A STUDY OF PLASMA AND SERUM PROTEINS IN MALIGNANCY

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I hereby declare that this thesis entitled "A study of pl ma and erum protein in malignancy" is my own unaided work save that Dr. C Vos assisted me with the scoring of the amounts of blood group antigens pre ent in some of the extracts.

This thesis has not been and is not being submitted for a degree in any other Univer ity. The information used in this thesis was obtained while the author was a junior research officer in the group for research into organ transplantation at the University of the Witwatersrand, and also as a post-graduate student in the Department of Biochemi try at the same Institution.

D.J. Klatzow

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ABSTRACT

This study concerns itself with an examination of two proteins which are elevated in the serum of patients with cancer, using polyacrylamide gel electrophoresis (PAGE) under dissociation conditions. The molecular weights (MW) of the two proteins were 40000 - 50000d for the larger protein which was called A-band and 18000 - 22000 for the smaller protein which was called B-band. Using PAGE it was possible to show that the two bands were elevated in 76% of patients with cancer and in 34% of patients with benign conditions. They were also elevated in patients who had undergone renal transplantation. B-band was significantly raised in those patients whose grafts were undergoing rejection. Furthermore an elevated B band seemed to be a reliable early indicator of rejection. A-band was not significantly raised in the rejectors.

In order to identify the proteins in A-band and B-band a search was made for them in the various fractions resulting from the Cohn fractionation procedure. A-band was found in fraction 5. Gel filtration chromatography showed both A-band and B-band to reside in the high molecular weight peak (158 000 - 240 000d). A-band and B-band were compared on PAGE with carcino-embryonic antigen (CEA) and beta$_2$-microglobulin and were shown to be unrelated to either. From the molecular weight of A-band, its presence in fraction 5 and its elevation in acute conditions and in cancer, it was thought that A-band might be alpha$_1$-acid-glycoprotein (orosomucoid). Accordingly alpha acid glycoprotein was prepared using a perchloric acid isolation technique and was found to correspond to A-band. The identity of B band is not yet known.

The similarity between alpha$_1$-acid-glycoprotein and haptoglobin of

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The depressed immunity of patients with cancer has long been of interest. Alpha acid glycoprotein extracts from sera of most patients were able to depress PHA-induced lymphocyte transformation. This was most marked in the preparations from patients with Hodgkins lymphoma and the effect was dose dependent.

In summary, serum from patients with cancer was investigated and two proteins shown to be elevated. These two proteins were shown to be of limited value in diagnosis of cancer. The smaller of the two proteins was found to be useful as a marker of rejection in transplant patients. The larger of the two proteins was investigated, identified and shown to be related to blood group antigens. It was also shown to affect blood coagulation and lymphocyte blastogenesis.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The terms cancer and malignant neoplasia refer to a group of diseases that have the properties of: - (1) biological autonomy; (2) cell proliferation; (3) a-social cell behaviour; (4) invasiveness; and (5) metastasis (Wallach, 1976).

There have been many attempts to explain malignancy in general biochemical terms (Bodansky, 1975). However, although cancer must be considered a disease resulting from a deep seated biochemical defect, it is apparent that invasiveness, (i.e. infiltration of normal tissues by malignant cells) and metastatic spread constitute critical features of cancer (Wallach, 1976). Non-invasive tumours, even if rapidly growing, are clinically benign. Invasiveness is a characteristic of some normal growth patterns. For example, the implanting trophoblast shows distinctly invasive properties. However, in this instance, the invasion is limited only to certain stages of development. It would be true to say that if it were not for the fact that cancers invade and metastasise, cancer would not present the same foreboding clinical problem that it does.

1.1. EVIDENCE FOR TUMOUR ASSOCIATED ANTIGENS AND A HOST IMMUNE RESPONSE DIRECTED AGAINST THE TUMOUR.

Effectively tumours constitute parasitic diseases (Huxley,1958). The success of this parasitism is interesting because tumours may represent immunologically foreign tissue (Haddow, 1965; Old & Boyse, 1964 and 1966; Baldwin, 1967; Southam, 1967; Klein, 1968; Oettken and Hellström, 1971). One of the important unanswered questions in the molecular biology of tumours concerns the mechanism or mechanisms whereby tumour cells escape destruction by the immune system. During

/invasion..

invasion and metastasis, tumour cells bearing foreign antigens are exposed to all aspects of the immune system yet they are not always destroyed. One of the early demonstrations of immunity against tumours was by Foley (1953), who showed that prior exposure of a syngeneic recipient to a tumour graft would result in protective immunity on rechallenge with the same tumour. The particular model he used was a methyl cholanthrene-induced tumour in mice.

Much evidence has accumulated showing that a common feature of neoplastic cells was the expression of foreign antigens on the cell membranes of the tumour cells. These antigens may best be referred to as tumour-associated antigens. Southam et al., (1962), injected live autologous tumour cells under the skin of patients suffering from cancer. He found that at least 10^6 cells were needed to form a tumour nodule. He also showed that if the tumour cells were mixed with the patients own lymphocytes, their transplantability was markedly reduced. These experiments were suggestive of the existence of tumour-associated antigens on tumour cells and of an immunological response directed against them.

Old and Boyse (1964,1966) review the data showing the existence of tumour-associated antigens in experimental animal tumours while Southam (1967), discusses in great detail the evidence for cancer-specific antigens in man. Clinical observation, experiments based on the natural course of tumour growth, tumour transplantation and vaccination, cellular mechanisms of immunological response,

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serological methods of antigenic analysis and skin tests are all reviewed and evaluated in this paper. Southam concludes that "Most of the studies which purport to show cancer-specific antigens have probably shown only differences in the tissue antigens of different people. However, some of the more recent and stringently controlled studies have so greatly reduced this source of error, that it seems quite probably that the more recently observed antigenic differences are actually cancer-specific. Further investigations comparing normal and neoplastic tissues from the same individual are necessary".

1.2 ANTIGENS IN VIRALLY-INDUCED TUMOURS

Tumour-associated antigens can be demonstrated for both virally and chemically-induced cancers. In all virally induced tumours in animals a common antigen appears whose identity remains unaltered in different species as long as the same virus is used to produce the tumour (Deichman, 1969). For example, a cross reaction between chick embryo cells and mouse cell lines is evident after transformation (Gelderblom and Bauer, 1973). Similarly a common antigen was found on the cell surfaces of several cell lines transformed by mouse sarcoma virus (Aoki et al., 1973).

1.3 ANTIGENS IN CHEMICALLY-INDUCED TUMOURS

Chemically-induced tumours also show tumour-associated antigens (Fitch 1962). Thomson et al.,(1973), have shown the presence of tumour-specific membrane antigens in the serum of rats with methyl cholanthrene-induced sarcomas. Dickinson et al., (1972) have shown that tumour-associated

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antigens are present on the surface of tumour cells and can be removed by mild trypsin treatment. Baldwin and his co-workers have isolated immunogenic cell surface components from amino-azo dye-induced rat hepatomas (Baldwin and Glaves 1972) and have shown that these components include a tumour-specific antigen (TSA) with molecular weight (MW) of 35,000d (Baldwin et al.,1973a).

Chemical carcinogenesis is distinct from viral carcinogenesis induces antigens which differ from one tumour to another in the same animal induced by the same carcinogen and also from animal to animal (Sjogren 1955). However, it has been observed that the induction of tumour-associated antigens on the liver cell surface maybe accompanied by a loss of normal liver-specific antigens. (Baldwin et al,1971).

1.4 IMMUNITY IN HUMAN TUMOURS

The aeteologies of human cancers are less well defined than those in animals, but there is evidence of tumour cell changes which induce an altered immune reaction towards the tumour. In many cases lymphocytes from cancer patients are cytotoxic to cultured autologous tumour cells. Of great interest is the finding of common antigens between human melanomas, neuroblastomas and a ganglioneuroma (Old and Theilkaes, 1973). All these tumours are thought to be derived from neural tube. The antigenic material has a MW of between 42,000 - 60,000d. Others have found a tumour associated antigen in human serous cystadenocarcinoma of the ovary (Bhattacharya and Barlow, 1973). This antigen has a high MW and is a glycoprotein with beta electrophoretic mobility. It was not related either to CEA or HLA antigens.

/Qualitative.

Qualitative differences in plasma membrane antigens may reside in the carbohydrate components of these antigens rather than the peptide fragments (Perdue, et al., 1973) The antigenic determinants of the membrane glycoproteins are orientated on the external face of the membrane and it appears that changes involving these molecules are important in the pathogenesis of malignant neoplasms. They may be involved in the altered response to various regulators; phenomena such as loss of contact inhibition; and may play a role in the life cycle of the tumour by allowing either escape from or destruction by the immune system.

One interesting aspect of antigenic changes associated with transformation of cells to the malignant state is the appearance of antigens which are normally associated with the embryo. In 1967 Prehn showed that immunization of mice with embryonic tissues would protect them against subsequent challenge with methyl cholanthrene-induced tumour cells.

The fact that membrane changes are known to be closely linked to the malignant change of tumour cells (Emmelot 1973; Wallach 1975), is of importance in understanding the relationship between host and tumour. The documented evidence of antigenic changes associated with neoplasia lends credence to the suggestion by Burnett, and others that one function of the immune system is to prevent the development of clones of cells with malignant potential (Deichman 1969; Pasternak 1969; Baldwin 1973). Generally these experiments have involved transplantation of tumour cells and the

/demonstration.

demonstration that a specific immunity could be induced against them. Not all tumours, however, possess neoantigens (Baldwin 1971). Many mammary tumours in rats whether spontaneous or carcinogen-induced appear to be deficient in tumour antigens.

In man, for ethical reasons, evaluation of tumour immunity by transplanting tumour cells is not desirable, although Southam (1967), did perform such experiments and demonstrated that large numbers of tumour cells were required to establish even limited tumour growth.

The development of transplantable tumours in syngeneic animals has allowed a study to be made of the roles played by the various effector arms of the immune system, in tumour cell destruction (Hellström and Hellström, 1974). The Hellströms demonstrated convincingly that the cellular arm of the immune system was of primary importance. They were able to show that specific immunity could be transferred from immune animals to non-immune syngeneic animals by lymphoid cells. (Lymph node cells, spleen cells, thoracic duct cells and peritoneal exudates). Tumour immunity can also be transferred by cells from tumour-bearing hosts (Deckers et al., 1971).

From many in vitro studies, differences have emerged between the types of antigens expressed on animal tumours and those found in human tumours. Chemically induced neoplasms in animals usually express neoantigens which are characteristic for that tumour alone and may be different from antigens expressed on other tumours induced by the same carcinogen in the same animal. Viral tumours on the other hand tend

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to express antigens which are characteristic of the virus irrespective of host or histology of the tumour. By comparison in man, it would appear that tumours express antigens which are characteristic for the histological type of the tumour (Carrel and Theilkaes, 1973).

1.5 MECHANISMS OF IMMUNITY AGAINST TUMOURS

Several immunological mechanisms of tumour cell destruction have been described.

1.5.1 T-Lymphocytes

It has for some time now been held that the cytotoxic T-cell constitutes the major effector mechanism in immunity towards tumour cells (Wagner et al., 1973).

In most cases of specifically immunized hosts, the T-lymphocyte has been widely shown to be the effector cell in the rejection of allografted tissue (Cerrotini and Brunner, 1974). The role of the T-lymphocyte in tumour immunology, while less definite than in the transplantation context, has been shown in the case of murine plasmacytoma and in murine sarcoma virus-induced tumours. However, it would appear that the T-cell response is a relatively short-lived phenomenon whilst the non-T effector arms of the immune system remain operational long after regression of the tumour (Lamon et al, 1973). In fact Lamon was able to demonstrate a cytotoxic effect not dependent on

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the presence of T-cells. The T-cell requires contact with the target cell for killing and one effector cell per target seems to be required. The target cell killing appears to proceed without assistance from B-lymphocytes (Cerottini & Brunner, 1974).

Investigations with the mouse DBA/a mastocytoma indicate that the lesion inflicted upon the target cell by the T-lymphocytes is due to the formation of a gap-junctional complex, lysolecithin does not play a role (Ferluga and Allison, 1974).

The involvement of T-lymphocytes in tumour immunity was strengthened in view of the inability to immunize athymic mice with tumour grafts which were subsequently excised. Also T-cells were found to be essential for the adoptive transfer of immunity to this tumour in normal mice (Rouse et al., 1973).

1.5.' Macrophages

The role of macrophages in tumour immunity was originally suggested from experiments where peritoneal exudate cells from tumour-immune hosts were shown to be highly effective in transferring immunity to syngeneic recipients. This effectiveness of macrophages has been demonstrated in vitro as well by adding murine lymphoma cells to monolayers of specifically sensitized macrophages whereupon the tumour cells

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are destroyed (Baldwin & Robins, 1976) The exact mechenism is not yet clear. Macrophages can be activated in vitro by co-culturing them with spleen cells from immune donors and target cells. A factor present in the supernatant of spleen cells cultured in the presence of antigen can be used to arm macrophages. This factor is known as specific macrophage arming factor (SMAF) when the armed macrophages come into contact with the specific antigen, they become activated and can then kill tumour cells in a non-specific manner (Evans and Alexander, 1972).

Antibody dependent cell-mediated cytotoxicity

The killing of tumour cells by lymphocytes in the presence of specific antibody has been shown for several systems (MacLennan, 1972, Forman and Möller, 1973, Hakala and Lange, 1974). Non-sensitized spleen cells could be rendered cytotoxic to PARA-7 tumour cells by sera from tumour-bearing hamsters. This activity could be absorbed out by PARA-7 cells but not by cytomegalovirus-transformed cells. If the serum was obtained more than 10 days after the tumour transplant, it blocked immune spleen cells cytotoxicity and did not mediate tumour target-cell killing by non-specific spleen cells. However, serum obtained 3-6 days after transplantation of the tumour transplant could mediate cell destruction by non-sensitized lymphocytes (Lausch et al., 1975). Virally or chemically-induced murine sarcomas

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could be specifically destroyed by non-sensitized lymphoid cells when the cytotoxicity was mediated by antibody released from presensitized lymphocytes in short term cultures (Nelson et al., 1975).

The cytotoxic effect is expressed only if the antibody has an intact Fc portion. There is, however, considerably controversy as to the nature of the effector cell involved. It is believed not to be a T-cell and has long been held to be B-cell with immunoglobulin on its surface. However, recently it has been proposed that the participating cell is a null cell or a K-cell or perhaps a macrophage (Greenberg and Shen, 1973). The work of O'Toole et al.,(1973, 1974) has demonstrated quite clearly that there is no cytotoxicity in the T-cell fraction of blood lymphocytes They showed their effector population to consist of B-cells or macrophages with Fc receptors. This work was confirmed by others (de Vries et al., 1974).

Complement-dependent cytotoxicity

In some forms of malignancy, particularly leukemia, antibody directed against tumour associated antigens is demonstrable. Specific cytotoxicity for appropriate target cells can be demonstrated in vitro. Sensitivity to complement-dependent antibody

/destruction..

destruction appears to be related to a number of factors including the amount and density of cell surface antigen and its stability on the cell membrane. Several murine leukemias are highly sensitive to complement-dependent antibody lysis. However, many hepatomas and sarcomas are very insensitive to such lysis (Baldwin & Robins, 1976). This mechanism of tumour cell destruction is not thought to be of any importance in vivo.

In the Moloney leukemia-lymphoma system, a correlation between regression of tumour and high titres of complement-dependent antibodies directed against tumour cells and the presence of antibodies which could mediate ADCC were noted. Serum from animals with rapidly progressing tumours did not possess such activities (Harada et al., 1973). Recently selective destruction of neoplastic cells have been observed in leukemic mice, cats and dogs following infusion of normal serum, plasma or whole blood. In AKR mice this anti-leukemic activity of serum is mediated by the C5 component of complement. It has been suggested that complement may be a crucial limiting factor in immune destruction of tumour cells (Kassel et al., [illegible]

SERUM FACTORS WHICH MODIFY THE IMMUNE RESPONSE IN MALIGNANCY

In the light of all the evidence for tumour-associated antigens together with the fact that an immunological response against tumours can occur, we are still faced with the fact that tumours do arise. In order to explain the fact that

/tumours

tumours do develop despite an immune response which is potentially lethal, many authors have postulated that the immune response towards a tumour is blocked by factors in the serum. The first and probably the best known demonstration that serum factor could interfere with cellular immunity came from the laboratory of the Hellströms (Hellström and Hellström,1969) They were able to show that pre-treatment of Moloney sarcoma cells with heat-inactivated serum from tumour-bearing mice prevented them from being killed by specifically immune lymphocytes. Such blocking activity has been demonstrated in the serum of many animals, including man, with various chemically and virally-induced tumours (Boyse et al., 1962, Bubenik and Turano, 1968, Ferrer and Mihich, 1968).

It was originally proposed that the serum blocking factor was antibody in nature, and that the antibody coated the tumour-associated antigens and prevented their recognition by suitable lymphocytes. This suggestion was supported by the finding that the blocking activity could be localised in the 7S immunoglobulin fraction of serum (IgG Class). Also it was shown that blocking activity in the serum of mice, bearing virally-induced sarcomas could be adsorbed onto intact tumour cells. It was also shown that IgM antibodies were potentially cytotoxic to tumour cells in the presence of complement and did not participate in blocking (Baldwin et al., 1974 a and b). The hypothesis that blocking activity in the serum of tumour-bearing animals, was due to antibody became untenable when it was shown that this

/activity...

activity was rapidly lost if the tumour was surgically removed (Baldwin et al., 1973a). Not only was cell mediated cytotoxicity no longer blocked, but these sera from animals which had tumours surgically removed were able to neutralise the blocking activity in tumour bearer serum. This was demonstrated for the Moloney-virus induced sarcomas in mice (Hellström and Hellström, 1970) whose tumours showed spontaneous regression, in animals whose tumours had been surgically removed (Baldwin et al., 1974 a and b) and also in clinically symptom free human cancer patients (Hellström and Hellström, 1974). In all the studies so far mentioned the unblocking effect showed specificities comparable to those of the neoantigens associated with the tumour in question (Baldwin et al; 1974b).

The phenomenon of unblocking can best be interpreted as a neutralization of blocking factor in tumour bearer serum by antibody, suggesting the involvement of tumour antigen in the blocking process. This would explain the rapid loss of blocking factors subsequent to removal of the tumour. In this situation tumour associated antigens would no longer be produced and the available antigens would be rapidly removed from the serum. In the light of these findings it was proposed that the blocking factors were antigen-antibody complexes. This suggestion received support from some workers who showed that antigen and antibody were required for blocking and that neither would block alone (Sjogren et al., 1971 and 1972). Direct proof of antigen-antibody involvement in blocking has been provided by Baldwin et al., (1972). These authors were able to show that specific antibody from hepatoma bearing rats would not block. If this was added to tumour antigen, solubilized

from...

from the hepatoma cell with papain, the blocking did occur. The exact mechanism underlying blocking by such immune complexes is, however, not clear.

1.7 THE ROLE OF ANTIGEN IN BLOCKING IMMUNITY TO CANCER CELL.

It is possible that the tumour-associated antigen itself may play a more specific role by inhibiti- sensitized lymphocytes. Evidence for this may be found in the literature. Currie and Basham (Currie and Basham, 1972; Currie 1973), have shown that lymphocytes from patients with advanced carcinomas need to be extensively washed before their cytolytic properties are detectable. They showed that the serum component responsible for inhibiting lymphocyte cytotoxicity had no detectable affinity for the target cells and appeared to act on the lymphocyte surface implying that tumour antigen may well be involved. Also when this inhibitory material was assayed in the serum of cancer patients the indications were that patients with small tumours had minimal inhibitory activity but in those with advanced disease it was readil detectable.

Further work sugg sting antigen involvement has been done on a rat hepatoma system. Baldwin had been able to show that the cytotoxicity of lymph node cells from tumour immune rats can be specifically inhibited by treatment with the serum from tumour-bearing animals (Baldwin et al., 1973b). This suggestion that the blocking factor involved is antigen, received further support from the finding that inhibitory activity can be neutralized by adding tumour immune-serum to the blocking serum

/Baldwin....

(Baldwin et al.,1973c, 1974a and b). In addition, other studies have shown that the cytotoxicity of lymph node cells from rats immunized against a hepatoma is specifically inhibited by short exposure to solubilized tumour-specific antigen (Baldwin et al.,1973b). The possibility that circulating tumour antigen may play an important and perhaps a major role in modifying the relationship between host and tumour is emphasised by studies showing the in vitro cytotoxicity of peripheral blood lymphocytes from tumour-bearing patients can be specifically inhibited by contact with the appropriate tumour antigen. Similarly Currie showed that extensive washing was required to restore lymphocyte cytotoxicity to lymphocytes from patients with melanoma thus confirming his earlier work (Currie, 1973).

The probable role of circulating tumour antigen in serum inhibitory activity is suggested by several of its features i.e. Predilection for lymphocytes; Absence of affinity for the target cells and histogenic specificity. All these properties provide some evidence, albeit circumstantial, that the blocking activity is antigen (Vaage, 1973 and 1974).

The balance between antigen and antibodies in circulation could be critical in determining the nature of the reaction. In a microcytotoxicity or related assay, it is possible to observe blocking when antibody-containing sera from animals with regressing tumours was introduced into the system. Tumour cells exposed to these sera shed their antigens and thus immune complexes were formed with antigen excess. If antibody was in excess it appeared that the serum could exert an unblocking effect (Hayami et al., 1964). Tests for blocking serum factors

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in vitro were commonly done by inhibition of lymphocyte-mediated cytotoxicity (Hellström and Hellström, 1974). Convincing evidence has been provided in many reports for blockade at the level of effector cells. This mechanism prevents access of cytotoxic lymphocytes to the target cells (Kayli[illegible], 1962; Möller, 1965; Hutchins, 1968; Klein, 1971; Todd et al., 1973; Cohen et al, 1974).

Currie's work on melanoma is important (Currie, 1973) because it showed that blood lymphocytes from patients with early localised disease were actively cytotoxic in vitro and that this cytotoxicity was progressively lost as the disease progressed. Similar observations have been made with bladder carcinomas and sarcomas. This lymphocyte cytotoxicity may vary according to the phase of the disease. This, in turn, seems to be linked to the amount of tumour antigen, free or complexed, present in the host.

The data of Carrel and Theilkaes (Carrel and Theilkases,1973) is interesting. They have been able to demonstrate melanoma-specific antigen in the urine of 29 out of 32 cases of the disease, and in only three patients suffering from other diseases. The three false positives occurred in patients with tumours of the nervous system. This is an intriguing finding which suggests that these antigens are histogenically specific with reference to their embryonic origin. (Melanocytes are derived from the neural crest).

The discovery of CEA by Gold and Freedman (1965) has helped to emphasise the fact that the glycocalyx of tumour cells is shedding into the extracellular fluid and that some tumour cell surface constituents may appear in the serum. The glycocalyx

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of all cells is a dynamic state of flux and is constantly being synthesised and shed. This is true of malignant as well as non-malignant cells. Such cell surface antigenic components when in a soluble form, appear to retain their antigenic specificity but lose their immunogenic capacity. They can interact with and inhibit antibody or cytotoxic cells but are not capable of inducing an immune response. With increasing tumour size, the quantity of antigen shed must increase and find its way into the circulation in ever-increasing titres. At first this material will be rapidly complexed with circulating antibodies which will be in excess but at some stage it is conceivable that antigenic excess will occur with concomitant blocking activity. Such circulating blocking factors could be incriminated in the development of tumours at distant sites. In fact, Currie, and his co-workers,have shown in rats with spontaneously metastatic sarcomas, that the appearance of metastatic deposits is concomitant with the disappearance of antibody in circulation and the appearance of such blocking activity (Currie and Basham, 1972; Currie and Gage, 1973).

1.6 SERUM DERIVED IMMUNOSUPRESSIVE SUBSTANCES

The presence of naturally-occurring immunosuppressive substances in serum has long been known (Kamrin,1959). Kamrin showed that alpha-globulins in Cohn Fraction IV were capable of prolonging skin grafts in rats. (Mowbray (1962) was able to confirm and extend these results. He separated bovine alpha-globulin fractions on ion exchange chromatography obtaining a fraction that could prolong

skin grafts and prevent antibody production. It appeared to act in the inductive phase of antibody synthesis (Mowbray, 1962). Later they showed that the active fractions contained ribonuclease activity and purified ribonuclease was capable of exerting this immunosuppressive effect (Mowbray and Scholand, 1966. Mowbray, 1967; Milton and Mowbray, 1969). Other authors had also shown alpha-globulins with immunosuppressive activity to be present in normal blood (Mannick and Schmid, 1967) and showed that their fraction from human blood could prolong skin-allografts in rabbits. It could prevent PHA-induced lymphocyte transformation and also transformation induced by specific antigens (Cooperband et al., 1968).

Yet other authors (Phillips et al. 1975) investigated the immunosuppressive effects of alpha-globulins and found that they predominantly inhibited DNA synthesis and proliferation of lymphocytes. Antigen recognition was not blocked nor the release of macrophage migration inhibition factor from sensitized cells.

Milton (1971) has reviewed the literature and has compared Fraction C (FrC) described by Mowbray (1962) with immuno-regulatory alpha-globulin (IRA) described by Mannick and Schmid (1967). The comparison is shown in Table 1.

The current state of the literature on serum derived immunosuppressive factors has been reviewed by Miller (Miller,1976) and by Cooperband et al., (1976). On the basis of the available data it seems that the substance responsible for immunosuppressive activity in serum is an alphaglobulin which is not cytotoxic, does not contain cortisol and is heat

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stable. It may be associated with α_1-macroglobulin but lacks protease and ribonuclease activity. It appears to act across a species barrier and may act either by opportunistic limitation of available CO_2 or by inhibiting receptors on the lymphocyte surface.

The possibility that serum blocking factors are related to membrane components such as HLA antigens or blood group antigens must be considered.

TABLE 1.

SOME PROPERTIES OF Fr C AND IRA

SIMILARITIES

Alpha globulins present in normal serum with a similar method of preparation.

Immunosuppressive when administered to mice before immunisation with SRBC

Inhibitory to lymphocyte proliferation in vitro

DIFFERENCES

Fr C	IRA
RNase activity	No RNase activity
Eluted from DEAE with 0.5M acetate pH5	Eluted from DEAE with 0.2M acetate pH5
No effect on MIF production	Inhibits MIF production
No effect on E-rosette formation	Inhibits E-rosette formation

1.9 CHEMISTRY OF THE A,B,H BLOOD GROUP ANTIGENS

Difficulties in obtaining blood group substances from red cells prompted a search for them in other places (Kabat,1956). Morgan found that ovarian cyst fluid was a particularly rich source of A,B,H and Lewis blood group substances (Grubb and Morgan, 1949). Substances of specificities closely related to A,B,H are widely distributed in nature and are found in many micro-organisms and plants (Springer, 1958). The A,B,H and Lewis substances as they occur in tissue fluids and secretions are very similar and the same procedure can be used for the isolation of blood group substances from any of these sources. Extremes in pH are undesirable. The method which has found most general application is extraction of the freeze-dried tissue fluid or secretion with cold 95% phenol. A phenol-insoluble residue is obtained which possesses most of the activity of the original starting material. Further purification methods can be applied to the phenol-insoluble residue such as ethanol fractionation, ammonium sulphate fractionation, column chromatography on DEAE cellulose, extraction of polyacrylamide gel electrophoresis and density gradient centrifugation.
The A,B,H substances purified from secretions are glycoproteins consisting of approximately 80% carbohydrate and 20% amino acids. They are heterodisperse in size with an average MW of 300,000d but the molecules of different sizes have similar serological properties. The oligosaccharide chains are attached to the polypeptide by means of alkali-labile O-glycosidic bonds to serine or threonine. The sugar though to be involved in the linkage is N-acetyl-galactosamine (Fig. 1).

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The sugars found in blood group substances are found in Table 2. The amino-acid compositions of various blood group glycoproteins are similar to each other and appear to be unrelated to blood group specificity of A,B, H although some indication exists that this is not true for MN blood groups (Lissowska and Kordowicz,1976) The A,B,H and Lewis antigens possess a common basic structure and the blood group specificity resides in the sequence and linkage of sugars at the terminal non-reducing ends of oligosaccharide chains. There are two types of chains: (a) Type 1 in which galactose is linked Beta 1-3 to N-Acetyl glucosamine (Fig2), (b) Type II chains in which the linkage is beta 1-4 (Fig 3). (Watkins. 1972;1974).

TABLE 2

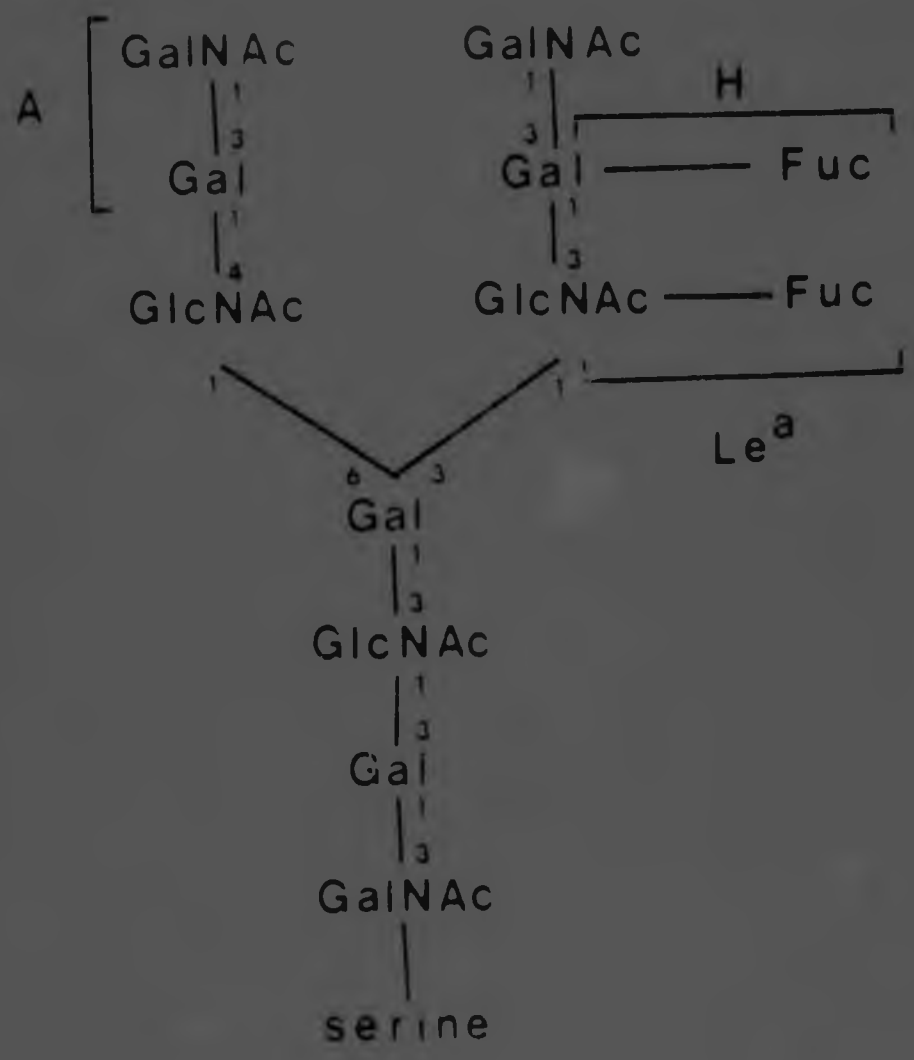
ANALYTICAL FIGURES FOR SPECIMENS OF HUMAN BLOOD-GROUP SPECIFIC SUBSTANCES ISOLATED FROM OVARIAN CYST FLUIDS

Group	No. of specimens	Nitrogen %	Fucose %	Hexosamine %	Reducing Sugars	Sialic acid %
A	4	4.7-5.3	17.0-19.5	27.8-36.3	51.0-57.0	1.3-2.9
B	4	4.5-5.1	16.2-20.8	23.9-27.5	50.0-60.1	1.9-5.1
AB	2	4.7-5.3	17.3-17.9	25.9-28.9	51.2-52.4	1 -1.7
H	4	4.7-5.9	16.7-21.6	23.0-26.0	45.0-57.2	1.5-4.3
Le^a	4	4.5-5.2	8.6-13 1	26.5-30.6	50.0-56.2	3.0-18.0
Inactive	1	5.4	1.6	28	49	

(Watkins, 1972)

FIGURE 1

GENERAL STRUCTURE OF THE ANTIGENIC DETERMINANTS OF BLOOD GROUP SUBSTANCE.



