spectrum Medical Industries, Los Angeles) with appropriate molecular weight cut offs. The tubings were prepared by heating at 80°C . for 30 min, in 0.1M $NaHCO_3$ followed by intensive rinsing with distilled water.

VI. CONCENTRATION OF PROTEIN FRACTIONS

Dry calcium chloride was used to concentrate very diluted protien fractions and to provide a system by which almost all the materials could be conserved during the process. This was done by placing the sample container in a desicator containing dry calcium chloride and evacuating with a vacuum pump.

To concentrate larger fractions (i.e. fraction volume of aboute 15-20 ml) an amicon concentrator Model 75 PSI was used. The pressure source was a nitrogen tank, and the power was set to reading of about 25 pound per square inch. A membrane filter with a molecular weight cut off of 50,000 daltons was also used.

VII. SOURCE OF TUMOR MATERIAL

Human malignant cells, tissue and sera were provided by the McGill University Cancer research Unit, Montreal, Quebec.

1. Methods of Obtaining

i. Tissue. cancer tissue was obtained either at surgery or authopsy and sent immediately to the laboratory. The specimens were placed in a clean petri-dish with a small amount of tissue culture medium 199 (T.C. 199), sealed with masking tape and placed in a walk-in deep freeze at -136°C . (Phillips and Lewis, 1970).

ii. Cells. tissue for cell suspension was minced as finely as possible in a small amount of T.C. 199 and filtered through sterile gauze. 1-2 drops of the cell suspension was mixed with 2 drops of trypan blue and placed on a Haemocytometer slide. The number of viable tumor cells was then counted using a light microscope. Suspensions containing cells with a high viability were frozen as follows: the desired number of cells in suspension was centrifuged, the supernatant was decanted and the cells were placed in 1-ml sterile ampoules containing 0.15 ml dimethylsulfoxide (DMSO), 0.15 ml fetal calf serum (FCS) and 0.7 ml T.C. 199 containing 10% FCS. The ampoules were then sealed and placed inside a tightly closed liquid nitrogen chamber for approximately 45 minutes and stored ~~at -136°C~~ , in vapour phase liquid nitrogen freezer (Phillips and Lewis, 1970; Lewis and Phillips, 1972).

iii. Sera. sera were obtained from blood samples which were collected only from malignant melanoma patients who has had surgical treatment. As a means of increasing the significance and specificity of the results, sera were absorbed by the following technique (Minden et al. 1974).

4.5×10^7 normal human spleen cells and 2×10^7 tumor cells pooled from a variety of non-melanotic tumors were prepared by washing in sterile PBS (0.01M KPO_4, 0.1M NaCl pH 7.2) and mixed with 1-ml of the serum in a tube which had been

brought up to 37°C . Absorption was performed at 37°C . for one hour and continued overnight at 4° C . with constant stirring of the suspension. After this period, the samples were centrifuged at 2000g for 30 minutes, and the supernatant was separated and kept at -20°C . until tested by membrane immunofluorescence.

VIII. SURFACE MEMBRANE IMMUNOFLUORESCENCE TECHNIQUES

Direct and indirect membrane immunofluorescence were performed by the method of Phillips and Lewis (1970) and, Lewis and Phillips (1972) as follows:

1. Indirect

About $1x10^6$ cells per sample were placed into small centrifuge tubes and spun down at 100g for 2-3 minutes. After removal of the supernatant, the cells were washed 4 times in PBS and added to tubes each containing 0.1 ml of 1:4 PBS diluted autologous patient serum, mixed properly, and allowed to stand at room temperature for 30 minutes for the antibody-antigen reaction to take place, and then washed 3 times in PBS by centrifugation at 100g for 2-3 minutes. At the end of the final wash, 0.01 ml of fluorescein isothiocyanate (FITC) conjugated antiserum (see below for preparation) was added to each tube and mixed throughly. The suspension was again left at room temperature for 20 minutes to allow reaction to take place between the conjugated antiserum and any antigenically bound gamma

globulins. Washing was repeated 5 times in PBS at 100g, the final precipitate being resuspended in 1:1 glycerol:PBS pH 7.6; a few drops were then placed on a microscope slide and topped with a No:1 grade coverslip. The slides were then viewed under an illuminated U.V. Light fluorescence microscope, and the number of cells showing positive membrane fluorescence were recorded.

2. Direct

To insure the specificity of the membrane reaction, direct immunofluorescence was performed as follows: Melanoma patients' cells were centrifuged and washed 4 times in PBS and incubated directly with the fluorescein conjugated antisera without being treated with the autologous serum. The rest of the procedure was followed as described above.

The basic steps of direct and indirect immunofluorescent techniques are summarized in Figs. 1 and 2.

3. Conjugated Antiserum

The conjugate used in this study was fluorescein isothiocyanate isomer I (FITC), conjugated to goat anti-human gamma globulins and was obtained from Behring Institute, Germany. To prepare the conjugate, one ampoule's content was dissolved in 1 ml of distilled water and 7 ml of PBS pH 7.3 to give the proper working dilution (Lewis and Phillips, 1972).

IX. ISOLATION OF MELANOMA CELL PLASMA MEMBRANES

Essentially the same method was used for isolation of membrane from both melanoma tumor tissue and viable tumor cells.

In the case of melanoma tissue, the tumor tissue was thawed rapidly in a 40°C . water bath and finely minced with stainless steel scissors. It was then suspended in a volume of 0.25 M sucrose containing 5 mM Tris -HCL, 0.2 mM $MgSO_4$, pH 7.5; homogenized with 10-12 strokes using a glass homogenizer, and passed through 4 layers of cheese cloth in order to remove unbroken materials.

Free tumor cells were also thawed at 40°C . , washed by centrifugation in the same buffer and homogenized as above.

In both cases, the suspension was centrifuged for 10 minutes at 700g in order to remove unbroken cells and nuclei. The pellet was resuspended in 2-5 ml of the same buffer, homogenized gently and centrifuged as before. The two supernatants were combined and centrifuged at 7,000g for 10 minutes to remove cell organelles such as mitochondria. The 7,000g supernatant was centrifuged at 10,000g for 10 minutes to precipitate the lysozymes. The 10,000g supernatant was then centrifuged at 200,000g for one hour. By doing so, a pellet of crude membranes was obtained. The 200,000g pellet was then washed twice in 0.01 M $NaHCO_3$ and suspended in 0.1 ml of the same buffer by gentle

homogenization in a loose-fitting homogenizer. The suspension was mixed with I.7 ml of 70.74% sucrose in 0.01M $NaHCO_3$. A gradient was created by layering successively I ml of 53.6% sucrose, 0.9 ml of 48.45% sucrose, 0.7 ml of 42.9% sucrose, and 0.6 ml of 37.4% sucrose, in 0.01M $NaHCO_3$, over the 70.74% sucrose-200,000g pellet suspension. Four (4) bands appeared on the gradient, these were collected with pasteur pipettes, resuspended in about 10 volumes of 0.01M $NaHCO_3$ and centrifuged at 200,000g for 1 hour. The membrane fractions as well as the sample fractions from homogenate, 700g, 7000g, 10,000g and 200,000g supernatants were dialyzed against 0.15M NaCl, frozen at -20° C. and stored.

X. PROTEIN CONCENTRATION DETERMINATION

Protein concentration was determined by the method of Lowry et al (1951), except that the final volume of the reaction mixture was decreased to 3.8 ml, and 1mg/ml BSA was used as a standard solution.

XI. ENZYMATIC PROPERTIES OF ISOLATED MEMBRANE FRACTIONS

The following enzyme markers were employed to follow the yield and the purity of plasma membranes during isolation procedure:

a. 5'- NUCLEOTIDASE

b. $Na^+ + K^+$ ADENOSINE - TRIPHOSPHATASE

c. GLUCOSE-6-PHOSPHATASE

1. Principle

The methods by which the activities of the above mentioned enzymes were determined are based on the incubation of the specific substrate with each sample fraction, and determination of the liberated inorganic phosphate.

2. 5'-Nucleotidase

5'-nucleotidase activity was determined by a technique adapted from the method of Schachter et al (1970) and modified by the method of Schimmel et al (1973) as follows.

An aliquot containing 50 µg of protein was added to an incubation mixture containing 125 mM glycine-HCl (pH 8.5), 12.5 mM MgCl, 0.2 mM EDTA and 7.5 mM AMP in a final volume of 0.3 ml.

After incubation at 37°C . for 60 minutes the reaction was stopped by addition of 0.3 ml of cold 10% TCA. The precipitated protein was removed by centrifugation, and the inorganic phosphate content of the supernatant was determined.

3. OUABAIN SENSITIVE Na^++K^+ ADENOSINE-TRIPHOSPHATASE

ATPase

Ouabain sensitive Na^++K^+ transport activity was determined by the method of Schimmel et al (1973) except

that the final volume was increased to 0.4 ml and sodium plus potassium salts were omitted from the mixture containing ouabain according to the method of Post and Sen (1967).

Into two test tubes was placed 50 µg of protein from each of the fractions. To the first and second tubes was added 0.1 ml of Imidazole-HCl buffer (pH 7.5) containing 16 mM $MgCl_2$, 2 mM ethylenglycol-bis (B-aminoethylether) N, 'N-tetraacetic acid (EGTA), and 20 mM NaN_3. To the first tube was added 0.1 ml of a solution of 0.4 M NaCl, 0.08 M KCl, and to the second tube was added 0.05 ml of 1.328 mM ouabain. To all tubes was added enough water to bring the volume to 0.4 ml. Both tubes as well as two blanks containing no protein were incubated at 37° C. for 5 minutes, and 0.05 ml of 40 mM ATP was added to each tube. The reaction was then allowed to continue for 60 minutes in a 37°C. water bath with occational shaking. At the end of the incubation time, the reaction was stopped by addition of 0.4 ml of cold 10% TCA. The precipitated proteins were removed by centrifugation and the inorganic phosphate content of the supernatant was determind. $Na^+ + K^+$ ATPase activity was calculated as the difference in ATPase activity in the presence and absence of ouabain.

4. Glucose-6-Phosphatase

The enzyme was assayed by the method of Swanson (1965) as follows: In a 0.5 ml incubation mixture was added o.3 ml of 116 mg/10 ml maleic acid-NaOH buffer (pH 6.5), 50 µg of protein and 0.1 ml of 0.1M G-6-phosphate (see below for preparation). The reaction was carried out for 60 minutes at 37° C . and terminated by addition of 0.5 ml of 10% cold TCA. After diluting the suspension to 2.5 ml, the precipitated proteins were removed by centrifugation in a clinical centrifuge, and inorganic phosphate content of the supernatant was determind.

G-6-phosphate stock solution (0.1M) was prepared by suspending 260 mg of the salt in 2 ml of distilled water and dissolving in a minimum amount of 1N HCl. This was followed by the addition of 72 mg of anhydrous Na_2SO_4. The precipitated $BaSO_4$ was removed by centrifugation and the supernatant solution was tested for complete precipitation with a very small amount of Na_2SO_4. The pH of the supernatant solution was then brought to 6.5 with NaOH and the volume was brought to 5 ml before use.

XII. ANALYTICAL METHODS

1. Inorganic Phosphate Analysis

Enzymatically released inorganic phosphate was measured by the method of Lowry and Lopez (1946) as follows:

To a known amount of each supernatant, usually 0.2 ml, was added 2.0 ml of of freshly prepared 2% ascorbic acid and 2.0 ml of 0.5% ammonium molybdate in 2N H_2SO_4. After proper shaking, the reaction was allowed to take place at 45°C for 20 minutes and stopped by immediate cooling. The optical density of the suspension was then determind at 820 um in a Beckman DB-G spectrophotometer. To perform the standard experiment, 1mM KH_2PO_4 solution was used.

A unit of activity was expressed as micromoles of inorganic phosphate released per 60 minutes, and specific activity was defined as unit per mg of protein.

2. SDS Acrylamide Gel Electrophoresis

SDS-gel electrophoresis was performed by the method of Fairbanks et al (1971) using the components indicated in table 1. A disc electrophoresis apparatus connected to a power-supply from Instrumentation Specialties Company was employed in these experiments.

To form a working solution for 3 gels, concentrated stock solutions were mixed (in the order and proportions

given in table 1, except that the addition of ammonium persulfate and TEMED was followed after deaeration of the mixture), and added to 13 cm glass tubes of 8 mm diameter that had been cleaned by soaking in chromic-sulfuric acid. Each column was gently overlaid with a solution of 0.1% SDS, 0.15% ammonium persulfate, and 0.05% TEMED. When polymerization was completed, the tops of the gels were rinsed and overlaid with about 0.5 ml of electrophoresis buffer. The gels were then covered and left to stand at room temperature for about 12 hours.

1. Sample Preparation

Sample loads of about 150 µg of proteins were first dialyzed overnight against 10 mM Tris-HCl, 1mM EDTA (pH 8) and prepared for electrophoresis by adding the following to the stated final concentration: 1% SDS, 10mM Tris-HCl (pH 8), 1mM EDTA (pH 8) and 40 mM DTT. The clear sample solutions were allowed to incubate at 37°C. for 30 minutes to promote reduction of disulfide bonds by DTT. The treated samples were then made 5% in sucrose plus a sufficient amount of 0.05% bromophenol brilliant blue and subjected to electrophoresis in the 5.6% polyacrylamide gels in the apparatus employed.

Each sample was taken up in a disposable micropipette and discharged gently beneath the upper buffer onto the top of the gel. Electrophoresis was carried out for about 3

hours at 8 mA/tube by which time the tracking dye had travelled about 10-12 cm. Molecular weight markers were also treated in the same manner except that they were incubated for 20 minutes at 45°C .

2. Staining and Destaining

When the electrophoresis was completed, the gels were stained for protein with Coomassie brilliant blue. They were placed in culture test tubes to which was added the fixing, staining and destaining solutions in the order and proportion given below:

A) 25% isopropyl alcohol, 10% acetic acid, and 0.05% Coomassie brilliant blue overnight.

B) 10% isopropyl alcohol, 10% acetic acid, and 0.005% Coomassie brilliant blue for 6 hours.

C) 10% acetic acid and 0.002% Coomassie brilliant blue, overnight.

D) 10% acetic acid several hours, until the background was clear.

3. Estimation of Molecular Weight

Except for BSA and β-galactosidase, the following lyophilized proteins, which were provided in the Pharmacia calibration kit for protein molecular weight determination, were used.

PROTEIN STANDARD	M.W
Ribonuclease (A)	13,700
Chymotrypsinogen (A)	25,000
Aldolase	40,000
Ovalbumin	45,000
BSA	68,000
β-galactosidase	135,000

Molecular weights of the stained protein bands were calculated from a standard curve constructed from the electrophoretic mobilities of the marker proteins versus the known molecular weights expressed on a semi-logarithmic scale, (Fig. 12). The mobility was calculated from the Weber and Osborn (1969) equation;

$$M = \frac{A}{B} \times \frac{D}{C}$$

Where

M = mobility

A = distance of protein migration

B = distance of dye migration

C = length before staining

D = length after destaining

XIII. MEMBRANE ELECTRON-MICROSCOPY

Electron-microscopic visualization of isolated cell surface membranes as well as the 200,000g pellet were performed at McGill Cancer Unit Microscopy department. To do so, membrane pellets were washed in distilled water by centrifugation and fixed by the additon of about 2 ml of 4% gluteraldehyde in 0.2M sodium cacodylate pH 7.2 and allowed to stand at 0°C . for about 60 minutes. Following this, membranes were pelleted down by centrifugation at 200,000g for 30 minutes and fixed for the second time at 0 C . in the same buffer for 2 hours.

Further preparations were done by the departmental electron-microscopist.

XIV. PURIFICATION OF IgG

IgGs were purified from preabsorbed sera showing tumor specificity by membrane immunofluorescence, as follows.

1. Ammonium Sulfate Precipitation

To a known amount of serum was added, with constant stirring, sufficient amount of saturated ammonium sulfate until a concentration of 40% was attained. Stirring was continiued for 5-10 minutes and the mixture was allowed to stand for 30 minutes. The precipitated proteins were removed by centrifugation at 20,000g for 10 minutes, and resuspended in a volume of the starting buffer, 0,01M KPO_4,

pH 7.5 equal to that of the original serum (Sapin et al 1975). The suspension was then equilibrated against the same buffer by dialysis, and chromatographed on diethylaminoethyl (DEAE)-cellulose.

2. Preparation of the Absorbent

DEAE-cellulose was precycled by treatment with 0.2M NaOH and 0.2M HCl. Slurries were washed with distilled water until close to pH 5, then they were neutralized using concentrated K_2HPO_4 and finally they were washed with the starting buffer, i.e. 0.01M KPO_4 pH 7.5. After the removal of fines by several decantations, the absorbent was poured as a slurry into a chromatographic column, from Pharmacia fine chemicals (20 x 1.5 cm), and allowed to settle under flow condition induced by gravity. When gravity settling was nearly complete, the absorbent was further compacted and equilibrated by mounting the column above a fraction collector (Minirac 17,000 from LKB ,Biocal, instrument), and passage of the starting buffer for about 24 hours prior to application of the samples (Sober et al 1956).

3. Development of the Chromatogram

The equilibrated protein sample was absorbed into the column with several 1 ml portions of the starting buffer before the continuous flow was begun. The column was then eluted with a salt gradient of 0.005M and 0.5M NaCl in 0.01M KPO_4 pH 7.5 at a flow rate of about 0.2 ml per minute

(Williams and Chase, 1967 and 1968). The eluting buffer gradient applied to the column wasformed by siphoning 200 ml of the high molarity buffer into a mixing chamber containing 200 ml of the low molarity buffer, keeping constant the volume of the mixing chamber.

4. Examination of the Effluent

The effluent fractions were routinely examined for protein at 280 mμ in a Beckman DB-G spectrophotometer. The eluted peak fractions were also routinely tested by either double-diffusion or micro-immunoprecipitation against goat anti-human IgG and/or anti-human serum proteins. The IgG rich fractions were then combined, concentrated, and stored at $-20^{\circ}C$. until used.

XV. IMMUNOLOGICAL METHODS

1. Immunodiffusion

Double-diffusion in 1% Nobel agar was carried out by the Ouchterlony technique in Gelman immunoplates using enlarged wells when necessary (Ouchterlony, 1958). Routinely, 1 gm of the agar was placed in 90 ml of 0.1% saline, melted, cooled to about $60^{\circ}C$. and then 10 ml of 15^{-5} M merthiolate solution was added and mixed. Immediately, 2 to 2.5 ml of melted agar were poured on each microscopic slide and allowed to solidify so as to form a perfectly level surface.

Wells were cut with a rounded metal cutter or by using a special gel puncher (Gelman Instrument Co.) and the agar plugs were sucked out through a pasteur pipette connected to a vacuum line. Finally about 10-15 µl samples were poured into each well using a 50 µl syring (Hamilton Co. Reno Nevada). A positive control of human IgG or anti-human IgG (Behringwerke AG Germany) was also included in each run. Precipitation lines were developed overnight and recorded either by photography after staining of the slides or graded 1+ to 3+ according to intensity of the lines.

2. Staining of Precipitation Lines in Gels

i. Washing and Drying of the Plates

After the development of the antigen-antibody precipitates, the gels were washed in (0.01M KPO_4, 0.015M NaCl pH 7.5) for 24 hours to remove the soluble nonreacting constituents.

The drying of the plates reduced the gel layer to a thin transparent film and was accomplished by placing a sheet of filter paper over the gel plate, which was then exposed to a current of air until dried. The filter paper was then removed and the plate was cleaned for a few seconds in running tap water (Uriel,1971).

ii. Staining Reaction

The slides were stained for the characterization of proteins in immunochemical precipitates using the following method (Uriel, 1971).

The dried plates were immersed in the staining solution, containing 0.1% ponceau red or amido black, 0.425M acetic acid and 0.042M sodium acetate for about 2 hours, and washed in 2% acetic acid until the gel background was decolorized. After decolorization they were rinsed in distilled water and dried under a current of air.

3. Immunoprecipitation

Microprecipitation in 50 µl capillary tube was performed for the identification of IgG rich fractions eluted from DEAE-cellulose column or, in some cases, to test the antigenic activity of the solubilized membranes, except that melanoma autologous serum was first centrifuged (Canlab Microeppindorf 3200 centrifuge) for 10 minutes and the clarified serum was removed with a pasteur pipette before being used.

In both cases about 20 µl of either clarified autologous serum or goat anti-human IgG was placed on a glass coverslip coated with wax and inserted into a capillary tube followed by the addition of 20 µl of either solubilized membranes or IgG fraction . After mixing the two phases, the tube was

allowed to stand at room temperature for about 1 hour and viewed for the presence of formed Ag/Ab precipitates. As a control experiment, instead of the antigen fraction, 20 µl of 0.01M Tris-HCl buffer pH 7.4 containing 0.15M NaCl was used.

4. Counter Current Immunoelectrophoresis

Crossover immunoelectrophoresis in agarose was accomplished by the method modified by Dr. D. Hartmann of the McGill University Cancer Research Unit (Meerovitch et al 1977)

The electrophoretic plates, cells and power supply employed in these experiments were products of Hyland , California, U.S.A

i. Preparation of Agarose Plates

Routinely, 0.5 gm of Sigma type II agarose medium EEO was dissolved in 50 ml of veronal-acetate-HCl buffer (0.5% Na-barbiturte, 0.333% CH_3CooNa. 3 H_2O and 0.1% NaN_3 pH 8.5) and allowed to boil on a hot plate. As soon as the boiling started, the flask was removed and 25 ml of the melted agarose was poured into a precleaned plate, covered and allowed to solidify so as to form a perfectly level surface and give a diffusion layer of suitable thickness.

ii. Procedure for Making the Wells

A specially made template containing cylindrical openings was placed on top of the plate and secured by means of two side holders. The wells were marked by pressing a loose-fitting rounded steel cutter through each hole and finally cut by using a square metal cutter. They were punched to the desired size and shape to accommodate the required number of tests and located within the lines of flow of electerical charges, 1-2 centimeter apart from the top and bottom sides of the plate. It is important to leave a sufficiently large space on the cathode and anode side for the conductive sponges. The agar plug was sucked out through a flattend end of a pasteur pipette connected to a vacuum line.

iii. Samples Preparation and Application

Frozen sera and antigen fractions were thawed rapidly in a 40° C . water bath and a required amount of each fraction was transfered into small plastic tube. This was allowed to stand at room temperature for at least 30 minutes. The serum was positively charged by 1/8 dilution in 0.2M glycine- HCl buffer pH 2.2 . As a positive control human IgG and anti-human IgG were also diluted 1/8 in the veronal acetate-HCl and glycine-HCl buffer respectively. After another 30 minutes of incubation at room temperature, the acidified sera or anti-human IgG were placed into the wells

on the anode side, and the antigen or the buffered IgG fraction on the cathode side of the plate. It is advisable to use between 20-25 µl of sample per well so as to facilitate proper electerical conduction.

iv. Electrophoresis

An electrophoretic cell containing the veronal-acetate-HCL buffer was mounted properly above the power supply. Into each chamber was placed a connective metal electrode and covered with the conductive sponge which was presoaked in the same buffer. The filled plate was then inverted and fitted above the electrophoretic cell so that the antibody-containing wells were near the anode side of the power supply.