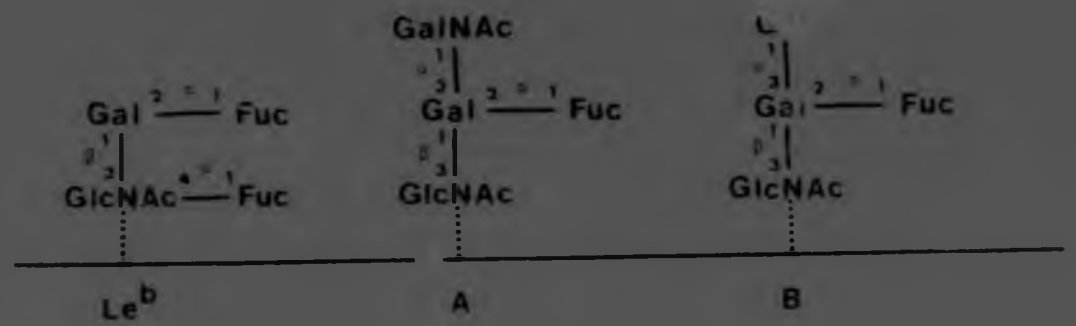
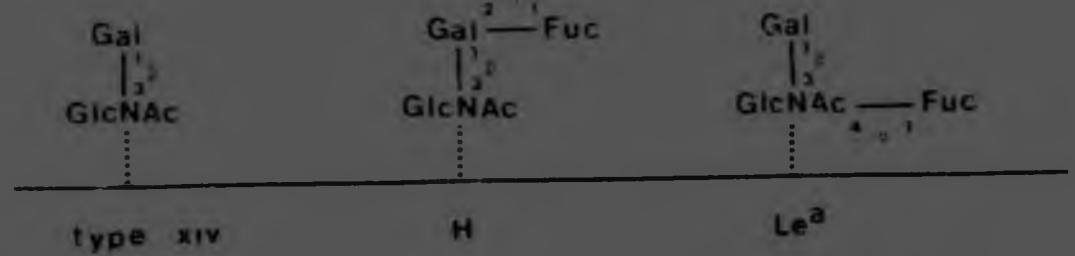


FIGURE 2

TYPE 1 CHAINS



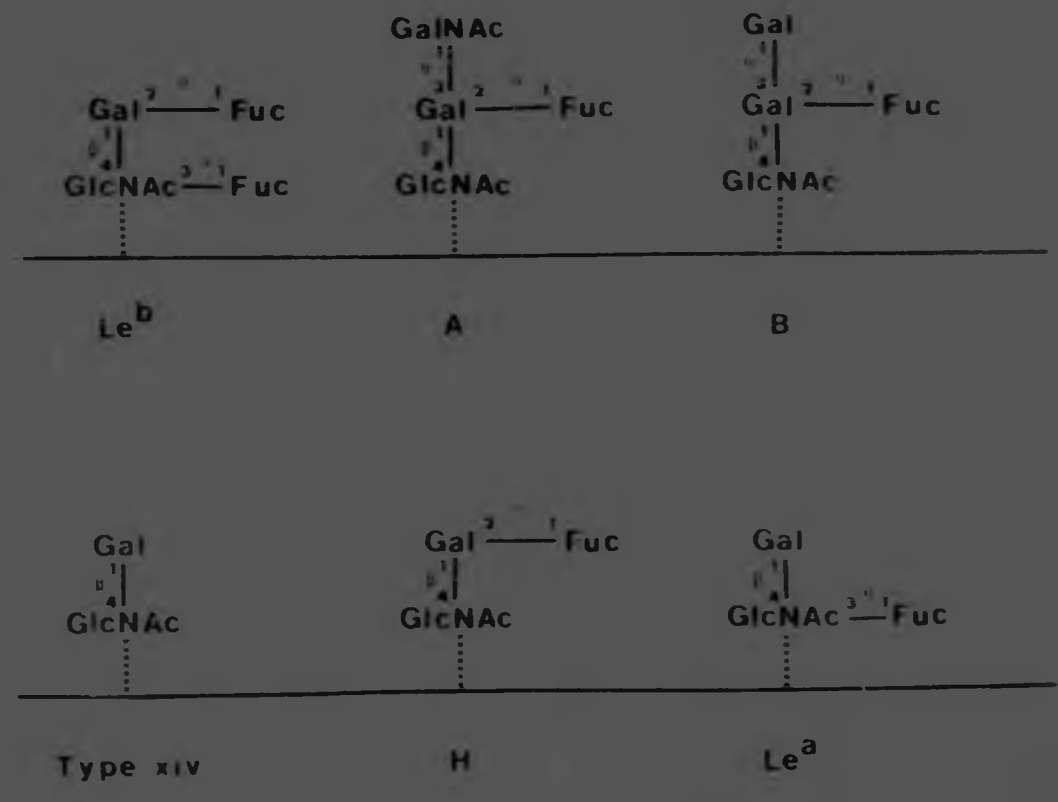


FIGURE 3
TYPE 1 CHAINS

1.10 THE CHEMISTRY OF THE M,N and T BLOOD GROUP ANTIGENS

In 1958 it was shown that treatment of red cells with neuraminidase destroyed the M and N activity of the cell (Springer and Ansell, 1958). The isolated substance behaved in a similar way when treated with neuraminidase thus sialic acid was shown to be of importance in this antigenic determinant. It is of interest that although removal of sialic acid destroys the reactivity of the M, N antigens towards human and rabbit antisera, it does not destroy the reactivity towards the anti-N lectin of Vicia Graminea (Lissowska and Kordowicz,1976). The reactivity towards Vicia Graminea lectin appears to reside in the galactose moeity (Springer et al.,1966). The structures which are thought to be important in the antigenic determinants of this group are shown in Fig 4.

Recent research indicates that the specificity of the M and N antigens may be a result of more complex interactions between the carbohydrate portion of the molecule and the amino-group of lysine on the polypeptide portion (Lissowska and Kordowicz, 1976). According to these authors M and N activities are confined to the major glycoprotein of human erythrocyte membranes, and are thought to be transported by high and low density lipoproteins from where they are acquired by the erythrocyte. This is very similar to the J antigen of cattle (Stone, 1966) and the R antigens of sheep (Rasmussen,1962). The fact that A B M and N antigens can be extracted from red cell blood ghosts in the aqueous phase using the butanol extraction procedure of Maddy as modified by Rega et al.,(1967), provides substantial evidence of their glycoprotein nature.

/change...

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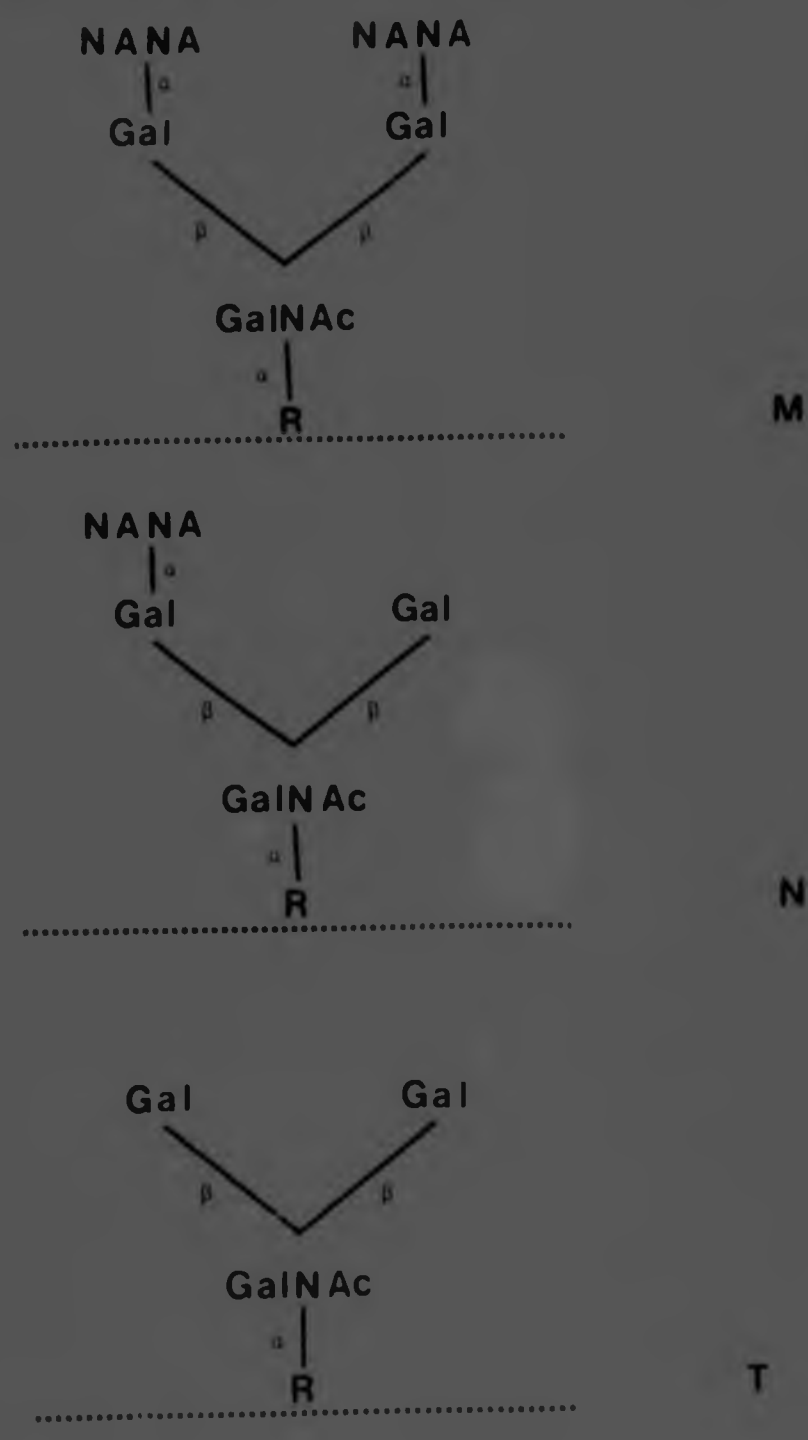
/changes..

FIGURE 4

THE STRUCTURE OF THE M N AND T ANTIGENIC DETERMINANTS.



(Springer et al., 1975b)

1.11 CHANGES IN BLOOD GROUP ANTIGENS ASSOCIATED WITH MALIGNANCY

Changes in blood group substances, associated with the malignant change have been known for a long time. The Tja antigenic determinant was first described in association with a gastric carcinoma (Levine et al., 1951). Later work by Marcus confirmed the earlier work by Levine et al., namely that malignant cells of a gastric adeno-carcinoma may express serologically active red cell antigenic markers different from those inherited by the host (Marcus,1971; Naiki and Marcus,1974). This refers to the first case of Tja produced by a 66 year old woman of genotype pp (Sanger,1953). Only this group of antibodies was absorbed specifically by the dried tumour tissue made available for study. Similar examples of blood group antigen changes were reported by others (Hakomori et al., 1967; Hakkinen, 1970). Hakomori and Andrews speculate that in malignancy there is aberrant and incomplete synthesis of A,B,H determinants (Hakomori and Andrews,1970). Hakkinen found blood group A activity in gastric cancer cells of patients of groups O and B. In addition to the A B H and Tja antigens, another group, the M and N antigens have recently been shown to be intimately involved with neoplasia. Springer et al., have demonstrated the presence of the M N precursor T antigen in malignancies of breast tissue. The T antigen was shown not to be present in normal breast tissue. Furthermore, they showed the anti T antibody titres were diminished in the sera of cancer patients (Springer et al., 1975 a,b,1976). These authors were also able to show the precursor of the T antigen viz. the T_n antigen, in malignant tissue. The T

/antigen..

antigen is known as the Thomsen Friedenreich antigen (Friedenreich,1930). The association between malignancy and blood group substances has been given further support by the finding of blood group A activity on carcinoembryonic antigen (Gold and Gold, 1973; Simmons and Perlmann,1973). Recently a paper linking blood group carbohydrate chains with both ontogenesis and oncogenesis has appeared (Watanabe and Hakomori, 1976). These authors suggest that a genetic or epigenetic programme for synthesising blood group determinants and their carrier carbohydrate chains develops step by step during the process of ontogenesis and that the programme of synthesis is blocked or modified in the process of oncogenesis. This concept is supported by the finding of loss of blood group specificity in a number of tumour types (Kay and Wallace, 1961; Davidson et al., 1966, 1969).

Also blocked synthesis of A and B determinants in human epithelial and endodermal tumours have been described based on immunochemical grounds (Prendergast et al., 1968; Davidson et al., 1969) and even enzymatically (Stellner et al.,1973). Changes in Lewis blood group hapten and accumulation of Lewis like antigens in some adenocarcinomas have been described (Yang and Hakomori,1971).

Immunohistological studies have shown that blood group determinants appear and disappear in a certain order during development (Szulman,1960). The H determinant was shown to be a marker of cellular differentiation (Pann and Kuhns,1972). Also changes in I,i antigens have been associated with malignant change (Feizi et al., 1975, 1976).

/The..

The picture of tumour associated antigens being blood group precursors is not quite so simple. High levels of A B H and Le^a substances have been reported in cases of mucus secreting or gastric carcinomas (Barber and Dunsford,1959; Saeed and Fine,1968). The loss of suppression of red cell A_1 antigen has been observed in a number of patients with leukemia (Stratton et al., 1958). There is also a report of loss of B activity in patients with leukemia (Richards,1962) and a decrease in the strength of the I antigen on red cells in leukemia (Race and Sanger,1975).

1.12 EMBRYONIC ANTIGENS ASSOCIATED WITH MALIGNANCY

Foetal substances designated antigens (since they are detected immunologically) are frequently re-expressed in neoplastic tissue. Besides CEA, many other oncofoetal antigens have been described including alpha foetoprotein (Abelev,1968). sulphoglycoprotein (Hakkinen and Viikari,1969), $alpha_2$ H ferroprotein (Buffe et al.,1970), gamma foetoprotein (Edynak et al.,1972), leukemia associated antigen (LAA) (Harris et al., 1971) and the Regan isoenzyme (Nathanson and Fischman,1971). Although it was originally thought that these substances were specific markers for the malignant state, this was later shown not to be the case. They are now known to be present in normal tissue in small amounts. It would seem that they are increased in many conditions in which cell proliferation occurs. Evidence for this being provided by increased levels of alpha foetoprotein in patients with nodular regeneration of the liver (Thomson et al.,1969). Also alpha foetoprotein increases in rats after liver injury with carbon tetrachloride (Jerry et al.,1976).

/Carcino....

1.12.1 Carcino-Embryonic Antigen (CEA)

It has already been seen that neo-antigens exist on tumour cells and that shedding of these antigens in soluble form may interfere with the immune response and so aid the tumour cell in its avoidance of destruction by the immune system of the host. Carcino-embryonic antigen is an example of a glycoprotein component of the tumour cell periphery which is shed from the tumour cell surface in greater amounts than from the normal cell surface (Gold, et al., 1968,1970). This shed material finds its way into the circulation and can be measured there by a variety of methods.

CEA was first described by Gold and Freedman (1965). The subject has been recently reviewed by Coligan et al., (1975) and Martin et al., (1976). Gold and Freedman prepared antisera in rabbits against extracts of colonic cancer. These antisera were adsorbed with an excess of normal colonic tissue obtained from the same patient. By double diffusion in agar it was demonstrated that human adenocarcinomata arising from endodermally-derived digestive system epithelium contained a common tumour associated antigen. They were able to show that this antigen was present in normal embryonic gut liver and pancreas during the first two trimesters of gestation. It has been classified as a protein polysaccharide complex (Gold,1967; Gold et al.,1968,

/Krupey....

Krupey et al., '967,1968). At first its presence in the adult was thought to be specific indicator for the presence of colonic carcinoma (Thomson et al.,1969). However, it has now been shown that CEA is elevated in many benign conditions (Khoo et al., 1973; Kupchick and Zamcheck,1972; Lo Gerfo et al.,1971a,b; Lurie et al,1974; Munjal et al.,1974; Zamcheck et al.,1972) and also in non-endodermally derived tumours (Denk et al., 1972; Lo Gerfo et al.,1971a,b).

Of interest is the finding that elevated CEA levels are found in 30% of diseases associated with regeneration or inflammation (Laurence et al., 1972). Some of its properties may be summarised as follows; it has a MW of 200 000d and a sedimentation constant of 7-8S; it is a glycoprotein containing 50-60% carbohydrate; it is soluble in perchloric acid up to concentrations of 1M and is also soluble in 50% ammonium sulphate and it moves is a beta globulin on immunoelectrophoresis. That CEA is produced by tumours is supported by several lines of evidence: The presence of CEA in digestive tumours which have been maintained in organ culture; the presence of CEA in human colonic tumours serially propagated in hamsters; secretion of CEA by some permanent colonic tumour cell lines; and in vitro biosynthesis of CEA as measured by microincorporation of radio-active glucosamine

/Goldenberg...

(Goldenberg et al.,1972; Laing et al.,1972; Peiper et al.,1973).

Recently blood group A activity has been described associated with CEA (Gold and Gold,1973). These two authors found that the majority of human sera which contained anti-A-antibodies, were capable of binding to purified CEA. They showed that the antibody concerned was predominantly IgM.

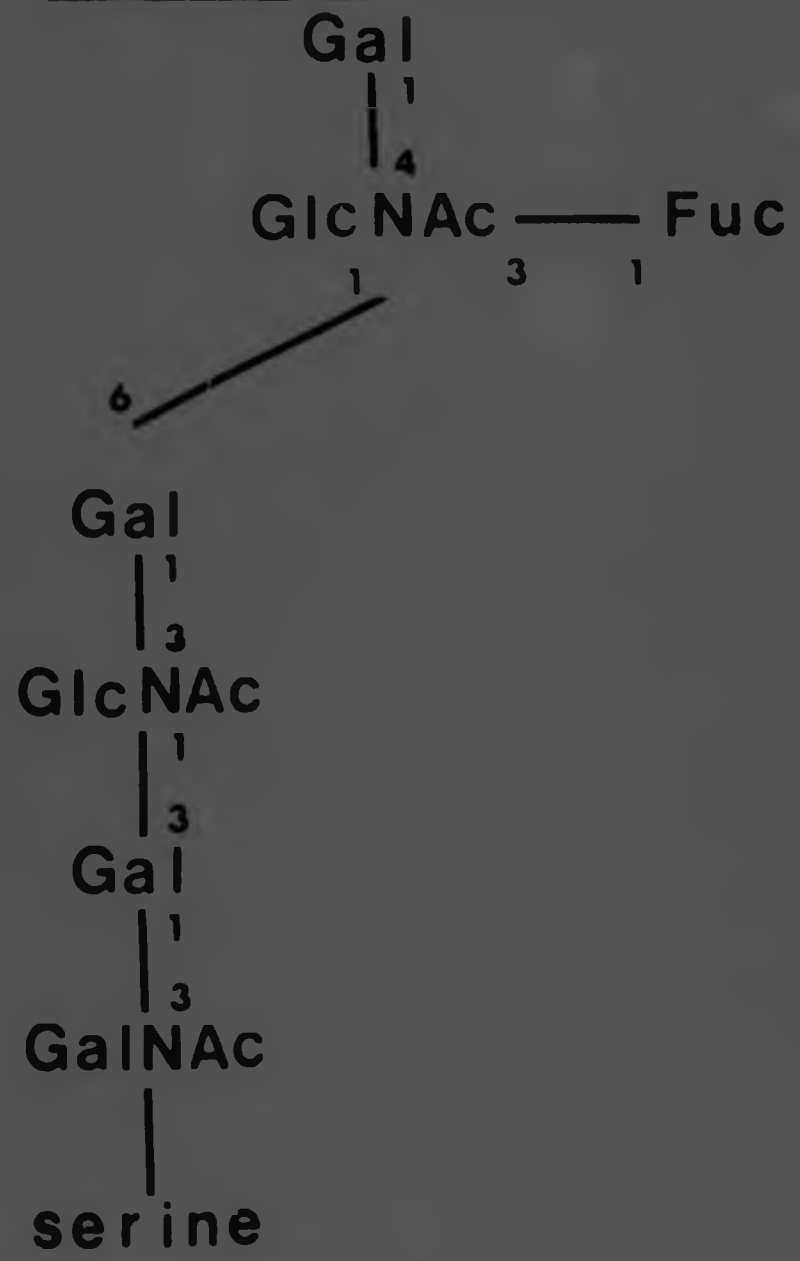
The carbohydrate attached to CEA has been structurally determined and is identical with the molecule which carries the human blood groups (Fig 5) (Simmons and Perlmann,1973). Although CEA were qualitatively indistinguishable from blood group substances, they were quite distinct in two important aspects: The carbohydrate in blood group substance accounts for some 80% of the molecule whereas in CEA it only accounts for some 50% and generally the fucose content of the tumour antigens was lower than in blood group substance.

Simmons and Perlmann have shown (Simmons and Perlmann, 1973) that CEA from entodermally derived adenocarcinomas are incomplete blood group substances of the ABO system. The deficient antigens have a tumour-specific activity due to unmasking of structural sequences that are cryptic in the normal situation. This suggestion is interesting in the light of findings that there is loss of blood group specificity in a number of tumour types (Kay et al.,1961; Davidson et al.,1966 and 1969;

/Feizi....

FIGURE 1

STRUCTURE OF CARBOHYDRATE CHAIN ATTACHED TO CARCINOEMBRYONIC ANTIGEN



(Simmons and Perlmann, 1973)

Teizi et al.,1975). There appears to be more variability in the carbohydrate composition of CEA depending on the particular tumour from which the material is isolated (Colligan et al., 1975). It has been suggested that the heterogeneity may, in fact be related to variations in sialic acid content (Coligan et al., 1973) However, other carbohydrate differences do exist (Coligan et al., 1975).

CEA evokes a weak immune response in the human host and repeated attempts to demonstrate cell mediated immunity to CEA in human hosts have met with failure. Circulating IgM antibodies to CEA in low titre may be detected.

Generally CEA is extracted from tissue by perchloric acid treatment. CEA has been shown to reside in the cell membrane glycocalyx of the colon cells (Krupey et al.,1967,1968; Hansen et al., 1971a; Hansen et al., 1971b; Lo Gerfo et al., 1971a; Lo Gerfo et al.,1971b; Laing et al.,1972). However there has been at least one suggestion that CEA is not a membrane component but rather a secretory product (Denk et al.,1972)

CEA is found in normal individuals to a concentration of 2.5 ng/ml It has been shown to be elevated in carcinomas of the gastro-intestinal tract (Martin et al.,1976) It was originally hoped that CEA was cancer specific but subsequently it has been

/shown..

shown that serum CEA i elevated in malignant condition other than gastro-intestinal malignancies and also in many benign condition (Laurence et al., 1977) Elevated serum CEA has also been reported in heavy moker (Stevens and MacKay,1973). Normal serum CEA levels occur in 60-80% of Dukes-A colon carcinoma and 45% of Duke and low CEA level have been reported for poorly differentiated tumour (Martin et al.,1976). It was originally hoped that CEA would provide an accurate di iostic tool for cancer. However its non- pecif.. elevations and variability have reduced its value in thi respect. It has, however, found a ne use in following up pat.ent who have had definitive treatment.

1.12.3 Alpha Foetoprotein (AFP)

AFP may be considered an antigen only in as far as it is detected by its pecific eaction with an antibody. It is however, not a component of the cell surface. AFP i not normally detectable in the serum of adult It is a normal constituent of foetal ser (Gitlin and Boesman, 1967). The levels are low at birth and fall rapidly subsequently.

Situations in which AFP have been found in the serum of adult are embryonal tumours of ovary and testi Abelev, 1968; Hou k et al , 1968; Linder and Widholm, 1968; Masopust et al., 1968). Raised levels of AFP al so well documented in primary

/hepatocellular...

hepatocellular cancer of the liver (Tatarinov,1966). This reflects on the finding of Gitlin and Boesman (1967) that embryonic liver and yolk sack are the only tissues capable of synthesising AFP.

In DAB-induced hepatomas in rats, two peaks of AFP production are seen. The first co-incides with the hepatotoxic effect of the DAB and the second with the appearance of the hepatoma.

The percentage of patients with hepatoma who showed elevated levels of AFP varied from 18% in some series to 87% in others (Kew,1974).

It has been shown in experimental animals that factors other than the presence of a tumour can result in increased AFP synthesis such as laparotomy and turpentine injections (Abelev,1968; Sarcione,1967; Stanislawski-Birencwajg et al.,1967).

AFP elevations in the serum are found also in acute viral hepatitis (Kew et al.,1973). Serial samples obtained from these patients with viral hepatitis show that the levels of AFP are related to the severity of the hepatocellular necrosis as reflected by serum glutamate-pyruvate transaminase (SGPT) levels. The peak AFP levels occurred in the regenerative phase 5-16 days after peak SGPT levels.

1.12.3 Other Embryonic Antigens Associated with Malignancy

In recent years several systems demonstrating antigenic reversion in human cancers have been demonstrated. These, however, in comparison with AFP and CEA are of relatively minor importance

/In....

In this thesis, I have not considered human chorionic gonadotropin (HCG) as an antigen. It seems likely that as the subject expands and progresses, more of these antigens will be demonstrated and used together diagnostically and so will be a more powerful clinical tool than they are at present. The other antigens which have been associated with cancer will be discussed now for the sake of completeness.

1.12.6 Regan Isoenzyme (Placental Alkaline Phosphatase)

It has been shown that in some 12% of patients with various forms of malignant diseases an alkaline phosphatase can be demonstrated in the sera, which is indistinguishable from the placental form of the same enzyme. This is known as the Regan isoenzyme after a patient in whom it was first demonstrated (Nathanson and Fischman,1971).

1.12.7 Foetal Sulphoglycoprotein Antigen (FSA)

A foetal type of sulphoglycoprotein has been demonstrated in the gastric juice of about 90% of patients with gastric carcinomas. It may also be found in 10% of benign gastric disorders. This glycoprotein shows some immunological cross reactivity with CEA (Häkkinen and Viikari,1969).

1.12.8 Alpha$_1$ Ferroprotein

This ferritin-like protein of hepatic origin is normally present in human foetal organs and serum. It disappears, however, post-natally and is not detectable in children over the age of 2 years.

It reappears in approximately 80% of children with teratomas and a variety of other malignant diseases including those involving the kidney, central nervous system, liver and lymphoreticular system (Butle et al.,1970). Approximately 10% of children with non-malignant disease also show elevated level of this protein.

1.12.7 Gamma Foetoprotein

This protein is found in the serum and tissues of the normal human foetus. Its level is elevated in the serum of approximately 10% of patients with solid tumours or leukemia and is demonstrable in tissue samples from 75% of human tumours tumours both benign and malignant (Edynak et al.,1972). This antigen shows no species specificity and is found in sera from human, bovine, porcine and canine foetuses.

1.12.8 Leukemia associated antigens (LAA)

In patients with leukemia an antigen is found in about 30% of cases. This antigen is also present in embryonic tissues and sera (Harris et al.,1971) This leukemia associated antigen has been demonstrated to be a tumour cell surface component and is also found in the sera of patients with Hodgkin's disease.

1.13 METASTASIS

The two most important criteria of tumours which eventually determine their benignity or malignancy, are the ability to invade and metastasise.

/Metastasis ..

Metastasis can be regarded as a series of separate interlocking events which begin with local invasion by the primary tumour, and culminate in the establishment of progressively growing secondary tumours at distant sites. The natural history of metastasis may be shown as follows.

1. Initial development and growth of primary tumour
2. Invasion of adjacent tissues and direct spread into contiguous body cavities
3. Penetration of lymphatics or blood vessels near or within primary tumour
4. Dissemination of tumour cells in lymphatic and blood circulations
5. Lymphatic spread - regional lymph nodes, then to more distant nodes
6. Haematogenous spread: arrest of circulating tumour cells: penetration of vascular walls; entry into interstitial compartment of distant tissue
7. Initial survival of disseminated tumour cells in tissue spaces
8. Acquisition of fibrovascular stroma
9. Progressive growth
10. Macroscopic metastasis

(Carter, 1976)

Some idea as to the metastatic potential of a tumour can be gained from its histological pattern. Generally undifferentiated tumours are much more aggressive than well differentiated ones. However, the biological mechanisms underlying metastasis have not been well defined.

/Invasion

1.13.1 Invasion

Invasion may occur in two phases of the disease: invasion of normal tissue adjacent to the primary tumour (including blood vessels and lymphatics and invasion of an organ following systemic dissemination. It has been suggested that tumour cells are motile *in vivo* (Willis,1967). Also evidence for this has been provided by Wood and his co-workers (Wood *et al*.,1967) by studying the migration of tumour cells in the rabbit ear chambers and also by Easty (Easty and Easty,1963). The invasion was shown to be due to factors other than simple mechanical pressure within the tumour. Nicolson *et al* ,(1976a,b) have shown using an *in vivo* assay that purely mechanical factors such as pressure within the growing tumour as suggested by Eaves (1975) could not explain the invasive properties of a melanoma cell line. The system used by these authors was chick mesonephros in culture. Using this they were able to show that normal embryonic cells were also able to invade the mesonephros although their ability to infiltrate was lower than that of tumour cells and in addition no cell destruction was observed in the area of invasion. There are a number of factors which may be involved in the ability of cancer cells to invade and metastasise. One of which appears to be reduced adhesiveness of tumour cells.

/Cell..

1.13.2 Cell Adhesion

One of the most important aspects of differentiation is the ability of differentiated cells to form tissues which are functionally and hi tologically distinctive. The ability to form a solid tissue clearly depends on the ability of the constituent cells to adhere to each other in a specific way. The role of the cell membrane in this adhesion is important. In embryonic development the making and breaking of contacts between cells, and between cells and non-cellular surfaces, appears to be a fundamental part of the mechanism by which a simple array of cells gives rise to more complex structures

Cell interaction can be seen possibly in a continuum which encompasses the following processes. As the two cells approach each other they fall under the influence of each others electrostatic field. They will be subjected to either mutual attractive forces or repulsive forces depending upon the nature of the cell surfaces and their electrical properties. The possible attractive and repulsive forces in the adhesions of cells have been listed. These may be arranged as follows, in roughly descending order of specificity and strength; co valent bonding forces between surfaces; hydrogen bonding; ionic links e.g $(NH_3 \ldots {}^{-}OOC$ and $COO^{-} \ldots Ca^{++} \ldots {}^{-}OOC)$; charge fluctuation forces due to the ionization and de-ionization of individual groups which are

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at a pH at which they are partially ionized. These fluctuations set up an attractive force in a nearby surface. Forces due to charged mosaics on either surface arranged so that areas of opposite charge face each other; electrostatic interactions between like charges of differing surface potential and van der Waal's forces. A further requirement for the interaction of cells as an enmeshing of surface macromolecules on opposed cells such interaction would depend on an approach to less than 0.5nm of the 2 cell surfaces. This could be achieved by the protrusion of some molecular species a distance of 5-10nm from the lipid surface. Depending on the nature of the interacting molecules, the contact could be a highly specific one i.e. the interaction could easily constitute adhesive recognition by a mechanism of molecular complementarity (Roth et al.,1971). The nature of the interacting molecules is still the centre of controversy and some suggestions have been made that glycosyltransferases are involved (Roseman, 1970; Roth et al ,1971).

I.14 THE ROLE OF CELL SURFACE CARBOHYDRATES IN ADHESION AND CELL SURFACE RECOGNITION PHENOMENA

It is known that tissues from various animals if dissociated into single cell suspensions re-aggregate under appropriate conditions into structures strongly reminiscent of the original tissue (Steinberg,1970). It was first noted in

/marine

marine sponges and was shown that the information necessary for the re-ordering of single cell suspensions into their characteri tic architectural patterns, is carried by the individual cells themselves (Humphreys,1975). It was seen in warm-blooded vertebrates that cells from a given tissue would sometimes aggregate with cells from the same tissue of a completely different species to form a chimeric tissue. On the other hand cells of a given histological tissue type would not aggregate with those of a different tissue type.

These experimental approaches have been called into question by some authors however who question the relevance that they bear to real morphogenetic processes in vivo. Single cell suspensions can be obtained from tissues by several methods including mild proteolysis and treatment with EDTA (Steinberg et al.,1973). Cells which have been dissociated by chelating agents are able to reaggregate immediately in physiological solutions which contain divalent cations. This is in contrast to cells which have been dissociated using proteolysis. These cells require a resting period before they will reaggregate (Steinberg et al.,1973). It seems that cell surface glycopeptides/peptides are removed by the proteolytic action and are replenished only slowly by metabolic events within the cell Hughes,1972). It would appear that these components are essential for aggregation to occur; the molecules containing information for assembly of tissues must be carried by the individual cells and must be expressed at the cell surface.

(The...

The molecular species involved in the aggregation phenomenon have been isolated and partially characterised from a variety of species including chick embryos (Balsamo and Lilien, 1974a;b;1975), mouse teratomas (Oppenheimer and Humphreys,1971; Oppenheimer,1975), and slime moulds (Roth et al., 1973).

Despite the various experimental approaches it appears that complex polysaccharides are involved in all the systems so far investigated. Oppenheimer et al.,(1969) was able to show that teratoma cells growing as single cell suspensions will form aggregates in a medium containing glutamine, glucosamine or mannosamine, thus implying the involvement of hexosamines in the aggregation process. Oppenheimer and his colleagues have extended their studies to other cell types and have found similar requirements for aggregation. Humphreys working with sponges showed that an aggregation enhancing factor dissolved in the supernatant when the tissue of several species of sponges were dissociated in cold magnesium-free sea water (Humphreys,1963; Henkart et al., 1973). Unless this factor was added to the sea water the cells aggregated slowly or not at all. As to the species specificity of the factor, it was interesting to note that it showed the same specificity as the normal aggregation of the species from which it was isolated (Humphreys,1970). That is, if the cells of two species of sponge aggregated separately from a mixed suspension,then the aggregation enhancing factor from each of these two species did not affect the cells of the other. It however, the cells of

/the..

the two species tended to aggregate together from a mixed suspension then the factor from one species would promote some aggregation of the cells of the other species. Humphreys has succeeded in purifying and partially characterising the aggregation enhancement factor from the sponge Microciona parthena (Henkart et al.,1973 and Cauldwell et al., 1973). It was shown to be a large negatively charged proteoglycan of MW 20 x 10^6d. It proved to be comprised of 50% protein and 50% carbohydrate. If treated with EDTA the complex dissociated into sub-units of MW 200,000d. The sub-units also contained about 50% carbohydrate.

The molecular nature of the receptor site on the cell membrane is under extensive investigation. If the cells of Microciona prolifera are treated with hypotonic saline, a factor is released which inhibits the activity of the aggregation enhancing factor (Weirbaum and Burger,1973). The inhibitor did not appear to be species-specific. Further research as to the nature of these cell surface receptors will be discussed later.

The activity of the aggregation enhancing factor is sensitive to most proteases (Gasic and Galanti,1966). It is also sensitive to heat (56°C for 5 minutes), and low Ca^{2+} concentrations. These results indicate an important role played by the protein portion of the aggregation enhancing factor but do not rule out any activity by the carbohydrate portion. Some evidence of carbohydrate involvement is provided by the observation that glucuronic acid partially inhibits Microciona prolifera factor activity if the cells are incubated with it prior to

/the

the addition of the aggregation enhancing factor (Turner and Burger,1973) This suggests that glucuronic acid plays an important role in the active site of the factor. Recent advance in our knowledge of the distribution of complex carbohydrate in mammalian cell membranes particularly its presence only on the outer surface of the membrane (Benedetti and Emmelot,1967,1968; Rambourg and Leblond,1967) suggests a specific role played by these molecules in recognition phenomena. The carbohydrate in mammalian cell membranes may be of two types: glycolipid and glycoprotein. The glycolipids of [illegible] membranes are derivatives of the base sphingosine. The base is substituted through an amide group with a fatty acid. The carbohydrate is linked via a glycosidic linkage to the hydroxyl group. The fully substituted structure therefore contains separate regions of highly hydrophobic or hydrophilic nature. The hydrophobic portion serves to insert the structure into the membrane. Some well known antigens are carried on this glycosphingo-lipid viz. Forsmann antigen and the determinants of the ABO blood groups.