The electrophoretic run was carried out in the conventional way using a constant current of 30(mA) for 60 minutes.

Following electrophoresis, the plates were either washed and stained, employing the same method as described for immunodiffusion, and finally recorded by photography, or graded 1+ to 3+ according to the intensity of the lines.

XVI. EXPERIMENTS TO DETERMINE OPTIMUM CONDITION FOR MEMBRANE SOLUBILIZATION

1. Salt Extraction of Soluble Membrane Antigen

Hypertonic salt extraction of membrane-associated

antigens in soluble form with 1M KCl was performed by the method of Reisfeld et al (1971) as follows:

A known amount of isolated plasma membranes was suspended in 0.15M NaCl containing 1M KCl and dialyzed against the same solution for about 16 hours. The extracted membrane components were removed by centrifugation (Microeppendorf centrifuge for 30 minutes), the resulting sediment was resuspended in 0.15M NaCl, and finally both fractions were dialyzed against physiological saline solution overnight before being used.

2. Sonication and Salt Extraction

Membrane sonication was performed as described before; the sonicated membrane were then subjected to hypertonic salt extraction as above, the supernatant was removed by centrifugation, the pellet was resuspended in physiological saline followed by dialysis of the fractions against 0.15M NaCl.

3. Membrane Solubilization with SDS

Tumor cell membranes were solubilzed in 0.01M KPO_4 pH 7.4 containing 1% sodium dodecyl sulfate (SDS). Following solubilization an aliquot of the mixture was removed and stored until further use. The remainder was centrifuged, the supernatant and pellet were collected as described above and SDS was removed by dialysis against 3 changes of 0.15M

NaCl containing 1gm/liter of Dowex 50^{H^+}.

4. Membrane Solubilization with Triton X-100

Membrane solubilization with 1% Triton X-100 was performed as described above for SDS except that Triton was replaced for SDS and Dowex was omitted from the dialysis solution.

In order to select a suitable technique for membrane solubilization, all fractions were examined by double diffusion and crossover- immunoelectrophoresis as described before. In addition, a positive control of cytochrome oxidase and rabbit anti-cytochrome oxidase (Kindly provided by Dr. J. Kornblatt, Department of Biology, Concordia University) and also 1% Triton, 1M KCl , anti-human IgG, and whenever necessary 0.2M glycine-HCl buffer pH 2.2 were included in each run. Consequently, the following method of solubilization was used throughout.

5. Solubilization of Membranes

Isolated plasma membranes were dialyzed for 16 hours against 1M KCl and exposed to low frequency sonication for four sequential periods as mentioned earlier. To this was added sufficient quantity of 10% Triton-X100 to achieve a concentration of 1% . After 30 minutes of incubation with occasional stirring, unsolubilized constituents were removed by centrifugation using a microeppendorf 3200 centrifuge.

Following solubilization Triton was removed by extensive dialysis against 0.01M Tris-HCl pH 7.4 containing 0.15M NaCl.

XVII. REMOVAL OF MELANOMA CELL MEMBRANE BOUND IgG MOLECULES

In order to remove membrane-bound immunoglobulins, solubilized tumor cell membranes were chromatographed on Sephadex G-200 with or without acidification of membrane components according to the method of Phillips and Lewis (1971).

1. Membrane Acidification

Solubilized tumor plasma membranes were acidified by 1:3 dilution in 0.2M glycine-HCl buffer pH 2.2 prior to chromatography on Sephadex G-200 columns.

2. Molecular Sieve Chromatography on Sephadex G-200

Sephadex gels were washed at least three times in 0.1M KCl and allowed to swell in a boiling water bath under suction for about five hours, followed by incubation at room temperature overnight. Slurries were equilibriated with starting buffers (0.01M KPO_4 pH 7.4 or 0.2M glycine-HCl buffer pH 2.2), after removal of fines by decantation, columns (1x5 cm) were poured and packed under a flow condition of about 0.05 ml per minute. In order to stabilize and equilibrate the gel bed, 3 column volumes of

the starting buffer were passed through each column prior to application of the sample. Blue dextran 2000 (Pharmacia fine chemicals) at a concentration of 2 mg/ml was used for determination of the void volumes. Solubilized membrane components in 0.01M KPO_4 (pH 7.4) or, after acidification in 0.2M glycine-HCl, were then applied onto the specific column and eluted with the starting buffer.

The effluent fractions were examined in a Beckman DB-G spectrophotometer at 280 mμ, dialyzed against 0.15M NaCl, and finally examined by immunodiffusion and crossover-immunoelectrophoresis against autologous serum and anti-human IgG.

XVIII. AFFINITY CHROMATOGRAPHY OF SOLUBILIZED MEMBRANES

1. Preparation of Sepharose 4B-IgG

Partially purified IgG was coupled to Sepharose 4B (Sep 4B) with cyanogen bromide (CnBr) according to the methods of Cuatrecasas (1970) as modified by Thomson et al (1973) as follows:

10 ml of packed Sep 4B was centrifuged at 1000g for 5 minutes. The supernatant was discarded and the packed beads were washed three times in cold distilled water by centrifugation. The washed Sep 4B was resuspended in 10ml of cold water and then finely divided CnBr (1gm per 5ml of suspension) was added at once to the stirred suspension. The pH of the suspension was immediately raised to and maintained between 10.5-11.1 by the addition of 10% NaOH. At the same time the temperature of the suspension was held at about 20°C . by the addition of crushed ice, as needed. When the reaction was completed, as indicated by the cessation of proton release, a large amount of ice was added rapidly to the mixture which was then transferred quickly to a Buchner funnel and washed throughly with cold 0.2M citrate buffer pH 6.5.

The isolated IgG fraction was made 0.2M in citrate buffer pH 6.5 in a final volume equal to that of packed activated beads, added to the moist washed Sep 4B and mixed

immediately with a glass stirring rod. The suspension was then transferred to a 20 ml beaker containing a magnetic mixing bar, stirred gently for four hours, then allowed to stand at 4°C . for 16 to 20 hours. The entire procedure of activation, washing, and coupling usually took 9 to 12 minutes.

2. Chromatography of Solubilized Membranes on Sep 4B-IgG

Columns (1x5) cm were washed with about 50 column volumes of cold Tris-NaCl buffer (0.01 M Tris-HCl, 0.15M NaCl pH 7.4) until the OD of the elute was zero. Solubilized membranes in the same buffer were then absorbed onto the column and buffer flow was stopped for one hour. Following this, the column was again washed with the buffer until no more protein was eluted. This usually required about 20 column volumes. Finally the column was eluted with 3M KCNS and 0.6-1 ml fractions were collected. ODs of the fractions were determined at 280 mμ, the appropriate fractions were pooled and dialyzed against several changes of 1 to 10 diluted Tris-NaCl buffer overnight. The dialysate was concentrated to about 10-fold by using dry calcium chloride as previously described and stored at -20° C . until used. Aliquots of fractions isolated from Sep 4B-IgG columns were either electrophoresed on SDS acrylamide gels or used for counter-current immunoelectrophoretic assay. The basic steps of affinity chromatography technique

are summarized in Fig.3.

PART C

RESULTS

I. MELANOMA SPECIFIC ANTIBODIES IN PATIENTS SERA

1. Absorption

To establish specificity in determining the occurrence of antigen on a range of malignant melanoma tissue and to eliminate the contribution of antibodies to histocompatibility antigens, antibody absorption tests were performed (see Part B, VII,1, iii). For this purpose, positive patients' sera were mixed and incubated with normal human spleen cells and with a number of non- melanotic tumor cells. Supernatants were removed by centrifugation and retested by the membrane immunofluorescence technique.

2. Membrane Immunofluorescent Tests on Autologous Melanoma Cells (MIF)

During early investigation into the host response in human malignant melanoma by Lewis and co-workers (Lewis et al, 1969; Phillips and Lewis, 1970), there appeared to be a distinct class of antigen, in addition to other types, and a corresponding antibody class against it. The antibody system was shown to be directed against the plasma membrane of the patient's autologous tumor cells and present only in the serum of patients with early or localized disease. The presence of this class of antibody was shown by both immunofluorescent and tissue culture cytotoxicity.

In the present investigation, following the absorption of patients' sera the preliminary step was to select tumor from patients with an autologous antibody-antigen system. To confirm the presence of such a system, indirect membrane immunofluorescence tests were carried out (see Materials and methods). In these experiments, melanoma cells were washed in PBS and mixed with 1:4 PBS-diluted autologous serum. Antibody-antigen reaction was then allowed to take place. Following this, cells were washed once again and treated with fluorescein isothiocyanate isomer I (FITC) conjugated to goat anti-human gamma globulins, and the reaction was allowed to take place between the conjugated antiserum and any antigenically-bound gamma globulins. The formation of antigen-antibody complexes were then viewed under a fluorescence microscope.

The specificity of the membrane reaction was justified by the direct immunofluorescent technique by which cells were directly treated with the conjugated anti-human gamma globulins. Fluorescence microscopy was performed at the McGill University Cancer Research Unit according to the method of Phillips and Lewis (1970), and Lewis and Phillips (1972). The results of MIF tests on the sera and the melanoma cells of 16 patients are summerized in table 2. On the basis of serum reactivity as compared to the controls, three groups of patients can be defined. The majority , 6 out of 16 patients, showed no detectable antibody to

autologous melanoma cells . Five patients showed weak reactivity at 1:4 serum dilution (patients TR, CA, CO, OL, DC), and five patients (patients RO, VE, MC, DO, and DU) showed reaction with positive titer above 50%.

These results indicate that the binding of FITC conjugate to tumor cells which were pretreated with autologous preabsorbed serum is mainly dependent on the presence of two unknown factors in the system, namely the level of antigen and/or antibody. Consequently , sera from the above five patients were selected as relatively strong positive after being tested by MIF techniques. Tumor materials from these patients and others were used for further biochemical and immunological analysis throughout the investigation.

3. Partial Purification of IgG

Since the demonstration of tumor-specific antibodies in human malignant melanoma, two types of immunoglobulin components were found to be involved in the specific reaction against tumor antigens (Lewis et al , 1976a; Romsdahl and Cox ,1973; Seibert et al , 1977). The activity was characteristic of an antibody either of IgM and mainly of the IgG type. Therefore, 1-1.5 ml of serum from each of the six patients (patients VE, MC, OL, RO, DU, and DO) showing relatively strong tumor specificity by membrane immunofluorescence, were brought to 40% saturation by the

addition of saturated ammonium sulfate.

After centrifugation, the precipitates were dissolved in 0.01M KPO_4 pH 7.5 and dialyzed overnight against the same buffer followed by protein concentration determination (table 3) . The ammonium sulfate precipitated fractions were absorbed onto pre-equilibrated DEAE-cellulose columns, and immunoglobulins were eluted with a salt gradient of 0.005M and 0.5M NaCl in the starting buffer. The eluted fractions were then examined for protein by measuring their absorbance at a wavelength of 280 mμ (table 4-9). As it is shown in the effluent diagrams that have resulted (Fig. 4) , six (6) virtually identical chromatographic patterns were obtained, each showing a large unretarded peak followed by a well-separated small peak and two somewhat spread peaks of intermediate sizes.

By immunodiffusion tests done on the eluted peak fractions against anti-human IgG, as compared in two cases (i.e. Patients VE and MC) against anti-human serum proteins, IgG rich fractions were found to be eluted always in the first peak (tables 10-11) . Having established this, the chromatographic fractions from patients DO, DU, RO and OL were routinely checked by microprecipitation tests against anti-human IgG as described in Part B (tables 12-15).

Data presented in Fig.5 and Fig.6 show the results of double diffusion test of the eluted peak fractions from patients VE and MC when examined against anti-human IgG, which indicate the formation of single precipitation arcs with a line of identity to commercially prepared human IgG. In both cases, as expected, IgG positive fractions precipitate with anti-human serum proteins, but again single precipitation lines developed (Fig.7). By comparing these results, one could conclude that the DEAE-cellulose fractionation procedure is very reproducable and also that separated IgG fractions are fairly free of contamination with other types of serum proteins.

II. ISOLATION AND CHEMICAL ANALYSIS OF TUMOR CELL PLASMA MEMBRANES

Having demonstrated the presence of tumor specific antibodies in the patients' sera, the approach was to try to isolate and identify antigens which were associated with the tumor cell plasma membranes. For this purpose viable tumor cells or frozen tissue were homogenized in the Tris-HCl-sucrose buffer followed by differential centrifugation as described in Part B. The basic steps of isolation are shown in Fig.8 .

The final pellet (200,000g pellet) was then further purified by flotation through a sucrose gradient, (Fig.9) followed by ultra centrifugation. The bands which appeared

On the gradient were then collected and prepared for further studies.

1. Chemical Analysis

The recovery and purity of plasma membranes at each stage during the isolation procedure was procceded by assaying for protein, $Na^+ + K^+$ ATPase and 5'-nucleotidase. Contamination with cytoplasmic membranes was monitored by assaying for glucose-6-phosphatase. The purity and the integrity of the final preparation of plasma membranes were also checked by electron-microscopy as compared to the 200,000g pellet.

Protein concentration was determined by the method of Lowry et al (1951) on 10 µl aliquots of each fraction. The activity of the above-mentioned enzymes were measured by incubating 50 µg protein from each fraction in the specific buffer-substrate system and measuring the release of inorganic phosphate from the appropriate substrate at 37°C during the zero order portion of the reaction. The enzyme activites were corrected for phosphate release in the absence of the enzyme fraction. Since different metastatic tumor tissues were utilized during the course of the experiments, the enzyme markers were assayed for individual patient's plasma membranes isolation.

Tables 16-24 show the results of the representative experiments indicating that the plasma membrane fraction was

in all cases concentrated in Band #1 of the sucrose gradient (Fig.9) . A comparison of the enzyme marker activities in Band #1 over that of the homogenates is also shown in table 25.

In term of 5'-nucleotidase (commonly used as enzyme marker for plasma membranes) these experiments yielded between 1% to 6% recoveries in Band #1 over those present in the total tumor cell homogenates, with an average 11.5-fold increase in its specific activity. This indicates the highest enrichment of 5'-nucleotidase as compared to the mean values of 6.6 , 6.1 , and 6.0 for Band #2, Band #3, and Band #4 respectively. The recoveries of Na^++K^+ ATPase (an enzyme marker for plasma membranes) in Band #1 were between 1.2% to 5.6% , which closely parallel that of 5'-nucleotidase in all cases with an average 12.2-fold increase in its specific activity, which is also the highest degree of purification if compared with 6.3 , 5.6 , and 4.0 for the other three bands.

However, in Band #1, the recoveries of the cytoplasmic marker, glucose-6-phosphatase, were between 0.08% to 0.57% with an average enrichment value of 1.9 over that present in the homogenates, indicating that the plasma membranes were slightly contaminated. In these fractions (Band #1) the enrichment of glucose-6-phosphatase could be considered insignificant with respect to the average purification values of 5.5 for Band #2 and 11.75 for Band #3.

III. ELECTRON-MICROSCOPY

On electron-microscopic examination of isolated plasma membranes in Band #1, and of 200,000g pellet, it is clear that 200,000g pellet consisted of plasma membranes contaminated with a high level of rough and smooth membranes as well as free ribosomes, with no mitochondria, lysozymes, and other cell organelles (Fig.10). In contrast to this, an electronmicrograph of the tumor cell plasma membrane fraction seems to indicate the presence of only surface membrane with no cytoplasmic contamination; in particular, no endoplasmic reticulum was observed (Fig.11).

Consequently, the membrane fractions in Band #1 were collected as purified tumor cell plasma membranes and used in all subsequent experiments.

IV. PROFILES OF SOLUBLE PROTEINS FROM TUMOR CELL PLASMA MEMBRANES

Having completed tumor cell plasma membrane isolation from malignant melanoma patients, the number and the molecular weight of the constituent polypeptide chains of isolated membrane fractions (Band #1) were determind by SDS-acrylamide gel disc electrophoresis.

Sample loads of about 150 µg protein, from each of the patient's membrane fractions, were solubilized with SDS . Disulfide bonds were reduced by incubation with DTT, and finally prepared for electrophoresis by the addition of concentrated sucrose and tracking dye.

Electrophoresis was done in a system containing 5.6% polyacrylamide and 1% SDS, on separate gels, for about 3 hours at 8(mA/tube as described by Fairbanks et al (1971).

When the tracer (bromophenol blue) incorporated in the runs had migrated a distance of about 10-11 cm, electrophoresis was stopped, gels were removed and stained for protein with Coomassie brilliant blue. After removing the background stain with several changes of 10% acetic acid , gels were photographed and electrophoretic mobilities of protein bands were calculated according to the method of Weber and Osborn (1969).

An estimate of the size of the molecular species was then obtained from a standard curve constructed from the electrophoretic mobilities of SDS-denatured marker proteins plotted against the log of their molecular weights (Fig.12). A rough estimate of the size of the higher molecular polypeptides was obtained by extrapolation of the calibration curve.

The six(6) marker proteins (table 26) used were run on duplicate gels, except for aldolase and β-galactosidase, but

are shown together in Fig.12 . The molecular weights of the marker proteins were taken from the Pharmacia calibration kit, except for serum albumine and β-galactosidase which were taken from Weber and Osborn (1969).

The reproducibility of the system could be seen from the data in table 26, indicating a deviation of less than 4% over the average mobilities calculated for the same proteins which were run in duplicate.

Tables 27-35 show the number and estimated molecular weights of constituents protein-contaning molecules of the isolated plasma membranes from nine (9) melanoma patients (patients CA, CO, DU, DO, MC, OL, RO, TR, and VE).

By comparison of electrophorograms of the isolated membranes (Band #1), only 20-28 bands could be distinguished in all cases, with molecular weights ranging from 360,000 to 13,700 daltons. There may be additioal minor components that have not been seen because the amount of samples applied is kept low to prevent the distortion of the major bands which usually occur when the gels are overloaded.

A large portion of the proteins in this membrne system apparently are of unusually high molecular weights. This can be seen in tabels 27-30 and corresponding Fig.13 which could be due to noncovalently-bound polymers to subunits.

With respect to the different type of metastatic tissue that was originally used, the variation in the characteristic patterns detected for these membranes could be insignificant, because not only the resulted protein bands are within the same range of molecular weights in all cases, but also more than 70% of them appeared to have the same sizes with a variation of ±500 daltons.

V. DEVELOPMENT OF A MORE EFFECTIVE METHOD FOR MEMBRANE SOLUBILIZATION

As indicated in Part B (Part B, XVI, 1-4) of this thesis, four(4) different solubilization techniques were employed in order to find an effective method of solubilizing the tumor cell plasma membranes, desirable from the standpoint of yield and recovery as well as the immunological activity of the membrane antigens.

The initial stimulus was the finding by Reisfeld et al (1971) that hypertonic salt extraction with 3M KCl alone or in conjunction with sonication, efficiently solubilized HLA antigens from cultured lymphoblasts.

Since in this investigation a limited amount of isolated membrane proteins were available, and since high salt concentration does not extract all the plasma membrane proteins, an attempt was also made to use SDS and Triton X-100 solubilization as well for development of the method.

For this purpose an aliquot of 0.4 ml of patient VE plasma membrane fraction (Band #1) equivalent to 0.8 mg protein was divided into four(4) equal portions and treated as follows:

One sample was subjected to 1.0M KCl extraction as described in Part B. Extracted membrane proteins were removed by centrifugation and the resulting sediment was resuspended in physiological saline followed by dialysis.

The second membrane fraction was first depolymerized by sonication and dialyzed against KCl solution as above. After removing an aliquot of about 20µl, the remainder was centrifuged and the resulting supernatant and pellet were collected as described in Materials and Methods.

SDS solubilization of the third plasma membrane fraction was performed as mentioned in Part B. Following the removal of a 20 µl sample from the mixture, unsolubilized components were sedimented by centrifugation, the supernatant was collected, and SDS was removed by extensive dialysis against Dowex in physiological saline.

The fourth fraction was subjected to solubilization with 1% Triton X-100 as described in Materials and Methods.

Finally, antigenic activity of all nine fractions was established by immunodiffusion and crossover-immunoelectrophoresis tests against autologous preabsorbed

serum of patient VE, using 1M KCl, 1% Triton X-100 , cytochrome oxidase / anti-cytochrome oxidase, and anti-human IgG as controls.

On double diffusion assay only a single precipitation arc was observed for anti-human IgG against the patient's serum (table 36). Furthermore, no detectable precipitation was observed for cytochrome oxidase with anti-cytochrome oxidase, or between any of the solubilized fractions and the autologous serum. The resulting negative reactions in this case could possibly be due to the presence of a low level of tumor antigens or antibodies, or both, in the system, a lack of proper diffusion through the gel bed, or finally the sensitivity of the technique.

On the other hand, by crossover-immunoelectrophoresis positive reactions were seen with those fractions which were treated with Triton X-100, sonication and high salt extraction, and with salt extraction alone Fig.14 . No reaction was observed between SDS-solubilized samples and the patient serum. Similarly, a reaction was never seen in the fractions containing unsolubilized materials. All controls resulted in a negative reaction except for cytochrome and anti-cytochrome oxidase, as well as for IgG and anti-human IgG.

Based on these resultes (table 36), a solubilization method was developed which consisted of a) high salt

extraction for 16 hours followed by b) sonication for four sequential periods and finally c) treatment with 1% Triton X-100 for total solubilization of the membrane components (see Part B).

This method was used during further investigation.

VI. IMMUNOGLOBULINS IN MALIGNANT MELANOMA CELL MEMBRANES

The presence of immunoglobulins in saline eluates of human malignant tissue was suggested by the findings of Romsdahl and Cox, (1973) and Gupta and Morton,(1975).

In this investigation, during the search for melanoma antigens, it was noticed, particularly in the case of patient VE, that solubilized plasma membranes produced a positive reaction on double diffusion with anti-human IgG. Therefore, the isolation of this IgG molecules was thought to be important for the following reasons: (1) to trace back the presence of melanoma specific antigens and their isolation by affinity chromatography and (2) to inhibit their interference during the isolation procedure.