The glycoproteins of mammalian cell membranes are an interesting group of molecules. Much work has been done on the major glycoprotein of the red blood cell membrane glycophorin, which serves as a prototype for other membrane glycoproteins.

1.15 GLYCOPHORIN

Glycophorin has been extensively studied for many years. It has been isolated from red blood cell membranes (Moraweiecki,1964; Greirath and Reynolds, 1974).

Unresolved controversy exists as to the molecular

/weight..

weight of glycophorin. The molecular weight for the monomeric molecule was determined by sedimentation analysis and diffusion to be 31,000d. However, independent measurements also using sedimentation equilibrium gave a molecular weight of 41,000 d (Zvilichovski et al.,1971). Molecular weight determination of glycophorin is usually complicated by the tendency of the molecule to polymerise and form aggregates with molecular weights from 0.3-1 x 10^6 (Bezkorovainy et al.,1966, Morawiecki, 1964; Springer,1967). Estimation of the molecular weight of glycophorin from its electrophoretic mobility on SDS-containing polyacrylamide gels has given consistently higher values of about 50,000 d (Segrest,1971). However, this result must be treated with reservations in view of the known anomalous behaviour of glycoproteins on SDS-PAGE (Bretscher, 1971; Segrest et al.,1971). Generally a molecular weight of 31,000 is accepted for glycophorin.

The complete amino-acid sequence of human erythrocyte glycophorin is known (Tomita and Marchesi,1975). It is composed of 131 amino acids and approximately 16 oligosaccharide chains. Of these 15 are linked to serine/threonine residues via an O-glycosidic bond and one more complex unit is attached to asparagine. The localization of the oligosaccharides and the complete sequence has been determined by Edman degradation. Glycophorin appears to be organised into 3 distinct domains on the basis of the position of the carbohydrate and the positions of hydrophobic and hydrophilic amino acid residues. These domains are: a glycosylated

/segment..

segment of about 64 amino acid residues at the N-terminal; a hydrophobic segment of approximately 32 largely non-polar amino acids (Winzler,1972) and a C-terminal sequence containing hydrophilic amino acids (Winzler,1972; Tomita and Marchesi,1975). Glycophorin has been extensively characterized in terms of its membrane orientation and antigenicity by many workers (Table 3) (Marchesi et al.,1972). It is known to carry the carbohydrate determinants for the M and N antigens (Marchesi et al.,1972, Tomita and Marchesi,1975; Winzler, 1972; Marchesi and Andrews, 1971: Zvilichovsky et al., 1971; Cleve et al.,1972).

Glycophorin is also known to have the receptor site for the influenza virus and this is also known to be closely associated with the M and N determinants (Springer,1970). Furthermore glycophorin bears the receptors sites for PHA and wheat germ agglutinin (Springer et al.,1966). The proposed structure for the M, N, T and antigens are as shown in the (Figure 4 p.23). (Springer and Desai,1974).

1.10 CELL SURFACE ALTERATIONS AND TRANSFORMATION

Many of the basic functions of normal cells are maintained by tumour cells. It is therefore not unreasonable to suggest that certain of the altered surface properties of tumour cells could result from very subtle departures from normal. Such changes could involve additions or deletions,qualitative changes or alterations in topographical distribution of normal surface components. The majority of work to date on comparisons of normal and transformed cells has been done on in vitro systems. There are considerable problems in the interpretation of such studies. Some of these problems may

TABLE 3.

ANALYSIS OF VARIOUS PREPARATIONS OF THE MAJOR HUMAN ERYTHROCYTE GLYCOPROTEIN

Property	Preparation			
	a	b	c	d
Lipid %	1.0	ND	Present	10-20
Protein, %	37.5	40	25	40
Carbohydrate, %	64.8	60	75	40-50
$S_{20,W}$	2.15	ND	ND	ND
D x 10^{-6} $cm^{-2}s^{-1}$	0.575	ND	ND	ND
MN antigens	+	+	+	+
AB antigens	ND	+	+	-
Extraction method	phenol	LIS*	pyridine	chloroform-methanol
MW	31 000	50 000	22 000	58 000
				36 250
				24 000
MW of polypeptide	13 500	20 000	3 500	14 500

be briefly discussed

Many aspects of cellular organization are significantly altered by growing the cells *in vitro*. The initial dissociation of cells by enzymic means and subsequent passage may produce long term selective effects which may result from sub-lethal damage to the cell surface. Studies of transformation *in vitro* will always be open to question if strictly comparable normal and tumour cell populations are not used. The type of medium, pH, serum supplement, cell density, frequency of passage can all exert important effects on the properties of the cells. Population geometry is another factor which has not been considered in the extrapolation of *in vitro* results to the *in vivo* situation. The culture of cells as monolayers attached to solid surfaces allows for growth essentially in 2 dimensions. Growth in the third dimension being limited. Such cultures grow continually provided there is sufficient area for expansion. A more accurate representation of tumour cell growth may be obtained by culturing cells in suspension in soft agarose or methylcellulose. In this instance population growth occurs in 3 dimensions to form a spheroid. However, in this case the size of the spheroid is limited by the ability of nutrient to diffuse to the centre of the mass. This is partly reflected in tumours where growth exceeds the ability of the blood supply to keep up and necrosis occurs in the tumour centre. Clearly however blood supply of *in vivo* tumours and the production of tumour angiogenesis factor *in vivo* make cultures of tumour cells in suspension but an approximation to the *in vivo*

/situation..

Perhaps the most vital question regarding the relevance of *in vitro* of transformation is the adequacy of criteria used to define normal and transformed. By common usage transformed refers to a population of cells which have arisen either spontaneously or following exposure of the cells to tumour viruses or carcinogens, and which display certain stable properties such as potential for infinite growth *in vitro*; the ability to grow to high densities when grown in suspension culture; loss of contact inhibition; alteration in surface antigenicity; change in lectin agglutinability and loss or simplication of cell surface glycolipids and/or glycoprotein.

Unfortunately in many studies of transformation alterations such as those mentioned above have been used as the sole basis for assuming that the cells have undergone neoplastic conversion. However, very few of the available papers have correlated these events with tumorigenicity. That is the ability of these cells to produce tumours *in vivo*.

Table 4 lists cell surface changes associated with viral transformation. However the correlation between these changes and tumorigenicity has not been extensively investigated.

An example of the confusion that can arise from the use of these in vitro growth properties for classifying cells as normal or transformed is provided by the BHK-21 hampster cell line and the BALB/c 3T3 cells from mice. Both cell types have been used as normal control cells for comparison with cells of the same type after transformation by tumour viruses. In these systems cells showing any of the alterations listed in

/Table 4.

Table 4 as a result of viral infection are referred to as transformed, whereas uninfected BHK or 3T3 cells are referred to as normal. However uninfected BHK-21 cells can cause tumour when transplanted in vivo (Jarrett and Macpherson, 1968). Similarly BALB/c 3T3 cells are tumorigenic in histocompatible mice when implanted attached to glass beads. Cells derived from these tumours were capable of producing lethal tumours on subsequent transplantation in the absence of beads (Boone, 1975; Boone et al.,1976).

No single membrane abnormality is a single invariant feature of neoplasia (Wallach,1976). However one concept which is beginning to emerge is that certain of the surface alterations may result from topographical rearrangement of the existing surface components found in normal cells. Certain transformed characteristics such as loss of LETS (Large External Transformation Sensitive) protein, enhanced Lectin agglutination, altered antigenicity can be detected on normal cells during mitosis and on normal interphase cells after brief treatment with proteases.

A very important observation suggests that the organisation of membrane-associated cytoskeletal elements involved in transmembrane control processes, is altered following transformation (Nicolson,1976). This could lead to alteration in distribution of cell surface receptors (Poste 1975; Poste and Nicolson,1976). The finding that many surface alterations can be induced in tumour cells by brief proteolysis raises the question as to whether increased protease activity at the cell surface might not account for the properties seen in malignant cells.

(There

TABLE 4 Cell Surface changes associated with neoplastic transformation of cells cultured in vitro.

Property
1. Changes in cell behaviour influenced by cell surface organization
a. Decreased sensitivity to contact inhibition of movement.
b. Decreased sensitivity to density-dependent growth inhibition.
c. Loss of "anchorage dependence" and acquisition of capacity for growth in suspension.
2. Changes in cell-to-cell and cell-to-substrate adhesiveness
a. Status uncertain.
3. Reduced cell-to-cell communication due to changes in the formation and organisation of gap junctions.
a. Status uncertain.
4. Changes in the structural organisation of the plasma membrane.
a. Increased mobility of integral membrane proteins and glycoproteins.
b. Reduction in membrane-associated microfilaments and/or other cytoskeletal elements.
5. Changes in cell surface charge
a. No consistent correlation between zeta potential and malignancy.
6. Changes in plasma membrane transport activities
a. Enhanced transport of sugars
b. Enhanced transport of amino acids.
7. Changes in membrane-associated enzymes
a. Reduced adenyl cyclase activity
b. Enhanced Na^+-K^+-ATPase activity and loss of growth-dependent changes in enzyme activity
8. Changes in plasma membrane lipid composition and alterations in membrane "fluidity".

TABLE 4 continued...

Property
a. Status uncertain
9. Changes in plasma membrane glycolipids
a. Incomplete glycosylation of glycolipids
b. Reduced activity of specific glycolipid glycosyl-transferases
c. Increased reactivity of membrane glycolipids to anti-glycolipid antibodies.
10. Changes in plasma membrane glycoproteins
a. Incomplete synthesis of certain glycoproteins, especially lack of terminal sialic acid resulting from reduced sialytransferase activity.
b. Increased production of fucose-containing sialo-glycopeptides
c. Loss of a high molecular weight (ca. 200,000-250,000 daltons) membrane glycoprotein (LETS protein; Z protein; galactoprotein A; L1 protein).
d. Changes in glycopeptidyl-transferases.
11. Changes in membrane mucopolysaccharides
a. Increased synthesis of hyaluronic acid
b. Increased production of sulfated mucopoly saccharides
12. Changes in susceptibility to agglutination by plant lectins
13. Antigenic changes.
a. Novel tumor-associated transplantation rejection antigens
b. Novel tumor-associated antigens not necessarily capable of evoking rejection.
c. "Embryonic" or "oncofoetal" antigens.
d. Altered expression (deletion, reduction, increase) of normal cell-or tissue-specific antigens.

(Poste 1977)

There have been several reports indicating that tumour cells release significantly greater amounts of proteases and glycosidases than their normal counterparts (Roblin et al., 1975). In this context it is interesting to note that many transformed cells release a factor that activates serum plasminogen to generate the protease plasmin, (Roblin, et al., 1975). Treatment of normal cells with purified plasmin results in a loss of a high molecular weight glycoprotein (reminiscent of the LETS protein). Experiments designed to test the effect of protease on the cell surface using protease inhibitors are complicated by the cytotoxicity of these inhibitors. However such experiments tend to support the postulate that some cell surface changes result from increased proteolytic activity at the cell surface (Poste,1975).

Emmelot has reviewed the evidence for all surface changes in malignancy (Emmelot,1973) and considers malignancy as a problem in cell sociology and suggests that the altered behaviour of the tumour cell may stem from a chemical alteration at the cell periphery. He suggests that the tumour cell surface is incomplete. A recent paper from the group (van Beek et al.,1975) shows a difference in a fucose-containing glycopeptide isolated from virally transformed fibroblasts. It was shown that the difference was caused by the increased sialic acid content of the glycopeptide. Previous work also implicates sialic acid in the process of malignant transformation (van Beek et al.,1973).

1.17 METASTASIS AND CELL SURFACE ALTERATIONS

Metastasis involves the sequential release of cells from the primary tumour, their dissemination to distant sites and their

/arrest..

arrest, survival and proliferation at these new locations. It appears that three aspects of cellular function may be important in these processes; Motility and Adhesiveness, and production of lytic substances which may modify surrounding tissue.

1.17.1 Motility and Adhesiveness

It has long been held that infiltration and metastasis are processes involving single cells. However it seems more likely that multi-cellular cords of tumour cells may be involved in invasion and that this may be mediated by movement of cells at the edges of the sheet in a fashion analogous to epiboly in the early embryo (Trinkhause, 1976). The exact mechanisms whereby cells or multicellular aggregates detach from the primary tumour are not well defined There has been a general acceptance of the suggestion that cancer cells are less adhesive than their normal counterparts, probably stemming from Coman's work (Coman,1944). However his experiments suffered from a number of limitations, e.g. the cells were pulled apart with small hooks in a traumatic fashion. Trauma was not rate controlled and very few cell types were investigated. There appears to be a deficiency in the number of gap junctions in malignant tissues but this is by no means a general finding (Weinstein et al.,1975).

/Several...

1.17.2 Lytic Enzymes

Several studies have shown that the fluid surrounding solid tumours contains enzymes which modify cell surface properties and facilitating cell separation and invasion. One group of enzymes which may be involved is the lysosomal hydrolases These have been shown to promote cell detachment *in vitro* and to promote metastases *in vivo* (Weiss and Poste,1975). Protease and collagenase activities have been investigated (Strauch,1972). Despite the interest of such work, it has proved difficult to generalise. Observations of tumour cell invasion have revealed a broad pattern of behaviour ranging from complete lack of lytic activity (Carr *et al.*,1976) to massive destruction of host tissue (Willis, 1967).

The results on plasminogen activator production are also difficult to generalise. Studies on plasminogen-activator production by various malignant cell lines of high and low metastatic potential have failed to reveal any general correlation between plasminogen activator level and metastatic potential (Poste,1977).

Also tumorigenic clones of human fibrosarcoma cells have been described with low levels of plasminogen activator (Jones, *et al.*,1976), whereas non-tumorigenic diploid lung and kidney cells may produce high levels of plasminogen activator (Astrup,1975; Barlow *et al.*, 1975).

/The..

The paper by Jones, et al.,(1976) was interesting in that the factor which correlated best with tumorigenicity was the ability to grow in soft agar. With regard to the effect of proteolytic enzymes on surface structures they showed that treatment of normal fibroblasts with proteolytic enzymes resulted in cleavage of large surface protein (MW 250,000d) there was no correlation with fibrinolytic activities and the loss of this large protein. They conclude that it seems unlikely that fibrinolytic activity is responsible for its removal or degradation. Once metastisizing tumour cells have gained entry into the circulation their fate will depend on surface properties such as charge and/or foreigness. The cell surface properties will influence the interaction of the tumour cell emboli with the host immune system and with the vascular endothelium and may assume considerable importance in determining both the extent and location of the metastatic foci. It would appear that the finding of circulating tumour cells does not necessarily correlate with the development of metastases (Salsbury,1975). It seems as though the cells must first attach to the vascular endothelium and subsequently leave the vessel and move into the tissue. The factors which influence the interaction between these circulating tumour cells and the vascular endothelium are not well understood. It appears however that the arrest of tumour emboli in capillary beds is in

/some...

some way related to or associated with thrombogenesis.

1.16. ALPHA-ACID GLYCOPROTEIN

Alpha acid glycoprotein (orosomucoid) is a globulin which is isolated from human plasma and has a number of distinguishing characteristics, high carbohydrate and sialic acid content; peripheral microheterogeneity, very low isoelectric point; high solubility in water and a degree of homology with the immunoglobulins (Schmid,1975).

It may be prepared from serum by a variety of methods which include bulk precipitation of the majority of serum proteins with perchloric acid leaving orosomucoid in solution (other acids may be used, e.g. sulphosalicylic acid and trichloracetic acid); ammonium sulphate may be used to deproteinise the serum at low pH, leaving orosomucoid in solution and DEAE cellulose ion exchange chromatography may be used to prepare the protein in pure state. (Schmid 1975; Jeanloz, 1972). It is present in Cohn Fraction V (Jeanloz,1972). The chemical composition of alpha-acid glycoprotein is shown in Table 5 and in Table 6.

A number of interesting observations emerge from a study of the literature. Possible functions of this protein are unknown. It is hard to accept that a protein of this complexity which is evolutionally conserved should have no function. Also all investigations of the structure of alpha-acid glycoprotein have been performed on pooled material. Consequently subtle individual variations may be obscured (Schmid,1975).

Analysis of the carbohydrate moeity on the molecule reveals the following sugars to be present: D-mannose, D-galactose,

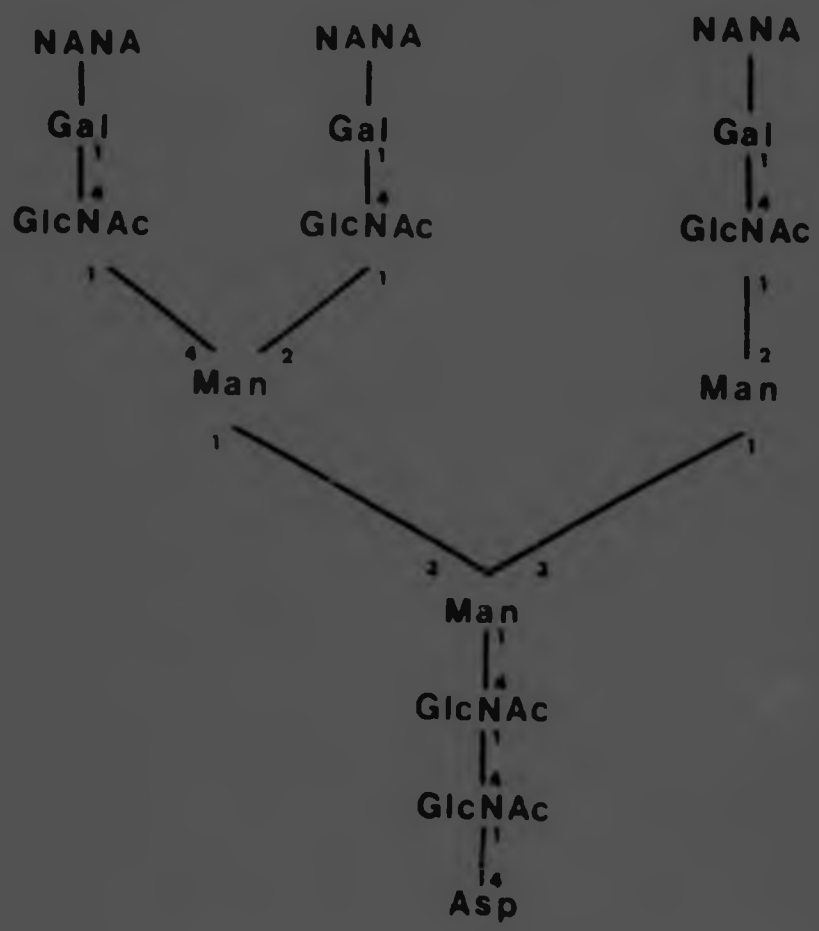
/L-fucose..

L-fucose, D-glucosamine and sialic acid. The quantitative analysis of the sugars present is shown in Table 7.

A tentative structure for alpha-acid glycoprotein has been put forward by Schmid (1975), Fig 6. This structure is essentially similar in many respects to the carbohydrate structure proposed for the major glycoprotein of the red cell membrane. See Fig 7. Another similarity between orosomucoid and the major glycoprotein of the red cell membrane is that in both, the carbohydrate moieties are attached to the N-terminal end of the molecule (Schmid,1975). Of considerable interest is the fact that alpha-acid glycoprotein shows peripheral microheterogeneity (Schmid,1975). These features of the molecule viz. sugar content microheterogeneity and localization of the carbohydrate to the N-terminal end prompted a study of this molecule and its relationship to known surface antigenic components such as blood group antigens.

FIGURE 6

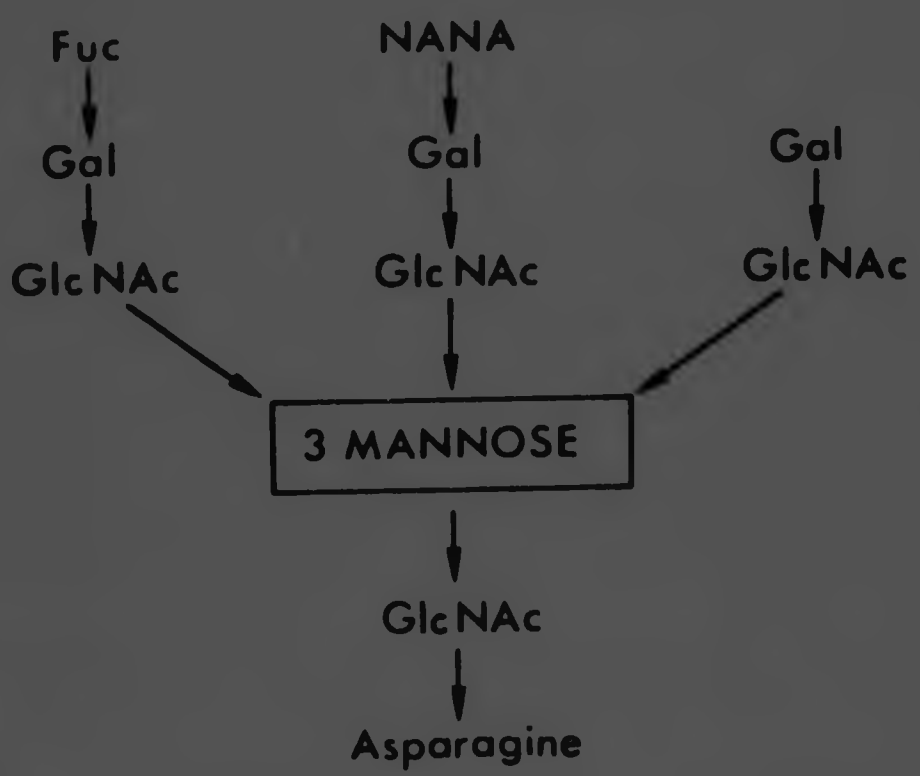
STRUCTURE OF CARBOHYDRATE ON ALPHA ACID GLYCOPROTEIN



(Wagh, et al., 1969; Schmid, 1975)

FIGURE 7

TENTATIVE STRUCTURE OF A GLYCOPEPTIDE RELEASED FROM RED CELL MEMBRANE GLYCOPROTEIN



(Winzler, 1972)

TABLE 5

CHEMICAL COMPOSITION OF ACID, GLYCOPROTEIN OF HUMAN PLASMA

Properties	Values
Nitrogen, %	10.7
Protein moiety,%	55
Terminal amino acids Amino terminal	Pyrrolidone-carboxylic acid (1 mole/mole)
Carboxyl terminal	Serine (1 mole/mole)
Carbohydrate moiety, %	45
Sialic acid	11 - 12
Neutral hexoses	13 - 17
Hexosamine	12 - 15
Fucose	0.7 - 1.5

Adapted from Schmid (1975)

TABLE 6.

PHYSICAL PROPERTIES OF ALPHA-ACID GLYCOPROTEIN OF HUMAN PLASMA

Physical Properties	Values
Isoelectric point :	
Phosphate buffer (I=0.1)	pH 2.7
electrofocusing	1.82
Electrophoretic mobility	
phosphate buffer (I=0.1),	
at pH 2.4	$+0.72 \times 10^{-5}$ cm^2, V^{-1}, sec^{-1}
at pH 7.6	-6.67×10^{-5} cm^2, V^{-1}, sec^{-1}
acetate buffer (I=0.1),	
at pH 9.5	-3.8×10^{-5} cm^2, V^{-1}, sec^{-1}
Molecular weight :	
by sedimentation	41100 d
by sedimentation-diffusion	40000 d
by sedimentation-viscosity	41600 d
by light-scattering	43000 d
by dodecyl sulphate polyacrylamide gel electrophoresis	40000 d
Coefficient of friction, f/fo	1.78
Viscosity increment, V	10.2
Optical rotation, $(\alpha)_D^{25}$:	
at pH 3,4	-53.1^o
at pH 5,8	-28.8^o
at pH 9,4	-21.1^o
Absorption value, $E_{1\,cm}^{1\%}$	
at 278 nm	8.93

TABLE 7.

CARBOHYDRATE COMPONENTS OF ALPHA-ACID GLYCOPROTEIN ISOLATED FROM HUMAN PLASMA

Methods of preparation	2-Acetamido-2-deoxy-D-glucose ( )	D-Mannose (%)	D-Galactose (%)	Total hexoses (%)	L-Fucose (%)	N-Acetylneuraminic acid (%)
Heavy metal-ethanol	15.2	4.8	6.5	14.2	0,9-1.1	10.8
Ammonium sulphate-gel electrophoresis	12,4-14,3	4,7-5,6	9,4-11,2	13,6-16,8	0,7-1.1	11,0-14,7
DEAE-cellulose-ion-exchange resin	12,2			14,4	1.	12,6
Heavy metal-ethanol heat-ion-exchange resin	15,3	6,5	6,7	13,2	0,7	10,9
Perchloric acid-gel electrophoresis	13,9	4,9	9,8	14,7	0,7	12,1
Heavy-metal ethanol	13,2	5,5	7,6	13,1	0,7	10,9

1.19 SOME EFFECTS OF SIALIC ACID ON THE PROPERTIES OF NORMAL AND TUMOUR CELLS.

Cellular mechanisms which control growth and development do not operate as effectively in transformed cells as in normal cells. Emmelot (1973) suggests that this is due to changes in surface chemistry. One such change is in sialic acid-containing molecules (van Beek et al.,1973,1975). Changes in sialic acid containing molecules at the cell surface can lead to many changes which may be associated with malignancy. One such change is in the transplantability of a mouse mammary carcinoma cell line which has been studied by Codington and his co-workers (Codington,1975). This line shows a loss of strain specificity and could grow in both allogeneic and xenogeneic animals, i.e. it was able to grow across both strain and species histocompatibility barriers. They suggest that the allotransplantability of this TA3-Ha mammary cell line may be the result of masking of the histocompatibility antigens by a glycoprotein which they called epiglycanin.

Sanford has shown (1967) that removal of sialic acid from the surfaces of the TA3-Ha cells with neuraminidase reduced the transplantability of this cell in an allogeneic mouse strain. The mechanism of action of neuraminidase in producing reduced transplantability is not well understood, both an enhanced cell mediated immune response, and the cytotoxic effect of an IgM antibody have been suggested. In this respect it is interesting to note that both the Thomsen Friedenreich effect and the reduced transplantability require the unmasking of a beta-D-galactopyranosyl residue.

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The effect of removing charge, thus allowing easier approach of lymphocytes cannot be ignored. However, removal of surface charge is not the whole story. In some systems, removal of the cell surface sialic acid has no effect on the adsorption of antisera to surface histocompatability antigens.

One problem which complicates the issue in such experiments is the rapid regeneration of cell surface sialic acid (Hughes et al.,1972). Suggestions that removal of sialic acid may increase the deformability of cells, enhancing their susceptibility to phagocytosis, have been made (Weiss,1965). Currie and Bagshawe (1967), reported that removal of sialic acid from mouse trophoblasts resulted in exposure of antigens which were previously masked. However, Simmons et al., (1971), were unable to confirm this finding. Neuraminic acid plays a role in the homing of lymphocytes (Gestner and Ginsburg,1964; Woodruff and Gesner,1969).

Sialic acid is also important at the cell surface as a component of the receptors for lectins, viruses, hormones, antibodies(and also circulating desialated glycoproteins) (Jeanloz and Codington, 1976).

ALPHA-ACID GLYCOPROTEIN AND ITS EFFECT ON COAGULATION

The exact function of alpha-acid glycoprotein is obscure Nilsson and Yamashina (1958) showed that alpha acid glycoprotein influenced the blood clotting mechanism. They found that the addition of alpha-acid glycoprotein to normal

plasma...

plasma resulted in a prolongation of the coagulation time. A solution of 1% alpha-acid glycoprotein in imidazole buffer showed no thrombin or prothrombin activities. It did not delay the clotting of fibrinogen by thrombin and showed no plasmin or plasminogen-activator activity. They claim that alpha-acid glycoprotein inhibited plasma thromboplastin and they suggest that the anti-coagulant effect of a alpha-acid glycoprotein could be attributed to an inhibition of the transformation of prothrombin to thrombin by thromboplastin in a competitive manner. The result is surprising in view of the fact that conditions in which alpha-acid glycoprotein is elevated, are associated with intravascular clotting disorders.

Of possibly related interest is the finding by Niewiarowski et al., (1975) that alpha-acid glycoprotein can reverse the effects of the drug "Persantin" (dipyridamole) on platelet aggregation. It is also known that platelets carry significant amounts of alpha-acid glycoprotein lightly bound to their membranes (Schmid, 1975). Moreover platelets can adhere to collagen, which adhesion can also be inhibited by dipyridamole. This inhibition can also be reversed by alpha-acid glycoprotein.

[7] SIALIC ACID AND PLATELET AGGREGATION

The adhesion of platelets to each other is an essential step in the formation of a hemostatic plug. Many materials cause aggregation of platelets such as thrombin, collagen, fatty acids, immune complexes, 5-hydroxytryptamine and adrenaline. Most of these compounds appear to exert their effect through the mediation of ATP or ADP (Jeanloz and Codington, 1976). The treatment of platelets with neuraminidase results in their

/spontaneous.

spontaneous aggregation when they are resuspended in citrated plasma. Incubation of washed human platelets with cytidine N-acetylneuraminic acid monophosphate in the presence of sialyl transferase increased the number of sialic acid residues at the surface of the platelet and resulted in an increased aggregation with 5-hydroxytryptamine. However, the same treatment led to a decrease in ADP-mediated aggregation. Injection of neuraminidase into mice resulted in a marked thrombocytopenia (Jeanloz and Codington,1976). It would thus appear that sialic acid - containing molecules are an integral component of the platelet surface and these play an important role in the interaction of the platelet with its environment.

1.22 THE RELATIONSHIP BETWEEN METASTASIS AND COAGULATION

Many observations of both human and animal tumours suggest that the arrest of tumour emboli in capillary beds is in some way associated with the coagulation process (Donati et al.,1977; Poste,1977; Hilgard,1973; Hilgard and Thornes,1976). Small clumps of tumour cells generally 6-10 attach to the vascular endothelium. They are then surrounded by fibrin, platelets and polymorpho-nuclear lymphocytes. Subsequently local defects in the endothelium form and the tumour cells pass out of the vessel and form new centres of growth. The site of adherence of the tumour cells to the endothelium is determined by interactions between the tumour cell surface and the vascular endothelial surface. This in turn seems to be influenced by micro-coagulation around the tumour cells. It is well known that anti-coagulants may decrease metastasis (Hilgard and Thornes, 1976; Carter, 1976 a and b). However some

/caution.

caution must be exercised in interpreting these results. Usually large doses of anticoagulants have been given over long periods of time. Also tumour cells were usually given directly int the bl od stream. Also consideration must be given to the effects of highly charged molecules such as heparin on the tumour cell surface as well as its effect on coagulation.

SERUM PROTEIN ABNORMALITIES IN PATIENTS WITH CANCER

The serum of patients with different types of malignancies is often characterized by an increase in the alpha-globulin serum component and changes in the carbohydrate content of serum glycoproteins (Petermann and Hogness, 1948; Almquist and Lausing, 1957; Rosato and Ravdin, 1968; Ablin et al., 1975). Similar changes have also been reported in the serum proteins of animals with transplanted or naturally occurring tumours (Schwenke and Kujawa,1967; Hadji-Azimi,1969; Ove et al.,1974).

Recently DNA-binding proteins were found in the serum of patients with various neoplasms (Brehm et al.,1975; Hoch et al.,1975). These proteins migrated in the alpha and beta regions. There were two major protein components of MW 94,000 and 126,000d. These authors ascribe a scavenger-function for these proteins (Brehm et al ,1975). Other proteins which undergo changes in malignant conditions are the acute-phase proteins (Fischer and Gill,1975). These consist of an electrophoretically heterogeneous group of proteins which migrate between the albumin and gamma-globulin fractions. They are functionally diverse and consist of a

/protease..

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/protease

protease inhibitor (alpha-antitrypsin); carriers for hemoglobin and copper (haptoglobin and ceruloplasmin); a coagulation component (fibrinogen) and proteins with undefined functions such as C-reactive protein and alpha-acid glycoprotein. A list of the acute phase proteins and some of their properties is shown in Table 8.

The mechanism of the elevation of proteins during the acute phase response is in considerable doubt and at least one suggestion is that they are released locally from affected tissue has been put forward (Bole and Leutz 1967). These authors showed that extrahepatic tissues were able to incorporate labeled N-acetyl glucosamine into acid-soluble glycoproteins found in serum. However the general suggestion is that these proteins are produced in the liver alone (Winzler,1965;Sarcione,1967). Such an experimental model which relies upon total hepatectomy of rats is in my opinion questionable. It would appear that such a massive operative procedure would result in experimental data which would be extremely difficult to evaluate and would not exclude the extrahepatic origin of serum glycoproteins. A totally hepatectomized animal is in effect moribund and the author prefers to treat such a model with extreme caution. Other evidence for extrahepatic origin of seromucoid may be found in the literature. Some authors have found higher seromucoid levels in the venous effluent of large transplanted fibrosarcomas than in the arterial supply to the tumour (Hoskins et al.,1974). There is also evidence that tumour cells which have a thin glycocalyx are more aggressive than those with a thicker glycocalyx. One interpretation of this

/is. .