For this experiments , 0.4 ml of 2.7 mg/ml solubilized membrane from patient VE was divided into two equal fractions. One fraction was acidified by 1:3 dilution with glycine-HCl buffer pH 2.2 in order to dissociate any IgG-bound complexes in the system, and the second was made 0.1M in KPO_4 pH 7.4 followed by chromatography on Sephadex G-200

columns. (see Part B, XVII, 2).

The chromatograms were developed with the specific starting buffers at a flow rate of 0.05 ml per minute, the eluate was collected in 3-drop fractions, and dialyzed against 0.15M NaCl overnight.

On immunodiffusion tests against anti-human IgG, fractions 2, 3, and 4 eluted from the acidified column had positive reactions shown by a line of identity to human IgG (Fig.15). The results in table 37 show that only upon acidification the IgG molecules could be separated and identified by the above techniques.

The entire procedure was repeated using the total amount of isolated solubilized plasma membranes (0.9 ml of 2.7 mg/ml) from the same patient. Following acidification, the suspension was applied to a column (2x10 cm) of Sephadex G-200, the chromatogram was developed with glycine-HCl buffer and 0.5 ml fractions were collected and dialyzed as above.

On immunodiffusion with anti-human IgG; separated IgG molecules were found to be eluted in peak #1 of Fig.16 . In the run for Fig.16. the distribution of the antigen(s) was determined by crossover-immnuoelectrophoresis against the patient's own serum. The results in table 38 and Fig.17 show that, antigens were eluted in peaks 2, 3, and 4, in a total volume of about 5 ml. The chromatographic pattern may suggest the presence of a different molecular weight species

of the antigens or it could be due to poor separation of the membrane proteins.

The corresponding positive fractions were then combined and tested against the membrane-bound isolated IgG fraction by double diffusion in agar; no detectable precipitation line was observed. The remainder of the antigen fraction was stored at -20° C., before further affinity chromatography studies.

VII. PURIFICATION OF THE AUTOLOGOUS MELANOMA SPECIFIC ANTIGENS

1. Affinity Chromatography of The tumor Solubilized Plasma Membranes

Having demonstrated the presence of autologous melanoma antigens in the solubilized membrane fraction of patient VE, the affinity chromatography technique of Cautrecases (1970) as modified by Thomson et al (1973) was employed for the final purification of the antigen(s) from a number of isolated tumor cell plasma membranes.

For this purpose, partially purified IgG fractions at a protein concentration indicated in table 39 from each of the five(5) melanoma patients was coupled to Sepharose 4B with cyanogen bromide (CnBr). Sepharose 4B was activated by using 1 gm cyanogen bromide per 5 ml of suspension, and the

pH was held between 10.5 and 11.1 by the addition of 10% NaOH. When the reaction was completed, the activated beads were washed with citrate buffer and gently mixed with the IgG fraction in the same buffer. Following this, the coupled batches of Sep 4B-IgG were then packed into 5 different columns (1x5 cm), washed with the Tris-NaCl buffer until the OD of the eluate was zero.

Autologous solubilized membranes in the same Tris-Nacl buffer were then absorbed onto the appropriate column and the Ab/Ag reaction allowed to take place by closing the flow for about 1 hour. After all the unretarded membrane componets had been flushed from the column by the Tris-NaCl buffer, dissociation of the antigens from Sep 4B-IgG was brought about by washing the column with freshly prepared, 3M KCNS. (See Part B, XVIII, 1-2). The eluted fractions were then examined for protein by measuring their absorbance at a wave length of 280 mμ, see tables 40-44.

Affinity chromatography patterns of the autologous solubilized membranes from the 5 patients (patients DO, MC, OL, RO and DU) are also shown in Fig. 18. Five somewhat identical and reproducible chromatographic profiles were obtained, each having three well-separated peaks. The first large peak is the unbound IgG molecules, followed by the second large peak which contains unretarded plasma mambrane components. The bound antigenic materials which were eluted with 3M KCNS resulted in the formation of the third, very

small peak.

In each case, materials eluted in peak #3 were pooled, dialyzed, and concentrated as described in Part B. Table 45 shows the volume and protein concentration of the isolated antigenic fractions. Aliquots of fractions isolated from Sep 4B-IgG columns were either electrophoresed on SDS acrylamide gels or used for crossover-immunoelectrophoretic test against the autologous serum.

2. SDS-Acrylamide Gel Electrophoresis of Purified Tumor Cell Plasma Membrane Antigens

To determine the number and molecular weight species of the antigen fractions isolated by affinity chromatography, samples of about 0.25-0.30 ml of each antigen fraction eluted from the Sepharose 4B-IgG column were prepared for and electrophoresed as described in Part B (Materials and Methods).

After the completion of the electrophoretic run, gels were stained with Coomassie brilliant blue and molecular weights of the stained bands were calculated from a standard curve (Fig.12).

The number and estimated molecular weights of the plasma membrane antigens from five(5) melanoma patients' tumor cells are shown in table 46 and the corresponding Figs. 19 and 20.

As the results indicate, there were six (6) protein bands in the isolated antigen fraction from patients DO, RO, and DU with molecular weights ranging from 50,000 to 185,000 daltons. This was also the case for patients MC and OL, except that these fractions contained seven (7) and four (4) proteins respectively.

By comparison of the molecular weight species in the isolated antigen fractions, at least four (4) protein bands with approximate molecular weights of 175,000 daltons, 75,000 daltons, 66,000 daltons, and 57,000 daltons were found to be common in all cases with a variation of less than 200 daltons. The other proteins could also be considered to be similar molecular weight species but with a higher variation, i.e. Of less than 1000 daltons. This could be due to experimental error, but most likely to a slight difference in the amount of the cross-linker (methylene-bisacrylamide) which was used during the preparation of the gels for each electrophoretic run.

The above autologous molecular weight species isolated by affinity chromatography were also originally found in the corresponding solubilized melanoma plasma membranes by SDS-gel electrophoresis, tables 27-31 and Fig. 13, as well as in the solubilized tumor cell plasma membranes of four (4) more melanoma patients whose membrane antigens were not isolated, tables 32-35.

VIII. SPECIFICITY OF THE MEMBRANE ANTIGENS

To determine the specificity of the isolated membrane antigens, crossover-immunoelectrophoresis was performed with sera from eight (8) normal people (obtained from the Montreal General Hospital) as well as sera from autologous and allogeneic melanoma patients as described in Part (B, XV, 4). The results presented in table 47 and Figs. 21-24 demonstrate that, in addition to the control experiments, all the melanotic sera precipitated the autologous as well as the allogeneic antigens whereas there was no reactivity against normal sera.

Based on the above findings, it could be concluded that there is at least one type of antigen, located on the tumor cell surface of each of these patients (patients DO, DU, LO, MG, and RO) which cross-reacts with sera from other melanoma patients tested in these experiments, and may possibly be melanoma-specific antigen(s).

PART D

DISCUSSION

I. SEROLOGICAL STUDIES ON AUTOLOGOUS MELANOMA CELLS

Since auto-antibodies against the surface determinants of melanoma cells were found to be highly stage dependent and circulate in the sera of patients in the early stages of the disease (Lewis et al 1969, 1973a, 1973b ; Morton, 1971 ; Bodurtha et al 1975), an indirect membrane immunofluorescence technique was employed in order to select patients with positive antibody activity for plasma membrane fluorescence.

This method has been widely used in a number of laboratories (Lewis et al,1969, 1975 ; Lewis and Phillips, 1972 ; Phillips and Lewis, 1970 ; Morton et al, 1968 ; Romsdahl et al, 1970 ; Potra et al, 1971 ; Leong et al, 1977a, 1977b) which have reported that sera from patients with malignant melanoma react with surface antigens. Some of these reports deal with the allogeneic specificity of the reaction (i.e. Reaction between sera and cells from different patients). Since it is somehow difficult to demonstrate or prove the specificity of an allogeneic reaction due to the presence of antibodies to histocompatibility antigens, pre-absorbed patients' sera were tested and used in autologous systems (i.e. Reaction between sera and cells from the same patient).

Absorption of the sera with normal human spleen cells as well as with a variety of nonmelanotic cells was also

performed to insure the specificity of any positive reaction, in particular when antigens were being tested against sera from other melanoma patients as well as normal controls. However, the immunofluorescence data in this study showed positive reaction in 10 out of 16 patients (i.e. In 62.5% of the tests), when viable melanoma cells were tested with the patients, autologous, preabsorbed sera (table 2). The 62.5% frequency of the positive reactions were found to be in a region which ranged from 24% to 100%, as reported by several investigators (Lewis et al, 1969; Phillips and Lewis, 1970; Morton et al, 1968; Romsdahl et al, 1970; Potra et al, 1971, Leong et al, 1977a, 1977b). This low frequency could either be due to the sensitivity of the technique or most probably to the possible influence of the blocking factors described in section (A,X). Additional factors that might account for the absence of the autologous reactions in patients (MU, UR, GE, PR, TU, and FE) could be due to the presence of a lower number of antigenic determinants on these melanoma cells, or to the presence of embedded antigens in the phospholipid bilayers of the cell membranes (Leong et al, 1977a,1977b). It is also possible that patients in the advanced stages of the disease have less antibody against suface membrane antigens (Lewis et al, 1969, 1973a, 1973b; Morton,1971; Bodurtha et al, 1975).

Moreover, the specificity of the positive membrane fluorescence reactions were further documented by direct

immunofluorescence tests (Lewis and Phillips, 1972) in which addition of the patient's own serum was omitted from the fluorescence staining steps, and consequently inhibiting the binding of fluorescein conjugated goat anti-human gamma globulin to the cell suface antigens (Table 2).

Since the results reported from a number of laboratories indicated that IgG was the major immunoglobulin species involved in the specific reaction with melanoma antigens (Lewis et al, 1969; Leong et al, 1977a, Romsdahl and Cox, 1973; Gupta and Morton, 1975) the IgG fractions were isolated from patients VE, MC, DO, DU, RO and OL sera (all showed relatively strong melanoma tumor specific activity) by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose ion exchanger. The elution of the immunoglobulin fractions was achieved by incorporation of a salt gradient in the eluting (phosphate) buffer. IgG rich fractions were found to be eluted always in the first peak when examined by double diffusion or micro-immunoprecipitation against anti-human IgG and anti-human serum proteins, tables 10-15 and Fig. 4. It is noteworthy that a decrease in the pH or ionic strength of the phosphate buffer was necessary for the higher yield of fractionated IgG molecules. A more distinct separation of immunoglobulin-type molecules could also be achieved by using a very slow flow rate during the entire isolation.

Using the above criteria, fractionated IgG molecules were found to be free of contamination from other serum proteins, because only single precipitation arcs were developed, with a line of identity to commercially prepared human IgG, on Ouchterlony tests against rabbit anti-human IgG (Fig. 5 and 6) as well as anti-human serum proteins (Fig. 7). Of course this does not rule out the co-elution of protein (s) with a similar net charge or isoelectric point. The yields of the partially purified IgG from individual patients were between 9.7 to 12.9 mg/ml of sera which are within the range reported for IgG content of normal human serum (Roitt, 1974).

II. ISOLATION AND CHEMICAL ANALYSIS OF THE TUMOR CELL PLASMA MEMBRANES

Methodologically, the isolation of tumor cell membranes described in Part B was a variant of that employed by Preddie *et al* (1978a, 1978b). Thus, a change was made in the sucrose gradient preparation used for suface membrane purification, from total volume of 26 ml to 4.9 ml. As a result, it could be used when only a small amount of crude membrane preparation was available. Plasma membranes were isolated from tumor cells or from tissue by homogenization in buffered isotonic sucrose followed by differential and sucrose discontinious density gradient centrifugation. Since mostly frozen metastatic tissue was used, the most

crucial step was found to be homogenization of tumors from different patients. It was noted that over-homogenization could result in a poor separation of plasma membranes from internal cytoplasmic membranes.

The results presented in tables 16-25 appear to show that melanoma cell plasma membrane fractions were always concentrated in Band #1 of the sucrose density gradient (Fig. 9). This band contained the highest enrichment of ouabain sensitive $Na^+ + K^+$ ATPase and 5'-nucleotidase activities and showed no significant G-6-phosphatase activity, indicating that the plasma membranes in Band #1 were not contaminated to any significant extent with cytoplasmic membranes, since a high concentration of G-6-phosphatase, as indicated by its specific activity, is present in Band #2 and Band #3. These results are in agreement with the electron-microscopic visualization of the protein fraction in Band #1 and 20,000g pellet (Fig. 10 and 11).

Although the average specific activities of the enzyme markers for plasma membranes has increased 11.5 and 12.2 fold (table 25) for 5'-nucleotidase and $Na^+ + K^+$ ATPase respectively, the average yields of these enzymes were found to be between 3.5% and 3.4% of that present in the initial homogenates. This low value may account for the very low yield of plasma membrane proteins during the isolation steps involving washing and flotation which could eleminate those

membranes with adhering mitochondria, nuclei, and other cytoplasmic components.

Determination of the molecular weights of the protein constituents of the tumor cell plasma membranes by SDS-polyacrylamide gel disc electrophoresis (tables 27-35) and (Fig. 13), showed that these membranes are made up of a complex mixture of 20-28 different molecular weight classes of proteins which indicate the heterogeneity of melanoma cell plasma membrane proteins.

These results indicate that no single polypeptide chain present in melanoma cell membranes has a larger molecular weight than about 360,000, or smaller than 13,700 daltons. Solubilization of the membrane proteins in 2% SDS followed by incubation of the mixture at 95°C . in a water bath for about 3 minutes did not change the destribution pattern of these proteins. There may, however, be additional minor components that have not been seen, because the amount of samples applied is kept low to prevent the distortion of the major bands which occurs when the gels are overloded.

It should be noted that the differences in the pattern and the number of protein bands, are due to the fact that plasma membranes were prepared from different metastatic deposits and therefore, the presence of several other types of tissue such as vescular and nerve elements as well as normal cell constituents may complicate the electrophoretic

pattern. However, similar difference has been revealed by polyacrylamide gel electrophoresis of KCl extracts of different primary metastatic melanoma tissues as reported by Hollinshead (1975).

III. DIFFICULTIES IN THE DETECTION OF MELANOMA ANTIGENS

Membranes, because of the difficulty with which they diffused in agar, could not be used in double diffusion, crossover-immunoelectrophoresis tests, or in affinity chromatography for obvious reasons. Isolated membranes were therefore solubilized by the technique described in Part (B, XVI, 5). However solubilized membranes did not give positive results when tested against either autologous or allogeneic sera in double diffusion.

The presence of IgG molecules in the solubilized membrane fraction of melanoma patients in particular patient VE (tables, 37, 38) and (Fig. 15), was thought to be the factor that prevented the formation of lines of identity by double diffusion. Since no IgG molecules were found in the supernatants after washing the membrane sediments, it is suggested that these proteins had been firmly bound to the tumor tissue. Although it could in fact be the main factor involved, elution of the "in vivo"-bound IgG and possibly other immunoglobulin molecules from solubilized tumor membranes by accidification and Sephadex G-200 chromatography did not improve the production of any

precipitation arc when tested against either autologous serum or separated IgG molecules by double diffusion technique.

It is possible that the low concentration of either antigens or antibody, or both , in the system was responsible for the negative results observed, although the involvement of other types of blocking or interfering factors present in whole membrane or sera could not be ruled out.

However, the observation of the positive results by crossover-immunoelectrophoresis, (tables 36, 38) and (Fig. 17), was a good indication that there are still antigeneic molecules in the solubilized membrane fraction which could be possibly isolated even though they seemed to be present in a very low concentration, or somehow blocked. This latter possibility could make the detection and the isolation of these antigens of low concentration next to impossible.

It was therefore decided to isolate the antigens directly, by an autologous affinity chromatography system which allows selective isolation of tumor antigens without trying to elute any bound immunoglobulins, for the following reasons: 1) to eliminate denaturation of membrane proteins which would possibly be caused upon acidification, and 2) since isolated membrane fractions were available in a

limited amount (table 39), dilution of these fractions during the steps of IgG separation would cause elimination of some proteins including antigenic molecules.

However, the presence of coating immunoglobulins on the plasma membrane of melanoma tumor cells have been also reported by other investigators (Romsdahl and Cox, 1973 ; Gupta and Morton, 1975). This could possibly be a factor involved in the mechanism by which antigenic tumor can escape from immunological destruction by host lymphocytes, and will require further consideration when studying the immunology of malignant conditions, such as malignant melanoma.

IV. ISOLATION OF MELANOMA CELL SURFACE ANTIGENS

The above studies described the detection and isolation of tumor antigens on the surface of human malignant melanoma cells. This was performed by isolation of cell surface membranes by differential and sucrose density gradient centrifugation and finally chromatography of the solubilized membranes on autologous affinity columns constructed from preabsorbed patients' sera and Sepharose 4B. The identification of the antigens was done by crossover-immunoelectrophoresis which has been widely used both for antigen and antibody detection (Kohn and Weaver, 1974).

The major advantage of this apporoach is that it permits the isolation of antigens present in the whole cell membrane

even if some antigens may be embedded in the phospholipid bilayer (Leong et al, 1977a, 1977b). There are a number of reports which indicate cell surface antigens provoke specific "in vivo" and "in vitro" cellular and humoral immune responses. These antigens have been detected by a variety of techniques including indirect membrane immunofluorescence (Lewis et al, 1969, 1975 ; Lewis and Phillips, 1972 ; Phillips and Lewis, 1970 ; Morton et al, 1968 ; Romsdahl et al, 1970 ; Potra et al, 1971 ; Leong et al, 1977a, 1977b) ; complement dependent cytotoxic effects of the patients' sera (Lewis, 1967b ; Lewis et al, 1969 ; Bodurtha et al, 1975) or of xenoantisera (Fritze et al, 1976 ; Stuhimiller and Seigler, 1977 ; Hakim, 1977) against surface antigens. Immunoadherence assay of cultured melanoma cell lines (Hiroshi et al, 1976 ; Seibert et al, 1977), and lymphocyte cytotoxicity assay of melanoma patients against either melanoma cells from established cultures, or surgical specimen (Potra et al, 1971 ; De Vries et al, 1971 ; Narin et al, 1972 ; Hellstrom and Hellstrom, 1973 ; Bodurtha et al, 1976). Nevertheless, these methods could not possibly detect tightly bound antigens which are not fully exposed and are well inserted in the membrane matrix.

A variety of methods has also been used to identify antigens once they are solubilized, such as delayed cutaneous hypersensitivity reaction (Stewart, 1969 ; Fass et

al, 1970 ; Hollinshead et al, 1974 ; Roth et al, 1976a, 1976b) ; and leucocyte migration inhibition (Cochran et al, 1972 ; Segall et al, 1972 ; Flak et al, 1973). The solubilization techniques included salt extraction of the cell surface antigens and/or sonication of the extracted membrane components. Moreover, detergent extraction of lactoperoxidase iodinated human melanoma cells in cultures (Bystryn and Smalley, 1977) and also trypsinization of melanoma-associated antigens (Leong et al, 1977a)

The disadvantages of the above techniques are the following : 1) since cytoplasmic (Lewis et al, 1969 ; Morton et al, 1968 ; Oettgen et al, 1968 ; Phillips and Lewis,1970 ; Romsdahl et al, 1970) and nucleolar (Mcbride et al, 1972 ; Bowen et al, 1976) melanoma-associated antigens have been reported to be present in melanoma cells, they would be also released with cell surface antigens when 3M KCl is being used (Bystryn and Smalley, 1977), and, in this condition, most probably only loosely-bound antigenic species would be released; 2) iodination procedure would most possibly detect those antigens which are accessible as well as suitable (i.e. Having sufficient number of tyrosine residues) for lactoperoxidase reaction; 3) enzymatic treatment would digest portions of membrane antigens since active melanoma cell membrane antigenic determinants were apparently retained after trypsinization (Leong et al, 1977a); and finally 4) since the whole membranes are not being isolated

and utilized, some antigens may not have been detected. It is also possible that in the case of whole membrane isolation, some antigenic materials may be lost during isolation and washing steps.

Consequently, the whole cell surface membranes used in this study may permit the identification of more antigenic determinants not detected by the above methods. In addition, the solubilization technique (see Part B, Materials and Methods) allows solubilization of almost 100% of membrane proteins since it has the advantage of salt extraction, sonication, and detergent solubilization.

From the foregoing results, the antigens isolated in this study appear to be melanoma specific antigens shared by all melanoma patients involved in the experiments reported here. With respect to the five (5) autologous system by which melanoma antigens have been isolated and used (tables 40-44), it is highly unlikely that they are HLA or other alloantigens. Moreover, the absence of any detectable reaction between the isolated antigens and the sera from normal controls confirms the above consideration, (table 47 and Figs. 21-24). On the other hand, the nature of the detected antigens is still a question of considerable importance. Since HLA antigens are the strongest histocompatibility antigens in man (Cunningham, 1977 ; Gill et al, 1978), they could be considered responsible for the observed cross-reactivity, but, this possibility has been

ruled out by the fact that antigens have been isolated in autologous systems using pre-absorbed sera with normal human spleen cells as well as with a variety of non-melanotic tumors. Melanoma cell have often been shown to carry both melanoma-specific and fetal antigens on their surface (Lewis et al, 1973a ; Hollinshead et al, 1974 ; Hollinshead, 1975 ; Irie et al, 1976 ; Fritze et al, 1976 ; Hiroshi et al, 1976 ; Bystryn and Smalley, 1977). In addition, the presence of virus-like particles, with some properties in common with RNA tumor viruses have been also reported in some human melanoma cells (Parsons et al, 1974). It is therefore possible that these antigens are responsible for cross-reactivity among melanomas. There is however no evidence to support the relationship to fetal antigens or to virus-related antigens. It could be useful to test additional patients in the same manner, as well as patients with cancer other than melanoma, and also fetal membranes to determine if such a possibility exist.

Several investigators (Lewis, 1976b ; Lewis and Phillips, 1972 ; Lewis et al, 1969, 1975 ; Everson and Cole, 1966 ; Phillips and Lewis, 1970 ; Bodurtha et al, 1975 ; Seibert et al, 1977) have identified autologous (i.e. Patient specific) melnoma antigens which do not cross-react with sera from other melanomas. Since isolated membrane antigens in this study have been shown to react specifically against patients' own sera as well as allogeneic sera from

other melanomas, (table 47 and Figs. 21-24), the possibility that patient specific antigens have also been isolated but could not be detected with this approach, can not be ruled out. For this reason absorption of each patient's serum with autologous and allogeneic melanoma cells should have been included before testing the sera by crossover- immunoelectrophoresis , but this requires the continuing availability of the target cells, which was not possible at the time.

The results presented here show the molecular weight species of the purified plasma membrane antigens from five(5) melanoma patients to be in the range of 50,000 to 185,000 daltons, as revealed by SDS-polyacrylamide gel electrophoretic studies (table 46 and figs. 19,20). There are seven (7) molecular species in the isolated antigen fraction from patient MC, six (6) from patients DO, DU, RO and four (4) molecular species from patient OL. These differences could possibly be due to the fact that the corresponding autologous sera used for purification of the antigen by affinity chromatography had different titers of membrane specific antibodies, specially in the case of patients MC and OL, as shown by membrane immunofluorescence technique (table 2). The changes in the antibody titer has apparently reflected the protein concentration of the isolated antigens (table 45).

Regardless of the above differences, the detected protein species from all five (5) patients appear to have similar molecular wieghts, with a variation less than 200 daltons in most cases; furthermore, the isolated molecular weight species have also been detected in the original plasma membrane preparations, (tables 27-31).

Because of the small quantity of the tumor cell plasma membranes available from other melanomas involved in this study, it has not been possible to isolate and compare antigens from these patients, but similar molecular weight species have been identified in soluble membrane preparation from four(4) other melanotic patients , (tables 32-35). These proteins have been assumed to be melanoma specific antigens, although they could be subunits of one or more major proteins .

It should be mentioned that the molecular weight of partially purified melanoma cell surface antigens have been described by some workers to be lower and in the range of about 10,000 to 40,000 for antigens prepared from 3M KCl extracts of melanoma tumor cells (Hollinshead, 1974 ; Hollinshead et al, 1975) and of 40,000 to 60,000 for antigens isolated by chromatography of urine from some melanoma patients (Carrel et al, 1973).

There are some other investigators who have reported the molecular weight of melanoma antigens to be greater than

200,000 daltons for antigens found in the tissue culture medium of a single cell line after partial purification by chromatography (Grimm, et al, 1976), and of the similar size for antigens isolated by the same technique as Hollinshead et al, (1974), from a fresh surgical specimen. In addition, Bystryn and Smalley (1977) have reported the molecular weight of lactoperoxidase-iodinated culture melanoma cell surface antigens to be greater than 160,000 daltons.

Inconsistancies in the results could be due to 1) the different sources of tumor materials used, 2) the differnt effect of various solubilization techniques and 3) the existence of multiple patient-specific or melanoma-specific and associated antigens. From an another viewpoint, if fetal antigens are in fact found in melanomas the contradictory results could be due to the expression of different fetal cell membrane antigens in different patients.

The molecular weights of the antigens presented in this study seem to be close to some of the previous findings , but still a firm conclusion can not be drawn since proteins are dissociated into subunits during SDS-acrylamide gel electrophoresis. The present results however appear to confirm some of the previous findings regarding the cross-reactivity of membrane antigens (Morton et al, 1968 ; Romsdahl et al, 1970 ; Potra et al, 1971 : Hollinshead et

al, 1974 ; Hollinshead, 1975 ; Grimm et al, 1976 ; Roth et al, 1976a, 1976b ; Fritze et al, 1976 ; Hiroshi et al, 1976 ; Bystryn and Smalley, 1977 ; leong et al, 1977a , 1977b ; Seibert et al, 1977 ; Stuhimiller and Seigler, 1977), and also the recent observation of Preddie et al (1977, 1978a, 1978b) which described the isolation of individually specific and cross-reactive melanoma antigens from a number of melanoma patients by almost the same overall approaches , except that lactoperoxidase-iodinated tumor cells were used as starting material. The molecular weights of the iodinated antigens were found to be of about 80,000 and 124,000 by SDS-gel electrophoresis. The former molecular weight species does not differ significantly from one of the membrane antigens involved in this study.

TABLE 1

FORMULAS FOR STOCK SOLUTIONS, BUFFERS, AND GELS

A. Stock Solutions

CON ACBIS	10X CON BUFFER (PH 7.4)
Acrylamide (40g)	1.0M Tris (40ml)
Bis (1.5g)	2.0M Sodium acetate (10ml)
H_2O to 100 ml	0.2M EDTA (10ML)
	Acetic acid to pH 7.4
	H_2O to 100 ml

20% (W/W) SDS
1.5% (W/V) Ammonium persulfate
0.5% (V/V) TEMED

B. Electrophoresis Buffer (per liter)

10 x CON BUFFER (100 ML)
20% SDS (50 ML)
H_2O (850 ml)

C. Gels (per 10 ml of solution 5.6% in acrylamide)

Con ACBIS (1.4 ML)
10 x CON BUFFER (1.0 ML)
20% SDS (0.5 ML)
H_2O (5.6 ml)
1.5% Ammonium persulfate (1.0 ml)
0.5% TEMED (0.5 ML)

TABLE 2

SUMMARY OF MIF* RESULTS ON AUTOLOGOUS MELANOMA CELLS

	SERA		RESULTS OF MIF TESTS	
PATIENT	No. of SERUM SAMPLE	Month BETWEEN FIRST AND LAST SERUM samples	No. POSITIVE/ TOTAL TESTS	MIF INDEX + %POSITIVE
MU	4	2	0/4	0%
UR	3	3	0/4	0%
UR	3	3	0/3	0%
GE	4	1	0/4	0%
PR	4	6	0/4	0%
TR	5	8	5/5	6-8%
TU	0	1	0/3	0%
CA	3	2	3/3	6-9%
CO	2	1	2/2	13-14%
DU	3	1	3/3	39-14%
FE	1	-	0/1	0%
OL	4	1	4/4	15-25%
RO	3	1	3/3	55-66%
VE	4	2	4/4	20-60%
MC	3	3	3/3	60-75%
DO	4	1	4/4	35-65%
BG	3	1	3/3	39-40%
DIREC	-	-	0/16	0%

*MIF = Membrane Immunofluorescence.
+Index = Refers to the lowest and the highest percent of positively stained cells at 1:4 serum dilution.

TABLE 3

AMMONIUM SULFATE PRECIPITATION

PATIENTS	DATE OF SERA	MIF INDEX %POSITIVE CELLS	VOLUME OF SERA (ml)	CON.* PROTEIN mg/ml	VOLUME 40% AM.SUL. PPT.** (ml)	CON. PROTEIN mg/ml
VE	16-8 -74	60	1.5	32	1.5	24
MC	7-8 -74	70	1.0	40	1.0	25
DO	6-12-73	72	1.0	-	1.0	40
DU	20-10-76	50	1.2	31	1.2	26.6
RO	30-7 -75	25	1.2	45	1.2	30
OL	4-3 -75	55	1.0	-	1.0	39.6

* = Concentration
** = Ammonium sulfate

TABLE 4

PATIENT VE. EFFLUENT FRACTIONS OF 40% AMMONIUM SULFATE PRECIPITATED SERUM PROTEINS

FRCTIONS	OD 280	FRACTIONS	OD 280	FRACTIONS	OD 280
1	0.0	21	0.03	41	0.2
2	0.0	22	0.01	42	0.15
3	0.0	23	0.01	43	0.10
4	0.0	24	0.05	44	0.35
5	0.0	25	0.05	45	0.46
6	0.0	26	0.15	46	0.49
7	0.0	27	0.09	47	0.5
8	0.0	28	0.05	48	0.55
9	0.0	29	0.05	49	0.60
10	0.0	30	0.04	50	0.95
11	0.02	31	0.03	51	0.60
12	1.6	32	0.04	52	0.39
13	0.85	33	0.06	53	0.20
14	0.76	34	0.10	54	0.15
15	0.3	35	0.17	55	0.09
16	0.23	36	0.23	56	0.01
17	0.18	37	0.28	57	0.00
18	0.10	38	0.31	58	0.0
19	0.05	39	0.21	59	0.0
20	0.03	40	0.21	60	0.0

TABLE 5

PATIENT MC. EFFLUENT FRACTIONS OF 40% AMMONIOUM SULFATE PRECIPITATED SERUM PROTEINS

FRCTIONS	OD 280	FRACTIONS	OD 280	FRACTIONS	OD 280
1	0.0	11	0.5	21	0.03
2	0.0	12	1.2	22	0.01
3	0.0	13	0.3	23	0.02
4	0.0	14	0.12	24	0.04
5	0.0	15	0.08	25	0.06
6	0.0	16	0.06	26	0.08
7	0.0	17	0.04	27	0.17
8	0.0	18	0.03	28	0.06
9	0.0	19	0.02	29	0.04
10	0.09	20	0.03	30	0.02

TABLE 6

PATIENT DO. EFFLUENT FRACTIONS OF 40% AMMONIOUM SULFATE PRECIPITATED SERUM PROTEINS

FRCTIONS	OD 280	FRACTIONS	OD 280	FRACTIONS	OD 280
1	0.0	11	0.03	21	0.04
2	0.0	12	0.56	22	0.03
3	0.0	13	1.5	23	0.03
4	0.0	14	0.51	24	0.06
5	0.0	15	0.26	25	0.10
6	0.0	16	0.12	26	0.12
7	0.0	17	0.09	27	0.07
8	0.0	18	0.07	28	0.04
9	0.0	19	0.06	29	0.04
10	0.0	20	0.05	30	0.04

TABLE 7

PATIENT DU. EFFLUENT FRACTIONS OF 40% AMMONIUM SULFATE PRECIPITATED SERUM PROTEINS

FRCTIONS	OD 280	FRACTIONS	OD 280	FRACTIONS	OD 280
1	0.0	11	0.0	21	0.16
2	0.0	12	0.0	22	0.15
3	0.0	13	0.02	23	0.12
4	0.0	14	0.5	24	0.10
5	0.0	15	1.2	25	0.08
6	0.0	16	0.85	26	0.06
7	0.0	17	0.52	27	0.04
8	0.0	18	0.34	28	0.03
9	0.0	19	0.25	29	0.03
10	0.0	20	0.2	30	0.03

TABLE 8

PATIENT RO. EFFLUENT FRACTIONS OF 40% AMMONIOUM SULFATE PRECIPITATED SERUM PROTEINS

FRCTIONS	OD 280	FRACTIONS	OD 280	FRACTIONS	OD 280
1	0.0	11	0.12	21	0.06
2	0.0	12	0.4	22	0.06
3	0.0	13	0.61	23	0.08
4	0.0	14	0.7	24	0.13
5	0.0	15	1.6	25	0.08
6	0.0	16	0.4	26	0.06
7	0.0	17	0.12	27	0.04
8	0.0	18	0.04	28	0.05
9	0.0	19	0.03	29	0.05
10	0.02	20	0.02	30	0.05

TABLE 9

PATIENT OL. EFFLUENT FRACTIONS OF 40% AMMONIOUM SULFATE PRECIPITATED SERUM PROTEINS

FRCTIONS	OD 280	FRACTIONS	OD 280	FRACTIONS	OD 280
1	0.0	11	0.52	21	0.035
2	0.0	12	0.17	22	0.06
3	0.0	13	0.09	23	0.09
4	0.0	14	0.06	24	0.1
5	0.0	15	0.045	25	0.13
6	0.0	16	0.04	26	0.06
7	0.0	17	0.04	27	0.055
8	0.02	18	0.035	28	0.01
9	0.65	19	0.025	29	0.01
10	1.4	20	0.04	30	0.06

TABLE 10

PATIENT VE. IMMUNODIFFUSION ANALYSIS OF DEAE-CELLULOSE SEPARATED SERUM COMPONENTS (10µl/SAMPLE) AGAINST ANTI-H-S-P AND ANTI-H-IgG

FRACTIONS	AGAINST ANTI-H-S-P*	AGAINST ANTI-H-IgG+	FRACTIONS	AGAINST ANTI-H-S-P	AGAINST ANTI-H-IgG
11	-	-	38	+	-
12	+	+	39	+	-
13	+	+	40	+	-
14	+	+	41	+	-
15	+	+	42	+	-
16	+	+	43	+	-
17	+	+	44	+	-
18	+	+	45	+	-
19	+	+	46	+	-
20	+/-	+/-	47	+	-
21	+/-	-	48	+	-
22	+/-	-	49	+	-
26	+	-	50	+	-
27	+	-	51	+	-
28	+	-	52	+	-
34	+	-	53	+	-
35	+	-	54	+	-
36	+	-	55	+	-
37	+	-	IgG	+	+

* = Anti-human serum proteins
\+ = Anti-human IgG.

TABLE 11

PATIENT MC. IMMUNODIFFUSION ANALYSIS OF DEAE-CELLULOSE SEPARATED SERUM COMPONENTS (10µl/SAMPLE) AGAINST ANTI-H-S-P AND ANTI-H-IgG

FRACTIONS	AGAINST ANTI-H-S-P	AGAINST ANTI-H-IgG	FRACTIONS	AGAINST ANTI-H-S-P	AGAINST ANTI-H-IgG
9	-	-	18	+/-	-
10	+	+	19	+/-	-
11	+	+	20	+/-	-
12	+	+	25	+	-
13	+	+	26	+	-
14	+	+	27	+	-
15	+	+	28	+	-
16	+	+/-	29	+	-
17	+/-	-	30	+/-	-

TABLE 12

PATIENT DO. MICROPRECIPITATION ANALYSIS OF DEAE-CELLULOSE SEPARATED SERUM COMPONENTS (10µl/SAMPLE) AGAINST ANTI-H-IgG

FRACTIONS	ANTI-HUMAN IgG	FRACTIONS	ANTI-HUMAN IgG
11	-	17	+
12	+	18	-
13	+	19	-
14	+	20	-
15	+	21	-
16	+	IgG	+

TABLE 13

PATIENT DU. MICROPRECIPITATION ANALYSIS OF DEAE-CELLULOSE SEPARATED SERUM COMPONENTS (10µl/SAMPLE) AGAINST ANTI-H-IgG

FRACTIONS	ANTI-HUMAN IgG	FRACTIONS	ANTI-HUMAN IgG
13	-	21	+
14	+	22	+
15	+	23	+
16	+	24	+
17	+	25	-
18	+	26	-
19	+	27	-
20	+	IgG	+

TABLE 14

PATIENT OL. MICROPRECIPITATION ANALYSIS OF DEAE-CELLULOSE SEPARATED SERUM COMPONENTS (10µl/SAMPLE) AGAINST ANTI-H-IgG

FRACTIONS	ANTI-HUMAN IgG	FRACTIONS	ANTI-HUMAN IgG
8	-	14	+
9	+	15	-
10	+	16	-
11	+	17	-
12	+	18	-
13	+	IgG	+

TABLE 15

PATIENT RÖ. MICROPRECIPITATION ANALYSIS OF DEAE-CELLULOSE SEPARATED SERUM COMPONENTS (10µl/SAMPLE) AGAINST ANTI-H-IgG

FRACTIONS	ANTI-HUMAN IgG	FRACTIONS	ANTI-HUMAN IgG
10	-	17	+
11	+	18	+
12	+	19	+
13	+	20	-
14	+	21	-
15	+	22	-
16	+	IgG	+

TABLE 16

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NULEOTIDASE AND $Na^+ + K^+$ ATPase DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT TR.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ENRICHMENT	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit*/mg PROTEIN	ENRICHMENT
Homogenate	11.3	176.62	0.6	1	17.6	0.06	1
700g Supernatant	8.7	-	1.8	3	-	0.068	1.1
7000g Supernatant	4.5	-	2.0	3.3	-	0.097	1.6
10,000g Supernatant	3.75	-	2.0	3.3	-	0.117	1.9
200,000g Supernatant	2.65	-	1.12	1.8	-	0.07	1.1
Band 1	4.45	10.68(6)**	4.8	8.0	1.0(5.6)	0.49	8.2
Band 2	1.40	1.40	2.0	3.3	0.19	0.28	4.6
Band 3	1.50	0.9	1.2	2.0	0.21	0.29	4.8
Band 4	1.60	1.85	2.32	3.8	0.59	0.37	6.1

= About 2×10^7 tumor cells dated 14-3-77 were used to obtain a preparation of plasma membranes.

* = The unit of activity is defined in Part B (Materials and Methods).

** = The values in brackets show the percentage of recovery over that present in homogenate.

TABLE 17

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NULEOTIDASE AND $Na^+ + K^+$ ATPase DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT CA.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ENRICHMENT	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit*/mg PROTEIN	ENRICHMENT
Homogenate	n.d	-	-	-	-	-	-
700g Supernatant	18	52.1	0.138	1.0	283.5	0.75	1
7000g Supernatant	11.6	-	0.181	1.3	-	1.1	1.4
10,000g Supernatant	10.4	-	0.195	1.4	-	2.5	3.3
200,000 Supernatant	8.2	-	0.140	1.0	-	0.7	0.93
Band 1	2.65	1.8(3.4)**	1.41	10.2	9.6(3.3)	7.25	9.6
Band 2	2.6	0.9	0.75	5.4	5.3	4.15	5.5
Band 3	2.1	0.47	0.45	3.2	3.6	3.5	4.6
Band 4	3.1	1.0	0.65	4.7	7.5	3.2	4.3

= About 2×10^7 tumor cells dated 21-3-77 were used to obtain a preparation of plasma membranes.

* = The unit of activity is defined in Part B (Materials and Methods)

** = The value in brackets show the percentage of recovery over that present in the homogenate.

TABLE 18

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NULEOTIDASE AND $Na^+ + K^+$ ATPase DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT CO.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ENRICHMENT	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit*/mg PROTEIN	ENRICHMENT
Homogenate	17.4	110.57	0.91	1.0	22.1	0.062	1.0
700g Supernatant	8.7	-	0.58	1.7	-	0.089	1.4
7000g Supernatant	6.9	-	0.70	2.0	-	0.109	1.7
10,000g Supernatant	6.5	-	0.768	2.25	-	0.114	1.8
200,000g Supernatant	4.9	-	0.108	0.31	-	0.058	0.9
Band 1	2.3	4.69 (4.2)**	4.08	12.0	1.03 (4.6)	0.896	14.2
Band 2	0.85	1.44	3.4	10.0	0.21	0.50	8.0
Band 3	0.85	1.44	2.7	7.9	0.37	0.438	7.0
Band 4	0.85	1.44	3.6	10.0	0.12	0.29	4.7

= About 2×10^8 tumor cells dated 21-7-77 were used to obtain a preparation of plasma membranes.

* = The unit of activity is defined in Part B (Materials and Methods)

** = The values in brackets show the percentage of recovery over that of homogenate.

TABLE 19

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NUCLEOTIDASE $Na^+ + K^+$ ATPase AND G-6-PHOSPHATASE DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT DU.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase			G-6-PHOSPHATASE		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+
Homogenate	21	468	0.93	1	141.62	0.281	1	65.52	0.13	1
700g Supernatant	12.4	-	2.31	2.4	-	0.5	1.78	-	-	
7000g Supernatant	10.6	-	3.2	3.4	-	0.52	1.85	-	-	
10,000g Supernatant	9.2	-	4.1	4.4	-	0.65	2.32	-	0.43	3.3
200,000g Supernatant	5.0	-	1.4	1.5	-	0.5	1.78	-	-	
Band 1	3.2	15.12(3.2)	9.45	10	4.57(3.2)	2.86	10.2	0.3(0.4)*	0.19	1.4
Band 2	4.0	14.16	7.08	7.6	3.04	1.52	5.4	1.18	0.59	4.5
Band 3	4.2	17.01	8.1	8.7	3.78	1.8	6.4		-	-
Band 4	4.2	16.8	8.2	8.9	4.3	2.1	7.5		-	-

= About 5 gm of tumor tissue dated 21-2-75 was used to obtain a preparation of plasma membranes
\+ = Enrichment
* = The values in brackets show percentage of recovery over that of the homogenate.

TABLE 20

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NUCLEOTIDASE $Na^+ + K^+$ ATPase AND G-6-PHOSPHATASE DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT OL.#

FRACTIONS	PROTEIN mg/ml	5'-NUCLEOTIDASE. TOTAL ACTIVITY µmoles Pi/hr	5'-NUCLEOTIDASE. SPECIFIC ACTIVITY unit/mg PROTEIN	5'-NUCLEOTIDASE. ERT+	$Na^+ + K^+$ ATPase TOTAL ACTIVITY µmoles Pi/hr	$Na^+ + K^+$ ATPase SPECIFIC ACTIVITY unit/mg PROTEIN	$Na^+ + K^+$ ATPase ERT+	G-6-PHOSPHATASE TOTAL ACTIVITY µmoles Pi/hr	G-6-PHOSPHATASE SPECIFIC ACTIVITY unit/mg PROTEIN	G-6-PHOSPHATASE ERT+
Homogenate	19.2	1344	3.5	1.0	76.8	0.2	1.0	19.2	0.05	1.0
700g Supernatant	10.6	-	5.2	1.4	-	0.52	2.6	-	0.09	1.8
7000g Supernatant	8.8	-	7.2	2.05	-	0.7	3.5	-	-	-
10,000g Supernatant	8.2	-	7.3	2.08	-	0.7	3.5	-	0.2	4
200,000g Supernatant	6.4	-	2.9	0.82	-	0.09	0.45	-	-	-
Band 1	2.8	43.26 (3.2)	30.9	8.8	1.9 (2.4)*	1.36	6.8	0.11 (0.5)	0.08	1.6
Band 2	3.2	27.36	17.1	4.8	1.0	0.68	3.4	0.46	0.29	5.8
Band 3	2.4	18.72	15.6	4.4	0.79	0.66	3.3	3.39	0.33	6.6
Band 4	1.5	11.3	15.1	4.3	0.57	0.76	3.8	-	-	-

= About 5 gm of tumor tissue dated 21-2-75 was used to obtain a preparation of plasma membranes
+ = Enrichment
* = The values in brackets show percentage of recovery over that of the homogenate.

TABLE 21

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NUCLEOTIDASE AND $Na^+ + K^+$ ATPase DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT RO.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY* unit/mg PROTEIN	ENRICHMENT	TOTAL ACTIVITY µmoles Pi /hr	SPECIFIC ACTIVITY unit*/mg PROTEIN	ENRICHMENT
Homogenate	30.0	317.4	0.46	1.0	144.9	0.21	1.0
700g Supernatant	26.8	-	0.65	1.4	-	0.429	2.0
7000g Supernatant	26.2	-	0.66	1.4	-	0.47	2.2
10,000g Supernatant	23.4	-	0.74	1.6	-	0.53	2.5
200,000g Supernatant	22.0	-	0.56	1.2	-	0.06	0.28
Band 1	1.9	7.41 (2.3)**	7.80	16.9	2.07 (1.4)	2.18	10.30
Band 2	-	-	-	-	-	-	-
Band 3	4.1	8.8	4.3	9.3	1.14	0.56	2.6
Band 4	4.8	11.2	4.7	10.2	0.48	0.20	0.95

= About 5gm of tumor tissue dated 5-5-75 was used to obtain a preparation of plasma membranes.
* = The unit of activity is defined in Part B (Materials and Methods).
** = The values in brackets show the percentage of recovery over that present in homogenate.