TABLE 8

CHARACTERISTICS OF ACUTE-PHASE PROTEINS

Protein	Molecular Weight	$S_{20,w}$	Electrophoretic Mobility	Peptide Content (%)	Carbohydrate Content (%)	Serum Content Range (mg/100ml)	Biological Function
C-reactive protein	135,000-140,000	7.5	α_1-Globulin	100	0	0	Opsonin; acute-phase reactant
α_1-Acid glycoprotein	44,100	3.1	α_1-Globulin	62	41.4	55-120	Inactivation of progesterone; acute-phase reactant
α_1-Antitrypsin	45,000	3.4	α_1-Globulin	86	12.4	180-305	Protease inhibitor of trypsin and chymotrypsin; acute-phase reactant
Ceruloplasmin	160,000	7.1	α_2-Globulin	89	8.0	10-40	Copper-binding oxidase activity; acute-phase reactant
Haptoglobin 1-1 Haptoglobin 2-1 Haptoglobin 2-2	85,000	4.4 4.3-6.5 7.5	α_2-Globulin	81	19.3	85-213	Hemoglobin-binding peroxidase; acute-phase reactant
Fibrinogen	341,000	7.6	β_2-Globulin	97	2.5	200-450	Coagulable protein; acute-phase reactant

is that the glycocalyx is being continually shed. In this respect it is interesting to note that high seromucoid levels are found in more advanced metastasised tumours (Kim et al.,1975).

1.24 SEROMUCOID AND THE IMMUNE RESPONSE

It has been suggested that seromucoid may play a role in preventing an immune response against fetal antigens (Currie and Bagshawe,1967; Good 1975). It is also of interest to note that alpha-acid glycoprotein can act on the surface of Escherichia coli and Staphylococcus aureus in such a way as to prevent phagocytosis of these organisms by human neutrophils (van Oss et al.,1974)

1.2 SUMMARY OF THE INTRODUCTION AND THE AUTHOR'S INTERPRETATION OF THE LITERATURE.

It would seem that membrane alterations are an important part of the neoplastic transformation. At least one of these changes relates to the loss of glycoprotein material from the cell surface. These glycoproteins may find their way into the circulation and may interfere with a host immune response which is directed against neo-antigens on the tumour cell. Some of the shed material may be composed of surface antigens normally confined to the plasma membrane of the cell. This would include HLA and blood group antigens. Changes in coagulation profile which are associated with malignancy may in part relate to the change in circulating glycoproteins. The work of Baldwin and Robins (1976) and Vaage, (1973;1974) indicate an important role for tumour antigens in the blocking of immunity directed against tumour cells. The antigenic

/material...

material in Baldwin's study had a MW of between 30,000 and 50.000d. Or omucoid has been known to be elevated in the serum of cancer patients for some time. It is an alpha-globulin and has been thought to be entirely synthesised in the liver. However, it is in some respects similar to glycophorin, the major glycoprotein of the red blood cell membrane, in size, distribution and nature of the carbohydrate attached to it. It is also similar in that it tends to polymerize and has a binding site for the influenza virus as shown by its ability to inhibit influenza virus haemagglutination.

It is thus possible that seromucoid may in fact contain cell membrane components and that its elevation in the serum in acute conditions may result at least in part, from tissue necrosis or shedding (as in the case of CEA). It is of interest to note that although the peripheral sequences of the carbohydrate chains on orosomucoid appear to be similar to those on blood group substance, some differences are apparent such as attachment of the carbohydrate to the polypeptide, the amounts of the different sugars present, and the total percentage of carbohydrate in the molecule. Carbohydrate containing molecules are of importance in cell-cell interactions and tissue formation and may be important in allowing hormones to interact with the cell. Humphreys (1975), has shown that the molecules which promote sponge aggregation are also lycoproteins with a high percentage of carbohydrate.

1.26 THE AIM OF THIS STUDY

The aim of this study was to examine the sera of patients

/with...

with cancer and a spectrum of other diseases and conditions. To further characterise any changes and to investigate the possible effects of such changes.

To this end polyacrylamide gel electrophoresis was performed on the serum of patients with cancer and non-malignant conditions. These were compared with sera from normal controls. Two bands were shown to be elevated in various diseases. One of these protein bands was isolated and identified as orosomucoid (alpha-acid glycoprotein). The use of the two bands as a diagnostic tool was evaluated and the effect of orosomucoid on the immune system and the coagulation cascade was investigated. The similarity between orosomucoid and glycophorin prompted a search for blood group antigens in the orosomucoid preparations.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

2. MATERIALS AND METHODS

2.1 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

2.1.1 Purification of Sodium dodecyl sulphate (SDS)

SDS (5g) was added to 100ml of 95% ethanol. The solution was warmed to 60°C in a waterbath with constant stirring to dissolve, cooled to 20°C and vacuum filtered through Whatmans No.42 filter paper. The filtrate was retained and cooled to 5°C. This was filtered and the precipitate was retained, dried to constant weight and stored. The yield was between 15-20%.

2.1.2 Preparation of Working Solutions

1. Stock 0.06M sodium phosphate buffer, pH 7.2, containing 0.2% (w/v) SDS.

2. Monomer solution

Acrylamide m	20 g
Bis-acrylamide	0.5 g
Stock buffer to	100 ml

3. Ammonium persulphate solution 0.4% (w/v)

4. TEMED

2.1.3 Casting the Gels

The gel was cast using the following mixtures:

Water	7.5	ml
Stock buffer	3	ml
TEMED	10	µl
Stock monomer	7	ml
Ammonium persulphate	2.5	ml

Giving a final acrylamide concentration of 7%.

/The...

The gels were cast in running tubes (0.5 x 7.5cm) which were marked at one end to allow reproducibility of gel length. This mixture was transferred t the running tubes and carefully overlayed with stock buffer using a Hamilton syringe. Gelling time was 20-40 minutes.

2.1.4 Splitting Solution

A splitting solution was prepared containing 5M urea, 1% SDS and 1% mercaptoethanol.

2.1.5 Loading the Gels

Between 4 and 10 µg of protein was loaded onto the gel in a volume not exceeding 10 µl.

2.1.6 Running the Gels

The gels were run at 5 ma per tube for 60 mins. Bromophenol blue was used as a marker. Electrophoresis was stopped when the dye reached 1 cm from the end of the gel. Time taken was 1 hour.

2.1.7 Staining

Gels were removed from the running tubes and fixed in 10% (w/v)tric acetic acid for 1 hour. They were rinsed in running tap water and placed in staining solution overnight. The staining solution found to be most convenient was Coomassie brilliant blue prepared as follows:

Coomassie blue	0.1	gm
Acetic acid	70	ml
Methanol	400	ml
Water to	1000	ml

/Destaining...

2.1.8 Destaining

Gels were destained and stored in 7% acetic acid for 48 hours.

2.1.9 Photography of the Gels

All gels were photographed using a Canon FTb 35mm camera with a standard macro lens and orange filter. Exposure time of 1/60 sec and lens opening of f16 were found to give satisfactory negatives. Panatomic X (Kodak^{R}) film was used. A light box was used as a source of illumination with the gels placed directly onto the light box alongside a plastic length scale.

2.1.10 Periodic Acid Schiff (PAS) Staining of the Gels for Detection of Glycoproteins.

PAS staining was performed by a modification of the method of Fairbanks et al.,(1971). Many of the steps used in this method were found to be unnecessary and consequently a shorter procedure was devised as follows:

1. Gels were fixed in the same way as for Coomassie Blue staining.
2. They were then placed in 0.5% (w/v) periodic acid for 1 hour.
3. The gels were then washed exhaustively in 40% methanol, 7% acetic acid in water until the washings no longer gave a precipitate with silver nitrate.
4. The washed gels were then placed in Schiffs

/reagent

reagent for 1 hour.

Red bands appeared within 10 minutes.

2.1.11 Preparation of Schiff Reagent

1 gm Basic Fuchsin was dissolved in 200 ml of boiling distilled water. This was cooled to 50°C and to it was added 20 ml of 0.1M HCl. The mixture was then further cooled to 25°C and 1 gram of Sodum Metabisulphite was added. After mixing it was left in the dark for 20 hours. Charcoal was added and the solution was filtered to give a colourless solution which may be stored at 4°C in the dark.

2.1.12 Polyacrylamide Gel Electrophoresis of Serum

Serum was collected from freshly clotted blood and 0.1 ml diluted with splitting solution (2.0 ml). After mixing 10 µl was layered onto the gel. Loading of more than 10 µl resulted in a decreased resolution.

2.1.13 Quantification of the Bands after Staining

The amount of protein in each band was estimated by measuring peak dimensions of the band and relating these to known concentrations of bovine serum albumin run under identical conditions.

2.1.14 Molecular Weight Estimation using PAGE

The molecular weights of the various bands on PAGE were estimated from a comparison of their migration distance on PAGE with that of proteins of known MW according to the method of Weber and Osborn (1969). The standard proteins used

/were...

were bovine albumin egg albumin and cytochrome C. The migration distance from the origin was plotted against Log MW, see Fig.8.

2.2 IMMUNOELECTROPHORESIS

Immunoelectrophoresis was performed according to the method of Arquembourg et al.,(1970). Briefly the method is as follows:

2.2.1 Stock Buffer

The following barbiturate buffer, pH 8.3 was prepared and stored at 4°C with sodium azide as a preservative.

Diethylbarbituric acid	1.4 g
Sodium diethylbarbiturate	5.0 g
Sodium chloride	1.0 g
Water to	1000 ml

2.2.2 Preparation of the Electrophoresis Gel

Agarose of high purity was used to prepare the gels. A 1% agarose solution in stock buffer was made up and autoclaved for 15 minutes to ensure complete solubilisation of the agarose. The agarose solution was poured onto microscope slides (2.5 x 7.5 cm) in a holder which ensured constant gel thickness on the slide. The procedure was carried out on a levelling table.

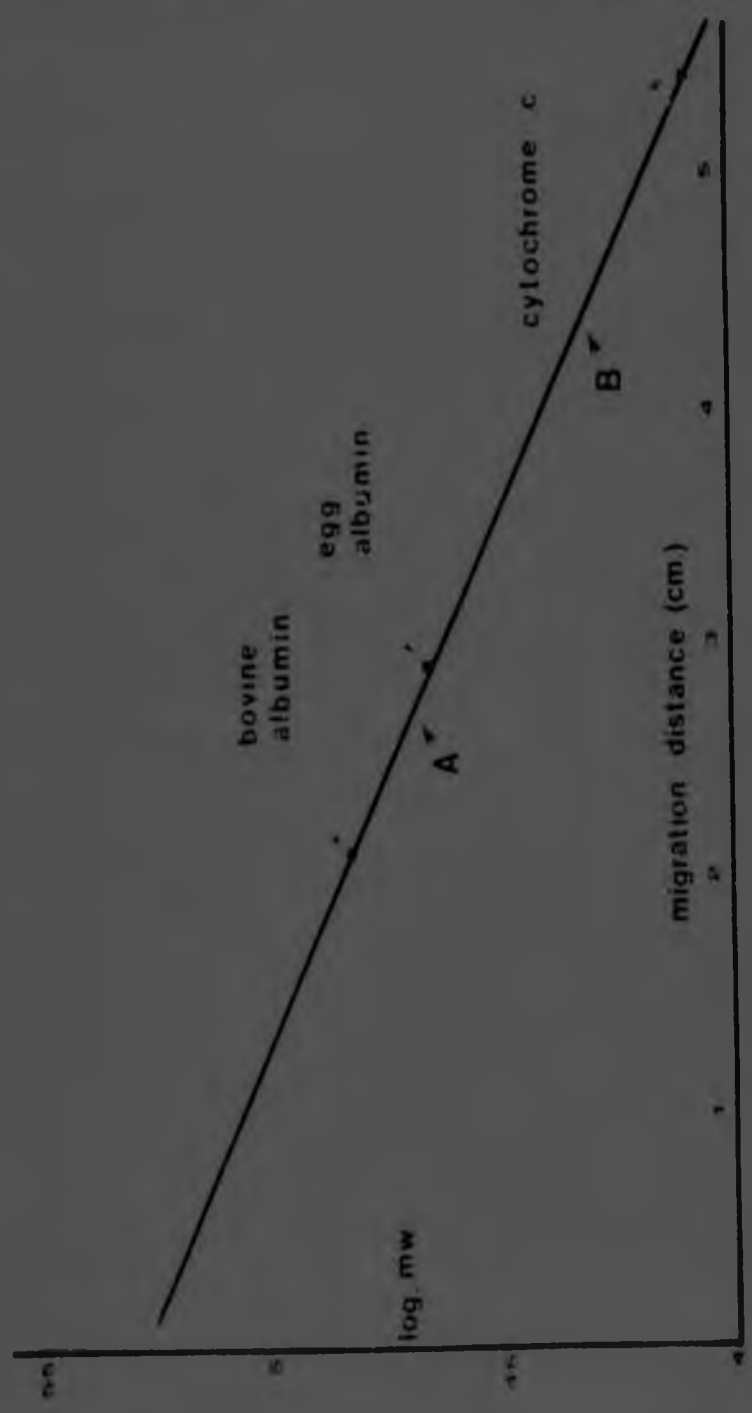
2.2.3 Cutting the well and trough

The wells and trough were cut using a template made to standard dimensions. The well cutter had a bevelled inner cutting surface and was attached to a vacuum pump. The trough was cut

/with..

FIGURE 8

MOLECULAR WEIGHT DETERMINATION OF A BAND AND B BAND ON PAGE



with a scalpel blade.

2.2.4 Electrophoresis

The gels were electrophoresised at 25 ma per slide until the bromophenol blue marker reached a satisfactory position.

2.2.5 Addition of antisera

The antisera were added to the trough using a tuberculin syringe and the gels were left overnight to allow the precipitin lines to develop.

2.2.6 Visualisation and Photography of the Gels

Examination and photography of the immuno-precipitin patterns was carried out under dark-field illumination.

2.3 RADIAL IMMUNODIFFUSION

Radial immunodiffusion was performed using the same buffer and agarose concentration as that used for immunoelectrophoresis (see 2.2.1 and 2.2.2 above). The mixture to be studied and the antisera were added to precut wells (2.5 mm diameter x 3 mm apart) in agarose in a petri dish. The pattern was allowed to develop overnight and was photographed as above (2.2.6).

2.4 SERUM PROTEIN FRACTIONS

2.4.1 Kistler Fractions

These were prepared according to Kistler and Nitschmann, (1962), and were a gift from Professor Peter Brain of the Natal Blood Transfusion Service.

2.4.2 Carcino-embryonic antigen

Radiolabelled CEA was obtained through the

/generosity...

generosity of Roche Ethical Company.

2.4.3 Alpha-acid glycoprotein

Serum and glycoprotein preparations were prepared from plasma by a modification of Winzler's original method. (Winzler et al.,1948). Blood was obtained from patients and normal volunteers after informed consent. Samples for serum were allowed to clot and the serum obtained by centrifugation of the clotted specimen. Blood for plasma extraction was collected into citrate phosphate dextrose (CPD). Each donation consisted of 450-500ml of blood collected into 70 ml of CPD. Plasma was obtained from the whole blood within 72 hours of collection. To either serum or plasma an equal volume of 1.4M-perchloric acid was added. All extraction procedures were performed at 4Ø°C. The perchloric acid/serum/plasma mixture was allowed to stand for 5 minutes and the precipitated proteins removed by centrifugation. The clear supernatant was dialyzed against running tap water for 24 hours, reduced in volume by evaporation in a dialysis tube and then dialyzed against distilled water for 4 hours. It was then lyophilized and stored in sealed ampules until used. This was referred to as the crude glycoprotein preparation. Further purification was achieved using Sephadex chromatography.

/2.5...

2.5 SEROLOGICAL DETERMINATIONS ON THE FREEZE-DRIED PREPARATIONS

Optimal concentrations of antisera were prepared as follows:

If anti-P_1 reacts to a dilution of 1:64 (e.g. undiluted ++++, 1:2 ++++, 1:4 ++++, 1:8 ++++, 1:16 +++, 1:32 ++, 1:64 +, 1:128 0) then 1:4 was selected as the optimum concentration of the specific antibody for the haemagglutination inhibition tests. To this optimum saline dilution of anti-P_1 was added an equal volume of saline (control) or seromucoid reconstituted in saline (to a standard concentration). The addition of saline should result in no loss of agglutinating ability of the antiserum. If, however, the reaction strength was lowered by the addition of the seromucoid extract then some inhibition of antibody activity has taken place. The inhibition of activity is recorded as follows: ++++ representing no inhibition, +++, ++, + - as partial or complete inhibition. Thus for any one sample of seromucoid which for example, does not lower the activity of the optimum concentration of antiserum, the pattern would read ++++ (4), ++++ (4) ++++ (4), or 4444 i.e. score = 16. If, however, the seromucoid sample when reconstituted had some inhibitory activity, the pattern in the four dilutions tested may be represented as follows: -, +, ++, +++ or - 1 2 3, meaning that there was activity in the extract undiluted, diluted 1:2,1:4 and 1:8. This would represent a score of 6. The inhibitory activity is expressed as the maximum score

/possible..

possible(4+4+4+4 = 16)minus the score actually obtained. Thus for our second example the inhibitory activity would be 10, .e. (16-6=10) referred to as the cumulative inhibition score.

Where groups of individuals were studied the cumulative inhibition scores were summed and a mean inhibition activity was calculated. Table 9 details the reactions of the various antisera selected for this study.

During the preliminary course of the investigation it was found that the inhibitory activity of various blood group determinants particularly the T factor, often spontaneously disappeared from the seromucoid preparations. To avoid this loss of activity, which is influenced by the presence of beta-galactosidase in the seromucoid (Kline and Issitt, 1970) phenyl-beta-D-thiogalactoside (10 mg/100 ml of serum extract) was added to the extract after dialysis and before freeze-drying the products.

2.6 COLUMN CHROMATOGRAPHY OF THE EXTRACTS

Column chromatography was performed to attempt to fractionate the preparation further and also to estimate the molecular weight using another method as the PAGE method is unreliable for glycoproteins (Segrest et al.,1971)

2.6.1 Sephadex G200 Gel exclusion chromatography

Sephadex G200 with a fractionation range of 5000d - 800,000 d was used. A column of dimensions 50 cm x 5 cm was used. The eluting buffer was 0.1M-Tris HCl, pH 8.0 with 1.0 M-NaCl. 10 ml aliquots were collected and the protein content

/monitored.

TABLE 9

REACTIONS OF VARIOUS REAGENTS

REAGENTS	Haemagglutination Activity at Dilutions											Optimal Temp. React. of Antisera	Dilutions of Antisera selected for AGGL-Inhib. Test.
	UND	2	4	8	16	32	64	128	256	512	1024		
Human-Anti- A	4	4	4	4	4	4	3	2	1	–	–	22°C	1.16
" " B	4	4	4	4	3	2	1	–	–	–	–	22°C	1.4
" " H	4	4	4	4	4	3	2	1	–	–	–	22°C	1.8
" " Le^a	4	4	4	3	2	1	–	–	–	–	–	22°C	1.2
" " Le^b	4	4	3	2	1	–	–	–	–	–	–	5°C	UND
" " P_1	4	4	4	4	3	2	1	–	–	–	–	5°C	1.4
" " $P+P_1+P^k$	4	4	4	4	4	4	3	2	1	–	–	5°C	1.16
" " I	4	4	3	2	1	–	–	–	–	–	–	5°C	UND
" " i	4	4	4	4	4	4	4	4	4	2	1	5°C	1.128
" " M	4	4	3	2	1	–	–	–	–	–	–	22°C	UND
" " N	4	4	4	3	2	1	–	–	–	–	–	22°C	1.2
lectin " T *	4	4	4	4	4	4	3	2	1	–	–	22°C	1.16

* Arachis Hypogoea Seed Extract Tested against T-activated Group O cells.

monitored by measuring A_{280}

2.6.2 Molecular weight estimation of the Protein Preparation

The molecular weight of the protein was estimated by measuring its elution volume off a calibrated gel filtration column. The partition coefficient (K_{av}) between the gel phase and the liquid phase was determined for ferritin, catalase, aldolase, bovine albumin and chymotrypsinogen K_{av} was calculated according to the following formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_t = Total bed volume = 981 ml

V_o = Void volume (determined by blue dextran) = 306 ml

V_e = elution volume of the protein under consideration and a plot of K_{aV} against log MW was constructed. (See Fig. 9).

2.7 LYMPHOCYTE CULTURE AND PHA STIMULATION

When lymphocytes are treated with various lectins, they are stimulated to undergo division. This response is usually accompanied by morphological change to a blast cell. The degree of lymphocyte stimulation may be assayed by determining the percentage of blast cells in a culture or by measuring the uptake of a radioactive DNA precursor, in this case tritiated thymidine

/(Oppenheim

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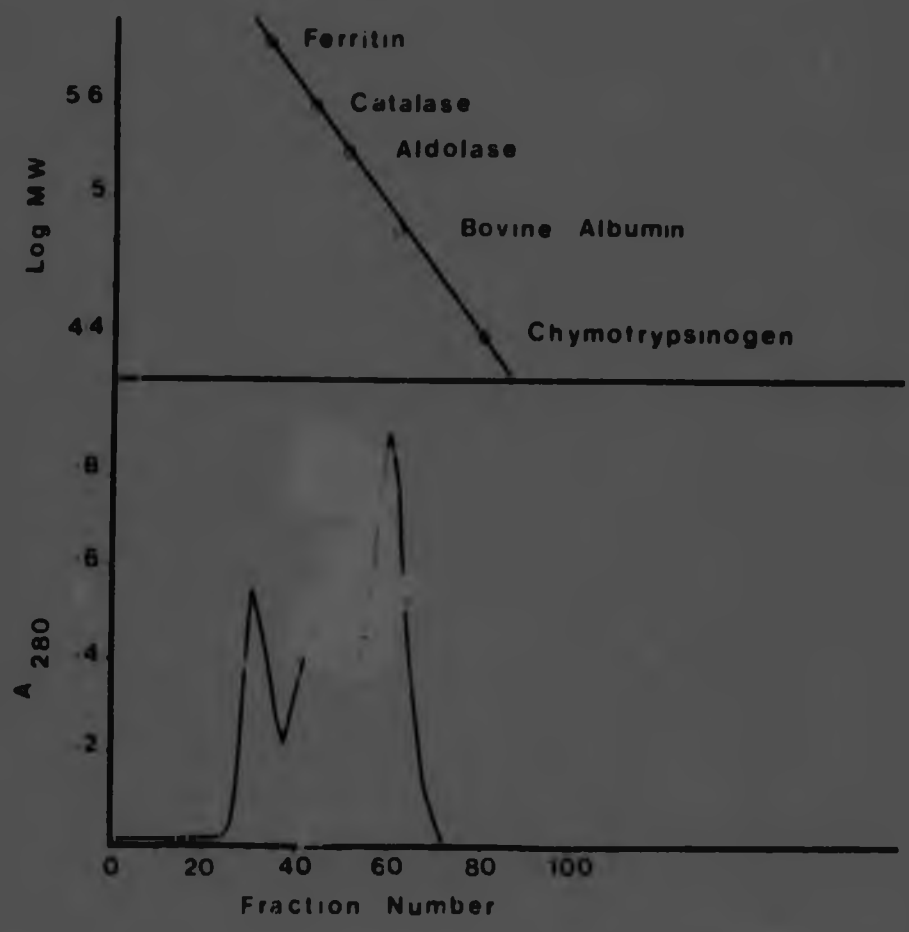
/(Oppenheim

FIGURE 9

ESTIMATION OF MOLECULAR WEIGHT OF A BAND BY COLUMN CHROMATOGRAPHY (WHOLE SERUM ELUTION PATTERN SHOWN BELOW)



(Oppenheim, 1968).

2.7.1 Isolation of Peripheral Blood Lymphocytes

Peripheral blood lymphocytes were isolated by flotation of defibrinated blood on Ficoll-Hypaque (10 parts 33.9% Hypaque and 24 parts 9% Ficoll, Sg 1.077). Two ml of defibrinated blood were gently layered onto 3 ml of Ficoll-Hypaque in a centrifuge tube (14 x 96 mm). This was centrifuged at 400 g_{av} for 30 min. Lymphocytes were harvested with a pasteur pipette and washed twice in Hanks Balanced Salt Solution (HBSS) followed by a wash in Eagles minimal essential medium (MEM) (Boyum, 1968).

2.7.2 Stimulation of Lymphocytes

Lymphocytes were isolated and washed as described above (2.7.1). The washed lymphocytes were counted and adjusted to give a final concentration of 2×10^6 cells/ml in MEM containing 20% autologous serum. The cells were cultured in microtitre plates. Each well contained 2×10^5 cells, 100 µl of MEM containing alpha-acid glycoprotein at the desired concentration. The optimum concentration of PHA was determined prior to the experiment and was added in 10 µl of MEM to the cultures.

2.7.3 Determination of Optimum Phytohaemagglutinin (PHA) Concentration.

Lymphocytes were cultured as described above (2.7.2) and different amounts of PHA were used to stimulate them. A plot of thymidine uptake vs PHA concentration

/was...

was constructed. See Fig.11.

2.7.4. Measurement of the Effect of Alpha-acid Glycoprotein on Blast Transformation.

In order to test the effect of the alpha-a glycoprotein extract on the blast transformation, the purified extensively dialyzed protein was added to a final concentration of 2.5 mg/ml. (This value was chosen because it represented a similar concentration to that found in the blood of patients with advanced tumours). In addition alpha-acid glycoprotein seromucoid was added in increasing concentrations to cultures of lymphocytes and the percentage transformation was measured in order to see if there was a dose dependent effect. The tests were run in quintiplicate.

2.7.5 Measurement of Blast Transformation

Blast transformation was measured by adding 10 µl of tritated thymidine (160 µci/ml) to each well on the second day of culture. The cells were harvested on the third day onto glass fibre filter discs and counted in a scintillation counter. Results were expressed as a percentage of the control of the same lymphocytes without alpha-acid glycoprotein.

2.7.6 Controls

Two controls were included with each experiment.

(1) Cells from the same donor, cultured under identical conditions without added alpha-

/acid.

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2.7.4 Measurement of the Effect of Alpha-acid Glycoprotein on Blast Transformation.

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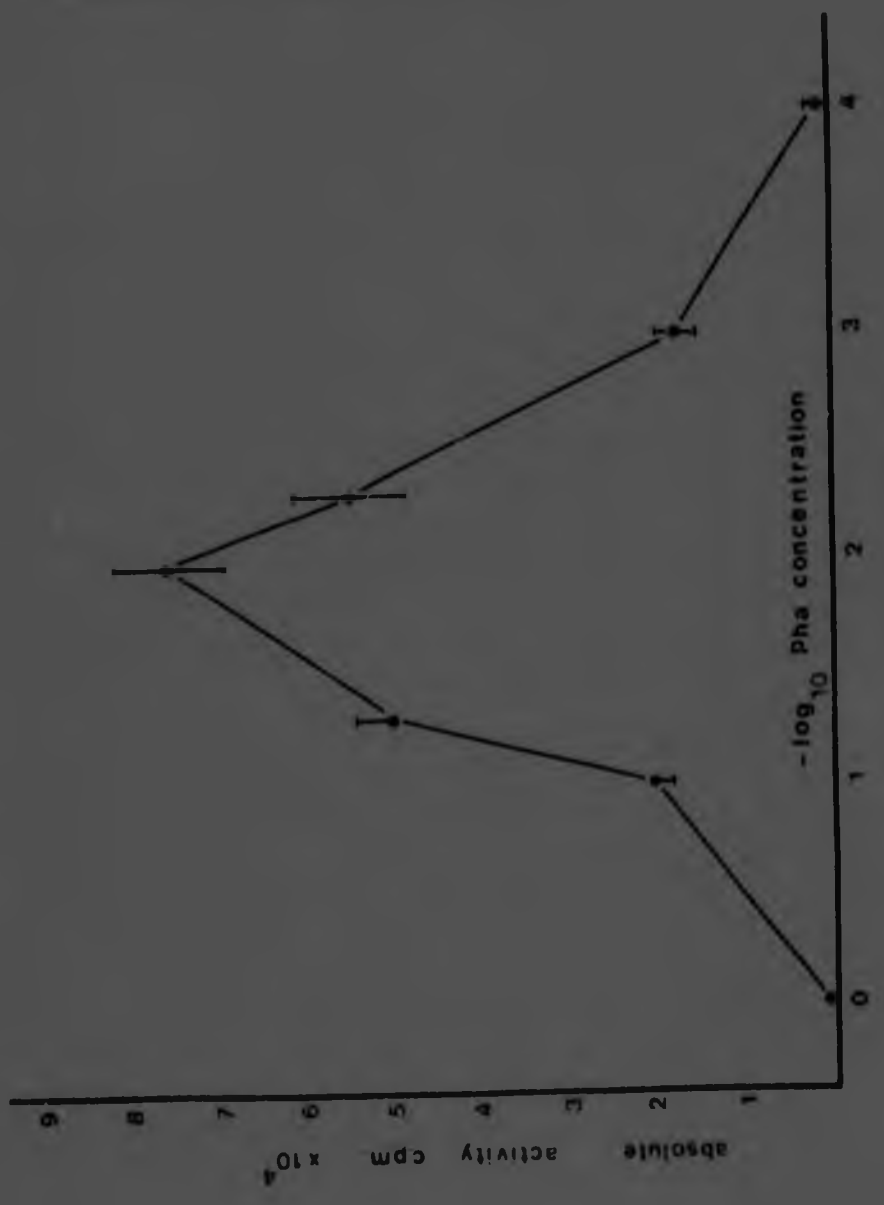
/acid ..

FIGURE 11

DETERMINATION OF THE OPTIMUM CONCENTRATION OF PHA



acid glycoprotein. These were taken as 100% and all results were expressed in terms of these cells. In this way it was hoped to minimise the inherent variability of the method. (2) Cells were cultured from the same donor under identical conditions without PHA. This was done in order to measure spontaneous blast transformation.

2.8 MEASUREMENT OF BETA$_2$-MICROGLOBULIN IN THE PREPARATION

Beta$_2$-microglobulin was measured using a radioimmuno assay in kit form (Pharmacia Diagnostics). The principle of the method is as follows: Anti-beta microglobulin antibodies are bound to particles of sephadex. The concentration of beta$_2$-microglobulin in an unknown sample is evaluated by its capacity to compete with a fixed amount of labelled beta$_2$-microglobulin for the binding sites of the anti-beta$_2$-microglobulin. The competitive capacity is then compared with that of standard beta$_2$ microglobulin preparations of a known concentration. When preparing the test, samples were first mixed with beta$_2$-microglobulin labelled with ^{125}I. Anti-beta-microglobulin complexed to sephadex was then added and the samples were incubated for 3 hr at room temperature with constant agitation. The bound and unbound beta$_2$-microglobulin were separated by centrifugation. Unbound beta$_2$-microglobulin in the supernatant was removed and the radioactivity bound to the sedimented sephadex particles measured in a gamma scintillation counter. The radioactivity taken up varies inversely with the quantity of unlabelled beta$_2$ microglobulin present.

Glycoprotein samples for beta$_2$-microglobulin extraction were passed through sephadex G200 columns prior to measurements to eliminate any free beta$_2$-microglobulin.

/2.9...

2.9 MEASUREMENT OF HISTOCOMPATIBILITY ANTIGENS IN THE GLYCOPROTEIN PREPARATIONS.

Antigenic activity was measured in the preparations by their ability to inhibit the complement-dependent lysis of ^{51}Cr-labelled target cells mediated by a polyspecific cytotoxic alloantiserum (PSS). Target cells were obtained from donors by Ficoll-Hypaque flotation as described previously. They were labelled with a solution of sodium chromate (^{51}Cr) in 0.15 M-NaCl (Radiochemical Centre, Amersham). Labelling was performed in 50% autologous serum using a cell concentration of 10^7/ml. To each ml of cell suspension was added 100μCi of sodium chromate in a total volume of 0.1 ml of 0.15 m-NaCl. The mixture was incubated for 45 minutes at 37°C. After this time the cells were washed five times with 0.15 M-NaCl and resuspended in the incubation medium.

The assay was performed in triplicate. PSS (25 μl) was used at a dilution sufficient to cause 70-95% target cell lysis. The antibody was incubated with an equal volume of the antigen preparation for 90 minutes at 37°C. Controls in which an equal volume of 0.15 M-NaCl was used were also run. The concentration of ^{51}Cr-labelled lymphocytes was adjusted to 5 x 10^6/ml and 25 μl of the cell suspension was added to 25 μl of antigen-antibody mixture/antibody-saline mixture. Rabbit serum (25 μl) was added as a source of complement. The combination was incubated for 60 minutes at 37°C. The reaction was stopped by the addition of 2 ml cold 0.15 M-NaCl. The cells were removed by centrifugation (1500 g x 10 min) and the radioactivity which had been released into 1 ml of supernatant was counted in a Packard Autogamma Spectrometer. A complement control was also included using 25 μl of the patient's own

/serum...

serum in place of PSS. Total release was established using 25 µl of anti-lymphocyte globulin in place of the PSS. Antigenic activity was expressed as the percentage inhibition of cytotoxicity calculated as follows:

$$\% \text{ inhibition} = 100 - \frac{(\text{CPM PSS + antigen}) - (\text{CPM C}' \text{control})}{\text{CPM PSS + saline}) - (\text{CPM C}' \text{control})}$$

CPM = counts per minute

C' control = complement control.

3.10 MEASUREMENT OF PARTIAL THROMBOPLASTIN TIME

This test is a development of the recalcification time in which variable platelet activity is eliminated by the addition to the plasma of a constant amount of platelet substitute before recalcification. It measures the same factors as whole blood clotting time with the exception of platelet activity. However, it suffers from the disadvantage of the effects of variable glass surface contact To a glass tube are added the following. The reaction is performed at 37° C.

Citrated Plasma	0.1 ml
saline (control)	0.1 ml
or seromucoid reconstituted in saline	0.1 ml
"Thrombofax"	0.1 ml
after exactly 1 minutes	
0.2M-$CaCl_2$	0.1 ml

and the time taken from the addition of $CaCl_2$ till clot formation is noted.

/The

The same plasma sample was used for the whole experiment to eliminate the chances of variability from sample to sample. Tests were run either in triplicate or quadruplicate. Means and SD were calculated for each.