TABLE 22

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NUCLEOTIDASE $Na^+ + K^+$ ATPase AND G-6-PHOSPHATASE DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT VE.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase			G-6-PHOSPHATASE		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+
Homogenate	29.6	523.3	0.52	1	60.3	0.06	1	37.2	0.037	1
700g Supernatant	25.6	-	0.68	1.3	-	0.23	3.8	-	0.039	1.05
7000g Supernatant	18.2	-	1.25	2.4	-	0.34	5.6	-	-	-
10,000g Supernatant	17.0	-	1.5	2.8	-	0.52	8.6	-	0.196	5.8
200,000g Supernatant	15.2	-	0.81	1.5	-	0.019	0.3	-	-	-
Band 1	2.7	6.8(1.2)	5.1	9.8	1.45(2.4)	1.02	17.0	0.13(0.3)*	0.1	2.7
Band 2	0.8	1.44	3.6	6.9	0.23	0.58	9.6	-	-	-
Band 3	3.0	4.95	3.3	6.3	1.2	0.8	13	0.96	0.64	17.2
Band 4	3.4	4.25	2.5	5.0	0.22	0.13	2.1	-	-	-

= About 5 gm of tumor tissue dated 13-8-76 was used to obtain a preparation of plasma membranes
\+ = Enrichment
* = The values in brackets show percentage of recovery over that of the homogenate.

TABLE 23

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NUCLEOTIDASE $Na^+ + K^+$ ATPase AND G-6-PHOSPHATASE DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT MC.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase			G-6-PHOSPHATASE		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+
Homogenate	30.8	426.8	0.66	1	71.1	0.11	1	20.69	0.032	1
700g Supernatant	22.4	-	1.11	1.6	-	0.26	2.3	-	-	-
7000g Supernatant	11.2	-	1.75	2.3	-	0.41	3.7	-	-	-
10,000g Supernatant	10.0	-	2.8	4.2	-	0.82	7.4	-	-	-
200,000g Supernatant	7.8	-	0.8	1.2	-	0.2	1.8	-	-	-
Band 1	2.4	11.0(2.5)	9.16	13.8	2.3(3.2)*	1.9	17.2	0.12(.75)	0.10	3
Band 2	2.0	4.8	4.8	7.2	0.66	0.66	6	-	-	-
Band 3	0.8	2.12	5.3	8	0.3	0.76	6.9	0.184	0.46	15.6
Band 4	11.2	0.9	1.5	2.2	0.28	0.47	4.2	-	-	-

= About 5 gm of tumor tissue dated 16-6-74 was used to obtain a preparation of plasma membranes
+ = Enrichment
* = The values in brackets show percentage of recovery over that of the homogenate.

TABLE 24

RECOVERY AND DEGREE OF PURIFICATION OF 5'-NUCLEOTIDASE $Na^+ + K^+$ ATPase AND G-6-PHOSPHATASE DURING ISOLATION OF TUMOR CELLS PLASMA MEMBRANES FROM PATIENT DO.#

		5'-NUCLEOTIDASE			$Na^+ + K^+$ ATPase			G-6-PHOSPHATASE		
FRACTIONS	PROTEIN mg/ml	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+	TOTAL ACTIVITY µmoles Pi/hr	SPECIFIC ACTIVITY unit/mg PROTEIN	ERT+
Homogenate	23.2	245.4	0.46	1	128.0	0.24	1	48.02	0.09	1
700g Supernatant	22.0	-	0.54	1.17	-	0.74	3	-	-	-
7000g Supernatant	8.4	-	0.9	1.9	-	0.9	3.7	-	-	-
10,000g Supernatant	6.4	-	2.2	4.7	-	1.5	6.2	-	0.28	3.1
200,000g Supernatant	6.0	-	0.4	0.86	-	0.3	1.2	-	-	-
Band 1	8.0	2.64 (1.0)*	6.6	14.3	1.64 (1.2)	4.1	17	0.04 (.08)	0.10	1.1
Band 2	0.7	1.22	3.5	7.6	0.49	1.4	5.8	0.199	0.57	6.3
Band 3	0.7	0.94	2.7	5.8	0.175	0.5	2.0	0.24	0.71	7.9
Band 4	1.2	1.38	2.9	5	0.456	0.76	3.1	-	-	-

= About 5 gm of tumor tissue dated 30-11-73 was used to obtain a preparation of plasma membranes

\+ = Enrichment

* = The values in brackets show percentage of recovery over that of the homogenate.

TABLE 25

ENZYME MARKERS ACTIVITIES IN PATIENT TUMOR CELL PLASMA MEMBRANES (BAND 1)

PATIENTS	FRACTIONS	5'-NUCLEOTIDASE *UNIT/mg PROTEIN	$Na^{+}+K^{+}$ ATPase UNIT/mg PROTEIN	G-6-PHOSPHATASE UNIT/mg PROTEIN
TR	HOMOGNATE	0.6	0.06	N.D
	BAND 1	4.8(8)+	0.49(8.2)	-
CA	700 G SUPER-NATANT	0.138	0.75	N.D
	BAND 1	1.41(10.2)	7.25(9.6)	-
CO	HOMOGENATE	0.31	0.062	N.D
	BAND 1	4.08(12)	0.896(14.2)	-
DU	HOMOGENATE	0.93	0.281	0.13
	BAND 1	9.45(10)	2.86(10)	0.19(1.4)
OL	HOMOGENATE	3.5	0.2	0.05
	BAND 1	30.9 (8.8)	1.36 (6.8)	0.08 (1.6)
RO	HOMOGENATE	0.46	0.21	N.D
	BAND 1	7.8 (16.9)	2.18 (10.3)	-
VE	HOMOGENATE	0.52	0.06	0.07
	BAND 1	5.1 (9.8)	1.02 (17)	0.1 (2.7)
MC	HOMOGENATE	0.66	0.11	0.032
	BAND 1	9.16 (13.8)	1.9 (17.2)	0.10 (3)
DO	HOMOGENATE	0.46	0.24	0.09
	BAND 1	6.6 (14.3)	4.1 (17)	0.10 (1.1)

* = The unit of activity is defined in part B (Materials and Methods)
\+ = The values in brackets represent an increase in specific activity over that of the homogenate

TABLE 26

MARKER PROTEINS FOR MOLECULAR WEIGHT DETERMINATION

PROTEIN	MOL. WT OF POLYPEPTIDE CHAIN	MOBILITY (CM)	AVERAGE MOBILITY (CM)
Ribonuclease(A)	13,700	0.98 0.99	0.985 + 0.5%
Chymotrypsinogen(A)*	25,000	0.86 0.86	0.86 + 0.0%
Ovalbumin	45,000	0.65 0.61	0.63 + 3.1%
Serum Albumin	68,000	0.52 0.51	0.515 + 0.9%
Aldolase	40,000	0.68	-
β-Galactosidase*	135,000	0.25	-

* = Indicates proteins which, under native conditions, exist as oligomers.

TABLE 27

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT RO

NO. OF BANDS	MOBILITY (CM)	MOLECULAR WEIGHT	NO. OF BANDS	MOBILITY (CM)	MOLECULAR WEIGHT
1	0.045	350,000	14	0.523	68,000
2	0.055	340,000	15	0.569	56,000
3	0.085	310,000	16	0.587	55,000
4	0.128	260,000	17	0.597	53,000
5	0.183	215,000	18	0.633	47,000
7	0.248	175,000	20	0.688	38,000
8	0.275	160,000	21	0.734	33,000
9	0.321	135,000	22	0.78	28,000
10	0.358	120,000	23	0.799	27,000
11	0.394	110.000	24	0.826	24,000
12	0.431	95,000	25	0.872	21,000
13	0.489	77,000	26	0.918	18,000

TABLE 28

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT DU.

NO.OF BAND	MOLECULAR WEIGHT	NO.OF BAND	MOLECULAR WEIGHT
1	350,000	15	85,000
2	325,000	16	73,000
3	320,000	17	62,000
4	275,000	18	58,000
5	220,000	19	51,000
6	20,0000	20	47,000
7	185,000	21	42,000
8	170,000	22	39,000
9	160,000	23	33,000
10	140,000	24	29,000
11	137,000	25	27,000
12	122,000	26	24,000
13	110,000	27	18,000
14	95,000	28	14,000

TABLE 29

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT OL.

NO.OF BAND	MOLECULAR WEIGHT	NO.OF BAND	MOLECULAR WEIGHT.
1	350,000	12	100,000
2	325,000	13	88,000
3	300,000	14	86,000
4	255,000	15	62,000
5	216,000	16	48,000
6	185,000	17	40,000
7	175,000	18	34,000
8	160,000	19	27,500
9	140,000	20	21,000
10	122,000	21	18,000
11	108,00	22	

TABLE 30

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT MC.

NO.OF BAND	MOLECULAR WEIGHT	NO.OF BAND	MOLECULAR WEIGHT
1	340,000	15	71,000
2	230,000	16	67,000
3	190,000	17	61,000
4	175,000	18	55,000
5	170,000	19	50,000
6	160,000	20	47,000
7	145,000	21	44,000
8	130,000	22	39,000
9	117,000	23	36,000
10	100,000	24	33,000
11	87,000	25	28,000
12	84,000	26	24,000
13	79,000	27	20,000
14	76,000	28	17,000

TABLE 31

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT DO.

NO.OF BAND	MOLECULAR WEIGHT	NO.OF BAND	MOLECULAR WEIGHT
1	360,000	11	93,000
2	275,000	12	76,000
3	227,000	13	64,000
4	185,000	14	59,000
5	180,000	15	50,000
6	160,000	16	41,000
7	140,000	17	34,000
8	128,000	18	29,000
9	108,000	19	24,000
10	100,000	20	22,000

TABLE 32

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT TR.

NO. OF BAND	MOLECULAR WEIGHT	NO. OF BAND	MOLECULAR WEIGHT
1	350,000	13	71,000
2	280,000	14	67,000
3	240,000	15	57,000
4	190,000	16	53,000
5	180,00	17	43,000
6	137,000	18	36,000
7	116,000	19	33,000
8	110,000	20	22,000
9	93,000	21	18,500
10	85,000	22	13,700
11	77,000		
12	74,000		

TABLE 33

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT CA.

NO.OF BAND	MOLECULAR WEIGHT	NO.OF BAND	MOLECULAR WEIGHT
1	350,000	14	66,000
2	255,000	15	62,000
3	230,000	16	56,000
4	190,000	17	46,000
5	170,000	18	41,000
6	150,000	19	38,000
7	140,000	20	32,000
8	130,000	21	29,000
9	115,000	22	26,000
10	107,000	23	23,000
11	97,000	24	20,500
12	92,000	25	14,000
13	75,000		

TABLE 34

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT CO.

NO. OF BAND	MOLECULAR WEIGHT	NO. OF BAND	MOLECULAR WEIGHT
1	350,000	14	87,000
2	330,000	15	81,000
3	315,000	16	74,000
4	288,000	17	62,000
5	235,000	18	55,000
6	220,000	19	48,000
7	195,000	20	42,000
8	170,000	21	39,000
9	145,000	22	33,000
10	125,000	23	28,000
11	110,000	24	24,000
12	100,000	25	21,000
13	97,000	26	17,500

TABLE 35

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF TUMOR CELL PLASMA MEMBRANES FROM PATIENT VE.

NO. OF BAND	MOLECULAR WEIGHT	NO. OF BAND	MOLECULAR WEIGHT
1	350,000	14	65,000
2	340,000	15	62,000
3	320,000	16	54,000
4	310,000	17	44,000
5	275,000	18	39,000
6	225,000	19	33,000
7	190,000	20	29,000
8	160,000	21	27,000
9	140,000	22	24,000
10	120,000	23	21,000
11	115,000	24	18,000
12	85,000	24	14,000
13	74,000		

TABLE 36

RESULTS OF DOUBLE DIFFUSION AND CROSSOVER IMMUNOELECTROPHORESIS ON SOLUBILIZED MEMBRANE FRACTIONS AND AUTOLOGOUS PATIENT'S SERUM

FRACTIONS	DOUBLE DIFFUSION	CROSSOVER IMMUNOELECTROPHORESIS
KCl extraction		
Supernatant	-	+
Precipitate	-	-
Sonication & KCl extraction		
Mixture	-	+
Supernatant	-	+
Precipitate	-	-
SDS-solubilization		
Mixture	-	-
Supernatant	-	-
Precipitate	-	-
Triton Solubilization		
Mixture	-	+
Controls		
1M KCl	-	-
1% Triton X -100	-	-
Glycine-HCl	N.D.	-
Anti-Human IgG	++	++
Cytochrome anti-cytochrome oxidase	-	++

TABLE 37

IMMUNODIFFUSION ANALYSIS OF SEPHADEX G-200 SEPERATED MEMBRANE PROTEINS (10µl/SAMPLE) AGAINST ANTI-HUMAN-IgG

FRACTION	DOUBLE DIFFUSION AGAINST ANTI-HUMAN-IgG (ACIDIFIED)	DOUBLE DIFFUSION AGAINST ANTI-HUMAN-IgG (UNACIDIFIED)	DOUBLE DIFFUSION AGAINST VE SERUM (BOTH)
1	-	-	-
2	+	-	-
3	+	-	-
4	+	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	-	-
19	-	-	-
20	-	-	-
21	-	-	-
22	-	-	-
23	-	-	-
24	-	-	-
25	-	-	-
26	-	-	-
27	-	-	-
28	-	-	-
H.IgG	+	+	-

TABLE 38

IMMUNODIFFUSION AND CROSSOVER IMMUNOELECTROPHORESIS ANALYSIS OF SEPHADEX G-200 SEPARATED MEMBRANE PROTEINS (20µl/SAMPLE) AGAINST ANTI-HUMAN-IgG AND PATIENT VE SERUM

FRACTION	OD 280	DOUBLE-DIFFUSION AGAINST ANTI-HUMAN IgG	IMMUNOELECTROPHORESIS AGAINST VE-SERUM
1	0.0	-	-
2	0.0	-	-
3	0.0	-	-
4	0.1	-	-
5	0.19	+	-
6	0.2	+	-
7	0.195	+	-
8	0.165	-	+
9	0.32	-	+
10	0.37	-	++
11	0.21	-	++
12	0.29	-	++
13	0.20	-	+
14	0.155	-	+
15	0.105	-	+
16	0.055	-	+
17	0.04	-	+
18	0.09	-	+
19	0.06	-	-
20	0.05	-	-
21	0.06	-	-
22	0.05	-	-
23	0.09	-	-
24	0.04	-	-
25	0.02	-	-
26	0.07	-	-
27	0.05	-	-
28*	0.05	-	N.D.
29*	0.05	-	N.D.
H-igG	-	+	-
ANTI-H-IgG	-	-	++

* = Peak Fraction 2 & 3 against eluted "in vivo"-bound
* = IgG molecules

TABLE 39

PROTEIN CONCENTRATION OF AUTOLOGOUS IgG AND TUMOR CELL PLASMA MEMBRANES USED FOR THE RUN OF AFFINITY CHROMATOGRAPHY

PATIENT	mg IgG USED FOR BINDING TO SEP 4B	VOLUME SOLUBILIZED MEMBRANES (ml)	CON. OF SOLUBILIZED MEMBRANES mg/ml	TOTAL PROTEIN (mg)
MA	12	1.0	2.4	2.4
DO	10.4	1.8	1.4	2.52
DU	12.96	0.6	3.5	2.1
OL	9.75	0.7	3.5	2.45
RO	13.5	0.6	4.3	2.58

TABLE 40

AFFINITY CHROMATOGRAPHY FRACTIONS OF THE AUTOLOGOUS SOLUBILIZED PLASMA MEMBRANES FROM PATIENT DO.

FRACTIONS	VOLUME (ml)	OD 280	FRACTIONS	VOLUME (ml)	OD 280
FIRST WASH					
1	3	0.57	26	3	0.0
2	"	0.15	27	"	"
3	"	0.05	28	"	"
4	"	0.005	29	"	"
5	"	0.0	30	"	"
6	"	"	KCNS-APPLIED		
7	"	"	31	0.7	"
8	"	"	32	"	"
9	"	"	33	"	"
10	"	"	35	"	"
11	"	"	36		"
12	"	"	36	"	0.06
13	"	"	37	"	0.09
14	"	"	38	"	0.02
15	"	"	39	"	0.005
AG-APPLIED	"	"	40	"	0.0
16	"	"	41	"	"
17	"	0.0	42	"	"
18	"	0.40	43	"	"
19	"	0.49	44	"	"
20	"	0.40	45	"	"
21	"	0.11	46	"	"
22	"	0.02	47	"	"
23	"	0.0	48	"	"
24	"	"	49	"	"
25	"	"	50	"	"

TABLE 41

AFFINITY CHOROMATOGRAPHY FRACTIONS OF THE AUTOLOGOUS SOLUBILIZED PLASMA MEMBRANES FROM PATIENT OL.

FRACTIONS	VOLUME (ml)	OD 280	FRACTIONS	VOLUME (ml)	OD 280
FIRST WASH					
1	3.5	0.4	26	3.5	0.0
2	"	0.2	27	"	"
3	"	0.15	28	"	"
4	"	0.0	KCNS-APPLIED		
5	"	"	29	0.6	"
6	"	"	30	"	"
7	"	"	31	"	"
8	"	"	32	"	"
9	"	"	33	"	"
10	"	"	34	"	"
AG-APPLIED					
11	"	0.02	35	"	0.09
12	"	0.32	36	"	0.06
13	"	0.53	37	"	0.06
14	"	0.33	38	"	0.03
15	"	0.03	39	"	0.02
16	"	0.2	40	"	0.02
17	"	0.1	41	"	0.0
18	"	0.1	42	"	"
19	"	0.0	43	"	"
20	"	"	44	"	"
21	"	"	45	"	"
22	"	"	46	"	"
23	"	"	47	"	"
24	"	"	48	"	"
25	"	"	49	"	"

TABLE 42

AFFINITY CHROMATOGRAPHY FRACTIONS OF THE AUTOLOGOUS SOLUBILIZED PLASMA MEMBRANES FROM PATIENT OL.

FRACTIONS	VOLIUME (ml)	OD 280	FRACTIONS	VOLIUME (ml)	OD 280
FIRST WASH					
1	3	0.3	24	3	0.0
2	"	0.25	25	"	"
3	"	0.15	26	"	"
4	"	0.1	27	"	"
5	"	0.005	KCNS-APPLIED		
6	"	0.0	28	0.5	"
7	"	"	29	"	"
8	"	"	30	"	"
9	"	"	31	"	"
10	"	"	32	"	"
11	"	"	33	"	0.04
12	"	"	34	"	0.09
13	"	"	35	"	0.04
14	"	"	36	"	0.04
15	"	"	37	"	0.02
AG-APPLIED	"	"	38	"	0.0
16	"	0.02	39	"	0.01
17	"	0.48	40	"	0.01
18	"	0.49	41	"	0.0
19	"	0.40	42	"	"
20	"	0.32	43	"	"
21	"	0.14	44	"	"
22	"	0.04	45	"	"
23	"	0.0	46	"	"

TABLE 43

AFFINITY CHOROMATOGRAPHY FRACTIONS OF THE AUTOLOGOUS SOLUBILIZED PLASMA MEMBRANES FROM PATIENT RO.

FRACTIONS	VOLIUME (ml)	OD 280	FRACTIONS	VOLIUME (ml)	OD 280
FIRST WASH					
1	3	0.07	25	3	0.0
2	"	0.04	26	"	"
3	"	0.2	KCNS-APPLIED		
4	"	0.1	27	0.6	"
5	"	0.05	28	"	"
6	"	0.0	29	"	"
7	"	"	30	"	"
8	"	"	31	"	"
9	"	"	32	"	0.01
AG-APPLIED			33	"	0.08
10	"	0.12	34	"	0.04
11	"	0.41	35	"	0.03
12	"	0.4	36	"	0.02
13	"	0.35	37	"	0.01
14	"	0.2	38	"	0.0
15	"	0.1	39	"	"
16	"	0.01	40	"	"
17	"	0.0	41	"	"
18	"	"	42	"	"
19	"	"	43	"	"
20	"	"	44	"	"
21	"	"	45	"	"
22	"	"	46	"	"
23	"	"	47	"	"
24	"	"	48	"	"

TABLE 44

AFFINITY CHOROMATOGRAPHY FRACTIONS OF THE AUTOLOGOUS SOLUBILIZED PLASMA MEMBRANES FROM PATIENT DU.

FRACTIONS	VOLIUME (ml)	OD 280	FRACTIONS	VOLIUME (ml)	OD 280
FIRST WASH					
1	3	0.13	24	3	0.0
2	"	0.27	KNCS-APPLIED		
3	"	0.14	25	0.5	"
4	"	0.05	26	"	"
5	"	0.0	27	"	"
6	"	"	28	"	"
7	"	"	29	"	"
8	"	"	30	"	0.05
9	"	"	31	"	0.09
AG-APPLIED			32	"	0.04
10	"	"	33	"	0.03
11	"	0.06	34	"	0.02
12	"	0.46	35	"	0.01
13	"	0.45	36	"	0.0
14	"	0.4	37	"	"
15	"	0.26	38	"	"
16	"	0.13	39	"	"
17	"	0.08	40	"	"
18	"	0.0	41	"	"
19	"	"	42	"	"
20	"	"	43	"	"
21	"	"	44	"	"
22	"	"	45	"	"
23	"	"			

TABLE 45

PROTEIN CONCENTRATION OF ISOLATED ANTIGEN FRACTIONS BY AFFINITY CHROMATOGRAPHY

PATIENT	VOLUME OF CONCENTRATED FRACTIONS	PROTEINS CONC.
	ml	mg/ml
DO	0.5	0.1
MC	0.5	0.22
OL	0.75	0.133
RO	0.5	0.22
DU	0.5	0.16

TABLE 46

MOLECULAR WEIGHTS OF THE ISOLATED TUMOR CELL PLASMA MEMBRANE ANTIGENS

PATIENT	NO. OF BAND	MOLECULAR WEIGHT	PATIENT	NO. OF BAND	MOLECULAR WEIGHT
DO	1	177,000	RO	1	185,000
	2	160,000		2	170,000
	3	75,000		3	91,000
	4	64,000		4	73,000
	5	57,000		5	66,000
	6	50,000		6	56,000
MC	1	177,000	DU	1	183,000
	2	160,000		2	175,000
	3	73,5000		3	93,000
	4	66,000		4	73,500
	5	61,000		5	66,000
	6	57,000		6	57,000
	7	50,000			
OL	1	185,000			
	2	176,000			
	3	75,000			
	4	66,000			

TABLE 47

CROSSOVER IMMUNOELECTROPHORESIS ON ISOLATED MELANOMA CELL MEMBRANE ANTIGENS

SERA FROM	PATIENT ANTIGEN FROM	MEMBRANE ANTIGEN µg	POSITIVE REACTION	SERA FROM	PATIENT ANTIGEN FROM	MEMBRANE ANTIGEN µg	POSITIVE REACTION
DU	DU	3.2-4	+	NORMAL(1)	DU	3.2-4	-
DO	DO	4.4-5	+	NORMAL(2)	"	"	-
MC	MC	2.4-3	+	NORMAL(3)	"	"	-
RO	RO	4.4-5	++	NORMAL(4)	"	"	-
OL	OL	2.6-3	+	NORMAL(5)	DO	4.4-5	-
DU	DO	4.4-5	+	NORMAL(6)	"	"	-
DU	MC	2.4-3	+	NORMAL(7)	"	"	-
DU	RO	4.4-5	+	NORMAL(8)	"	"	-
MC	RO	4.4-5	+	NORMAL(1)	MC	2.4-3	-
MC	DU	3.2-4	+	NORMAL(2)	"	"	-
MC	OL	2.6-3	+	NORMAL(3)	"	"	-
DO	DU	3.2-4	+	NORMAL(4)	"	"	-
DO	MC	2.4-3	++	NORMAL(5)	RO	4.4-5	-
DO	RO	4.4-5	+	NORMAL(6)	"	"	-
RO	MC	2.4-3	++	NORMAL(7)	"	"	-
RO	OL	2.6-3	+	NORMAL(8)	"	"	-
RO	DO	4.4-5	+	NORMAL(1)	OL	2.6-3	-
OL	DO	4.4-5	+	NORMAL(2)	"	"	-
OL	DU	3.2-4	+	NORMAL(3)	"	"	-
OL	MC	2.4-3	+	NORMAL(4)	"	"	-
				ANTI-H.IgG*	H-IgG		+++
				ANTI-C-O**	C-O		++++

* = Anti-Human IgG
** = Anti cytochrome oxidase

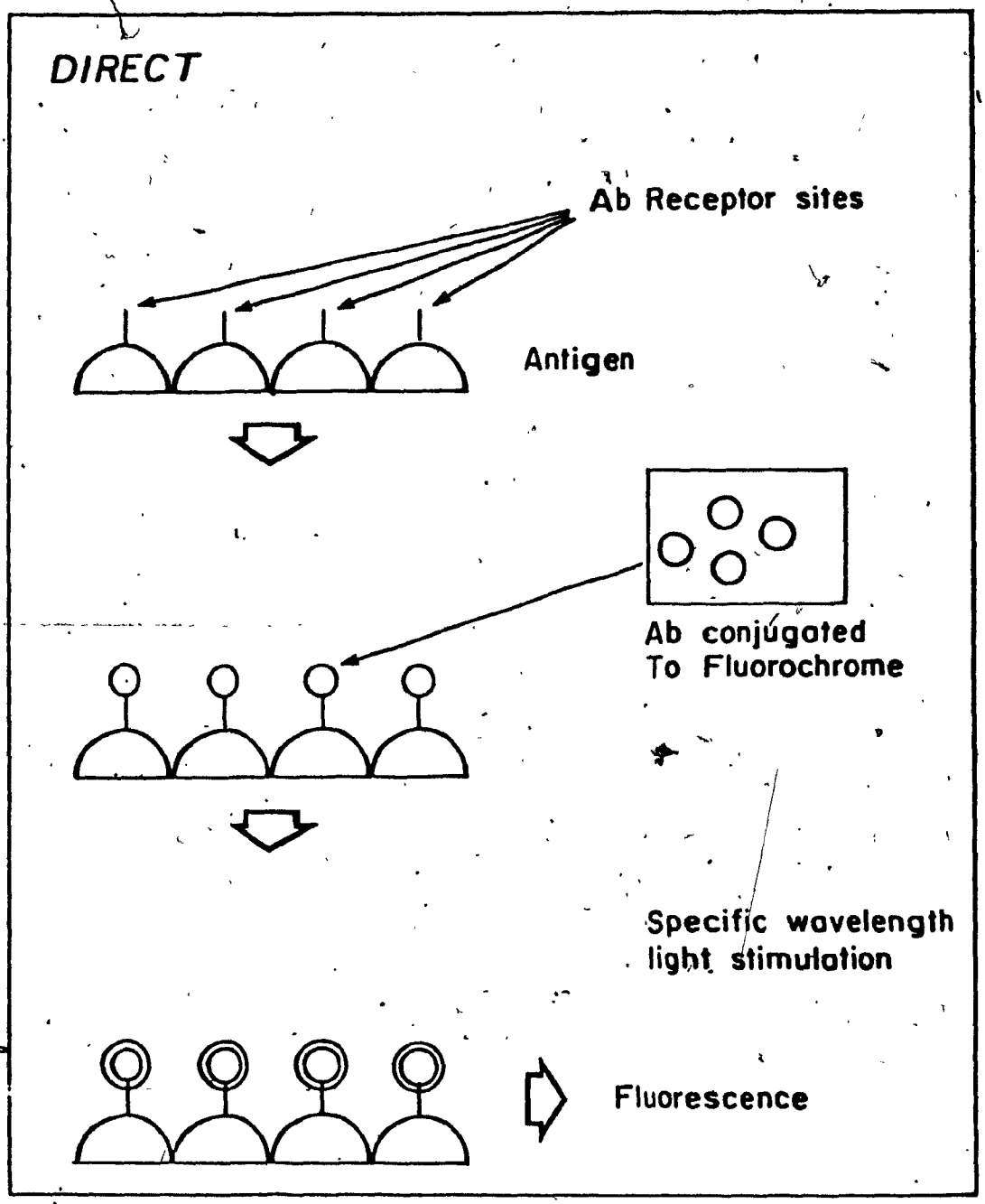

Fig.1. Diagramatic Summary of the Direct Immunofluorescence Technique.

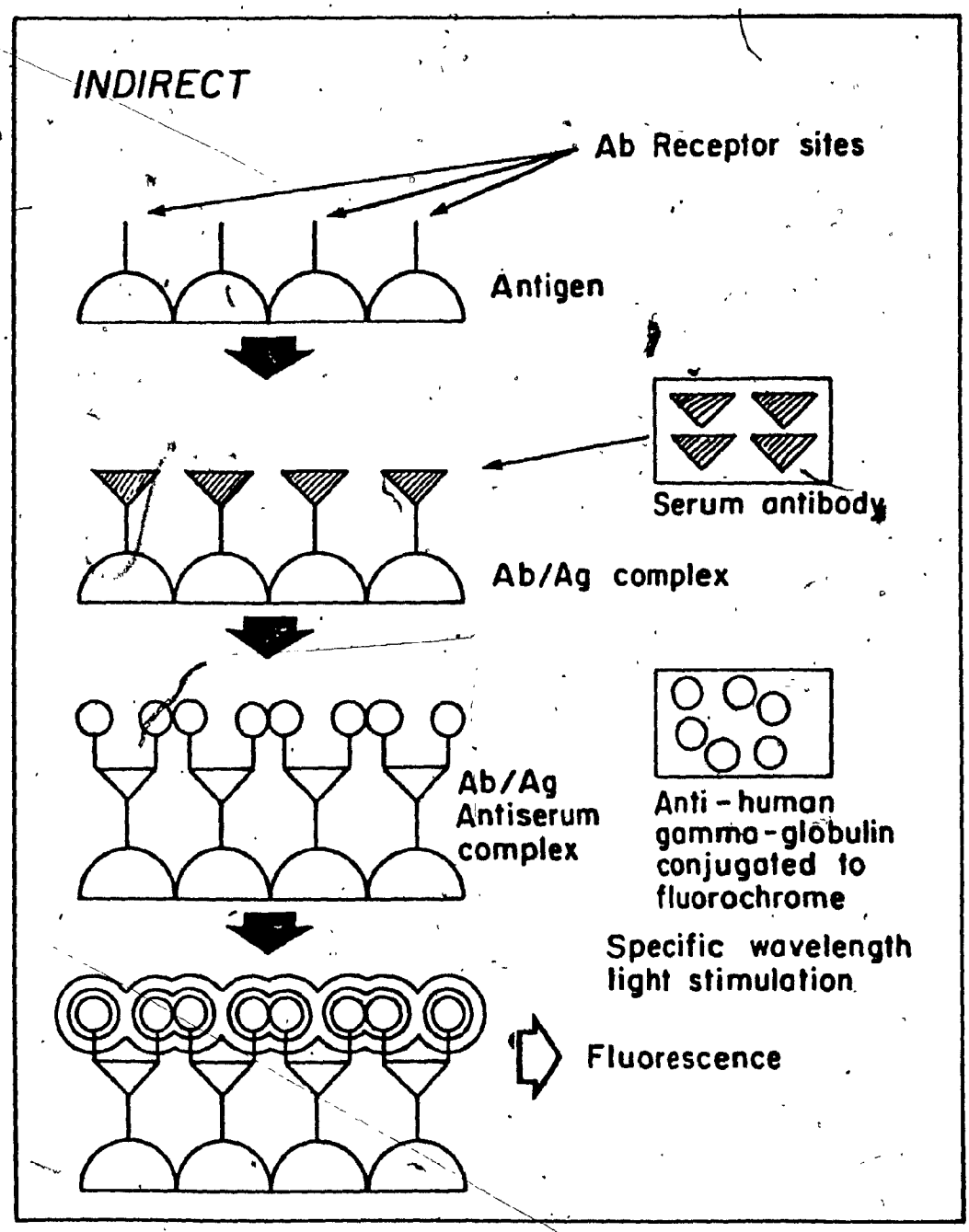

Fig.2. Diagrammatic Summary of the Indirect Immunofluorescence Technique.

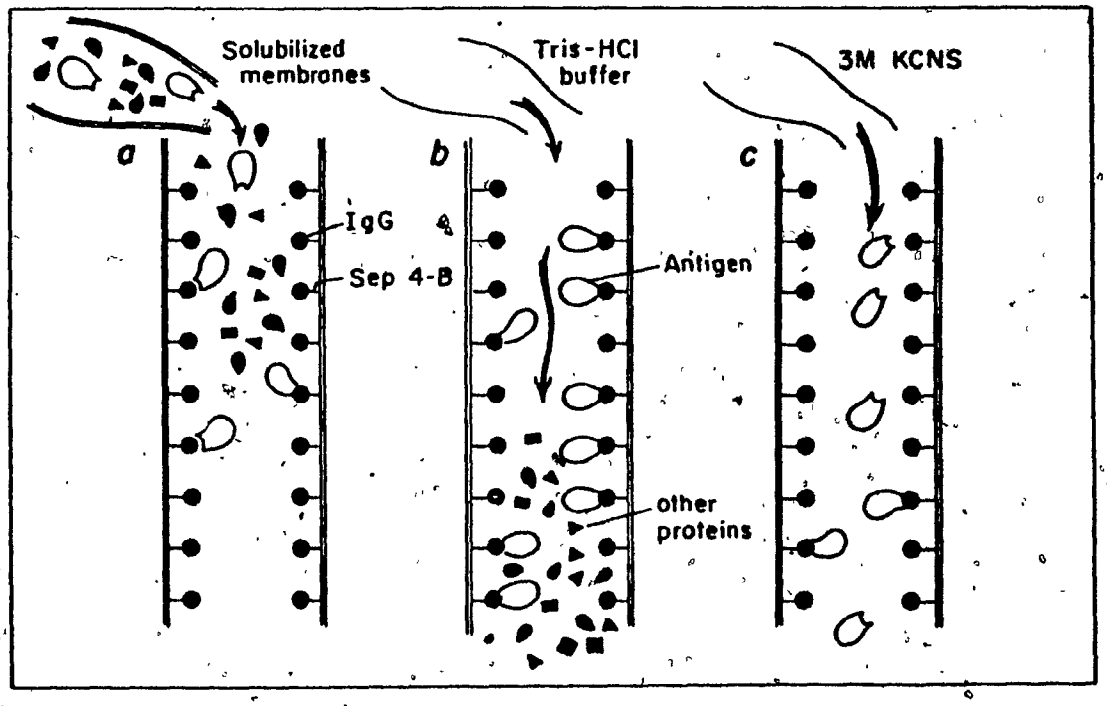

Fig.3. Diagrammatic Summary of the Affinity Chromatography.

Melanoma IgG was bound to CrBr activated Sep 4B, after washing the column; solubilized membrane was poured onto the column (a). The antigens were retained by the fixed IgG molecules, whereas all other proteins were washed away completly (b). Finally the antigens were freed from IgG molecules by eluting the column with 3M KCNS (c).

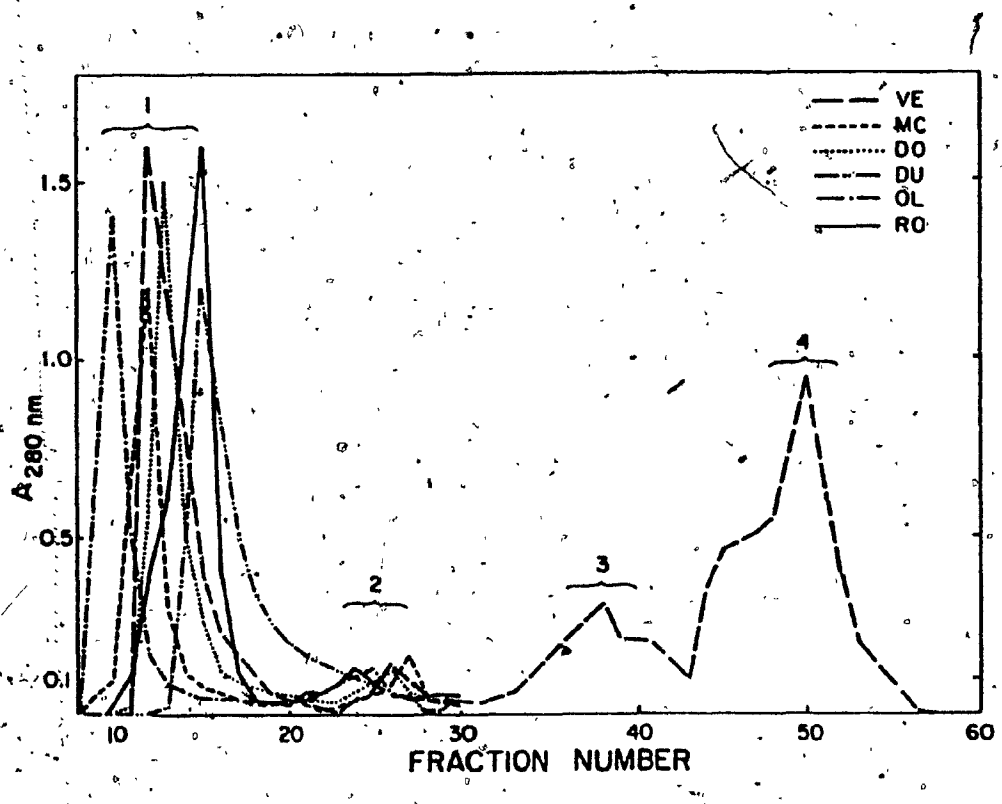

Fig.4. Salt Gradient Elution of Immunoglobulin G from DEAE-Cellulose Column.

The column bed was 20 X 1.5 cm, the starting buffer was 0.01 M KPO_4 pH 7.5, the gradient consisted of 0.005 M and 0.5 M NaCl in the starting buffer. The flow rate was of about 12 ml/hr. Anti-human IgG and anti-human serum proteins were used to localize IgG to its respective peak fraction (i.e. Peak No.1)

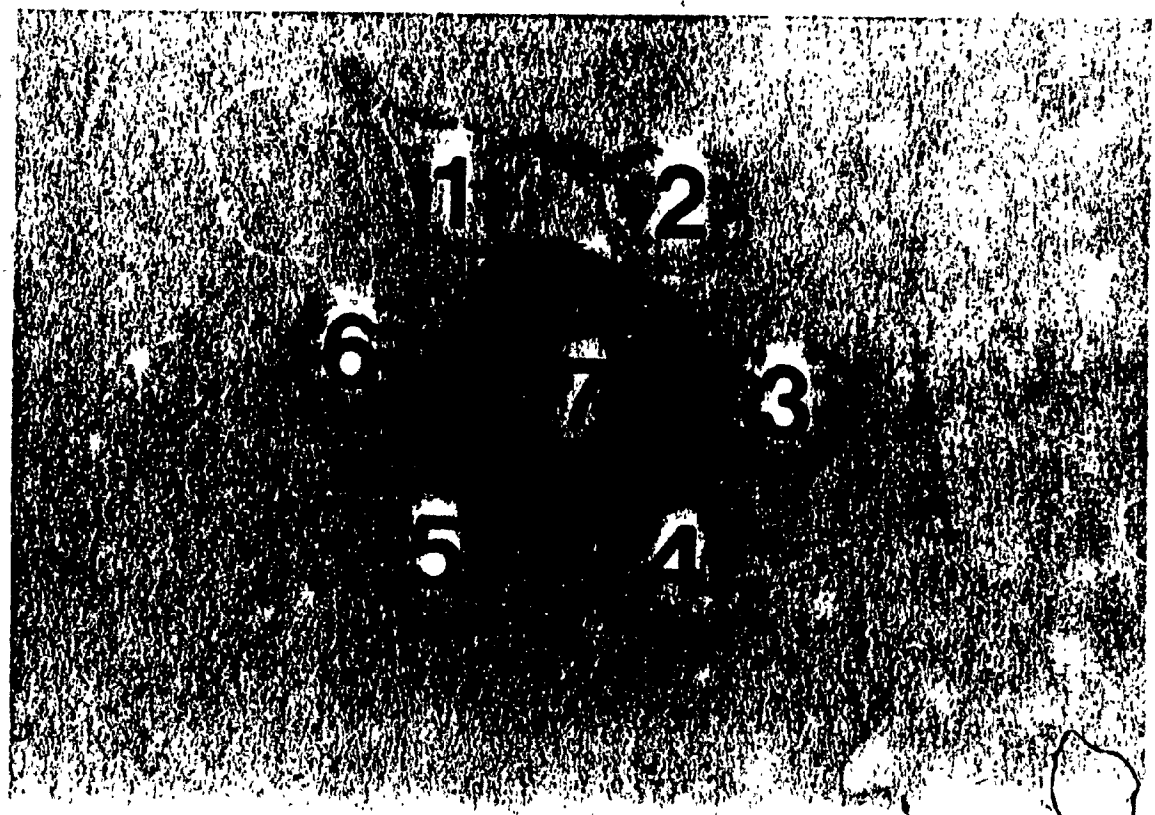

Fig.5. Immunodiffusion of Isolated IgG Peak Fractions (see Fig. 4) from Patient (VE) Serum Against Rabbit Anti-Human IgG.

1) fraction No.11; 2) fraction No.12; 3) fraction No.13 4) concentrated IgG positive fractions; 5) fraction No.15; 6) commercially prepared human IgG; 7) anti-human IgG.

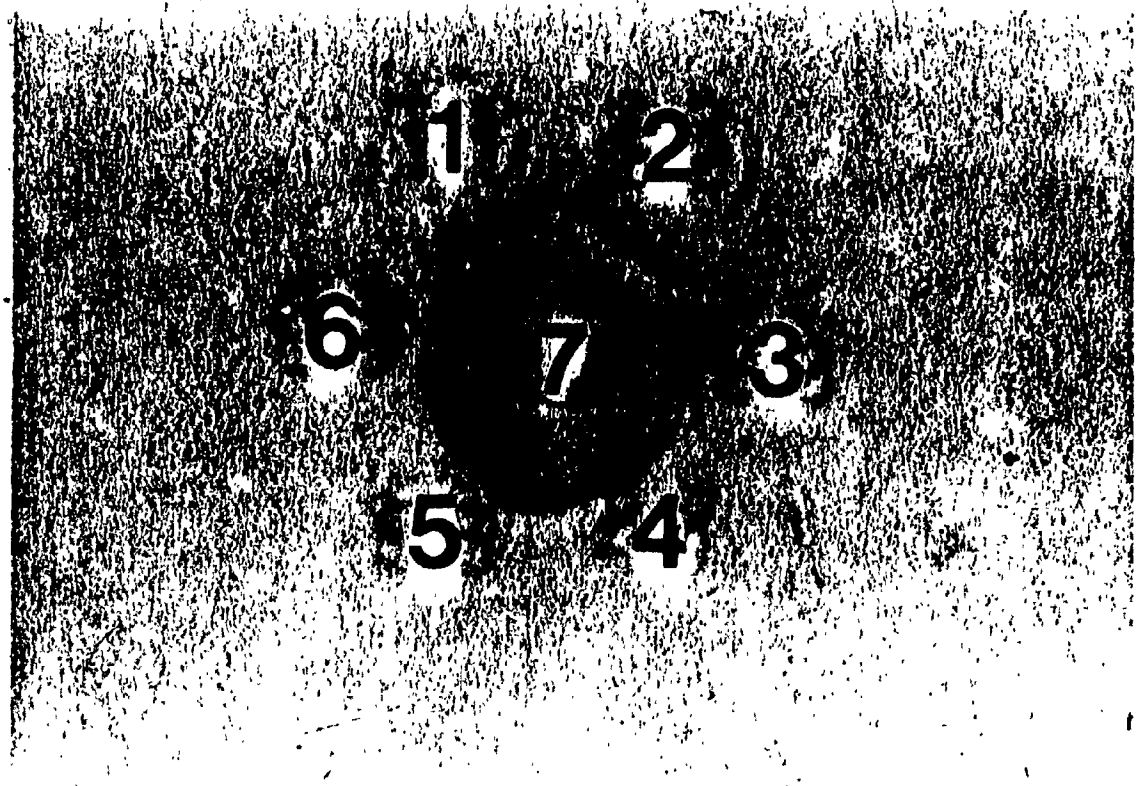

Fig.6. Immunodiffusion of Isolated IgG Peak Fractions (See Fig.4) from Patient (VC) Serum Against Rabbit Anti-Human IgG.

1) fraction No.10; 2) fraction No.11; 3) fraction No.12 4) concentrated IgG positive fractions; 5) fraction No.13; 6) commercially prepared human IgG; 7) anti-human IgG.