2.11 MEASUREMENT OF THE PROTHROMBIN INDEX

This was performed as follows.: Citrated plasma (0.1 ml) and brain extract (0.1 ml) were mixed in a glass tube and allowed to equilibrate at 37°C in a water bath. To this was added 0.2 Ml $CaCl_2$ (0.1 ml) and the time measured until a firm clot appeared.

2.12 STATISTICAL EVALUATION OF DATA

2.12.1 Measurement of Protein Bands on PAGE

The protein concentrations of the two low molecular weight protein bands demonstrated on PAGE was measured in patients with cancer, patients with non-malignant disease, transplant patients and age and sex matched normal controls. The groups were compared using an analysis of variance. The levels of the proteins did not fall into a normal distribution and had to be logarithmically transformed before the test could be applied to them.

2.12.2 The Effect of the Glycoprotein on PHA-Induced Blast Transformation.

The degree of inhibition of the glycoprotein on PHA-induced blastogenesis was measured using preparations from patients with Hodgkins lymphoma,

/trauma. .

trauma patients and normal age and sex matched controls. The results were evaluated using a Kruskal Wallis non parametric test and a Fischer exact test. All statistical calculations were performed on an IBM computer terminal.

2.13 PATIENT MATERIAL

All patients were from the teaching hospital complex. Blood was obtained by venipuncture after informed consent and were processed within 24 hours of withdrawal. The composition of our sample material is shown in Table 10.

TABLE 10

	No.	Age	SD
Normal	121	45	15
Non-malignant	80	38	12
Cancer	112	50	12
Transplant	77	-	-

/3...

CHAPTER 3

RESULTS

CHAPTER 3

3. RESULTS

3.1 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

3.1.1 PAGE of Sera from Normal Controls and Patients with Cancer Compared with Sera from Renal Allograft Recipients.

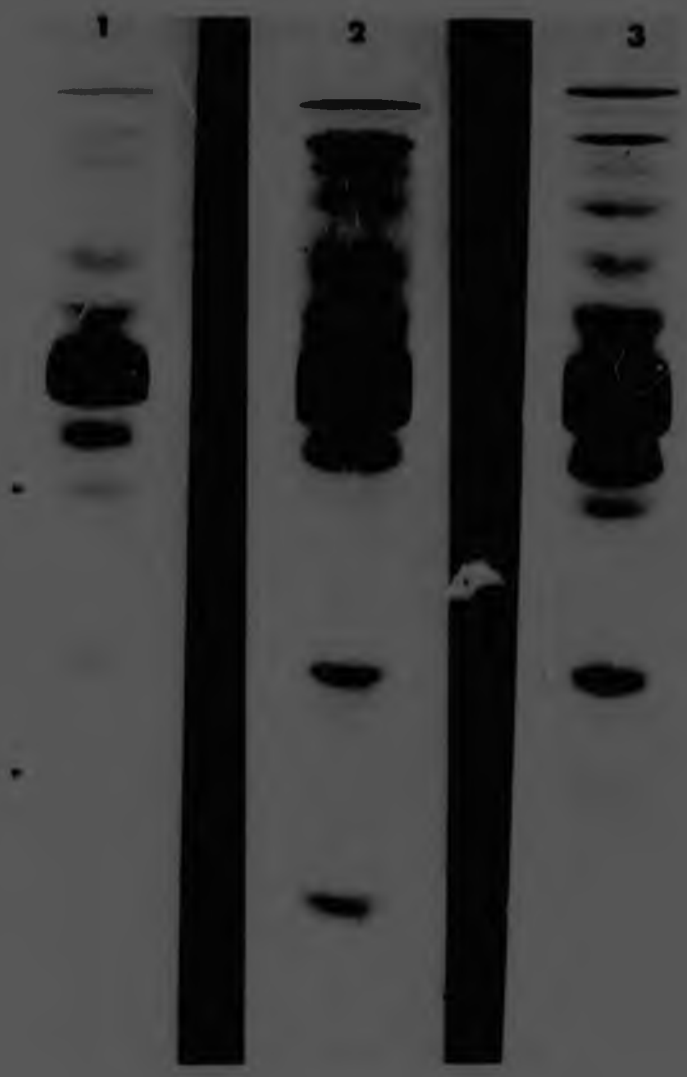
Sera were separated by PAGE as described in this thesis. Figure 12 shows a typical electrophoretic pattern of serum from a normal individual, a cancer patient and a transplant recipient. It will be seen that there are two extra bands in the transplant patient and cancer patien. (gels 1 & 3 arrowed) which are lower or absent in the normal control (gel 2). The heavy band at the bottom of gel 2 is haemoglobin from a haemolyzed sample. The upper band was called A-band and the lower B-band.

3.1.2 An Evaluation of the Levels of the Two Bands in Different Diseases.

For this evaluation 73 patients were bled and the sera were coded and evaluated on PAGE without knowledge of the diagnosis. Tables 11 and 12 show details of the patients selected and the numbers of these patients who showed increased levels of these two bands. Increased levels were taken as the mean for our controls plus two standard deviations. Of the 38 patients with cancer 29 showed increased levels of the two proteins (76%) whilst of the 35 non-malignant controls, 12 showed elevations (34%).

FIGURE 12

THE ELECTROPHORETIC PATTERN OF SERUM ON PAGE



1. Serum from transplant patient.
2. Serum from normal control.
3. Serum from patient with cancer

/Table 11.

TABLE 11

PRESENCE OF TWO PROTEIN SPECIES IN THE SERUM OF PATIENTS WITH KNOWN MALIGNANT DISEASES.

Tumour Type	No. of cases	No. of cases showing elevation of the two proteins
Melanoma	6	6
Renal carcinoma	1	1
Prostatic carcinoma	6	2
Bladder carcinoma	7	5
Carcinoma Breast	10	7
Carcinoma Rectum	3	3
Carcinoma Stomach	1	1
Lymphocytic lymphoma	2	2
Teratoma testis	1	1
Multiple myeloma	1	1
	38	29 (76%)

/Table 12.

TABLE 12

PATIENTS WITH NON-MALIGNANT DISEASE STUDIED		
Disease	No. of cases	No. of cases showing elevation of the two proteins
Benign Prostatic Hypertrophy	6	3
Renal Calculus Disease	2	
Pyonephrosis	1	
Ureteritis cystica	1	1
Renal artery stenosis	2	
Primary biliary cirrhosis	1	
Peripheral vascular disease	4	
Ulcerative colitis	2	
Cholangitis	1	
Major trauma	2	2
Obstructive jaundice	1	
Eosinophilic cystitis	1	
Urethral stricture	1	1
Cellulitis	2	2
Empyema	3	3
Multinodular goitre	1	
Pellagra	4	
	35	12 (34%)

/Table.

Table 13 and Figure 13 show the levels of the two proteins in the various groups studied.

TABLE 13

THE LEVELS OF THE TWO PROTEINS IN THE SERUM OF PATIENTS WITH CANCER AND VARIOUS OTHER CONDITIONS.

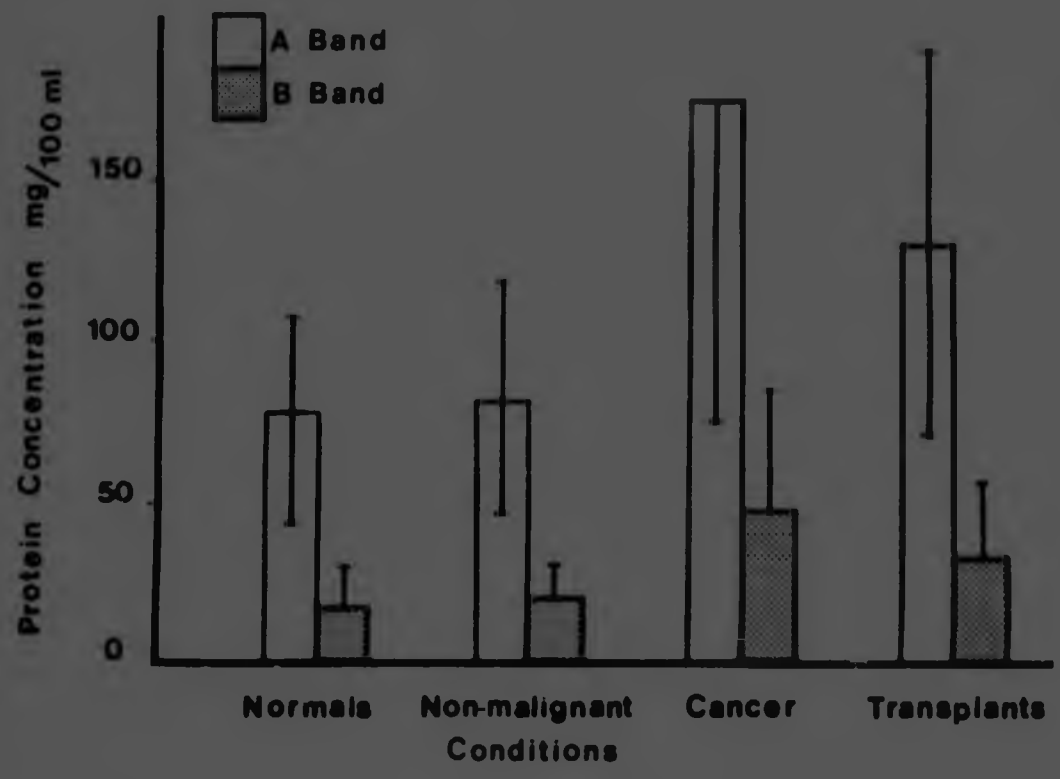
	No. of cases	A-band mg protein/ 100 ml	SE	B-band mg protein/ 100 ml	SE
Normal	81	73	3.8	15	1.4
Various diseases (non-malignant)	35	76	5.9	19	1.5
Malignant	38	160	15.0	54	10 9
Transplant	79	124	11.6	32	2.6

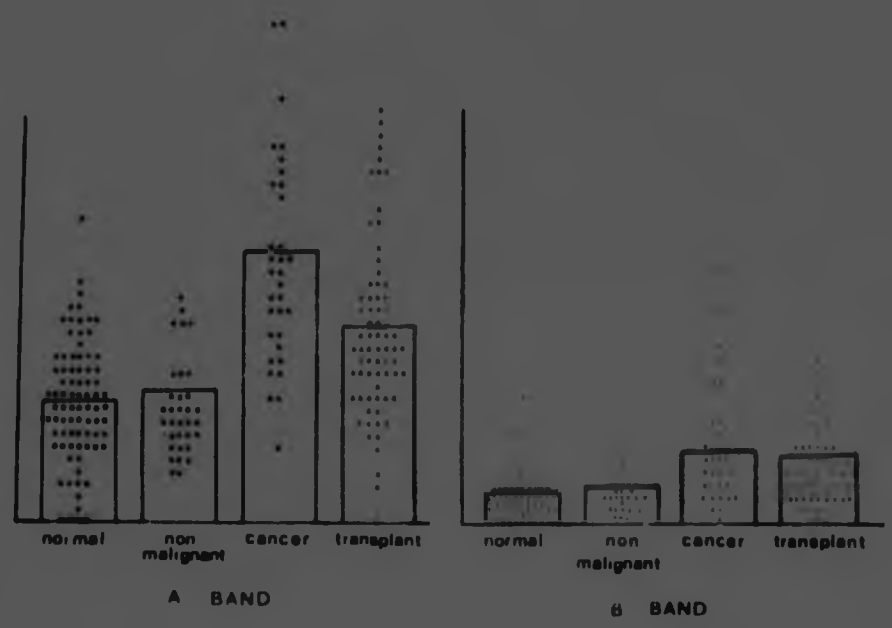
It may be seen from these results and Fig.13 that whilst the proteins are elevated in 76% of our sample of patients with cancer, they are also elevated in 34% of patients with non-malignant conditions including patients who have received a transplanted kidney. The scattergrams included in Fig.13 indicate the range of values to be expected for these two proteins in the various groups. The levels of the 2 proteins in our normals were compared with those in the cancer patients and patients with non-malignant diseases using an analysis of variance. The values in cancer patients were found to be significantly

/elevated.

FIGURE 13

LEVELS OF A BAND AND B BAND IN VARIOUS CONDITIONS





Scattergram of the above results.

elevated for both proteins when compared to the other 2 groups ($P < 0.05$). The levels in the non-malignant conditions were not significantly elevated above normal ($P < 0.05$).

3.1.3 An Evaluation of the Levels of the Two Bands in Renal Transplant Recipients.

It was noted that transplant recipients showed elevated levels of the 2 proteins (Fig.12). If the patients were divided into those with good graft function (serum creatinine less than 2mg/100ml and no chemical evidence of rejection) and those with poor graft function (serum creatinine greater than 4mg/100ml) it was noted that the smaller of the 2 proteins i.e. B-band was significantly elevated in patients with poor graft function ($P < .0001$). This elevation in B-band was demonstrated in 10 of 12 patients with poor graft function (83%). The A-band did not appear to be significantly raised in the rejectors.

TABLE 14

THE LEVELS OF THE TWO PROTEINS IN THE SERUM OF REJECTORS AND NON-REJECTORS

	n	A-band mg protein/ 100 ml	SE	B-band mg protein/ 100 ml	SE
Non-rejectors	65	117	7.8	29	3.9
Rejectors	12	152	15.6	59	7.8

/See.

See Appendix 4. The Presence of Two Protein Fractions in the Serum of Renal Allograft Patients.

SEE TABLE 14

3.1.4 Molecular Weight Estimations of the Two Bands Using PAGE.

It is apparent from the PAGE pattern that the proteins are smaller than human albumin (69,000 d). A calibration curve was set up using proteins of known molecular weight (see Methods 2.6.2) and the molecular weights of A-band and B-band were estimated from their migration distances compared to those of the 3 proteins of known MW used for calibration purposes.

The estimation that was obtained for A-band was 40,000-50,000 daltons and for B-band 18,000-22 000 daltons.

Some caution should be exercised in accepting these molecular weights as peri dic acid Schiff staining showed A-band to be a glycoprotein and the anomalous migration of glycoproteins on PAGE is well known.

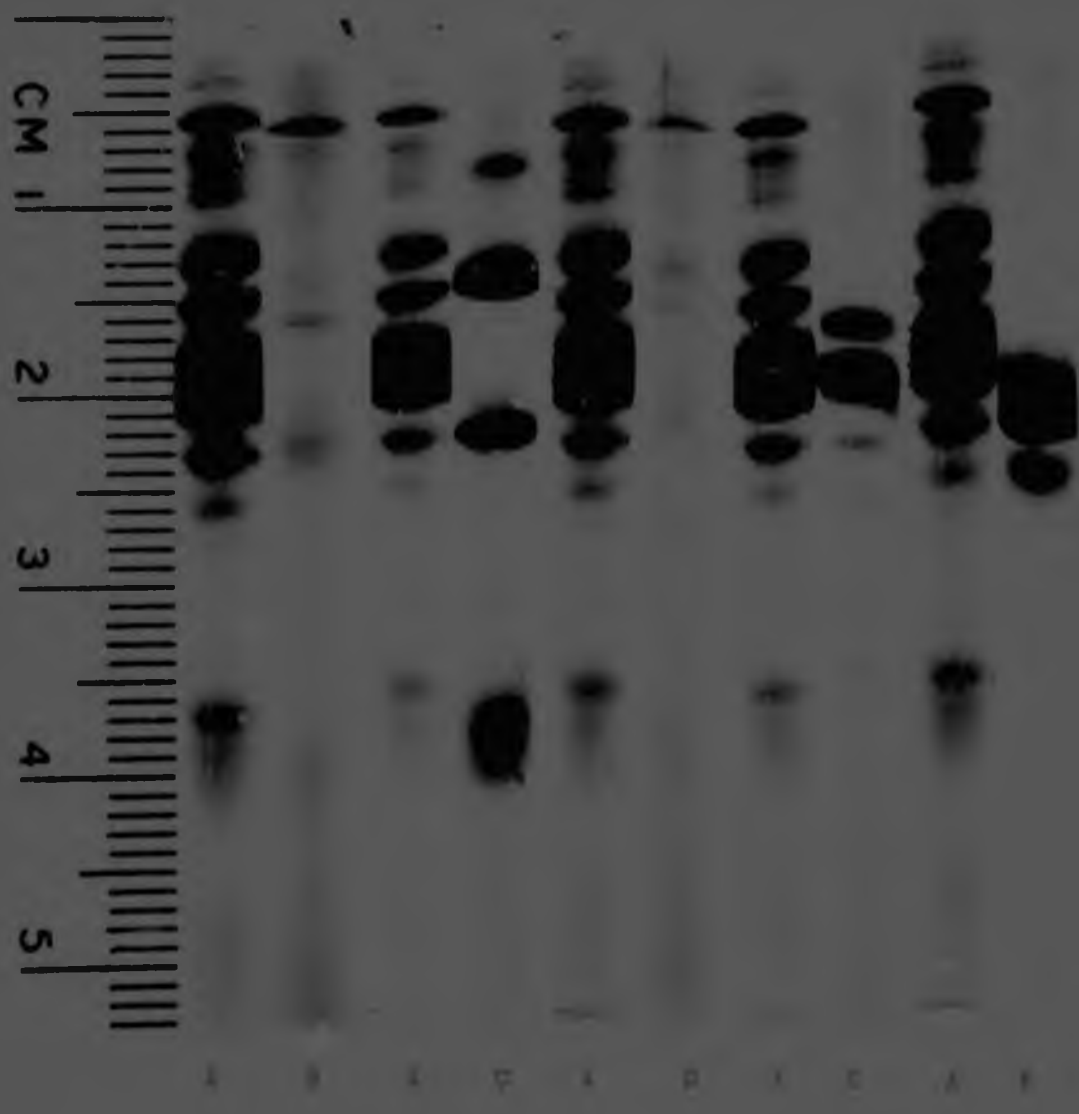
3.1.5 Further identification of A-band

In order to obtain further evidence on the identity of A-band, a search was made for this band in the various fractions of plasma proteins obtained by the Kistler fractionation method which is a modification of the Cohn fractionation procedure (Kistler and Nitschmann,1962). These fractions were a generous gift from Professor Peter Brain

/of...

FIGURE 14

PAGE OF KISTLER PLASMA FRACTIONS



A Whole Serum
B Fraction I
C Fraction II
D Fraction III
E Fraction IV
F Fraction V

of the Natal Institute of Immunology.

It may be seen from Figure 14 that A-band is found in fraction V, with a much smaller amount in fraction IV and nothing in fractions I to III.

3.1.6 Column Chromatography of Whole Serum

Whole serum was fractionated using Sephadex G200 and the fractions comprising each peak were collected, concentrated and separated by electrophoresis. Figure 15 shows the electrophoretic pattern of each peak. It can be seen that A-band and B-band were found predominantly in the first peak that is they appear to be of large molecular weight if not dissociated by SDS detergent.

3.1.7 Comparison of A-band with CEA

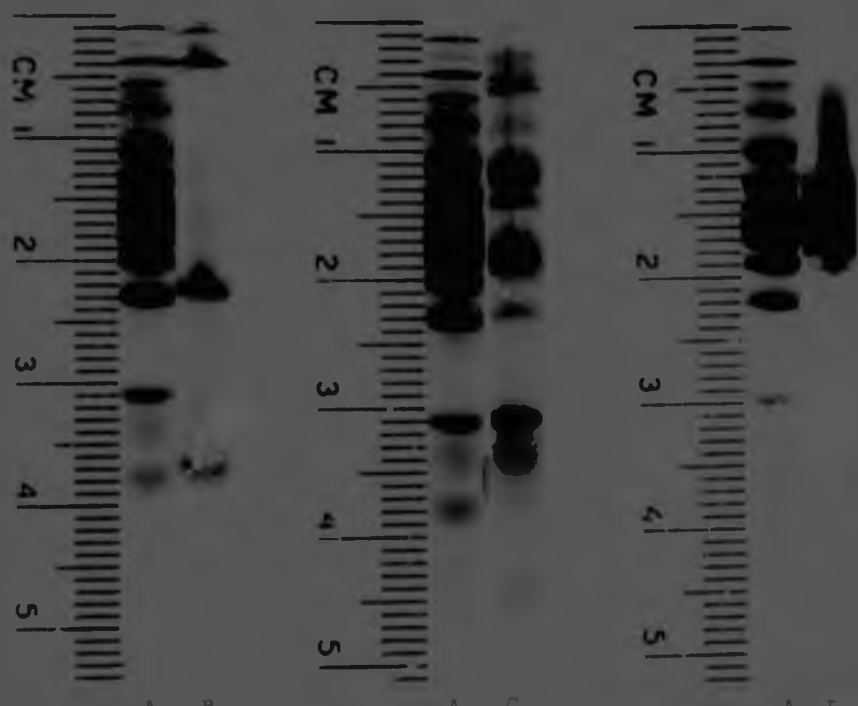
A sample of radio-labelled CEA was obtained from Roche Pharmaceuticals and was run on PAGE. The gel was then sliced into 35 slices of about 1.8 mm and each slice was counted. The sample did not migrate into the gel significantly and all the radio-activity was located in the first three slices thus confirming that the protein under consideration (i.e. A-band) was not CEA. This we had surmised from the differences in molecular weight.

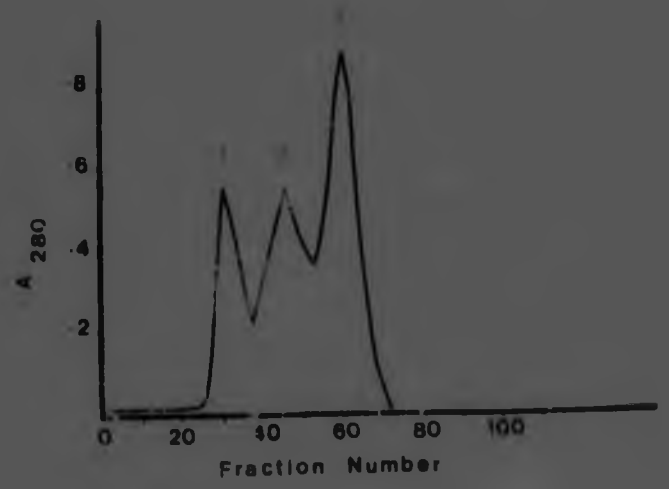
/Figure 15.

FIGURE 15

FRACTIONATION OF SERUM ON SEPHADEX G200 ELECTROPHORETIC PATTERN OF EACH PEAK.

A - Whole Serum
B - Peak I
C - Peak II
D - Peak III





3.1.8 Comparison of B-band with Beta$_2$-Microglobulin.

A sample of purified ^{125}I microglobulin was obtained from Pharmacia Diagnostics Inc. This compound was separated by PAGE electrophoresis and the gel was sliced and counted as in 3.1.7. above The results are shown in Fig. 16 Beta$_2$-microglobulin migrated in front of B-band and the radio-activity was located in the terminal 5mm of the gel. B-band ran at the 44mm mark and appeared therefore to be of higher molecular weight than beta$_2$-microglobulin (MW 11,900 daltons).

3.1.9 Comparison of Seromucoid and A-band

It is known that the acute phase proteins are elevated in malignancy. To investigate the possibility that A-band might be one of the acute phase proteins a sample of alpha-acid glycoprotein was prepared by a modification of Winzlers method (Section 2.4.3) and was separated on PAGE. The result is shown in Figure 17. It can be seen that the supernatant after perchloric acid precipitation of the other proteins,contained a single band on PAGE which migrated in a position corresponding to A-band.

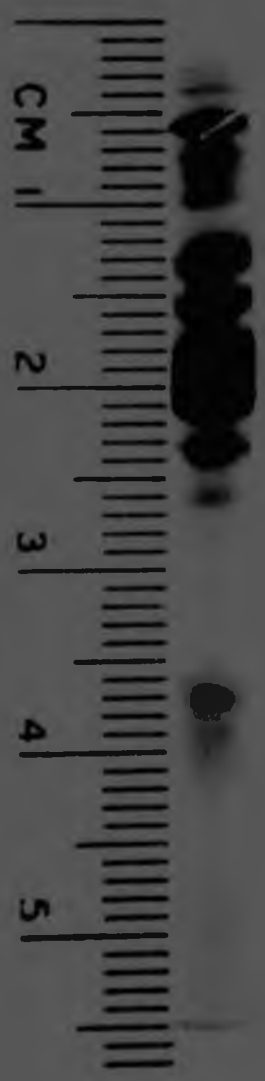
3.1.10 Immunoelectrophoresis of Perchloric Acid - Soluble Protein from Serum.

The perchloric acid soluble protein/proteins in serum were concentrated by dialysis and were analysed by immunoelectrophoresis. The precipitin lines obtained with rabbit antiserum to human serum

/is...

FIGURE 16

PAGE OF ^{125}I-BETA$_2$-MICROGLOBULIN



Slice No.	cpm
2	30
4	36
6	18
8	17
10	63
12	13
14	10
16	23
18	12
20	12
22	15
24	13
26	16
28	26
30	21
32	459
34	990
36	351
38	30

/Figure 17.

FIGURE 17

PAGE OF SEROMUCOID PREPARED BY PERCHLORIC ACID TREATMENT OF SERUM

is shown in Figure 18 a,b. A single strong line in the alpha$_1$ region was noted. Various monospecific antisera were used and Figure 18b shows the immunoelectrophoretic pattern obtained with anti-alpha$_1$ acid glycoprotein.

3.1.11 Discussion

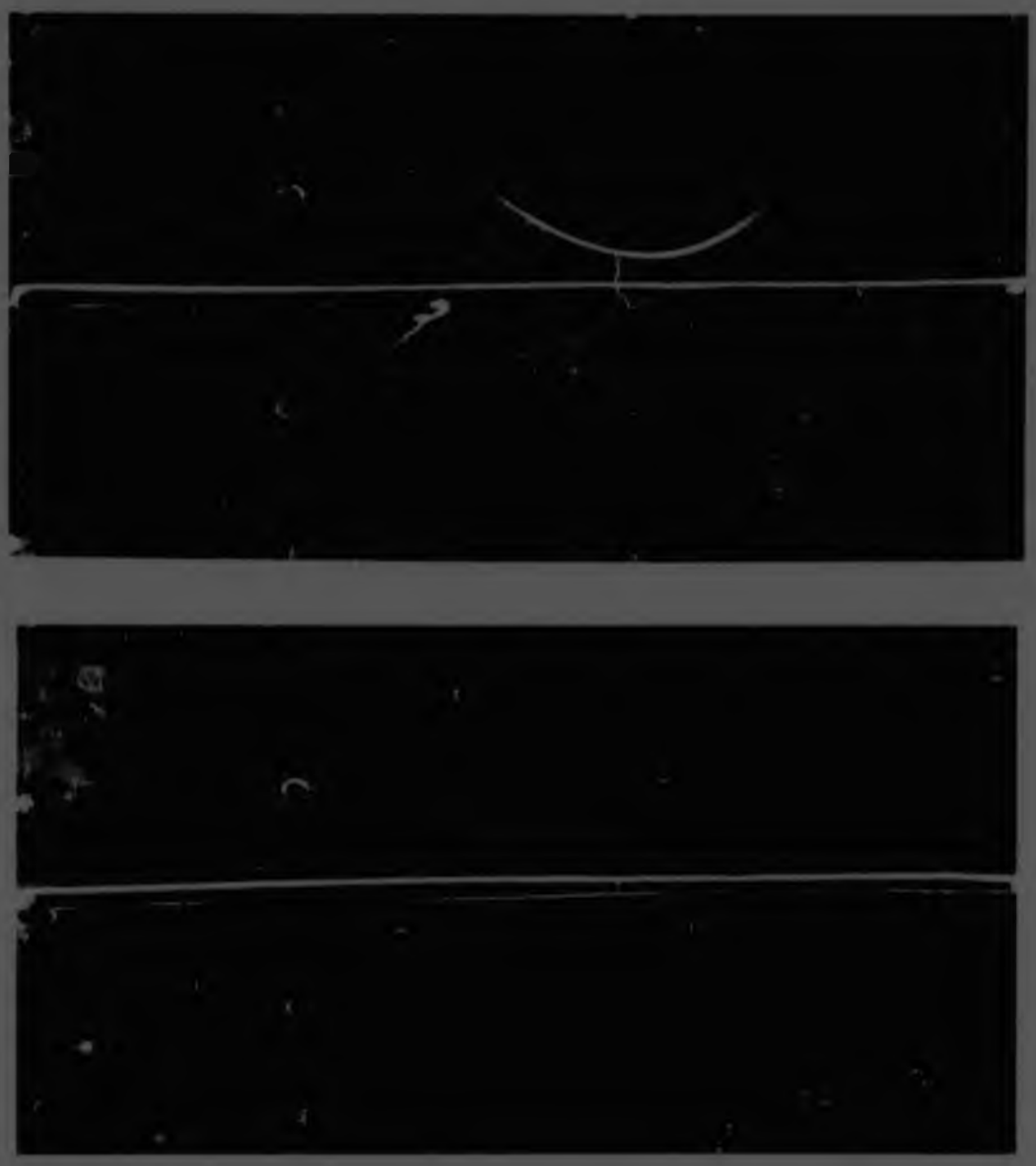
An examination of serum from patients with cancer showed the presence of 2 bands in considerably higher amounts than in the controls. These bands were named A-band and B-band respectively. Sera from 80 normal controls were evaluated in order to determine normal values for these 2 proteins. A-band was found to have a normal concentration of 73 mg/100ml of serum and B-band was shown to be present at a concentration of 15 mg/100ml. Using this information, abnormal values were defined as any value which was greater than the mean plus two standard deviations. Thus any value for A-band greater than 107 mg/100 ml was considered abnormal and any value larger than 27 mg/100 ml was considered abnormal for B-band.

The diagnostic value of these proteins was evaluated by quantitative PAGE electrophoresis. Sera of 75 patients were used in a blind study. Of the 38 patients with cancer, 29 (76%) showed elevated levels of both proteins whilst of the 35 patients with non-malignant disorders only 12 (34%) showed elevations. The two populations were shown to have statistically significant

/differences.

FIGURE 18

IMMUNOELECTROPHORESIS OF SEROMUCOID PREPARED BY PERCHLORIC ACID TREATMENT OF SERUM



1 and 4 ... Perchloric Acid-Soluble Protein from Serum
2 ..... Anti Whole Human Serum
3 and 6 .. Whole Serum
5 ..... Anti alpha-acid glycoprotein

differences in the levels of both proteins at a confidence limit of 5%.

It became apparent from this investigation that the proteins were elevated in conditions other than malignancy including trauma, infection and inflammation. Scattergram diagrams of the values showed that whilst the mean values for the 2 populations were significantly different there was considerable overlap. This would tend to diminish the value of the proteins for diagnosis of cancer. However, many of the non-malignant conditions in which the proteins are elevated do not present a diagnostic problem and the levels of these 2 proteins may be of some use in cancer diagnosis. They may furthermore be of use in situations in which the diagnosis of cancer has previously been established and definitive treatment has already been given, as an evaluator of reoccurrence of the disease. Used together with other biochemical investigations, these proteins may be of some value.

Recipients of kidney allografts may in some respects be likened to patients with cancer in that in both cases it would appear that there is an exposure of the patient to "foreign" antigens under conditions of prolonged immunosuppression. It became apparent during this study that both A-band and B-band were elevated in transplant

/patients..

fractionation process of plasma. A-band appeared to reside in a high molecular weight peak after Sephadex G200 separation. This might be taken to suggest that A-band was capable of aggregating with itself or that it was a component of a larger molecule. B-band was also shown to reside in the high molecular weight fractions. The significance of this is at present unclear. This might also indicate aggregability or that B-band is a component of a larger molecule. Both bands eluted in the molecular weight range of 158,000-240,000d. A-band was shown not to be CEA and B-band was shown not to be beta$_2$-microglobulin. Furthermore their molecular weights suggested that they were different from the proteins described by Hoch et al.

The molecular weight of A-band, coupled with its presence in acute conditions such as trauma and sepsis, as well as the fact that it was a glycoprotein and its presence in fraction V suggested that this might be alpha-acid glycoprotein (orosomucoid). To compare the proteins, alpha acid glycoprotein was prepared from serum by a modification of Winzlers method and electrophoresed on PAGE. The result as shown was a single band which migrated in a position corresponding to A-band. This preparation of alpha-acid glycoprotein from plasma was termed

/orosomucoid....

Seromucoid and was further evaluated using immunoelectrophoresis. A single precipitin line could be demonstrated on immunoelectrophoresis against a poly-specific serum. This could be shown to be alpha-acid glycoprotein using a mono-specific anti-alpha-acid glycoprotein (Behring). Thus A-band could be shown to be alpha-acid glycoprotein. It was not possible to demonstrate any protein other than alpha-acid glycoprotein in the preparations using PAGE and immunoelectrophoresis.

3.2 SEROLOGICAL STUDIES ON ALPHA-ACID GLYCOPROTEIN PREPARED FROM PATIENTS AND CONTROLS

3.2.1 Amount of Glycoprotein Recovered From Serum and Plasma.

As a preliminary to investigating the serology of the alpha-acid glycoprotein preparations, the amounts recovered from serum and plasma were measured. Table 15 details the amounts of alpha-acid glycoprotein obtained from 100ml of serum and 100 ml of plasma obtained from apparently healthy blood donors. Serum was prepared from the clotted specimen within 24 hours of collection. Plasma was prepared from blood donations of 450-500ml of blood collected in 70ml of citrate

/phosphate...

phosphate dextrose (CPD). The plasma was prepared within 72 hours of collection. Plasma was also prepared by using heparin as an anticoagulant and serum was prepared by defibrination of whole blood using glass beads. It can be seen that more protein can be obtained from serum than from plasma irrespective of the method used to prepare the plasma or serum. TABLE 15.

TABLE 15

AMOUNT OF ALPHA ACID GLYCOPROTEIN RECOVERED FROM 100ml OF SERUM AND PLASMA FROM HEALTHY BLOOD DONORS

	A	B	C	D
No. of subjects	50	4	60	4
Amount of alpha-acid Glycoprotein recovered (mg/100ml)	43.5	42.7	22.3	33
Range	31-59	33-43	14.32	28-42

A..... Serum from clotted blood

B..... Serum from defibrinated blood

C..... Plasma using CPD as anticoagulant

D..... Plasma using heparin as anticoagulant.