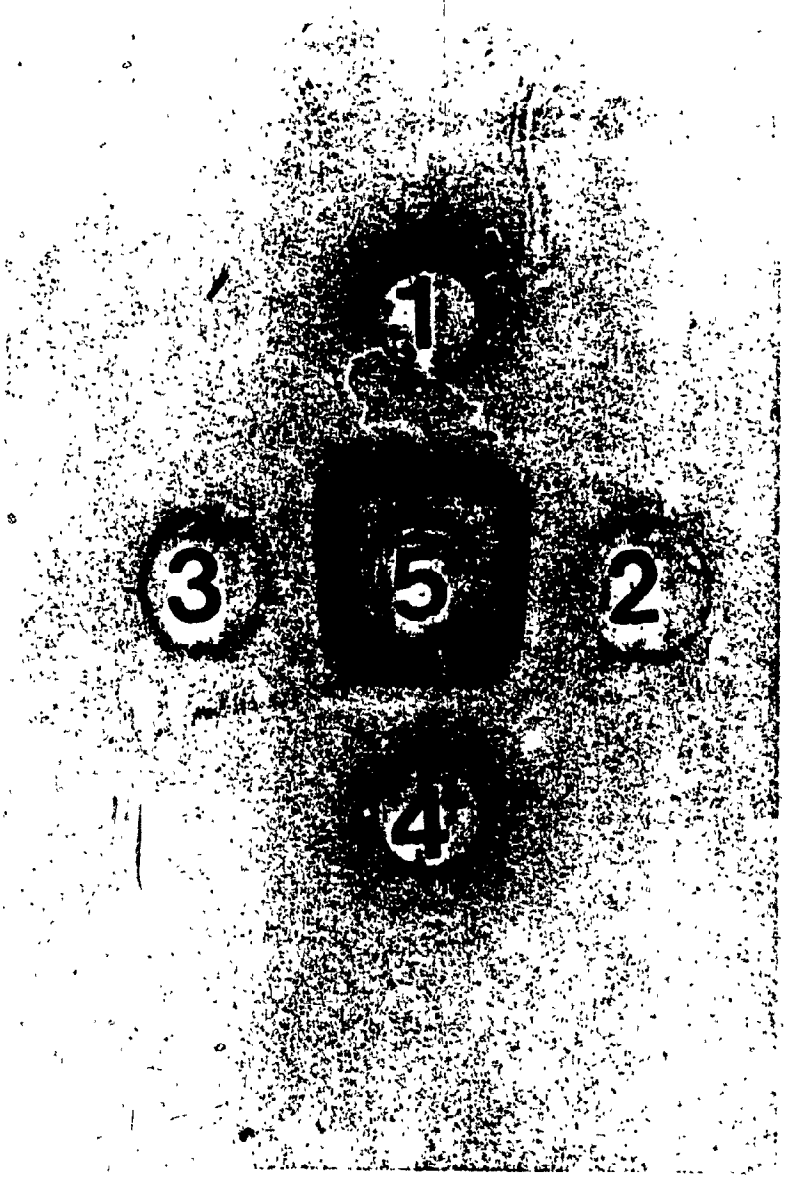

Fig.7. Immunodiffusion of Concentrated IgG Positive Fraction from Patients (VE) and (MC) Sera Against Rabbit Anti-Human Serum Proteins.

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1) Concentrated IgG positive fractions from patient (VE); 2 and 4) Commertially prepared human IgG; 3) Concentrated IgG positive fraction from patient (MC); 5) Rabbit anti-human serum proteins.

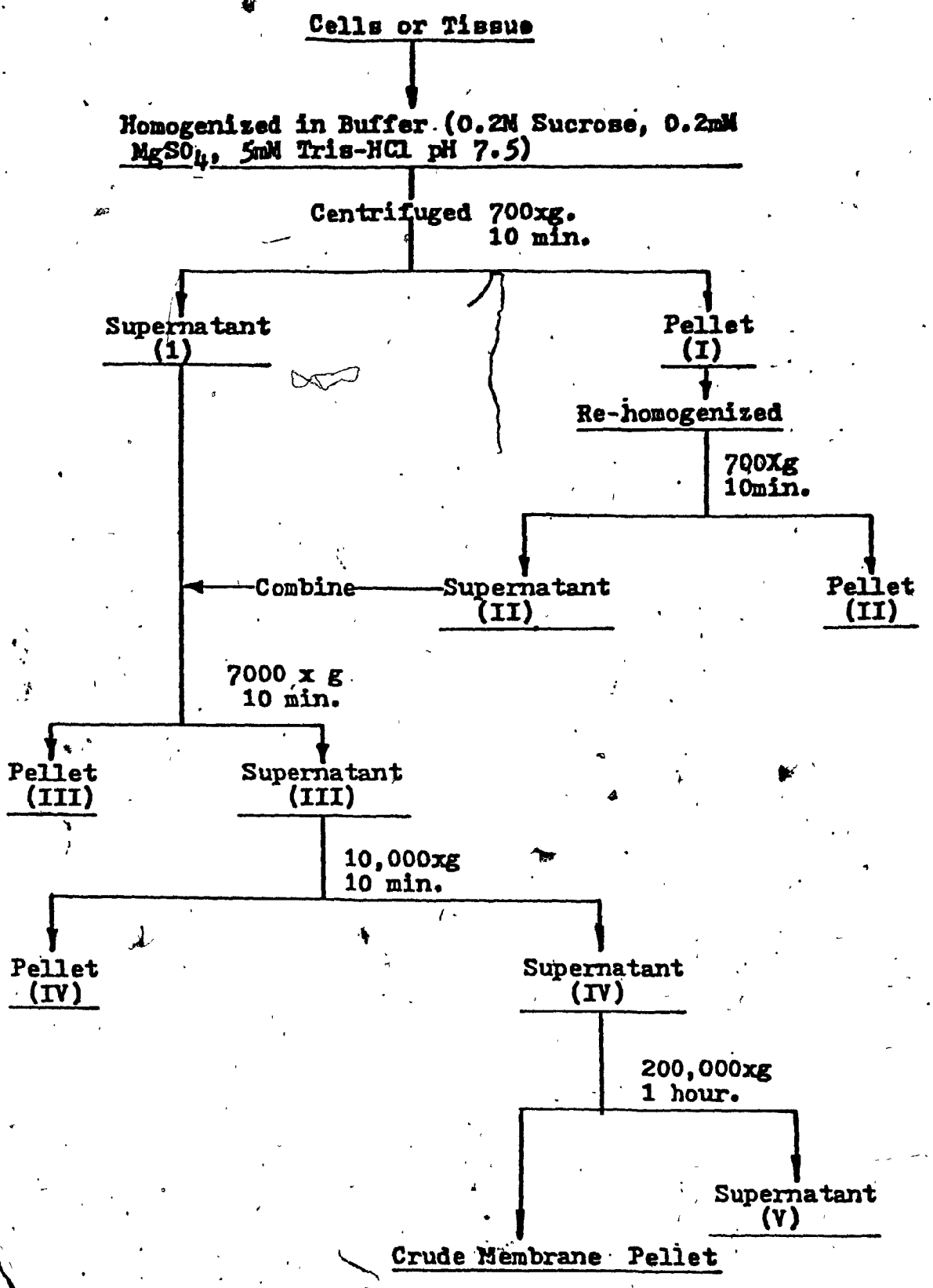

Fig.8. Diagram Illustrating Fractionation Procedure for the Preparation of Crude Tumor Cell Plasma Membranes.

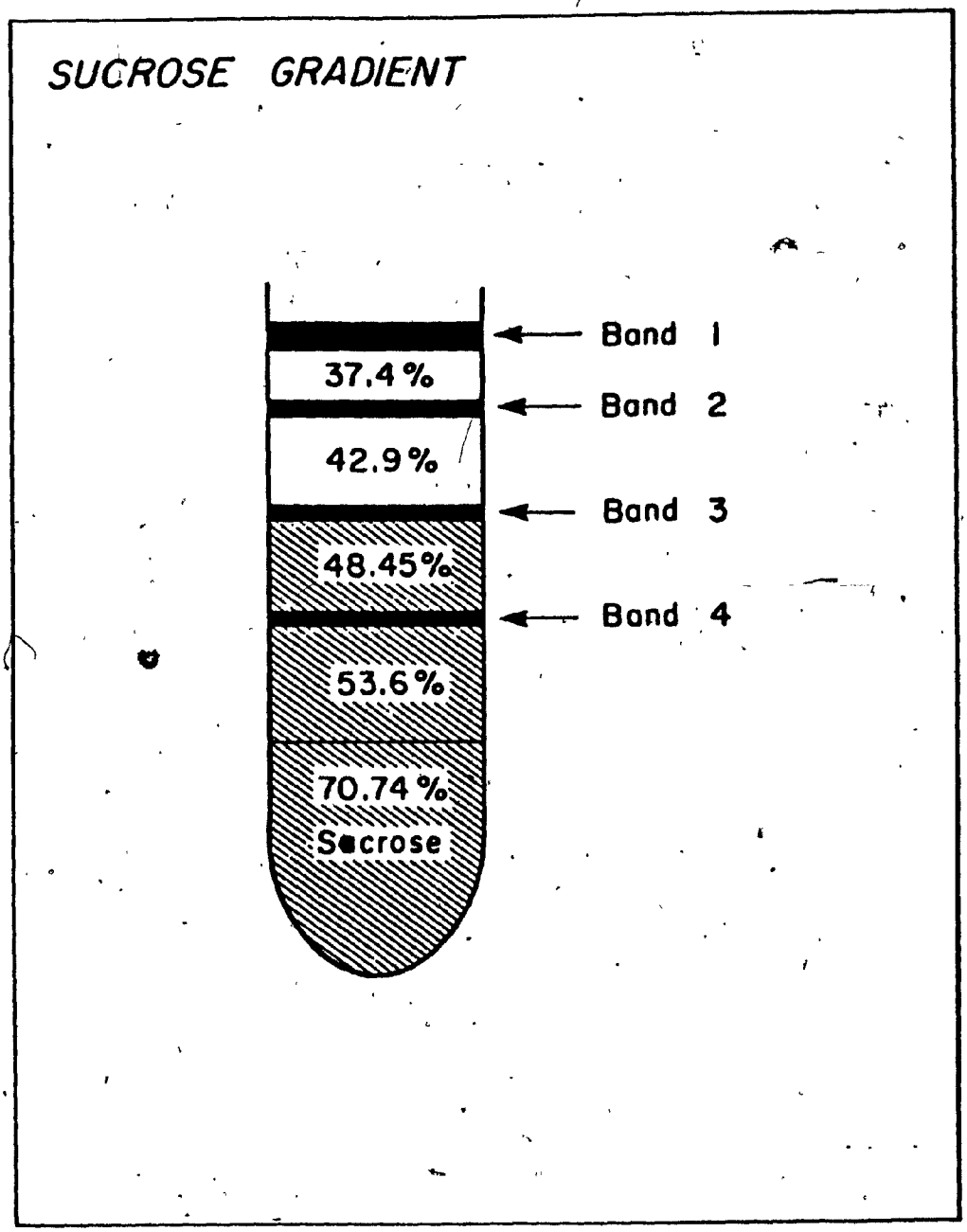

Fig.9. Sucrose Density Gradient Centrifugation of the 200,000g Precipitate (Plasma Membrane Enriched Fraction).

Centrifugation was performed at 66,000g for 75 min; protein bands were collected and examined for enzyme marker activities.

Fig.10. Electronmicrograph of a Cell Membrane Preparation of Melanoma Tumor Cells.

Fig.11. Electronmicrograph of a Crude Membrane Preparation (200,000g Pellet) of Melanoma Tumor Cells.

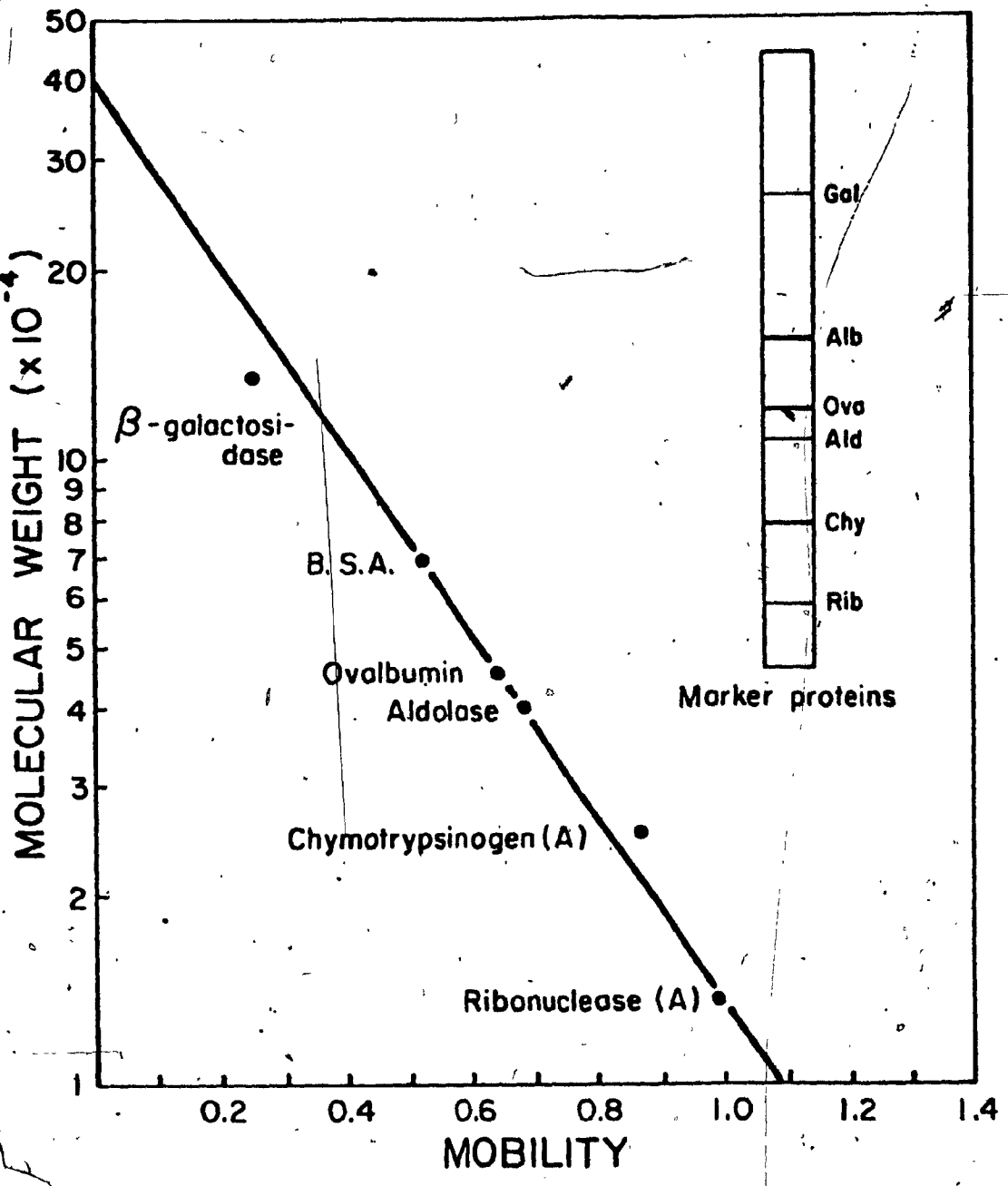

Fig.12. Calibration Curve for the Determination of the Molecular Weight of the Polypeptide Chains of Cell Surface Membranes.

The six marker proteins were β-galactosidase, BSA, ovalbumin, aldolase, chymotrypsinogen (A) and ribonuclease (A). All proteins were run on duplicate gels except β-galactosidase and aldolase. Higher molecular weights were estimated by extrapolation of the calibration curve.

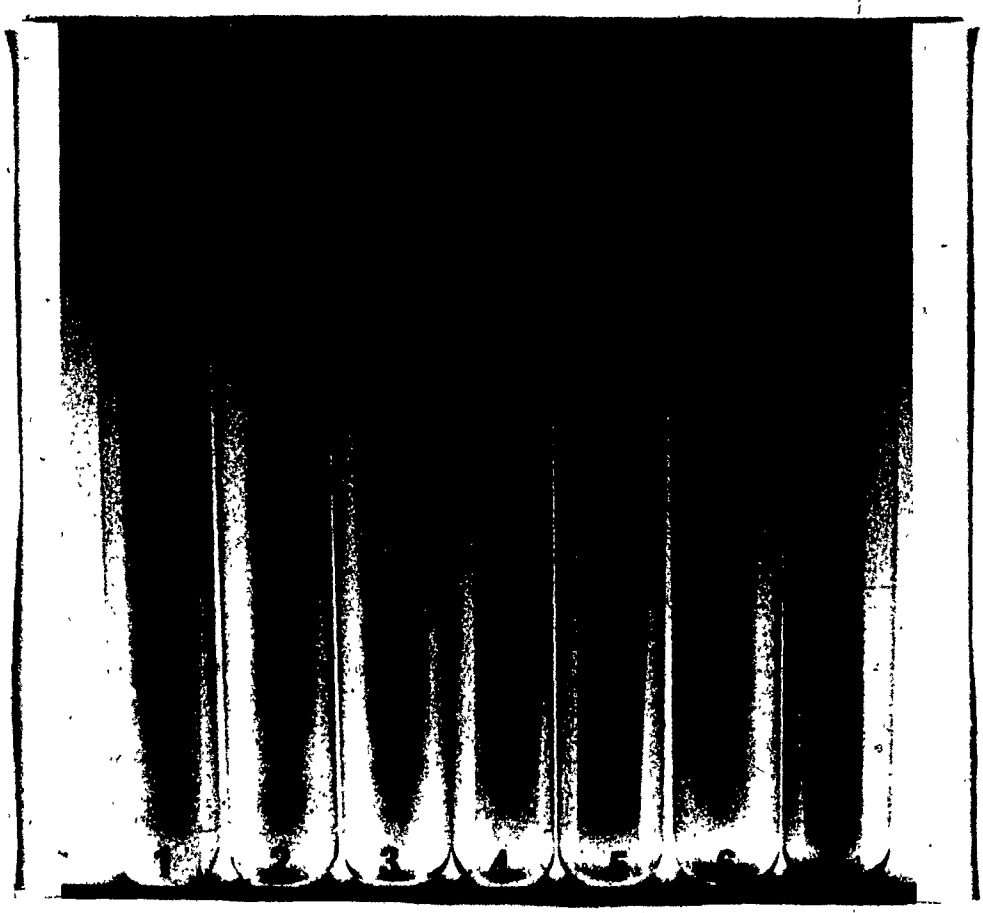

Fig.13. SDS-polyacrylamide Gel Electrophoresis Separation of Tumor Cell Plasma Membranes of Metastatic Melanoma Tissues.

Gels were stained with Coomassie brilliant blue. Sample loads were approx. 150 µg. From left to right melanoma cell membranes were from patients 1) RO; 2) VE; 3) DU; 4) TR; 5) CA; 6) CO; 7) MC.

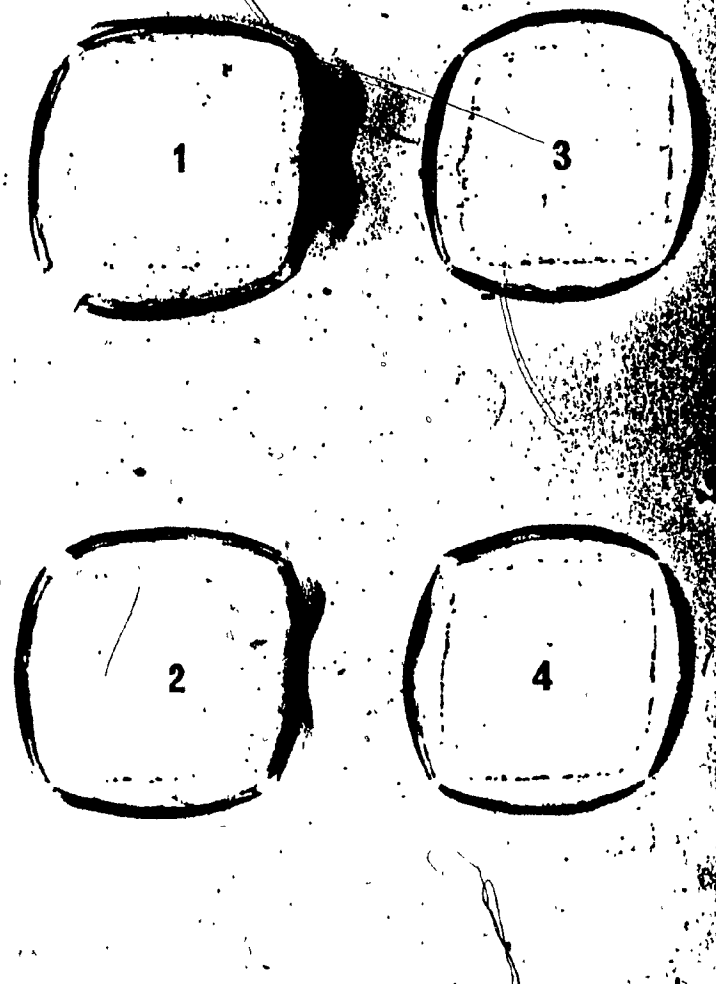

Fig.14. Crossover Immunoelectrophoresis of Solubilized Plasma Membrane Antigens from Patient (VE) Against the Autologous Preabsorbed Sera.

Well No.1 and 2, patient (VE) sera preabsorbed with normal human spleen cells and a variety of nonmelanotic cells; well No.4 1M KCl extract of plasma membranes from (VE); Well No.3 1M KCl extract of sonicated (VE) plasma membranes.