/3.2.2

3.2.2 Inhibitory Activity on Blood Group Antibodies of Alpha-Acid Glycoprotein Preparations from Serum and Plasma of Healthy Donors.

The inhibitory activity of the alpha-acid glycoprotein preparations from serum and plasma were measured in 102 donors. The cumulative and mean inhibition scores are shown in Table 16. It may be seen that although more protein could be obtained from serum than from plasma (43mg/% against 22 and 33 mg/%), more TABLE 15 inhibitory activity was noted in the plasma extracts (Mean inhibition score of 37.6 against 28.2 of serum). All the inhibition scores in plasma were increased with the exception of T which was decreased by 20%. No P,Tja, I or i antigens could be detected in serum whilst these were readily detected in plasma.

3.2.3 Measurement of Inhibitory Activity in Preparations from Donors of Differing Secretor Status.

The presence of blood group antigens in soluble form in secretions has been shown to depend on the secretor status of the individual for the major blood groups A, B, H and Lewis. It was thus decided to investigate the inhibitory activity in the alpha-acid glycoprotein preparations and to relate this to the secretor status of the individual from whom the protein was obtained.

/Table

TABLE 16.

CUMULATIVE AND MEAN INHIBITION SCORES FOR VARIOUS BLOOD GROUP ANTIGENS IN PREPARATIONS FROM SERUM AND PLASMA

	A	B	H	Le^a	Le^{bL}	P_1	Tja*	I	i	T	M	N	Total	
Serum	71	43	12[illegible]	89	79	0	0	0	0	942	10	51	1412	Cumulative inhibition scores
n = 50	(1.4)	0.9	2.5	1.8	1.6	0	0	0	0	18.8	0.2	1	28.2	Mean inhibitory activity
Plasma	81	84	229	180	134	53	19	164	36	776	83	88	1927	Cumulative inhibition scores
n = 52	1.6	1.6	4.4	3.5	2.6	1	0.4	3.2	0.7	14.9	1.6	1.7	37.6	Mean inhibitory activity

Table 17 details the results of this investigation. Four groups were investigated and denoted S L for secretors of A, B, H and Lewis substances, -L for secretors of Lewis substances only, S- for secretors of A B and H substances only and -- for non-secretors.

It was noted that the extracts from S L subjects contained A B H and Lewis activity as well as M N and T activity; those from L-contained Lewis activity and T and N activity; S-contained A and H activity as well as M N and T; whilst -- extracts contained only T activity, although in the latter case conclusions drawn from only 1 subject should be subject to cautious interpretation.

Inhibitory Activity of Preparations from Patients with Various Diseases Compared with Normal Controls.

In view of the elevated levels of alpha-acid glycoprotein associated with many diseases, inhibitory activity was measured in preparations from serum of patients with a spectrum of diseases. The results are detailed in Table 18 . The salient features of this investigation concern the P_1, Tja, I, i and T antigens. The first four of these antigens were increased in the patients with cancer, whereas T is decreased. P_1, Tja, I and i were not detectable in the preparations from the controls. The increase was most marked in patients with advanced cancer of the breast, lymphocytic lymphoma and Hodgkins lymphoma.

/Tja....

TABLE 17

CUMULATIVE INHIBITORY ACTIVITY OF HAEMOUCOID SUBSTANCES PREPARED FROM THIS SERA OF HEALTHY SUBJECTS

	SL(30)Subjects	*	-L(9)Subjects	*	S-(10)Subjects	*	--(1)Subject
A	37	(1.2)	0		43	(4.3)	0
B	43	(1.4)	0		0		0
H	74	(2.4)	0		44	(4.4)	0
Le^a	43	(1.4)	46	(5.1)	0		0
Le^{b1}	54	(1.8)	25	(2.7)	0		0
P_1	0	-	0		0		0
Tja	0	-	0		0		0
I	0	-	0		0		0
i	0	-	0		0		0
T	518	(17.2)	195	(21.6)	196	(19.6)	33
M	8	(0.2)	0		2	(0.2)	0
N	34	(1.1)	2	(0.2)	15	(1.5)	0

SL denotes subjects who secrete AH, BH, H and Lewis substances

-L denotes subjects who secrete only Lewis substances

S- denotes subjects who secrete only AH, BH, H substances

-- denotes subjects who do not secrete any substances.

* Figures in parentheses denote mean inhibitory score.

TABLE 18

GLYCOPROTEIN PREPARATIONS TESTED FOR INHIBITORY ACTIVITY AGAINST ANTIBODIES DIRECTED AGAINST BLOOD GROUP ANTIGENS.

	No. Tested	MEAN CUMULATIVE INHIBITION SCORES FOR ANTI-									
		A	B	H	Le^a	Le^{b1}	P_1	Tja	I	i	T
Controls	50	1.42	0.86	2.54	1.78	1.58	0	0	0	0	18.8
Patients with trauma, sepsis and other illnesses	45	0.68	0.57	0.24	0.73	1.31	0.22	0	0.86	0.40	0.2
Patients with ca breast, lymp. lymph.Hodgkin's lymphoma	41	1.73	0.29	0.82	1.95	1.82	3.75	2.82	3.29	2.41	1.5
Patients with other varieties of cancer	33	1.60	0.63	0	0.48	0.87	0.75	0.39	0	0.39	0.1

Tja activity was detected only in patients with malignant conditions and in none of the 95 controls. The number of individuals giving a positive result against a particular antibody was recorded and is shown in Table 18. The various groups were compared using a Fischer exact or non-parametric analysis. The number of individuals with cancer who had blood group antigens in their glycoprotein extracts were significantly higher for the following antigens P_1, Tja, I, i and T (P values < 0.05). Table 18. Significantly fewer patients with cancer showed T activity in the extracts ($P < 0.05$). Table 19.

3.2.5 The Effect of Storage on the Blood Group Activity of the Glycoprotein Preparations.

During the investigations it was noticed that the inhibitory activity of the various blood group determinant, particularly the T activity, spontaneously disappeared from the preparations Table 20 shows the loss of activity. The antigenic determinant of the T antigen is known to be galactose and it was thought that the loss of activity could be due to beta galactosidase activity in the preparations. Accordingly Phenyl (p-D)-thiogalactoside (an inhibitor of beta galactosidase) was added to the extracts at a concentration of . mg/ 50ml after dialysis. (Kline and Issitt, 1976). Table 21 shows the effect of this inhibitor on the loss of activity.

TABLE 19

STATISTICAL EVALUATION OF SEROLOGICAL DETERMINATIONS ON GLYCOPROTEIN PREPARATIONS FROM PATIENTS WITH MALIGNANT CONDITIONS NON MALIGNANT CONDITIONS AND CONTROLS.

MALIGNANT CONDITIONS WERE GROUPED INTO A SINGLE GROUP AND COMPARED WITH NON-MALIGNANT CONDITIONS AND NORMAL CONTROLS AS A SINGLE GROUP.

BLOOD GROUP	PATIENT GROUP				
	Hodgkins	Ca	rauma	Normal	Signif.
A	8/41	8/33	5/45	11/50	P = .44
B	2/31	4/33	4/45	8/50	P = .38
H	6/41	0/33	3/45	26/50	P = .03
Le^a	13/41	3/33	5/45	16/50	P = .04
Le^b	12/41	5/33	11/45	17/50	P = .03
P_1	24/41	4/33	2/45	0/50	P = .03
Tj^a	21/41	3/33	0/45	0/50	P = .02
I	21/41	2/33	6/45	0/50	P = .03
i	18/41	3/33	3/45	0/50	P = .02
T	8/41	1/33	3/45	30/50	P = .01

TABLE 20

STORAGE EFFECT ON LOSS OF KNOWN DETERMINANTS

Selected ABH and Lewis sitive Blood Donors	MEAN CUMULATIVE INHIBITION C[illegible] FOR ANTI-											
	Day 1						Day 10 (-20°C Storage)					
	A	B	H	Le^a	Le^{bl}	T	A	B	H	Le^a	Le^{bl}	T
Serum Alpha-Acid Glycoprotein 15 subjects	1.7	2.6	3.3	1.6	2.2	17.4	0.5	0.8	0.9	0.4	0.6	0.4
							Activity Reduced by Factor of 8.5					
Plasma Alpha-Acid Glycoprotein 12 subjects	4.4	3.0	9.2	5.0	6.0	28.5	2.1	1.3	4.2	2.2	3.1	2.6
							Activity Reduced by Factor of 3.6					

TABLE 21

THE EFFECT OF PHENYL BETA-D THIOGALACTOSIDE ON THE ENZYMIC DESTRUCTION OF T ACTIVITY

Inhibition Scores for T Activity

SERUM NORMAL DONORS		Day 1	Day 3 5°C storage	Day 6 °C storage	Day 10 5°C storage	Day 12 24 hrs at 37°C	Day 14 72 hrs at 37°C	Day 120 - 20°C storage
CONTROL	1	17	15	15	10	0	-	0
	2	29	27	24	18	0	-	0
	3	25	24	20	10	0	-	0
	4	10	10	9	7	0	-	0
Addition of Phenyl-Beta-D Thiogalactoside* Before Acid Extraction	1	24	-	-	20	21	22	15
	2	35	-	-	32	30	32	26
	3	36	-	-	34	32	30	22
	4	23	-	-	20	21	19	12
Addition of Phenyl-Beta-D Thiogalactoside* After Acid Extraction	1	26	-	-	25	-	24	21
	2	35	-	-	34	-	35	28
	3	38	-	-	36	-	37	25
	4	25	-	-	24	-	26	15

*10 mgs Phenyl-Beta-D-Thiogalactoside for every 50 ml of Plasma or Serum Extract.

7.6 Discussion

Measurement of the amount of protein obtained from perchloric acid extraction indicated that more material could be obtained from serum than from plasma. It was also apparent that the material obtained from plasma was considerably less pure than that obtained from serum (see later). The larger amount of protein obtained from serum could not be explained in terms of the diluting effect of the CPD in the plasma. The same results could be obtained irrespective of the method used to prepare the plasma and serum. Serological studies on preparations from plasma and sera showed that a wide range of blood group antigenic determinants were present, including A,B,H,Le^a,Le^b, P_1,Tja, I,i,Γ,M and N. A comparison of the amounts of these antigens present in serum and plasma showed that despite the greater amount of protein obtained from serum, more antigenic activity could be detected in plasma, for all the antigenic determinants with the exception of the T antigen. Furthermore it was not possible to detect P_1, Tja, I or i antigens in preparations from serum although these could be readily detected in the preparations from plasma. These results are of interest in that the P antigen systems including Tja appears to be a membrane bound system and the soluble form of the P blood group substances have been found only in hydatid cyst fluid, worm extracts, liver flukes

/and.

and fish roe (Cameron and Stavely, 1957; Prokop and Schlesinger, 1965; Race and Sanger, 1975). Furthermore no convincing case has been made for the presence of the I antigen in serum (Race and Sanger, 1975) although the i antigen has been demonstrated in a soluble form (Cooper and Brown, 1973). Similarly it has been thought that the M and N blood group activities are membrane bound (Lissowska and Kordowicz, 1976), and Springer et al., (1976), have shown these antigens on normal breast tissue. We have reported the presence of the M,N and T antigens in soluble form after acid extraction of urea (Klatzow and Vos, 1978). The studies on the correlation of secretor status with the antigens present in the extracted material indicated that for the A,B, H and Lewis systems the antigenic activity present in the extract was determined by the secretor status of the donor. This did not appear to be so for the M,N and T antigens, these being found in the sera whatever the secretor status. For this study only serum extracts were used and as no I, i, P or Tja activity was demonstrable in acid extracts of normal serum, no conclusions can be drawn regarding the secretor status.

Many authors have shown changes in blood group antigens associated with malignancy (Section 1,11 page 29). The suggestion has been made that there is an accumulation of blood group antigen precursors on cancer cells (Springer et al., 1975 a,b; 1976; Hakomori

/cont...

and Andrews, 1970; Davidson et al., 1969, Prendergast et al., 1968). However it would appear that blocked synthesis of blood group antigens is not the case in all tumours. High levels of A, B, H and Lewis substances have been reported in gastric carcinomas (Barber and Dunsford, 1959, Saeed and Fine, 1968). This study suggests that many of these antigens may be shed from the surface of blood cells in a variety of situations including storage (higher levels of antigens in plasma than in serum) and various pathological states. Whilst the higher levels of antigens in plasma compared with serum are suggestive of shedding, they are by no means conclusive and other possibilities such as release of serologically inactive material during coagulation, coprecipitation of antigen with fibrin, adsorption to fibrin or to altered membranes in the clot, cannot be excluded. Further evidence for shedding may be obtained from Table 18 in which the levels of P, Tja, I and i are higher in patients with cancer than in the controls or patients with non-malignant conditions. This is most marked in patients with carcinoma of the breast, lymphocytic lymphoma and Hodgkins lymphoma. The absence of I in the sera of patients with other varieties of cancer is difficult to explain. Of special interest is the presence of Tja activity only in our group of patients with malignant disease. Tja was present in 24/74

/(32%)

(32%) patients with cancer and in 21/41 (51%) of patients with Hodgkins disease. The diagnostic significance of this finding will need to be re-evaluated using a larger patient sample. Whilst the T antigen was the strongest reactant in all the preparations from normal plasma and serum, its activity was markedly reduced in all the patient groups. There are a number of possible explanations for this observation. It may be caused by insufficient inhibition of beta galactosidase. This is unlikely as the samples were prepared at random and the inhibitor was added to each sample in a similar manner. Another possibility is that the T antigen is present in lower quantities in the serum of patients and finally beta galactosidase may be elevated in the serum of patients with various disorders.

3.3 CHROMATOGRAPHY

3.3.1 Column chromatography of the glycoprotein preparations

Preparations were made from plasma (ACD and heparin) and from serum and were separated by gel filtration on Sephadex columns as described. The load applied to each column was 150 mg of dried preparation in 3 ml of saline. The elution patterns of glycoprotein preparations from serum and plasma separated on Sephadex G200 are shown in Figures 19,20,21, and 22. Molecular weights were calculated from the elution volume and by reference to standard

/proteins...

FIGURE 19

CHROMATOGRAPHY OF PERCHLORIC ACID EXTRACTS OF SERUM ON SEPHADEX G200.

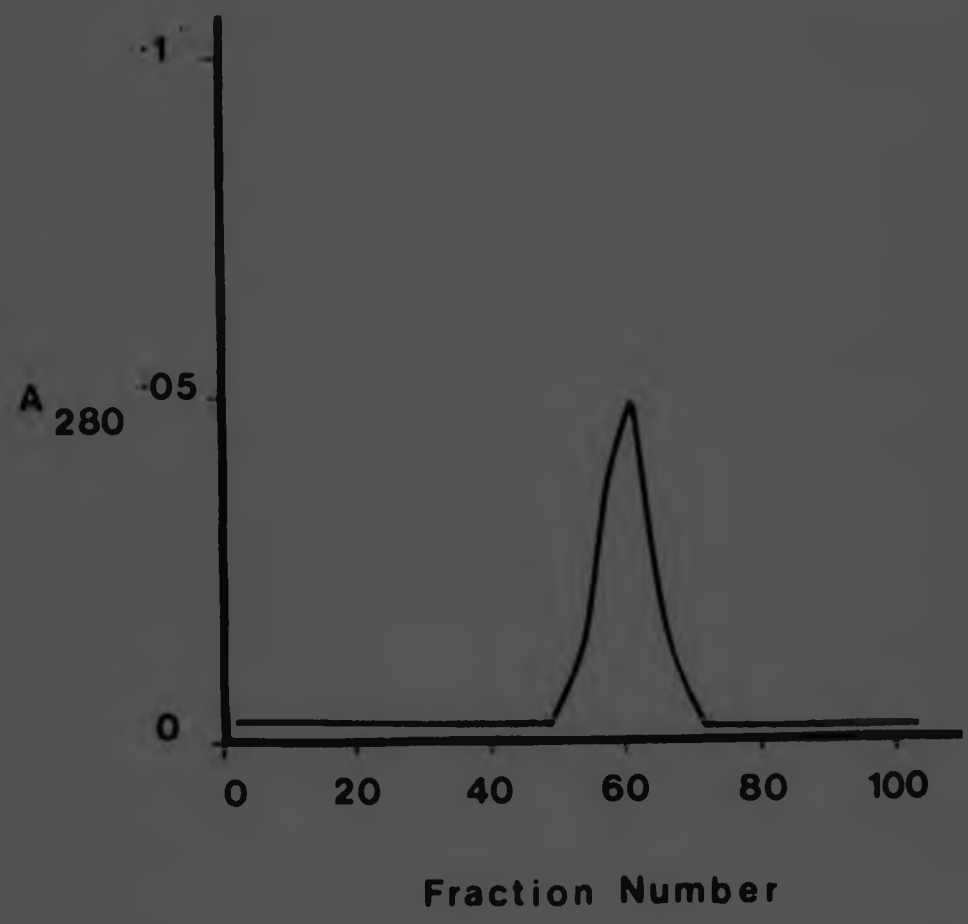


FIGURE 20

CHROMATOGRAPHY OF PERCHLORIC ACID EXTRACTS OF SERUM ON SEPHADEX G200

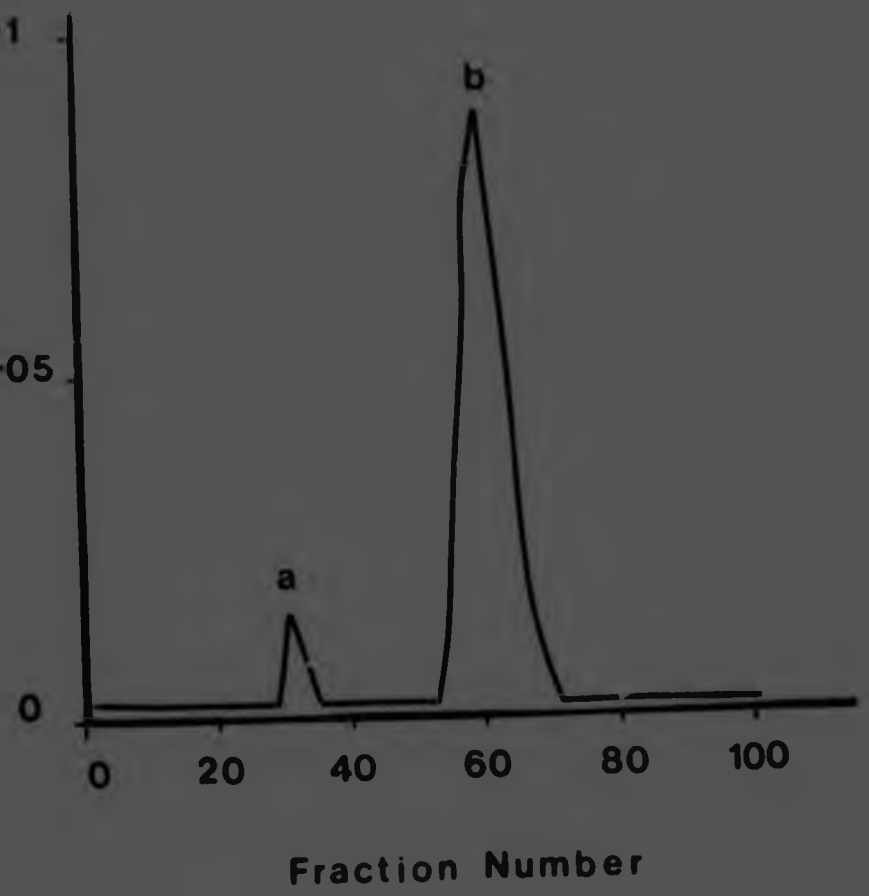


FIGURE 21

COLUMN CHROMATOGRAPHY OF PERCHLORIC ACID EXTRACT OF HEPARINISED PLASMA ON SEPHADEX G200

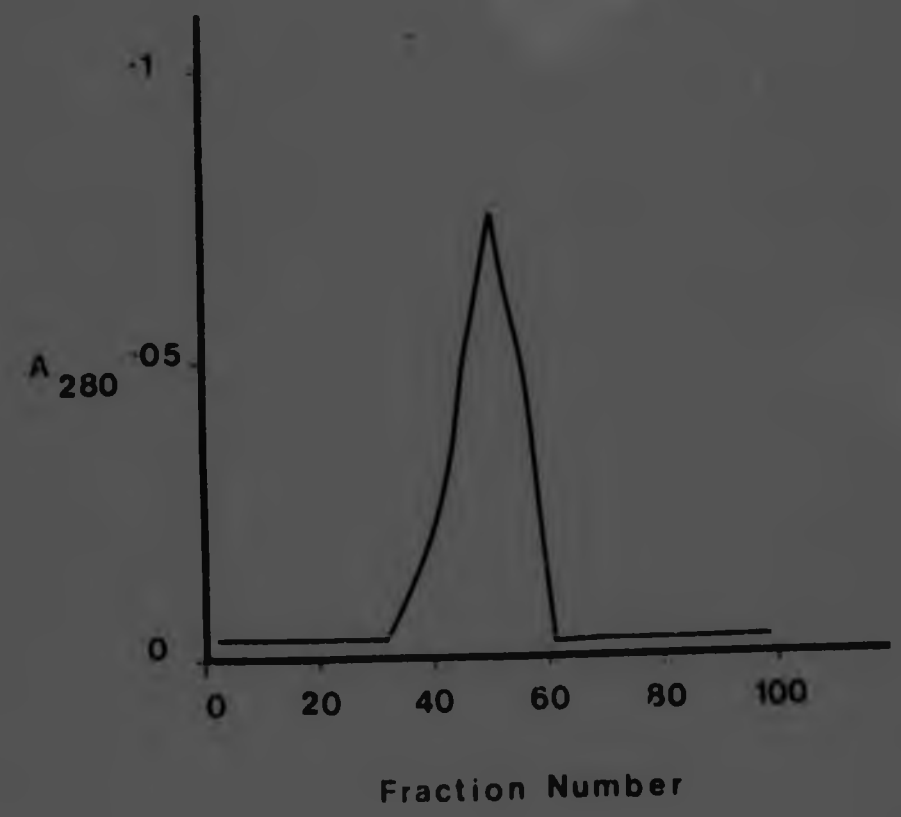
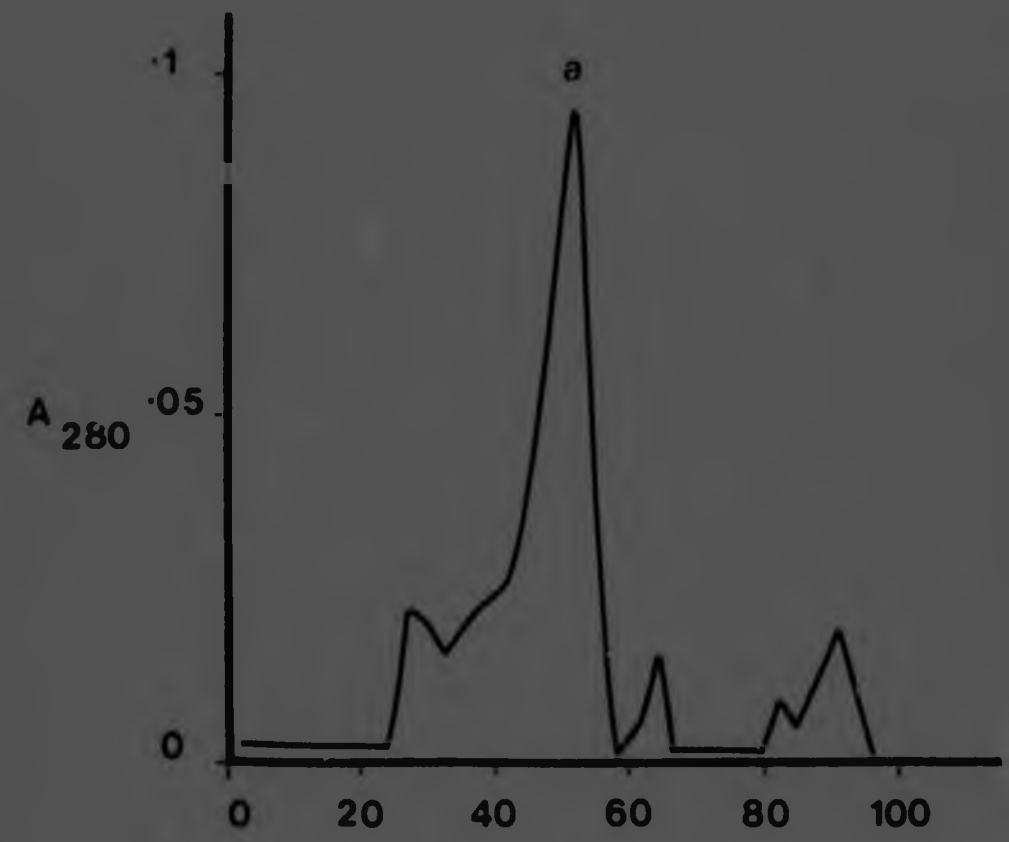


FIGURE 22

COLUMN CHROMATOGRAPHY OF PERCHLORIC ACID EXTRACT OF ACD PLASMA ON SEPHADEX G200.



proteins separated under identical conditions. It can be seen from Figure 19 that the preparation from serum gives a single peak on column chromatography with maximum absorption at fraction 60. It has a molecular weight of 55,000d by this technique which agrees with the value of 40,000-50,000d previously determined on PAGE. In some preparations a peak of higher molecular weight was noted, having an apparent molecular weight of 182,000d. (Figure 20). This is approximately three times the molecular weight of the smaller peak. Figure 21 shows the elution pattern of heparinized plasma extracts. A single peak is apparent with absorption maximum at fraction 0 and an estimated molecular weight of 81,000d. This appears to be 1.5 times the molecular weight of the preparations from serum. Figure 22 shows the elution profile from citrated plasma. Whilst this appears to be less pure than the preparation from heparinized plasma, it still contains a major peak with an estimated molecular weight of 81,000d. The major component in all these preparations was again shown to be alpha-acid glycoprotein, although citrated plasma could be shown to have other material present as well. (See below Table 23). Table 22 details the apparent molecular weights for the major components of each preparation.

In addition the secretor status of the individuals

/11m...

from whom the preparations were made, was investigated. The elution profile did not depend on the secretor status. The elution profile was also found to be unrelated to the blood group of the donor.

In the case of serum which showed an additional peak, Figure 20 both peaks were retained, concentrated and examined on immuno-diffusion. The results are shown in Figure 21.

TABLE 22

Molecular Weight of Proteins Extracted by Perchloric Acid from Serum and Plasma.

Source of Extract	Molecular weight of major peak.
Serum	55,000
Plasma (citrated)	81,000
Plasma (heparinised)	81,000

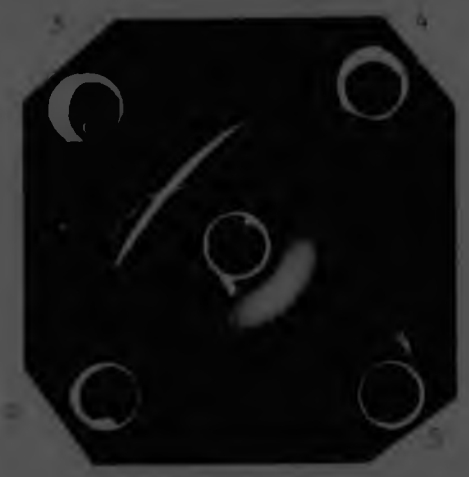
Both peaks gave single precipitin lines with rabbit anti serum against human serum and with anti serum against alpha-acid glycoprotein. It would thus appear that peak "a" in Figure 20 is a trimer of the species in peak "b". Table 23 shows the reactivity of the preparations from serum and citrated plasma against a panel of antibodies. From this it can be seen that the serum preparation contains alpha-

/acid...

FIGURE 23

CENTRE WELL, ANTI ALPHA ACID GLYCOPROTEIN

CENTRE WELL, ANTI WHOLE SERUM



Wells 1 and 3 Peak B

Wells 2 and 4 Peak A

Well 5 Whole serum

Wells O Empty

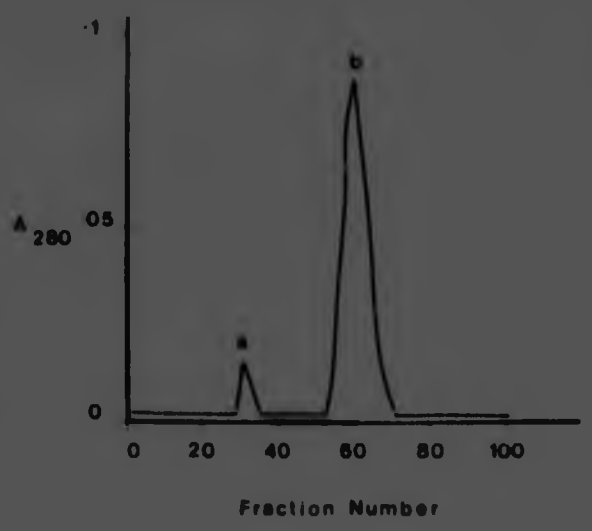


TABLE 23

RABBIT ANTISERUM TO HUMAN

	Plasma	Serum
1. whole serum	+	+
2. albumin	+	-
3. prealbumin	+	-
4. alpha-acid glycoprotein	+	-
5. ceruloplasmin	-	-
6. haptoglobin	+	+
7. alpha-HS glycoprotein	+	-
9. retinol-binding protein	-	-
10. alpha-lipoprotein	+	-
11. alpha-macroglobulin	-	-
12. alpha-anti trypsin	+	-
13. C.R.P.	+	-
14. sperm	-	+
15. C_1-inactivator	+	-
16. C_1 q component	-	-
17. C_1 S component	-	-
18. C_9 component	-	+
19. fibrinogen	-	-
20. inter alpha-trypsin inhibitor	-	-
21. IgA	-	+
22. IgD	-	+
23. IgE	-	-
24. IgG	+	+
25. IgM	-	-
26. G.C. globulin	-	-
27. plasminogen		

acid glycoprotein and no measurable other contaminants whilst the plasma preparations contain albumin, pre-albumin, alpha-acid glycoprotein, alpha-H-S, glycoprotein and alpha anti-trypsin.

3.0 Discussion

Column chromatography of the glycoprotein preparations was performed for two reasons. Firstly to determine whether or not the preparation could be fractionated any further, i.e. as an indication of the purity of the preparations and secondly to evaluate the molecular weight of the protein using a different technique. The elution profile of the glycoprotein preparation from serum indicated a single large peak with an estimated molecular weight of 55,000d. This is in good agreement with the molecular weight of 40,000 - 50,000d on PAGE. In some samples of preparations from serum a small amount of larger material was present having an apparent molecular weight of 182,000d. The material constituting this peak was isolated and shown to be alpha-acid glycoprotein. It appears therefore that the molecule may exist in an aggregated form possibly a trimer. Fractionation of whole serum shows alpha-acid glycoprotein to elute in the molecular weight range 158,000-240,000d. These results indicate that the protein is present in serum largely in the aggregated form and that

/perchloric. ..

acid glycoprotein and no measurable other contaminants whilst the plasma preparations contain albumin, pre-albumin, alpha-acid glycoprotein, alpha-H-S, glycoprotein and alpha anti-trypsin.

3.3.2 Discussion

Column chromatography of the glycoprotein preparations was performed for two reasons. Firstly to determine whether or not the preparation could be fractionated any further, i.e. as an indication of the purity of the preparations and secondly to evaluate the molecular weight of the protein using a different technique. The elution profile of the glycoprotein preparation from serum indicated a single large peak with an estimated molecular weight of 55,000d. This is in good agreement with the molecular weight of 40,000 - 50,000d on PAGE. In some samples of preparations from serum a small amount of larger material was present having an apparent molecular weight of 182,000d. The material constituting this peak was isolated and shown to be alpha-acid glycoprotein. It appears therefore that the molecule may exist in an aggregated form possibly a trimer. Fractionation of whole serum shows alpha-acid glycoprotein to elute in the molecular weight range 158,000-240,000d. These results indicate that the protein is present in serum largely in the aggregated form and that

/perchloric....

perchloric acid treatment may split the aggregate into its monomeric components Also treatment of the aggregate with urea SDS and mercaptoethanol will result in a breakdown of the aggregate into its monomeric components. An examination of the components of the extract from serum by immunodiffusion reveals that it consists of a single protein namely alpha-acid glycoprotein (which is in agreement with previous results).

An examination of the elution profile of the perchloric acid extract from plasma reveals some singificant differences. Plasma prepared using heparin as an anticoagulant showed a single peak with an apparent molecular weight of 81,000d that is 1.5 times as large as the preparation from serum. The elution profile of the preparation from citrated plasma showed many small peaks and also a major peak of molecular weight 81,000d.

Examination of the components of the preparation from citrated plasma revealed the presence of albumin, pre-albumin, alpha-anti trypsin. The elution profile of the extracts was found to be independent of secretor status or blood group of the donor.

The difference in size between the preparations from serum (55 000d) and plasma (81 000d) is as yet unexplained. One possible explanation is that the protein may be involved in coagulation and undergoes cleavage by proteases during the coagulation cascade. The

/different..

different amounts obtainable from serum and plasma suggest that the protein may be altered during coagulation. The contaminants in extracts from citrated plasma may be as the result of changes in the precipitation properties of the various contaminants in the presence of ACD anticoagulant. The differences noted between the preparations from plasma and serum prompted a study on the possible effects of these proteins on the coagulation cascade.

3.4 THE EFFECT OF ALPHA-ACID GLYCOPROTEIN ON COAGULATION

3.4.1 The Effect of Alpha Acid Glycoprotein on the Prothrombin Index (PI)

The effect of adding alpha acid glycoprotein to plasma on the PI is shown in Table 24.

TABLE 24

THE EFFECT OF ALPHA ACID GLYCOPROTEIN ON THE P.I.

	t (secs)			Mean (SD)
Control + 0.1 ml saline	17.3	17.1	17.8-	17.4 (0.36)
Alpha Acid Glycoprotein 42 mg/ml	17.8	17.8	18.0-	17.8 (0.12)

3.4.2 The Effect of Alpha-Acid Protein on the Partial Thromboplastin time (PTT)

Tables 25 and 26 show the results of the addition of different amounts of alpha-acid glycoprotein to plasma, on the PTT. IS WITHOUT EFFECT

Figures 24,25 and 26 show graphically the effect of increasing concentrations of alpha-acid glycoprotein on the PTT. It is apparent that an increase in the concentration of alpha-acid glycoprotein results in a marked shortening of the PTT. This is so for both plasma and serum extracts. At higher concentrations the extract has the reverse effect and results in an increased PTT. Figure 26 shows the effect of adding a preparation which has been stored for

TABLE 25

THE EFFECT OF STORED EXTRACT ON THE PTT

Amount of Extract mg/ml	Time (secs) 1	2	3	4	Mean	SD
Plasma alone	30.0	29.0	29.0	30.0	29.5	(0.58)
Control Plasma + 0.1 ml saline	33.0	33.0	32.0	-	32.6	(0.58)
106 mg/ml	70.0	82.0	63.0	-	71.6	(9.61)
94 mg/ml	63.0	61.5	64	-	62.83	(1.26)
60 mg/ml	64.0	50.0	50.0	-	54.6	(8.0)
55 mg/ml	48.5	51.0	44.5	-	48.0	(3.28)
42.5 mg/ml	66.5	66.0	52.0	62.5	61.75	(6.74)
20 mg/ml	53.4	51.0	52.4	-	52.13	(1.03)

TABLE 26

THE EFFECT OF FRESH EXTRACT ON THE PTT

Amount of Extract mg/ml	Time (secs) 1	2	3	4	Mean	SD
Plasma alone	30.0	29.0	29.0	30.0	29.5	(0.58)
Plasma + 0.1 ml Saline	32.8	33.0	33.5	33.5	33.2	(0.36)
Plasma + Serum Extract						
100 mg/ml	157	155	-	-	156	(1.41)
80 mg/ml	36.0	33.5	27.0	-	32.17	(4.65)
60 mg/ml	23.0	22.3	25.0	-	23.43	(1.40)
40 mg/ml	0	0	0	0	0	
20 mg/ml	0	0	0	0	0	

FIGURE 24.

THE EFFECT OF SEROMUCOID ON THE PTT

(FRESH EXTRACT FROM SERUM)

Each point is the mean of 4 determinations.

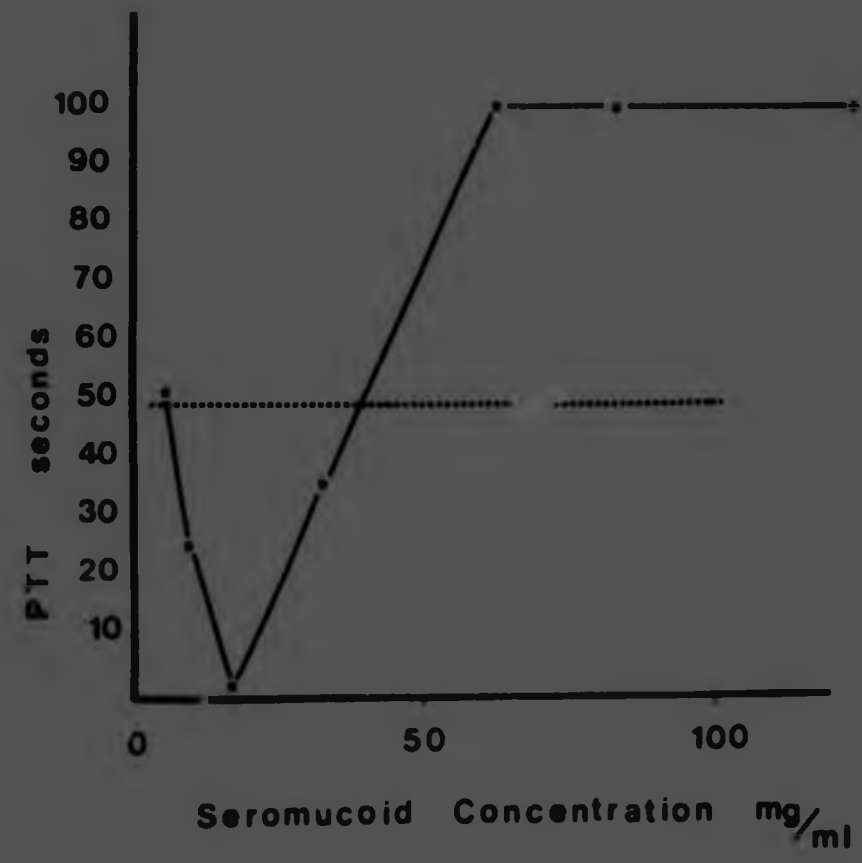
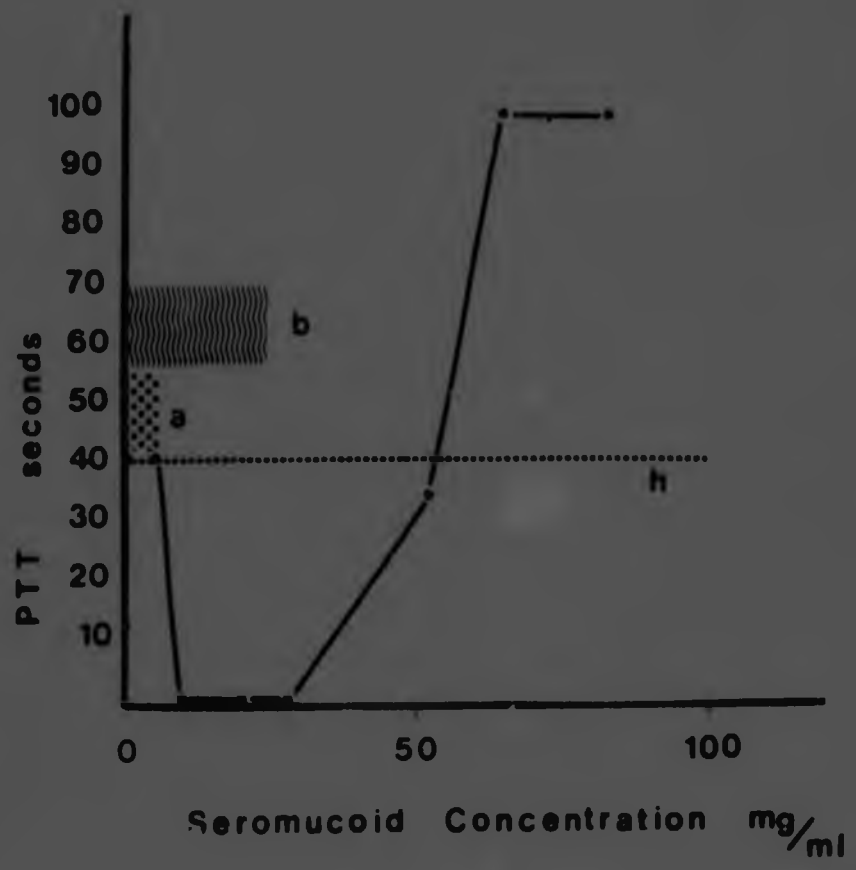


FIGURE 25

THE EFFECT OF SEROMUCOID ON THE PTT

(FRESH EXTRACT FROM PLASMA)

Each point is the mean of 4 determinations.



a.. normal levels

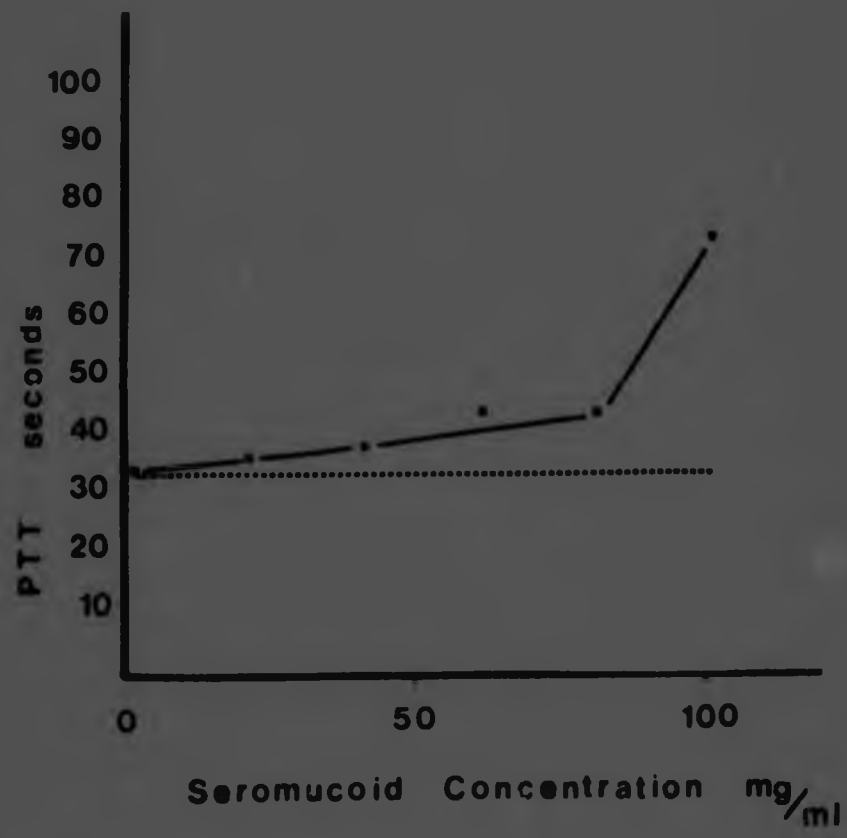
b.. levels which may be found in cancer patients

FIGURE 20

THE EFFECT OF SEROMUCOID ON THE PTT

(SEROMUCOID PREPARED FROM SERUM AND STORED FOR SIX MONTHS)

Each point is the mean of 4 determinations.



6 months freeze dried in sealed ampules.

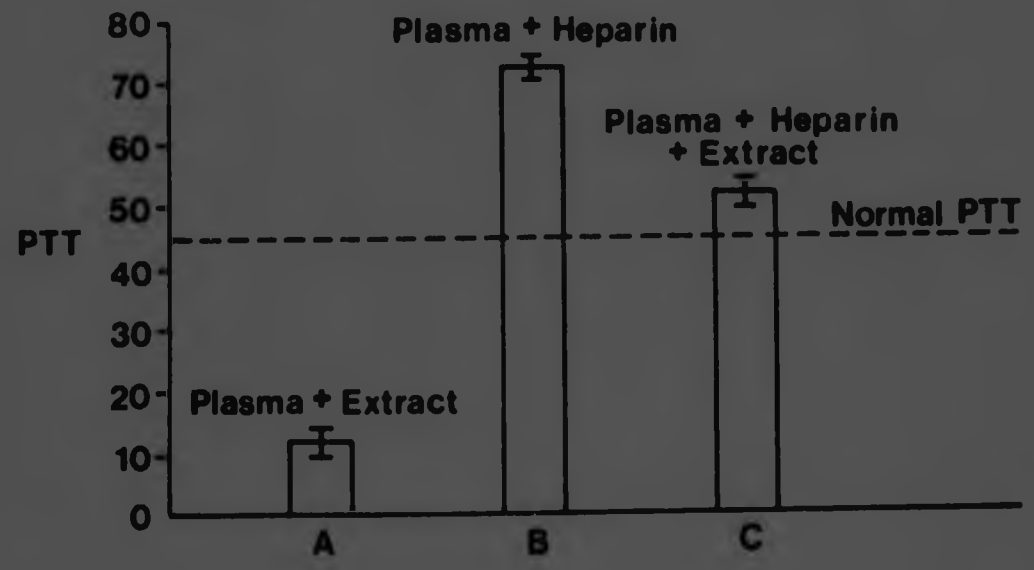
It is apparent that the ility of the extract to shorten the PTT is absent although it was still able to prolong the PTT. Figure 27 show the effect of alpha-acid glycoprotein on the anti-coagulant heparin. It will be seen that alpha-acid glycoprotein extract at the concentration which shortens the PTT is able to reverse the effect of heparin on the coagulation.

Discussion

The occurrence of multiple venous thrombosis is a well recognised complication of cancers (Pineo et al., 1974; Willi 1967). The reason for the increased incidence of thrombosis is not well known. Pineo et al.,(1974) have suggested that the production of mucus by the tumour may result in hypercoagulability of the blood. They were able to show that the proteins of mucus which were soluble in 5% trichloroacetic acid were capable of exerting a strong procoagulant effect. The active material had a high carbohydrate to protein ratio, exhibited a high degree of stability and did not appear to contain any phospholipid material. The partial thromboplastin time (PTT) of plasmas deficient in factors XI,IX,VIII,VII and X was corrected by addition of the mucus extract. They suggested that mucus entering the circulation was able to activate coagulation and this may explain the frequency

FIGURE 27

THE EFFECT OF ALPHA ACID GLYCOPROTEIN ON HEPARIN



of coagulation disorders in patients with carcinomas. Alpha acid glycoprotein is present in various body secretions including lung mucus and may well be present in their preparations. It is also significant that conditions in which alpha acid glycoprotein is elevated are associated with a high incidence of thrombosis (Trauma Sepsis Surgery and Cancer).(Myburgh J.A. Personal Commun.) . During the course of this study differences between alpha acid glycoprotein preparations from serum and plasma suggested that the protein may be in some way associated with coagulation. Other authors have noted that alpha acid glycoprotein may influence coagulation (Nilsson and Yamashina,1958). These authors found that alpha acid glycoprotein prolonged the clotting time of plasma. This is somewhat surprising in view of the high incidence of thrombo-embolic phenomena in cancer patients. Further evidence that alpha acid glycoprotein may be involved in the coagulation process comes from the observation that normal human platelets carry a significant amount of alpha acid glycoprotein tightly bound to their membrane. (Schmid, 1975) and are known to adhere to collagen. This adherence can be inhibited by the drug Persantin (dipyridamole). The addition of alpha$_1$ acid glycoprotein to the latter system restores the adhesive capacity of the platelets to collagen (Schmid, 1975).

/This...

Per anetin also inhibits the spontaneous clumping of platelets and this inhibition too can be reversed by the addition of alpha acid glycoprotein (Simmons et al., 1965 and Niewiarowski et al., 1975).

The role of platelets in metastasis is not clear but it appears that low platelet levels are associated with a lower incidence of metastasis than higher platelet levels. The relationship between metastasis and coagulation has been discussed elsewhere in this thesis (1.22).

The results which have been obtained in this study indicate that addition of alpha acid glycoprotein to plasma may result in a shortening of the PTT (i.e. a shift towards hypercoagulability. The protein has no effect on the extrinsic pathway (measured by prothrombin index), and appears to affect the intrinsic pathway only. Also alpha-acid glycoprotein is able to neutralise the anti-coagulant activity of heparin. The pro-coagulant activity appears to disappear on storage. The exact mechanism whereby alpha glycoprotein exerts these effects is not clear. It may act directly on the coagulation cascade, or via platelets.

THE EFFECT OF ALPHA ACID GLYCOPROTEIN ON LYMPHOCYTE FUNCTION

The effect of alpha acid glycoprotein on the ability of lymphocytes to undergo PHA - induced blast transformation is shown in Figure 28. It may be seen that with increasing concentration of alpha acid glycoprotein there is a decrease in the ability of the lymphocytes to undergo transformation. This effect was apparent in both the gross uptake of thymidine acid also on the stimulation index which was defined as:

$$\text{Stimulation index} = \frac{\text{CPM of PHA - stimulated cells}}{\text{CPM of unstimulated cells}}$$

The effect was noted with all of the preparations to some degree, however this effect was most marked in alpha acid glycoprotein preparations from patients with tumours. Figure 29 shows the effect of alpha acid glycoprotein preparations from various individuals, on the PHA induced blast transformation of lymphocytes. It can be seen that 9 of 14 preparations from patients with Hodgkins lymphoma, depressed blast transformation by greater than 80% (arbitrarily chosen value). Of preparations from trauma patients only 2 of 14 depressed transformation by 80% and of normal individuals now depressed the transformation by 80%. These results were evaluated statistically using a Kruskal Wallis non parametric analysis and were shown to be statistically significant ($P < .05$). The group means for the Hodgkins lymphoma patients was shown to be significantly higher than the means of the other 2 groups ($P < .05$).

FIGURE 28

THE EFFECT OF INCREASING SEROMUCOID CONCENTRATIONS ON BLAST TRANSFORMATION.

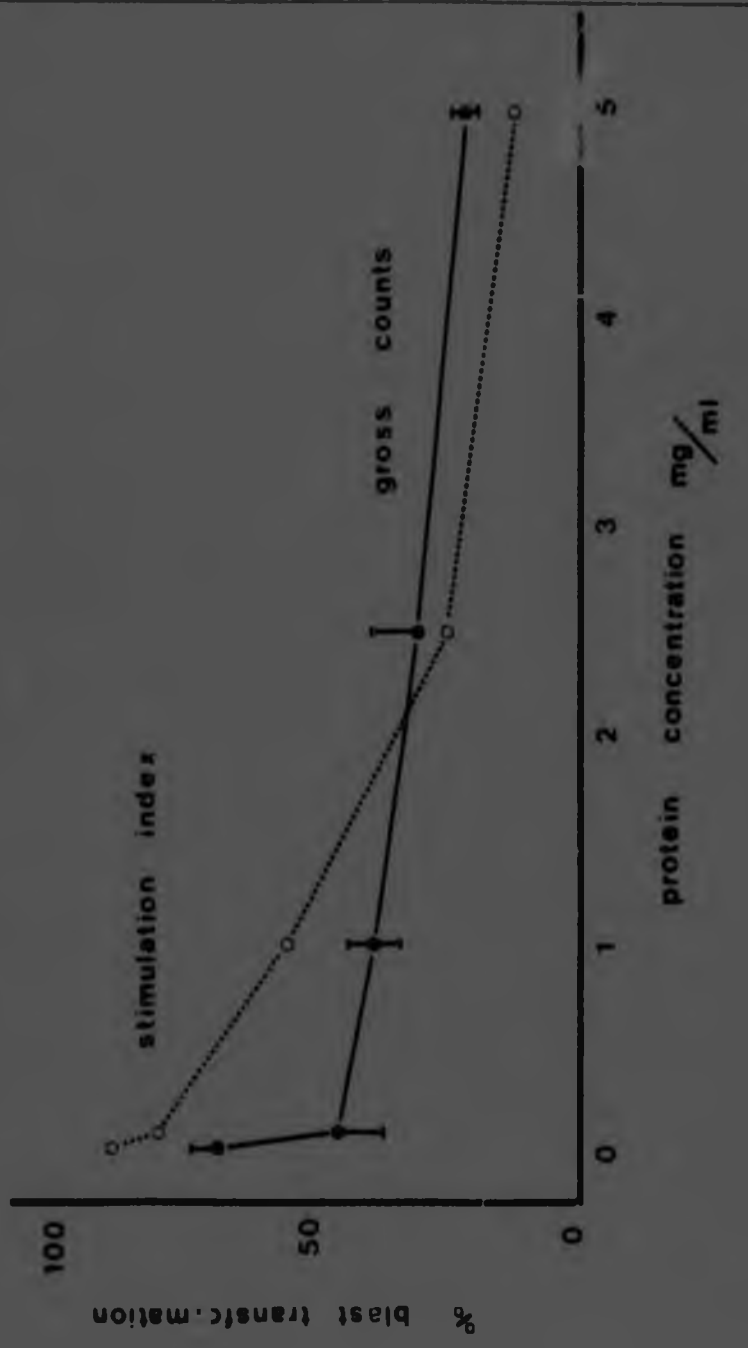
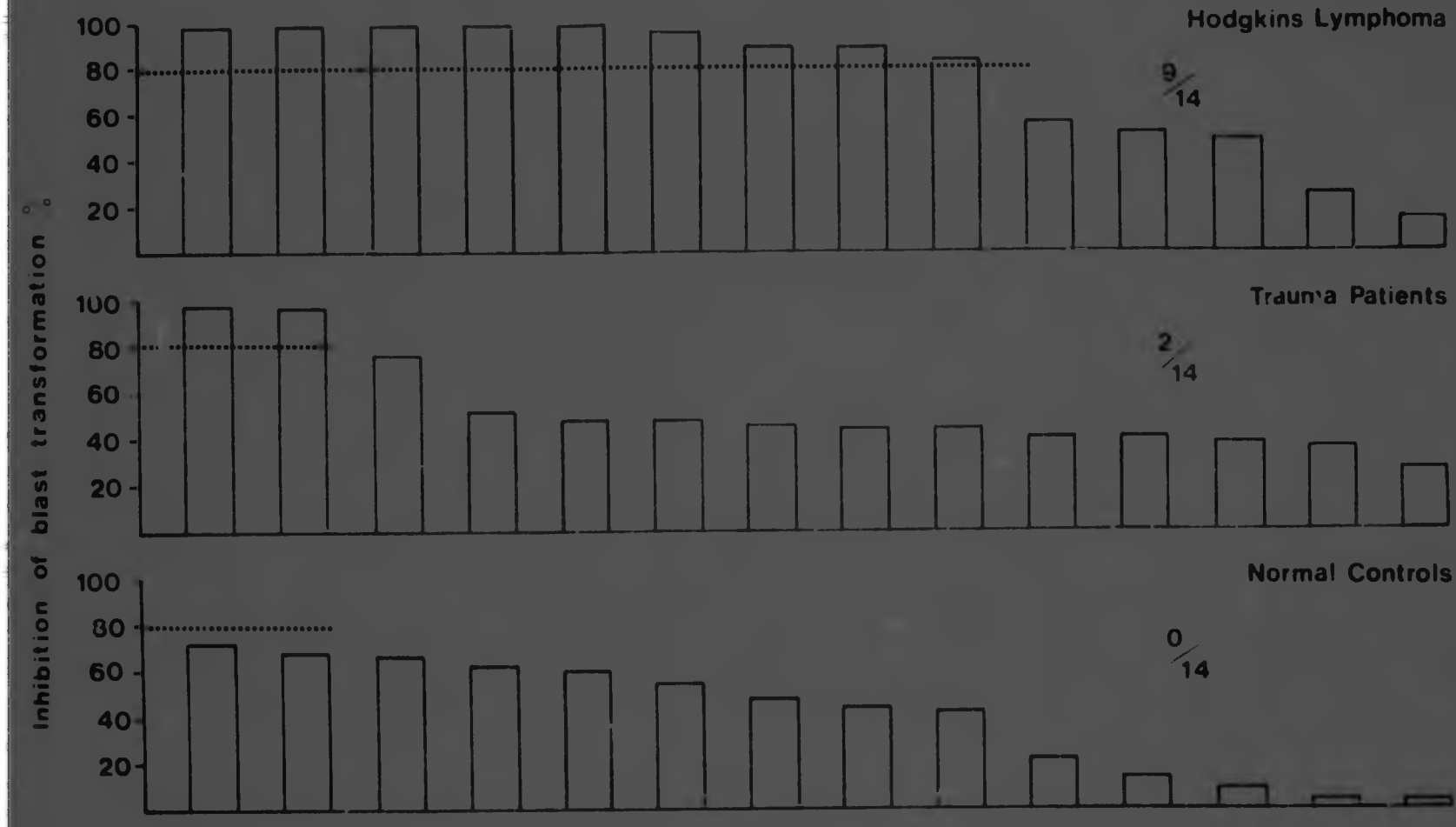


FIGURE 29
THE INHIBITION OF PHA-IND ED BLAST TRANSFORMATION BY SEROMUCOID FROM PATIENTS WITH ARIOUS CONDITIONS.



Discussion

The ability of alpha acid glycoprotein to inhibit PHA induced blast transformation is of interest in that depressed lymphocyte function and depressed immunity are features of advanced malignant disease. The mechanism whereby alpha acid glycoprotein exerts this effect is not as yet clear. It may bind to the lymphocyte surface and prevent the approach of the lectin or it may bind with the lectin. Other authors have suggested an immuno-regulatory function for sialoglycoproteins (Apffel and Peters, 1970; Codington 1975). The binding of a highly charged molecule such as alpha acid glycoprotein to a lymphocyte might interfere with its approach to a target cell either sterically or coulombically. Removal of such protein would be expected to result in an increased immune response. This has been noted with neuraminidase treated cells (Simmons and Rios,1971) and lymphocytes (Han,1975). The presence of antigenic material in these preparations is of interest in view of the possibility of specific blocking of lymphocyte receptor sites by such material. It appears that alpha acid glycoprotein is able to interfere with macrophage function as well (van Os et al., 1974). There are many substances in the circulation which can interfere with lymphocyte function and their elevation may possibly explain the depressed immunity seen in patients with cancer. The role of alpha acid glycoprotein in lymphocyte function needs to be

/evaluated..

1.5.1 Discussion

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/evaluated..

evaluated using for example mixed lymphocyte culture an experimental model. It would also be more meaningful to investigate the effect of this protein in vivo on tumour growth, lymphocyte function and the coagulation cascade.

3.6 MEASUREMENT OF HLA ANTIGENS AND BETA$_2$ MICROGLOBULIN IN ALPHA ACID GLYCOPROTEIN PREPARATIONS

3.6.1 Measurement of beta$_2$ microglobulin

Figure 30 shows the calibration curve used for the determination of beta$_2$ microglobulin. Points X,Y and Z denote the beta$_2$ microglobulin activity of 3 preparations of alpha acid glycoprotein, ranging from 3-10 µg/ml. The samples used for this had been passed through a Sephadex G200 column and the high molecular weight fractions only were retained. This would indicate that the beta$_2$ microglobulin present in the extract was bound as a high molecular weight complex possibly HLA antigens.

3.6.2 Measurement of HLA activity

Table 27 shows the strength of the polyspecific anti serum used in this work. (Ref).

Table 28 details the results of the inhibition of polyspecific anti HLA using alpha acid glycoprotein preparations from two patients. Target cells were obtained from the individual from whom the extract had been prepared.

/Table 27.-

FIGURE 30

CALIBRATION CURVE FOR DETERMINATION OF B_2 MICROGLOBULIN

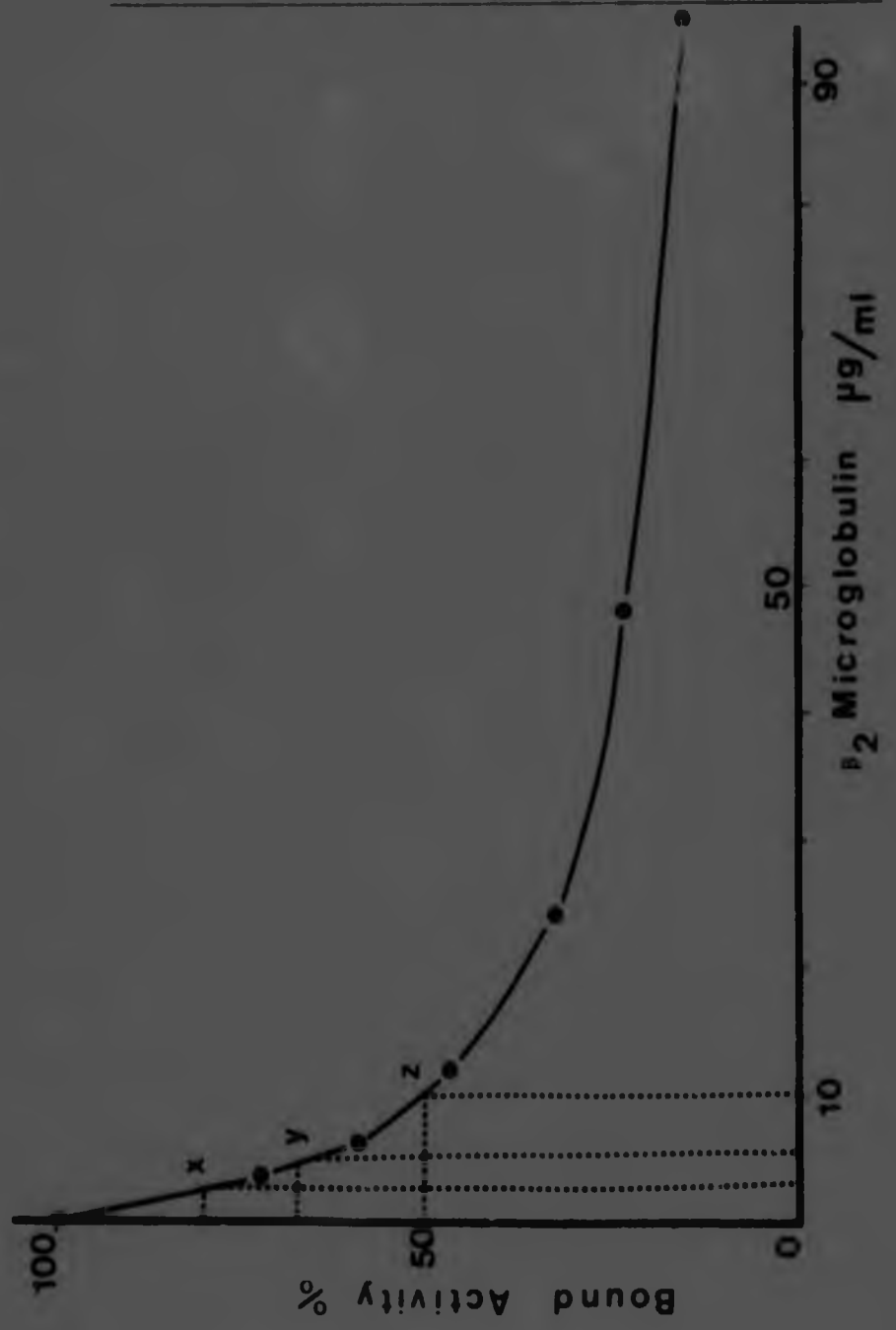

TABLE 27

DILUTION CURVE FOR POLYSPECIFIC ANTI HLA SERUM

Dilution	Mean of CPM	% Release
1	9974	98
1/2	10081	100
1/4	9363	90
1/8	9045	85
1/16	9266	88
1/32	6069	44
Complement Tese	2879	
Total Release	10063	
Titre chosen was 1/32		

TABLE 28

INHIBITION OF PSS BY ALPHA ACID GLYCOPROTEIN

	% ^{51}Cr release	
	Sample I	Sample II
PSS + 10 µl antigen	38	51
PSS + 25 µl antigen	34	64
PSS + 50 µl antigen	34	64
PSS + 100µl antigen	27	73
PSS + 200µl antigen	31	62
PSS	27	67

alpha acid glycoprotein was reconstituted in saline to a concentration of 10 mg/ml.

It can be seen that alpha acid glycoprotein showed no inhibitory activity against PSS.

3.6.3 Discussion

The demonstration of blood group antigens prompted a search for other cell surface antigens, i.e. HLA. It was possible to demonstrate the presence of beta$_2$ microglobulin in the perchloric acid extract of serum. The extract was passed through a Sephadex G200 column prior to the determinations. Beta$_2$ microglobulin activity was located in a high molecular weight fraction which eluted in the molecular weight range 40000 - 50000d. Thus the molecule was apparently part of a larger complex presumably HLA. However numerous inhibition tests failed to demonstrate any HLA antigen activity. This is not surprising in view of the low pH used to isolate the protein.

3.7 RADIAL IMMUNODIFFUSION OF PURIFIED OVARIAN CYST BLOOD GROUP SUBSTANCE

Radial immunodiffusion was performed on highly purified blood group substance derived from ovarian cyst fluid (a gift from Dr. W. Watkins). Figure 31 shows the pattern obtained against anti alpha-acid-glycoprotein. It can be seen that a strong precipitin line is formed.

3.7.1 Discussion

Analysis of pure blood group substance which was essentially free of other contaminating proteins (Watkins 1972) showed a strong precipitin reaction

/with...

with anti alpha acid glycoprotein. This provides further evidence for the relationship between soluble blood group antigens and alpha acid glycoprotein.

FIGURE 31

REACTIVITY OF PURIFIED BLOOD GROUP GLYCOPROTEIN AGAINST ANTI ACID GLYCOPROTEIN

4. CONCLUSIONS

A paper reporting serum lipoprotein apoproteins as major protein constitutuents of the red cell membrane has aroused considerable interest (Langdon, 1974). His conclusions were subsequently shown to be eroneous (Green, et al., 1975; Carey et al., 1975). Thus this question remains an open one. This thesis is concerned with identifying possible membrane components in human serum and studying the effects of such components on the immune system and coagulation cascade.

The finding of blood group antigenicity in glycoprotein preparations from serum is suggestive evidence of their membrane origin. Of interest is the finding of P, I, i, T and Tja antigens in the serum of patients with cancer. I have been unable to find reports in the literature of I, Tja and T antigens present in a soluble form in serum but the I antigen has been reported in human milk and saliva (Marsh et al., 1970). The presence of Tja may well be a clinically useful marker of malignancy and its usefulness in this respect needs to be fully evaluated. From this work it appears that orosomucoid or alpha acid glycoprotein appears to be a family of molecules of similar molecular weight but differing in the carbohydrate superstructure as evidenced by the different blood group antigens present in the preparations. The possibility that lipid present as a contaminant in these preparations cannot be overlooked, as it has been suggested that P and P_1 antigens are glycolipid (Naiki & Marcus, 1974). Lipid could not be detected in this study but this question requires to be further evaluated using exhaustive organic solvent extractions. The contribution of the liver to the synthesis of these glycoproteins cannot be excluded but it seems probable that at least part of the rise

/in

in alpha-globulins is due to contribution from sources other than the liver possibly by shedding from tumour cells or tumour cell debris from either necrosis or immune destruction of the tumour cells. These molecules would be very similar to CEA.

However, despite this, our preparations which were electrophoretically homogenous on PAGE and immunoelectrophoresis, were able to inhibit blood group antisera. The possibility that glycoproteins shed from tumour cells might interfere with lymphocyte function is of clinical importance. Accordingly the observation that alpha acid glycoprotein preparations can prevent PHA-induced lymphocyte blastogenesis is of interest. The author is aware that the conclusions drawn from PHA-induced blastogenesis have only slight bearing on the in vivo situation in man. In this respect it is noteworthy that conditions which are associated with elevated levels of alpha acid glycoprotein are also associated with a depressed immunity. (Constantian et al., 1977).