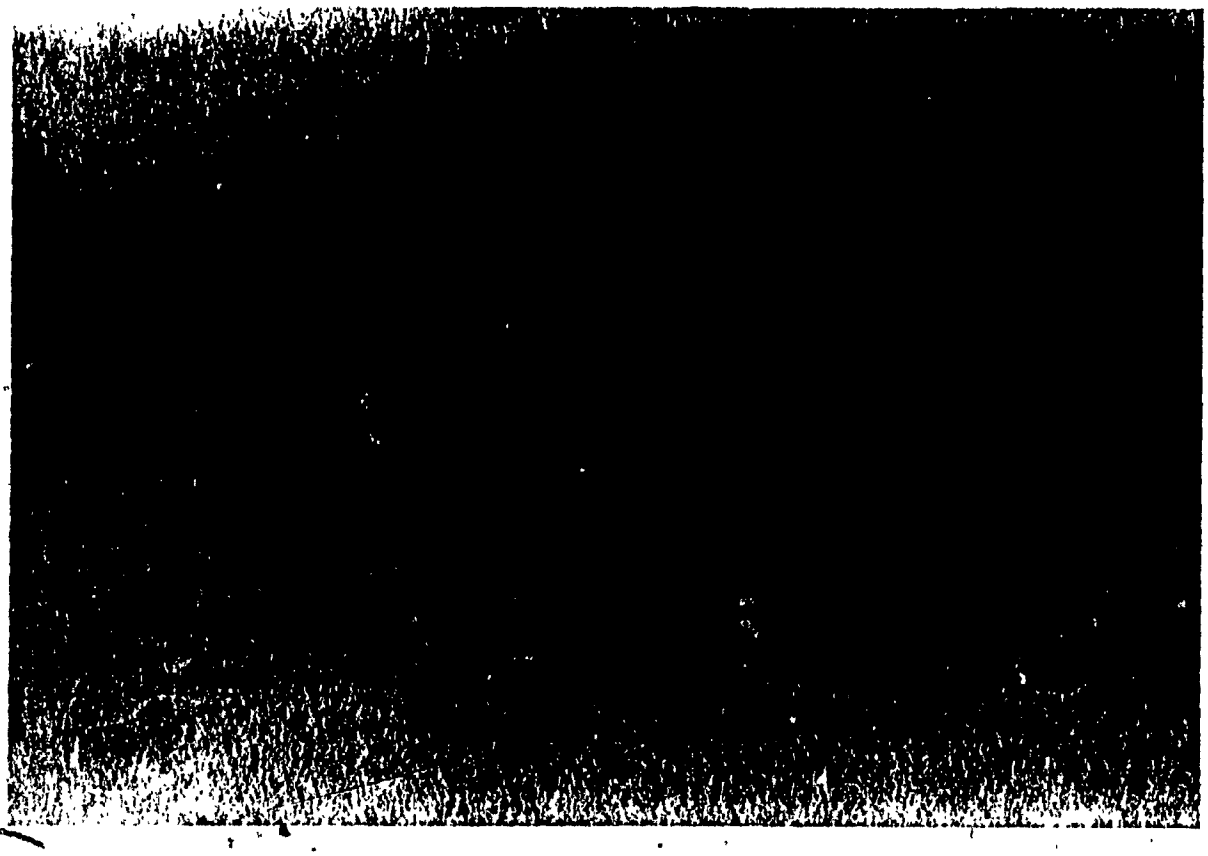

Fig.15. Immunodiffusion of Fractionated Membrane Bound IgG, Through Sephadex-G200, Against Anti-Human IgG.

Well No. 1 and 2 fractions; Well No. 3 human IgG; Well No.4,5, and 6, fractions 3,4 and 5; Well No.7, rabbit anti-human IgG.

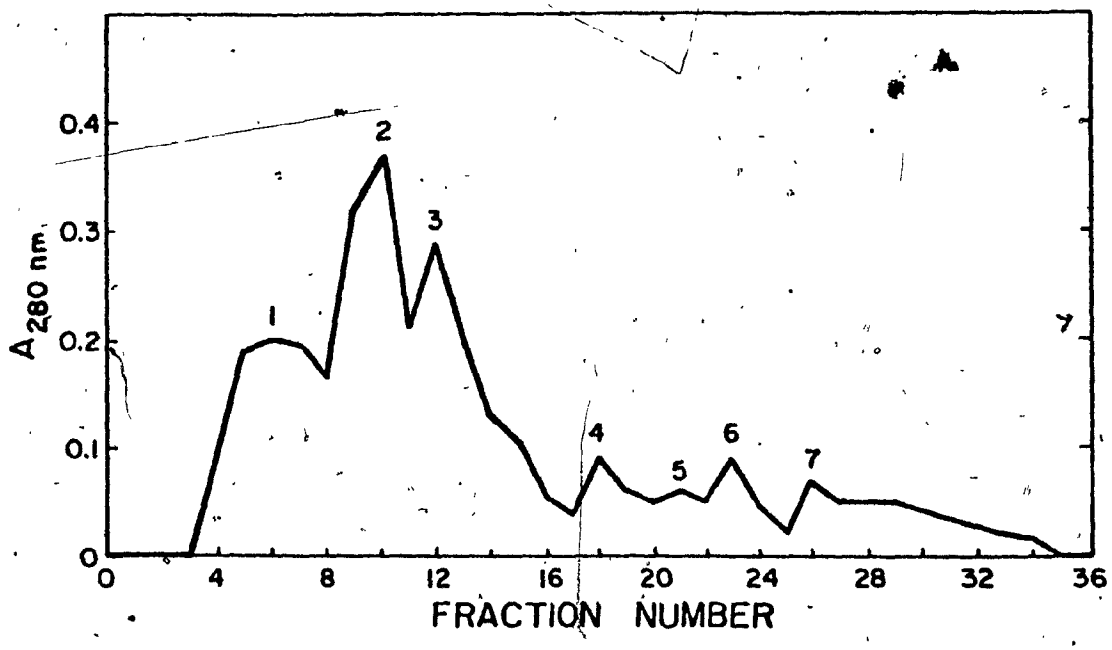

Fig.16.- Separation of Membrane Bound IgG of Patient (VE) Solubilized Membrane Fraction by Gel Filtration Through Sephadex G-200.

The column bed was 2 X 10 cm, The buffer was 0.2M clycine-HCl pH 2.2, and the flow rate was of about 2 drops/5 mins. Anti-human IgG and (VE) serum were used to localize IgG and antigenic components to their respective peak fraction.

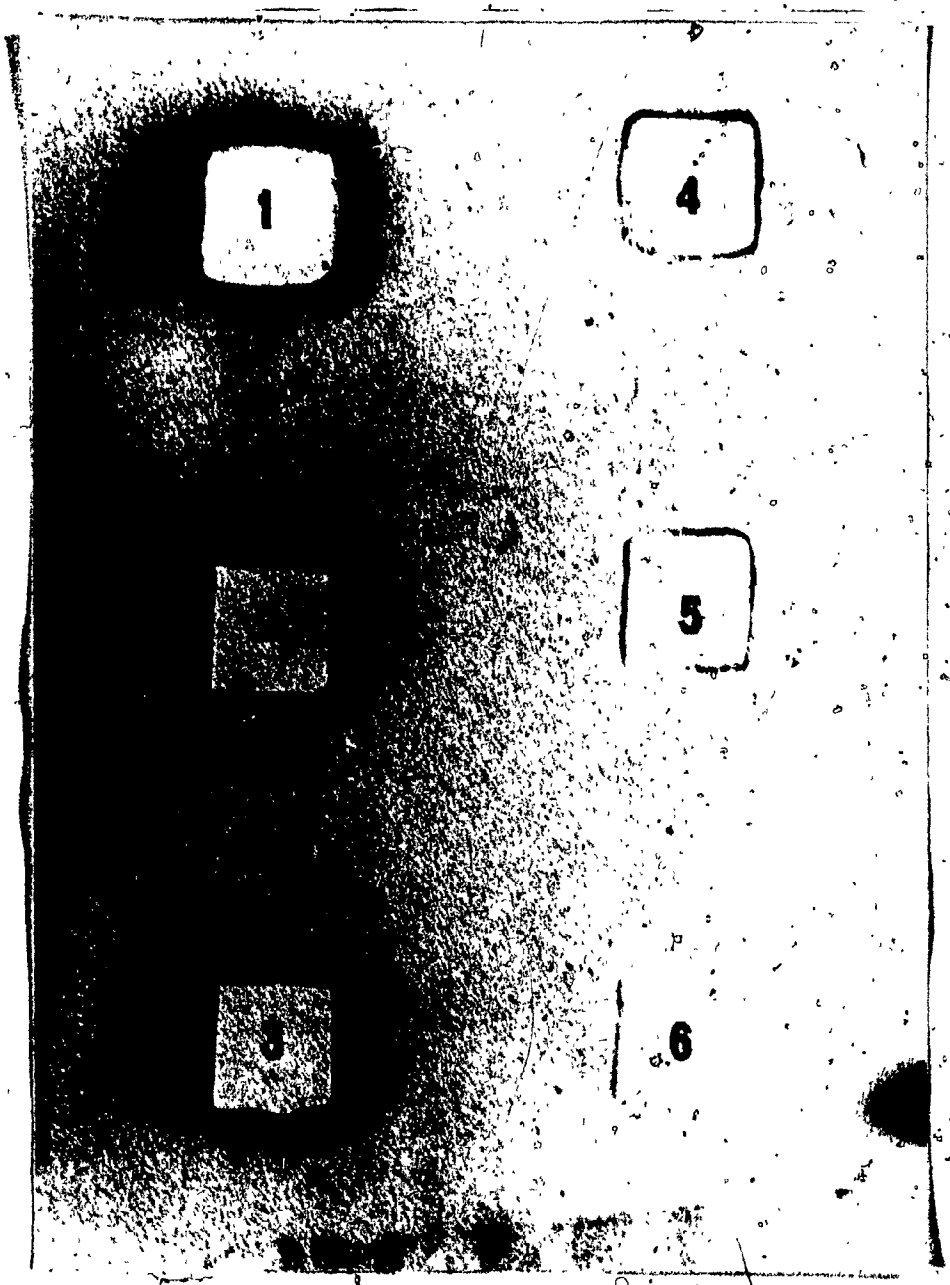

Fig.17. Crossover Immunoelectrophoresis of Plasma Membrane Proteins (Fractionated Through Sephadex G-200) of Patient (VE) against the autologous serum.

Well No.1, 2 and 3, preabsorbed sera from patient (VE); Well No.4, 5 and 6, contained fractions 10,12 and 18, eluted from Sephadex column.

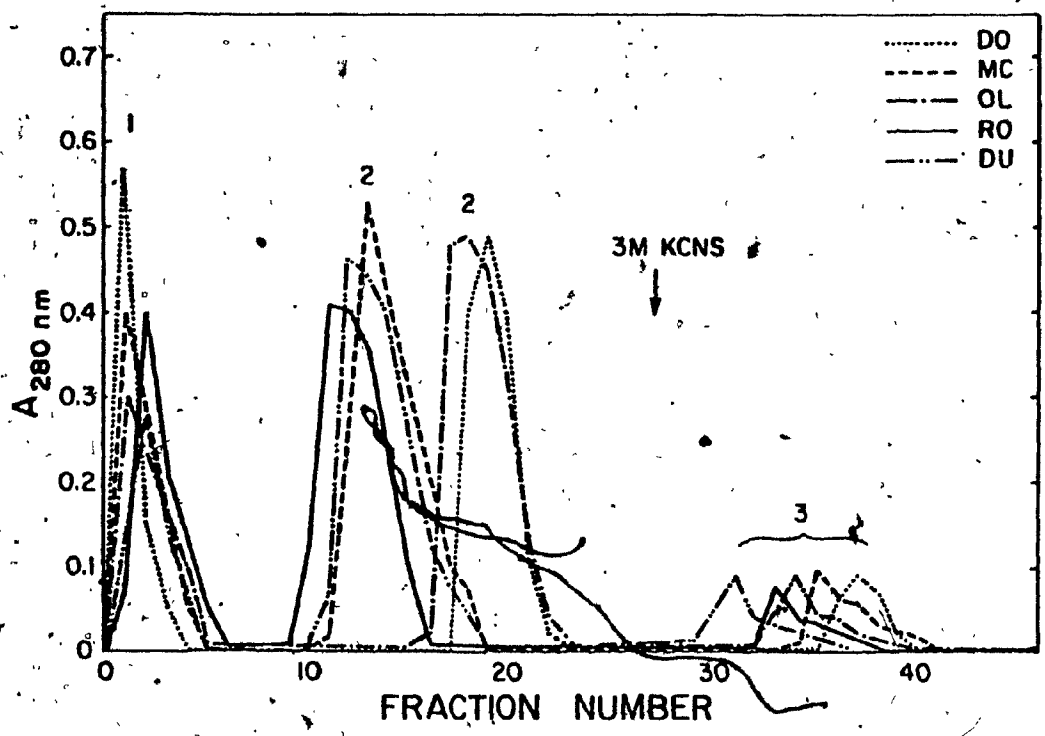

Fig.18. Affinity Chromatography Profiles of Cell Surface Antigens from (5) Melanoma Patients.

First peak is the unbound IgG molecules eluted after the first wash with 50 column volumes of 0.01M Tris-HCl pH 7.4 containing 0.15M NaCl. The second peak indicates the unretarded membrane components. The third peak is the antigenic molecules freed with 3M KCNS.

Fig.19. SDS-polyacrylamide Gel Electrophoresis of Melanoma Cell Surface Antigens Eluted From Autologous IgG Affinity Columns.

A bromophenol blue tracking dye was used in each preparation. Sample loads were approx. 25 µg. Bands were stained with Coomassie brilliant blue. From left to right antigens were isolated from patients 1) DO; 2) RO; 3) DU; 4) OL; 5) MC.

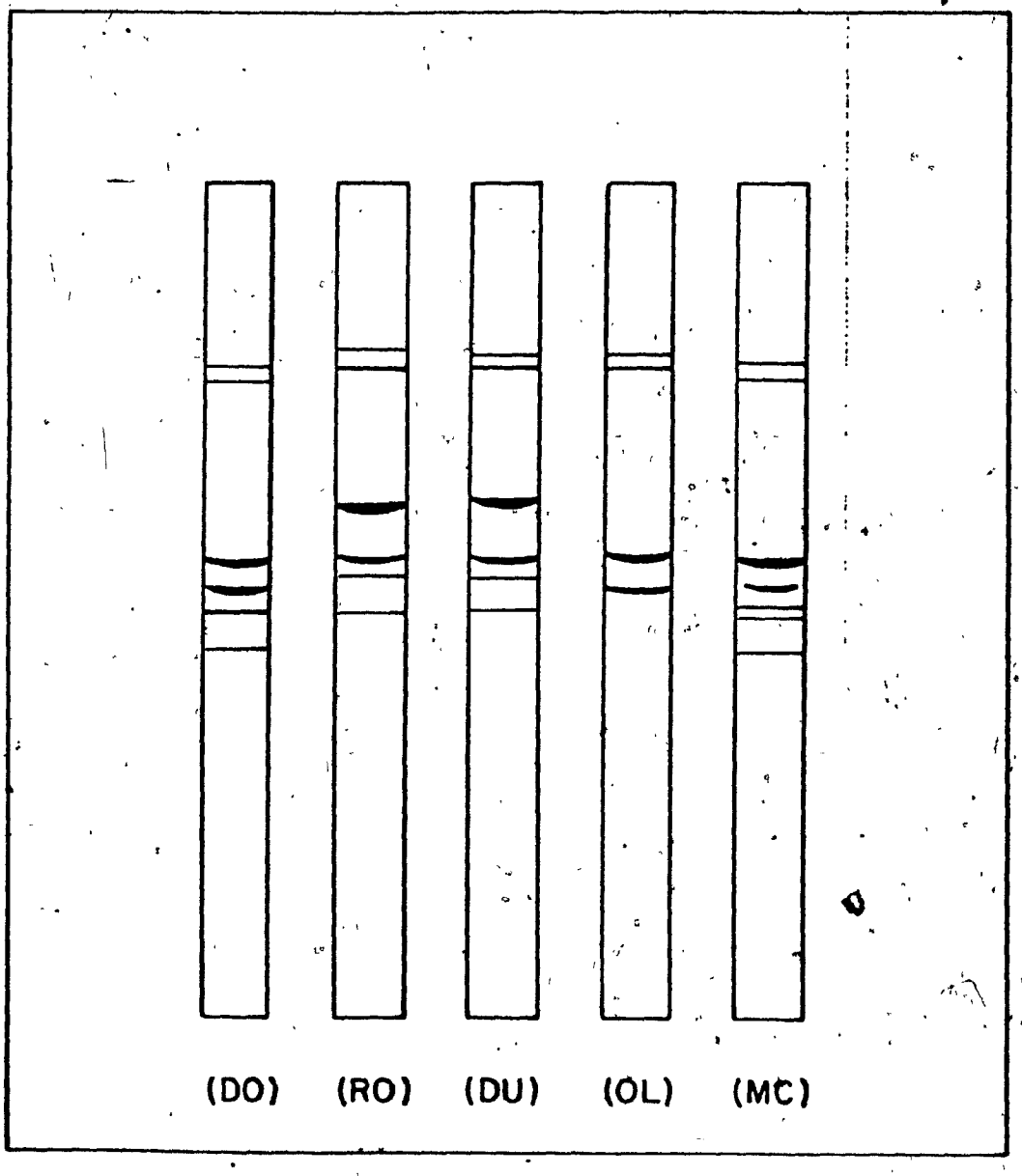

Fig.20. Diagramatic Presentation of Molecular Species of the Purified Tumor Cell Surface Antigens, Eluted from Autologous Affinity Columns, as Revealed by SDS-polyacrylamide Gel Electrophoresis for the Run in Fig.19.

Fig.21. Crossover Immunoelectrophoresis of Surface Antigens, Isolated From Patient (MC) Tumor Cell Plasma Membranes by Autologous IgG Affinity Column, Against Allogeneic Melanotic Sera.

1) Preabsorbed serum from patient (DU); 2) Preabsorbed serum from patient (DO); 3) Preabsorbed serum from patient (RO); 4) Preabsorbed serum from patient (OL); 5, 6, 7, and 8, purified melanoma antigens from patient (MC).

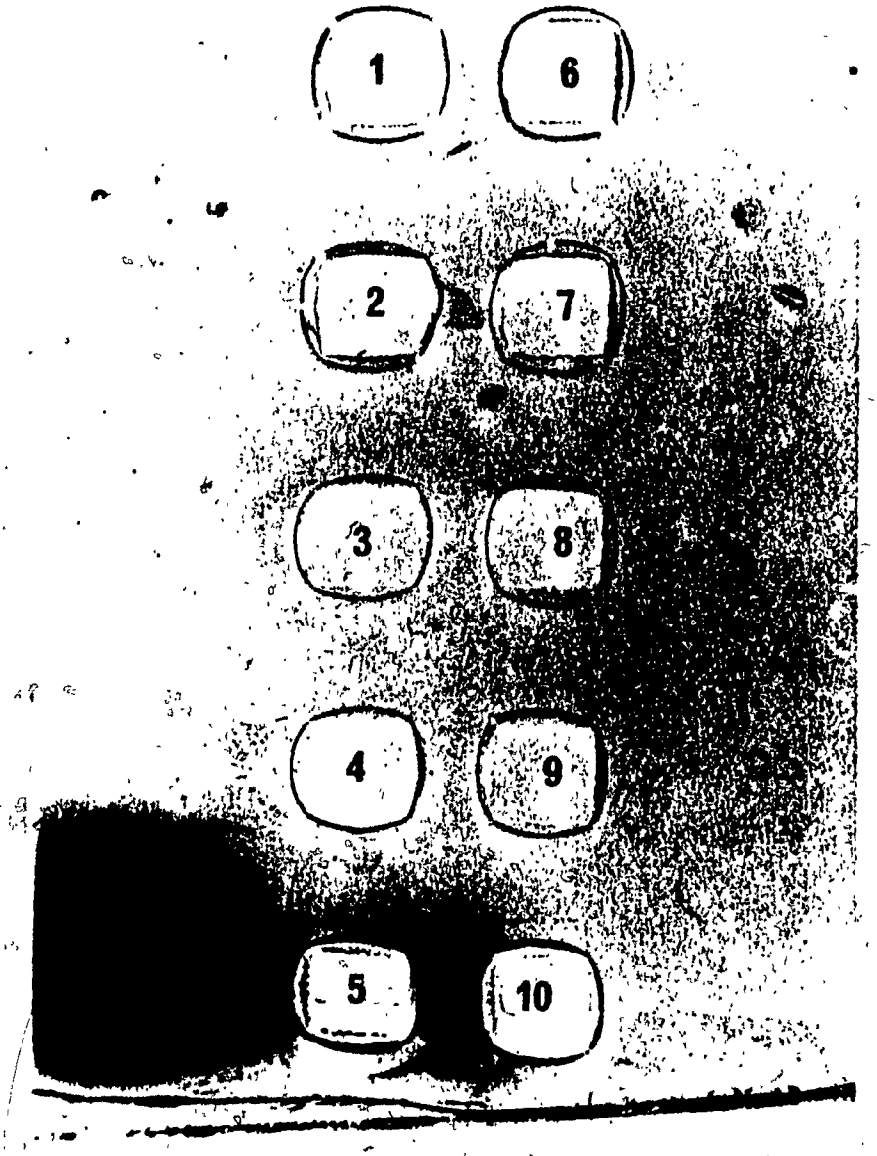

Fig.22. Crossover immunoelectrophoresis of Surface Antigens (Isolated from Patient MC Tumor Cell Plasma Membranes by Autologous IgG Affinity Column) Against Normal Controls.

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1) Normal serum (1); 2) Normal serum (2); 3) Normal serum (3); 4) Normal serum (4); 5) Anti-cytochrome oxidase; 6,7,8, and 9, purified melanoma antigen; 10) Cytochrome oxidase.

Fig.23. Crossover Immunoelectrophoresis of Surface Antigens, Isolated from Patient (DU) Tumor Cell Plasma Membranes by Autologous IgG-Affinity Chromatography, Against Allogeneic and the Autologous Melanotic Sera.

Well No. 1, 2, and 3, preabsorbed allogeneic sera from patients MC, DO, OL ; 4) Autologous sera from (DU); Well No. 5, 6, 7, and 8, purified antigens from (DU).

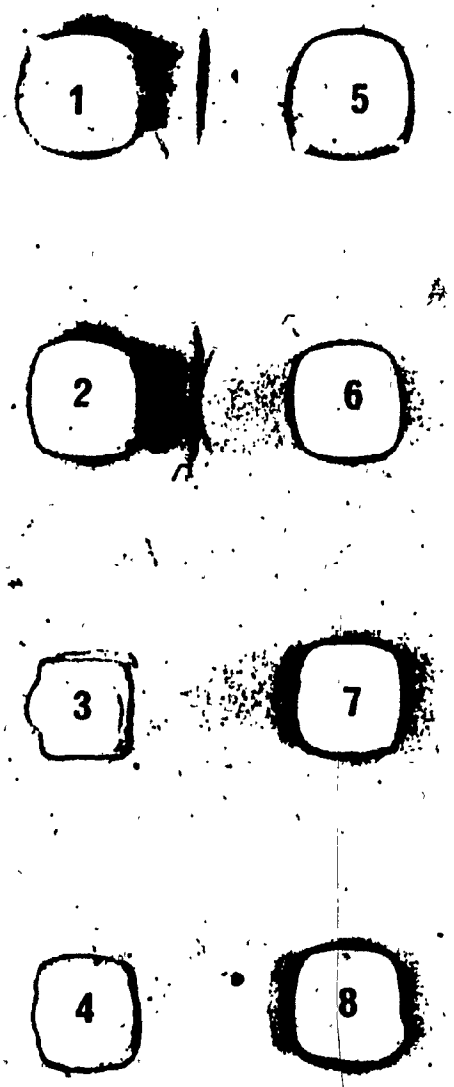

Fig.24. Crossover Immunoelectrophoresis of Surface Antigens (Isolated from Patient DU Tumor Cell Plasma Membrane by Autologous IgG Affinity Chromatography) Against Normal Controls.

1) and 2) Anti-human IgG; 3) and 4) Normal human sera (1) and (2); 5) and 6) Human IgG; 7) and 8) Purified melanoma antigens from patient (DU).

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