Elevated levels of A band and B band are of clinical relevance both in tumour diagnosis and in early detection of rejection in allograft recipients.

A1 a acid glycoprotein is known to be associated with the platelet membrane (Schmid, 1975). This fact, together with the differences between our protein preparations from serum and those from plasma and the high incidence of disseminated intravascular coagulation in patients with cancer, suggest that alpha acid glycoprotein might

/participate...

participate in coagulation in some way. The demonstration that elevated alpha acid glycoproteins shorten the PTT is of importance in this respect as it would be at least a partial explanation of the incidence of thrombo-embolic-phenomena in cancer patients. It is known that conditions in which alpha acid glycoprotein is elevated are associated with intravascular coagulation disorders. The mechanism whereby these glycoproteins exert these effects are not as yet clear. Charge may play a role of antigenic competition for the lymphocyte receptors or a combination of both. (Voorting, Hawking, and Michael, 1977). The *in vivo* effects of elevated levels of this protein require study.

CHAPTER 5

REFERENCES

5. REFERENCES

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CHAPTER 6

PUBLISHED WORK

Portions of this thesis have been published

in the following journals:

S.A.J. Surgery. 14: 7?-74. 1976.

S.A.J. Surgery. 14: 91-92. 1976.

S.A.J. Surgery. 15: 130-131. 1977

Vox. Sang. 1978. In pres .

Transplantation. 22: 7 78. 1976.

This study has confirmed that the muscle in clubfoot is grossly abnormal, and suggests that neurogenic factors are important.

DIFFERENTIATION OF MESOBLASTIC NEPHROMAS FROM NEPHROBLASTOMAS BY MEANS OF TISSUE CULTURE

I. Nuss, J. Duerma, D. J. Pudifin, D. Pirie, R. I. Mukel

Abstract

THE USE OF POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE EVALUATION OF SERUM PROTEINS IN PATIENTS WITH MALIGNANT DISEASE

D. J. K[illegible], D. Nathan, J. Rene Botha, and J. A. Myburgh
Department of Surg[illegible], University of the Witwatersrand, Johannesburg

Molecular weights of the two proteins have been shown to be 43 000 and 20 000 daltons respectively.

	No. of cases	A Band	B Band
Normal	81	4.3 (± 2.3) 12.0	(± 85)[illegible]
Non-Malignant Disease	35	9.8 (± 2.15) 2.4	± .06)
Malignant	38	[illegible]	(± 4.3)

We have not found these proteins in cord bloods.

The logarithms of the values were analysed by a one way analysis of [illegible].

This work was supported by a grant from Twins Pharmaceutical Pty Ltd and by a grant from the National Cancer Association.

† These values represent peak heights on a densitometer scan.

CELL-MEDIATED IMMUNITY IN BREAST CANCER

H. N. Desai

The effect of a[illegible] tumour extract on [illegible] own peripheral white blood cells [illegible] studied by [illegible] the leucocyte migration [illegible] after operation. Altogether [illegible] patients with breast carcinoma and 8 patients with benign [illegible] of the breast were investigated. The test was repeated between three and six months and between six and [illegible] months after operation. In the initial test 5 out of [illegible] patients with carcinoma and [illegible] patient with benign tumour had significant migration inhibition. Only one patient had migration inhibition [illegible] all [illegible] occasions. Our results of the initial test did [illegible] correlate with the degree of lymphocytic infiltration or with the clinical staging of the tumour. There was also [illegible] correlation with the leucocyte migration test and the lymphocyte [illegible] test. At the end of two years the clinical course of the patients with significant migration inhibition did not prove to be better than those who did not demonstrate migration inhibition of the leucocytes.

A CONTROLLED TRIAL OF ONCOLOGICAL THERAPY FOR GASTRIC CARCINOMA

D. M. Dent, B. N[illegible], P. Ch[illegible]verton and I. D. Werner
Combined Gastro[illegible] Clinic, University of Cape Town and Groote Schuur Hospital

Gastric carcinoma remains one of the most [illegible] and lethal gastro-intestinal malignancies. It is unlikely that new surgical techniques will alter the situation: one should rather look [illegible] diagnosis and [illegible] oncological therapy. The role of cytotoxic and radiotherapy, however, remains controversial with [illegible] bias towards these treatments having little influence on the disease. A trial seemed justified.

Methods: 127 new cases at all stages of disease were prospectively randomised into trial. Operations performed were at the discretion of the surgeon, being radical or palliative gastrectomy, [illegible] gastro-enterostomy or no procedure. Stratification into trial depended [illegible] on TNM staging and [illegible] operation.

(A) LOCALISED DISEASE (T[illegible], N[illegible], MO)

(i) [illegible] 2[illegible] [illegible] over 10 days; 5 FU 500 mg daily × 4 days, then 12.5 mg/kg for 5 days every month × 6 months.

(ii) No treatment.

(B) ADVANCED DISEASE (T4 or N3 or M1)

(i) As in A (i)

(ii) Thiotepa 45 mg i.v. × 3, then 15 mg i.v. monthly × 6

(iii) No treatment

Mean and median *survival* [illegible] were assessed. *Symptoms* were evaluated "blindly" on a 13 symptom questionnaire at regular intervals for all groups, quantification being on a 1-4 [illegible].

[illegible]			Survival (months)			Quality		
		[illegible]	Median	Mean	SD	Median	Mean	SD
A	(i)	[illegible]	[illegible]			[illegible]	[illegible]	[illegible]
	(ii)	[illegible]	[illegible]			[illegible]	[illegible]	[illegible]
B	(i)	[illegible]	[illegible]	[illegible]	1.7	[illegible]	[illegible]	[illegible]
	(ii)	[illegible]	[illegible]	[illegible]	[illegible]	15	30	[illegible]
	(iii)	[illegible]	[illegible]	[illegible]	[illegible]	14	[illegible]	[illegible]

[illegible]

high initial figure failure rate (28.8% in this series) may be reduced by improved techniques in construction of a standard 4.6 cm arteriovenous anastomosis and recognition of the common causes of early occlusion of the fistula. Arterial disease with intimal plaques and extensive thrombophlebitis from previous intravenous infusions, repetitive blood sampling and previous Scribner shunts were the most important predisposing causes for failure in the early period.

In the series of 78 patients a functioning fistula was established in 27 patients. One patient no longer requires haemodialysis because her renal allograft is functioning satisfactorily. Radio-cephalic fistulae (24 cases) were preferred because nearly all patients had already had one or more Scribner shunts inserted in the lower forearm. A standard side-to-side technique (4.6 cm diameter) was used in the series without distal venous ligation. The mean time interval before use in males was 4.4 weeks (range 2–7) and 15.5 weeks in females (range 5–33 weeks) with a mean initial flow rate of 247 ml/min in males and 242 ml/min in females. The low secondary failure rate (4%) is attributed to good needling technique and avoidance of premature use of the fistula, especially in females. Serial use of a bidirectional Doppler flowmeter will give a qualitative estimate of increasing flow through the fistula.

CELL MEDIATED CROSS-MATCHING ASSAYS FOR TRANSPLANT PATIENTS

J. V. Myburgh, J. A. Smit and J. R. Botha

MRC University Group in Renal and Organ Transplantation, Department of Surgery, University of the Witwatersrand Medical School, Johannesburg

Presensitisation to transplantation antigens may cause graft loss by accelerated or irreversible early acute rejection of renal allografts. Humoral evidence of presensitization may disappear with time and many patients with positive antibodies do well after transplantation. The interplay between cellular and humoral immunity complicates the picture even further. This is a study of 84 patients judged to be at high risk for graft rejection on the basis of HLA-DR locus incompatibility and/or presensitization detected by screening their sera against a panel of donor lymphocytes. Twenty-six received cadaveric kidneys and 6 others received grafts from live-related relatives.

Two assays were run concurrently: a conventional Complement Dependent Type Lymphocytotoxic (CDC) 4 hour ^{51}Cr release assay with recipient peripheral blood lymphocytes (PBL) and labelled donor PBL performed in vitro (CML-I); and CML-II performed in recipient serum to assess possible modulating effects of recipient humoral factors. Grafts were placed irrespective of the results. These assays and all patients have been followed up for 1 to 3 years.

A positive CDC-specific ^{51}Cr release assay and in particular a 10^{-4} ^{51}Cr release correlated strongly with graft rejection ($P < 0.05$). There was no correlation between CML-II and CDC rejection was not significant. Six patients had a positive CML-I and negative CML-II suggesting the presence of potentially beneficial blocking factors in their sera. However, 4 of these 6 patients rejected their grafts. Conversely, of 8 patients with negative CML-I but positive CML-II had an excellent long-term course. The use of CML-I pretransplant assays in selected high-risk patients is recommended.

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THE PRESENCE OF TWO NOVEL PROTEIN FRACTIONS IN THE SERUM OF RENAL ALLOGRAFT PATIENTS

J. R. Botha, D. J. K[illegible] and J. V. Myburgh

Department of Surgery, University of the Witwatersrand Medical School

Elevated levels of several glycoproteins have been demonstrated in malignant disease

[illegible] Clinical Chemistry

Some of these have been shown to be elevated in renal transplant patients. These include alpha fetoprotein (AFP), carcino embryonic antigen (CEA) and beta-2 microglobulin.

This present study reports the finding of two further low molecular weight proteins in the sera of patients undergoing clinical renal transplantation. Post transplant sera were obtained from 77 patients and 81 normal subjects served as a control group.

Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) two protein species of high (A Band) and low (B Band) molecular weights were demonstrated in significant quantities in the post renal transplant patients (P < 0.001). Estimated molecular weight of A Band is 16000 and of B Band 12000 Daltons. In the control group these proteins were either absent or present in minimal amounts. On densitometric scanning of the SDS polyacrylamide gels the mean peak heights of both these protein fractions were significantly higher in the transplant patients (P < 0.001).

The post transplant patients were subdivided into two groups. Group 1 – non rejectors – consists of 65 patients with a most of graft function and no clinical rejection (creatinine < 2 mg%). Group 2 – rejectors – consists of 12 patients – all of whom have lost their grafts from rejection and 3 with persistent deterioration of graft function (creatinine > 4 mg%). The mean peak heights of both protein fractions A and B are greater in Group 2 than in Group 1. The differences are not significant for the A band but are highly significant for the B band (P < 0.001).

The upper limit of the B band in normal controls (mean + 2SD). A rise above this for the B band was demonstrated in 10 out of 12 patients (83%) in Group 2. A similar rise of B band was seen in only 6 out of 65 patients (9%) in Group 1.

Although the serum and kidneys of these protein fractions are yet unclear a distinct correlation emerges between renal homograft rejection and rises in the low molecular weight (B band) fraction.

This study has been made possible by a grant from the MRC and the co-operation of the Tissue Bank (SAIMR, HPA) and Ewins Pharmaceuticals of Hoklmins (Pty) Ltd.

THE DEMONSTRATION OF THE INVOLVEMENT OF ANTIBODY IN THE IN VITRO LYSIS OF CHICKEN ERYTHROCYTES BY ALLERGISED MOUSE SPLEEN CELLS

S. MacPhail, *Department of Surgery, University of the Witwatersrand*

The result presented in this paper indicate that the in vitro lysis of chicken erythrocytes (CRC) by specifically allergised (primed) CRC up to five days before harvesting mouse spleen cells is an antibody mediated phenomenon revealing the incubation of target cells with the allergised spleen cells (ASC) a significant specific cytotoxic antibody is secreted into the surrounding culture medium to mediate lysis of the target cells by the normal effector cells of this type of cytotoxicity. Cytolysis is expressed as the percentage 51Cr released from the previously labelled target cells.

Testing of the supernatants presented by CRC with and without ASC in NSC for lytic activity

Supernatant from cultures containing	*Cr released*[a]
(diluted 1:)	(10)
	5.1
CRC	7.1
CRC + ASC	60.6
CRC + NSC	9.8
ASC	61.7
NSC	4.8

[a] Cr released from 5 × 10⁵ labelled CRC targets after incubation with effector cells at a ratio of 80:1 and the relevant supernatant for 20h.

RESULTS

(1)

Group	Survival Days	Median	P*
2 BM + CY	8, 12, 5, 57, 62, 95	56	~0,05
3 BM + II A	11, 20, 31, 32, 48, 150	31,5	~0,05
2 + 3		40	0,025
4 D Sp + CY	18, 19, 22, 31, 38, 63, 82	31	0,025
D Sp + II A	11, 23, 31, 48, 50, 81	39,5	0,025
4 + 5		31	0,01
Control	7, 8, 12, 12, 13, 14, 19, 19, 21, 22, 22, 23, 24, 27, 38, 40	19	

*Mann Whitney U test vs controls

Only one of the three animals in Group 1 (BM + CY) was transplanted successfully. It died five days after transplantation with florid histological rejection.

2. *Immunological Studies*. Baboons immunised with *BM* + CY formed predominantly B cell antibodies before and after transplantation. Four of the six had positive LMC before transplantation. Baboons immunised with *Bm* + II A formed no antibodies before transplantation but responded with brisk predominantly B cell antibodies after transplantation. Only two of the six developed a positive LMC before transplantation. All but two of the baboons immunised with *D Sp* developed antibodies before transplantation. Most consistently in the D Sp + Cy group B and T specificities were analysed in only five of the animals (D Sp + CY). In all five activity was strong against B and T cell preparations.

Conclusions. Significant enhancement of baboon liver allograft survival was achieved by active immunisation with donor spleen cells or bone marrow fractions rich in Ia like determinants. There was no evidence of superior efficacy of these bone marrow fractions over spleen cells and no evidence that enhancement in this model was the property solely of antibodies with Ia like specificities.

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A STUDY OF SERUM GLYCOPROTEINS IN MALIGNANCY AND THEIR POSSIBLE INFLUENCE ON THE IMMUNE SYSTEM OF THE HOST

D. J. Klatzow and J. A. Myburgh. *MRC University Group for Research in Organ Transplantation, Department of Surgery, University of the Witwatersrand.*

It is well known that serum glycoproteins are elevated in malignancy[1]. These are the acute phase reactant and are thought to be synthesised in the liver as a general reaction to non-specific insults such as trauma, infection and auto-immune disease. However, recent information casts some doubt on the statement that the liver is the sole source of the acute phase reactants.

In this study, a acid glycoprotein has been investigated in order to shed some light on its origin and function. Previous workers have shown that there is a factor in host blood serum which correlated with abnormal elevation of serum bound sialic acid. As α acid glycoprotein is a major contributor to the serum bound sialic acid we have investigated its effect on the PHA blast transformation of human lymphocytes.

Materials and Methods. A Perchloric acid (1.4M) was used to solubilise glycoproteins from serum (both normal and abnormal). Perchloric acid precipitates most proteins, leaving mainly glycoproteins (especially α acid glycoprotein) in solution. This perchloric acid extract after extensive dialysis and concentration was added to lymphocytes in increasing concentrations. The response of these lymphocytes to PHA was then measured by tritiated thymidine uptake. (Lymphocytes were prepared on a Ficoll Hypaque gradient). B. Perchloric acid was used to solubilise glycoproteins from a variety of normal and neoplastic tissues. These extracts were compared using polyacrylamide gel electrophoresis. The gels were stained for protein with Coomassie Blue and for glycoproteins using periodic acid Schiff stain (PAS).

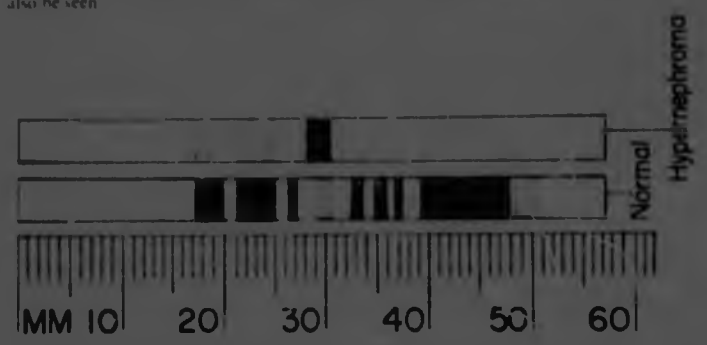
Results. The table shows the effects of increasing concentrations of the perchloric acid extract of serum on PHA blast [illegible]

Stimulation index for PHA blast genesis

Protein concentration µg/ml	0	1	10	50	100	1 000	2 500
Experiment No 1	70	52	41	42	30	30	
Experiment No 2	60	32	21	14	17	11	5
Experiment No 3	60	40	17	21	20	1	35

It can be seen that PHA blast transformation is inhibited by this extract.

The figure shows the difference between the perchloric acid extract of normal kidney and hypernephroma. Striking differences can be seen. Differences on PAS staining can also be seen.



We have extracted acid glycoprotein from tissues other than liver and we have excluded the possibility that this is merely contamination by serum within the tissue space.

We conclude therefore that acid glycoprotein is a component of several normal tissues including kidney and spleen. The sub-cellular location is speculated to be the cell membrane. It seems as though this protein may have some effect in depressing the immune system in patients with cancer.

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2 Watkins et al *Int J Cancer* 14:799 1974

SUTURE OF COLONIC WOUNDS IN THE RABBIT — AN EXPERIMENTAL STUDY

J C Robbs B Angorn. *Department of Surgery, Natal University Medical School*

The influence of different suture materials on colonic healing has been widely documented. This study was undertaken in an attempt to examine the breaking strength of incised colonic wounds with four currently available suture materials in the same animal and to correlate these results with histological changes in the colonic wall.

Four full thickness transverse incised wounds 2.5 cm in length and 3.5 cm apart were made in series along the length of the ascending colons of each of 19 adult rabbits. The wounds were sutured with chromic catgut, polyglactin 910, polypropylene and silk. Nine animals were sacrificed at five days after wounding, and two groups of five animals each at 14 and 21 days after wounding.

Breaking strength of the suture line was measured by determining the breaking strain following a constant rate of increase in tension across the suture line. Sections from the same wounds were taken for histological evaluation.

Silk was found to result in a significantly stronger wound when tested five days after suture. No difference was apparent when comparison was made at 14 days after operation. 21 days after operation, wounds sutured with absorbable material seemed to be weaker than those in which non absorbable sutures had been used. No definite correlation could be demonstrated between the breaking strength of the suture line and the degree of acute inflammatory or fibrous tissue reaction provoked by the suture material.

South African Journal of Surgery, Vol. 15, No. 3, September 1977

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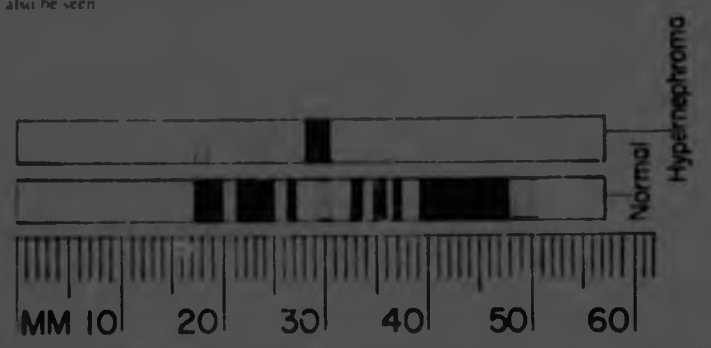

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STUDIES ON THE SEROMUCOID FRACTION IN HUMAN SERUM IN VARIOUS BENIGN AND MALIGNANT CONDITIONS

D.J. KLATZOW and G. VOS, MYBURGH, J.A. and SMIT, J.A.

We have investigated the perchloric acid - soluble proteins of human serum (seromucoid fraction) and have found it to contain a wide variety of blood group antigens and also β_2 microglobulin.

The blood group antigens represented in this fraction are A, B, H, I, i, Tj^a, P, N, T, Le^a and Le^b.

In our study of 50 normal controls, 45 patients with trauma, sepsis and other conditions known to elevate the acute phase reactants and 74 patients with various malignant conditions, we found quantitative and qualitative differences between the seromucoids from patients with cancer (particularly breast, Hodgkin's lymphoma and lymphocylic lymphoma) and those patients with non-malignant conditions. In particular we found elevations of Tj^a, I and i. In general the total extent of the seromucoid was increased in patients with malignant conditions.

ANTIGENIC CONTENT OF SEROMUCOID - [illegible] expressed as cumulative mean inhibition scores.

	P	Tj^a	I	i	T
Controls (50)	0	0	0	0	18.8
Non-Malignant (45)	0.29	0	0.86	0.40	0.2
Hodgkin's Lymph. Lymph. + Ca. Breast (41)	3.75	2.82	3.29	2.41	1.5
Other malignant conditions (33)	0.75	0.39	0	0.39	0.1

The seromucoids from patients with Hodgkin's lymphoma were found to strongly inhibit mitogen-induced lymphocyte blastogenesis.

Serological Studies of Seromucoids from Normal Subjects and Patients with Malignant and Non-Malignant Diseases

David J. Klatzow and Gerard H. Vos

Department of Surgery, University of the Witwatersrand, Medical School, Johannesburg, and Natal Institute of Immunology, Durban

Abstract. During investigations of serum from patients with carcinoma, elevated levels of two proteins were observed. The larger (MW ~44,000) was identified as (α-acid glycoprotein. The smaller MW ~20,000) has not yet been identified. Further investigation of α-acid-glycoprotein-containing seromucoid showed that many red blood cell and tissue antigens were present, for example, I, i, P_1, Tja ($P + P^k + P^{nk}$), H, A, B, Le^a, Le^{b1}, M and N. Differences in antigen content between normal serum and serum from carcinoma patients could be demonstrated quantitatively and qualitatively. Seromucoid preparations from patients with Hodkin's lymphoma inhibited phytohaemagglutinin-induced lymphocyte transformation more often than seromucoid preparations from normal subjects.

Introduction

The sera of patients with different types of malignancies often have increased serum α-globulin levels and changes in the carbohydrate content of serum glycoproteins [1, 2, 24, 27]. Similar changes have been reported in the serum proteins of animals with transplanted or naturally occurring tumours [12, 14, 23, 30].

One of us (D. J. K.) has also reported elevation of two protein fractions in the sera of 76% of patients with different kinds of malignancies. These proteins were found to have molecular weights of 44,000 and 20,000 daltons, respectively. The larger of the two was identified as α-acid glycoprotein (orosomucoid) [4, 17, 29].

Recent reports and reviews have proposed that the α-globulins may have immunosuppressive properties [6, 7, 15, 19, 20, 22]. The most prominent band in all the serum-inhibitory fractions was a glycoprotein which migrated with or slightly behind the albumin on electrophoresis [35]. Studies by *Occhino* [21] have suggested that the active principle of immunoregulatory α globulin is not the α-globulin itself but a polypeptide with a molecular weight of 6,000–8,000 daltons. He proposed that more than one serum protein may function as a carrier for this polypeptide.

Other authors have reported that the seromucoid fractions of human plasma may play an active role in the prevention of immune responses to fetal antigens [8, 11]. As well, the major component of seromucoid, an α_1-acid glycoprotein [29], can act on the surfaces of *Escherichia coli* and *Streptococcus aureus* and prevent phagocytosis of these organisms by human neutrophils [34]. Other investigators suggest a relationship between the metastatic potential of a tumour and the thickness of the glycocalyx surrounding the tumour cells [16]. Metastasizing tumours may shed their glycoprotein coat into their surroundings and thereby interfere with the immune response directed against them.

We have studied the blood group antigens present in seromucoid and its ability to interfere with immune processes, using preparations from plasma and sera of normal subjects and sera from patients with a variety of diseases.

Materials and Methods

Polyacrylamide gel electrophoresis was done as described in a previous paper [4].

Immunoelectrophoresis was done by the method of *Arquembourg et al.* [3]. Antisera, both monospecific and polyspecific, were kindly donated by Hoechst Pharmaceuticals through the good offices of Mr *H. Bester*.

Seromucoid extracts from serum and plasma were prepared by a modification of the original method of *Winzler et al.* [40]. Blood was obtained from patients and normal volunteers after informed consent. The blood samples for serum were allowed to clot and the serum was obtained by centrifugation. This was stored at -20 °C until used (never more than 24 h), in most cases, seromucoid was prepared immediately from the serum. For plasma extraction, 450–500 ml of blood was collected into 70 ml of citrate phosphate dextrose. Plasma was obtained from the whole blood within 72 h of collection.

To either serum or plasma, an equal volume of 1.4 *M* perchloric acid was added. All extraction procedures were carried out at 4 °C. The perchloric acid serum-plasma mixture was allowed to stand for 5 min and then the precipitated proteins were removed by centrifugation. The clear supernatant was dialyzed against running tap water for 24 h, reduced in volume and then dialyzed against distilled water for 4 h. Following this, it was lyophilized and stored in sealed ampules until used.

Haemagglutination Inhibition by Seromucoids

Optimal concentrations of antisera were prepared as in the following example:

If anti-P_1 reacted to a dilution of 1:64 (undiluted ++++, 1:2 [illegible] 1:4 ++++, 1:8 ++++, 1:16 +++, 1:32 ++, 1:64 +, 1:128 0) then 1:4 was selected as the optimum concentration of the specific antibody for the haemagglutination inhibition test. To this optimum saline dilution of anti-P_1 was added an equal volume of saline (control) or seromucoid reconstituted in saline (to a standard concentration of 10 mg/ml, from which 1:2, 1:4 and 1:8 dilutions were also prepared and tested). Any decrease in the reaction strength would indicate inhibition of antibody activity. Results were recorded as (4) for no inhibition, (3), (2), (1) for partial and 0 for complete inhibition.

For a sample which did not inhibit the antiserum, the pattern would be ++++ (4), ++++ (4), ++++ (4), ++++ (4) or 4444, i.e. reaction score 16. If the sample had some inhibitory activity, the pattern might be –, +, ++, +++ or – 1 2 3, for the extract undiluted and diluted 1:2, 1:4 and 1:8, giving a reaction score of 6. The inhibitory activity is expressed as the maximum reaction score possible (4 + 4 + 4 + 4 = 16) minus the score obtained. Thus for our second example the inhibitory activity would be 16 – 6 = 10, referred to as the cumulative inhibition score.

For the groups of individuals studied, the cumulative inhibition scores were summed and a mean inhibition activity was then calculated.

During preliminary investigation it was found that the inhibitory activity of various blood group determinants, particularly the T antigen, often disappeared spontaneously from the seromucoid preparations. To avoid this loss of activity, which

is due to the presence of β-galactosidase in the seromucoid [18], phenylbeta-D-thiogalactoside (10 mg/100 ml of serum extract) was added to the extracts after dialysis and before freeze drying.

Sephadex chromatography was performed using Sephadex G-200 and 0.1 m Tris-HCl buffer pH 8.2 with NaCl added to a concentration of 1 *M*. 5-ml fractions were collected. Column dimensions were 50 × 2.5 cm.

For studies of phytohaemagglutinin (PHA)-stimulated lymphocytes, 25 ml samples of blood from laboratory personnel were placed in sterile containers with glass beads and defibrinated by gentle rotation for 15 min.

Lymphocytes were prepared on a Ficoll Hypaque gradient and washed three times with Hanks balanced salt solution. They were then made up to a final concentration of $2 \cdot 10^6$/ml in Eagle's minimal essential medium (MEM) containing the seromucoid extract in a concentration of 2.5 mg/ml.

The cells were cultured in microtitre plates. Each well contained 2×10^5 cells, 100 µl of MEM (containing seromucoid), 50 µl of PHA. The optimum concentration of PHA was determined prior to the experiment.

For the seromucoid studies, on each patient, the test was run in quadruplicate.

Blast transformation was measured by adding 10 µl of tritiated thymidine (1 µCi/ml) to each well on the 2nd day. The cells were then harvested onto filter paper and counted in a Packard scintillation counter.

Control cultures that is cells from the same donor cultured without seromucoid extract were included with each experiment.

The isotope uptake of each culture was expressed as a percentage of the control.

Results

Figure 1 shows the elution profile of the perchloric acid extract of serum from Sephadex G-200. There was no high-molecular-weight component in the mixture. This extract was also analyzed by immunoelectrophoresis which confirmed that the major component was α-acid glycoprotein (fig. 2). A small amount of other protein was demonstrated by polyacrylamide gel electrophoresis (fig. 3).

Table I. The amounts of seromucoid recovered from 100 ml serum and 100 ml plasma of healthy blood donors

	Serum	Plasma
Number of subjects	50	60
Mean weight of seromucoid, mg	43.5	22.3
Range, mg	31–59	14–32

In control subjects, less seromucoid was isolated from plasma than from serum (table I). This variation was not merely due to the diluent effect of the anticoagulant. When assessing the number of different blood group determinants in the mucoids, more activity was found in the plasma extracts (mean inhibitory activity 37.6) than in the serum extracts (mean inhibitory activity 28.4, table II). The mucoids obtained from sera lacked inhibitory activity for the $P_1 + P_1 + P^k$ and the Ii blood group systems, while the plasma mucoid extracts were found to possess these determinants. This may indicate that the $P_1 + P_1 + P^k$ and Ii determinants found in the plasma mucoid extracts were derived from the membranes of red cells, white cells or platelets during storage. ABH and Lewis blood group substances were not found in the mucoid extracts of sera from A and Lewis non-secretors (table I); the presence of these blood group activities must therefore, be regulated by their secretor status. T substance activity which is not normally exposed in secretions or body fluids, was found

Table II. Cumulative haemagglutination scores for serum and plasma from healthy donors

	A	B	H	Le^a	Le^{b1}	P_1	Tja	I	i	T	M	N
Serum, n = 50												
Cumulative inhibitory scores	71	43	127	89	79	0	0	8	0	800	10	51
Mean inhibitory activity	1.42	0.86	2.54	1.78	1.58	0	0	0	0	16.0	0.2	1
Plasma, n = 52												
Cumulative inhibitory scores	81	84	229	180	134	53	19	164	36	776	83	88
Mean inhibitory activity	1.56	1.62	4.4	3.46	2.58	1	0.37	3.15	0.69	14.9	1.6	1.69

Tja = P + P_1 + P^k

Table III. Cumulative inhibitory activity (CIA) and mean inhibitory scores (MIS) of seromucoid substances prepared from the serum of healthy subjects

	SE n = 30		Le, n = 9		S−, n = 10		−, n = 1, CIA
	CIA	MIS	CIA	MIS	CIA	MIS	
A	37	1.2	0		43	4.3	0
B	43	1.4	0		0		0
H	74	2.4	0		44	4.4	0
Le^a	43	1.4	46	5.1	0	–	0
Le^{b1}	45	1.8	25	2.7	0	–	0
P_1	0	–	0	–	0	–	0
Tja	0	–	0		0		0
I	0	–	0		0		0
i	0	–	0	–	0		0
T	480	16	144	16	160	16	16
M	8	0.2	0		2	0.2	0
N	34	1.1	2	0.2	15	1.5	0

SE = Subjects who secrete A(H), B(H), H and Lewis substances; Le = subject who secrete only Lewis substances; S = subjects who secrete only A(H), B(H), H substances; − = subjects who do not secrete any substances; n = number of subjects.

in serum extracts from all subjects investigated and in the highest concentration of all the blood group determinants in all the normal subjects studied.

Table IV records the cumulative agglutination inhibition scores obtained for various blood group determinants in the seromucoids from sera of patients with various malignancies and other illnesses together with the controls. There was marked reduction in T substance for all patients investigated. Similar results were recorded for H, and to a lesser degree for A and B antigens in some groups of patients. On the other hand, increased P_1, P + P_1 + P^k and Ii activities were observed in the seromucoids of

Table IV. Seromucoid extracts from serum tested for their inhibitory [illegible]ity against antibodies to carbohydrate antigens

Subjects	Number tested	Mean cumulative inhibition scores for anti-									
		A	B	H	Le[a]	I [illegible]	P_1	Tja	I	i	T
Controls	50	1.42	0.86	2.54	1.78	1.58	0	0	0	0	16
Trauma, sepsis and other illnesses	45	0.68	0.57	0.24	0.73	1.31	0.22	0	0.86	0.40	0.2
Breast cancer, lymphocytic lymphoma Hodgkin's lymphoma	41	1.[illegible]	0.29	0.82	1.95	1.82	3.75	2.82	3.29	2.41	1.5
Other varieties of cancer	33	1.60	0.63	0	0.48	0.87	0.75	0.39	0	0.39	0.1

patients particularly those with Hodgkin's lymphoma, breast cancer and lymphocytic lymphoma.

Figure 4 shows the effect of adding the seromucoid extract from patients with Hodgkin's lymphoma to normal lymphocytes stimulated with PHA. In 60% (9/15) of the cases, blast transformation was depressed by more than 70% in the presence of the extract. Only 2/16 extracts from normal controls (12.5%) showed more than 70% inhibition of lymphocyte transformation (fig. 5). The effect of the seromucoid extract on blast transformation was found to be dose-dependent (fig. 6).

Discussion

This investigation has shown that α-globulin-containing seromucoid fractions of human serum have a variety of blood group antigen activities. The ABH and Lewis antigens appear to come from the secretory organs and not from the membranes of blood cells, as they were absent from the mucoid extracts of non-secretor subjects.

However, mucoids extracted from plasma may contain a combination of blood group determinants derived from the secretory organs with others from blood cells. Our result suggest that membrane-derived fragments of blood group antigens (ABH, Lewis, MN, P + P_1 + P^k and Ii) may be released from apparently intact cells when blood is collected and stored in citrate-phosphate-dextrose solution. However, other reasons for the differences between serum and plasma cannot be excluded, such as release of serologically inactive α-acid glycoprotein from cells during clotting, co-precipitation of antigens with fibrin, their adsorption to fibrin or adsorption to altered membranes in the clot.

The finding of carbohydrate-containing antigenic determinants in the mucoids is not surprising in view of the similarities in carbohydrate structures found on α-acid glycoproteins and on blood group substances [28, 36].

ABH, Lewis, P + P_1 + P^k and Ii determinants are demonstrable on cell surfaces of many tissues besides red blood cells [26]. Differences exist, however, in the distribu-

tion of these determinants in the secretory organs. ABH, Lewis and Ii determinants are detectable in saliva whereas P + P_1 + P^k is primarily a membrane-bound blood group system. The soluble form of P blood group substances has so far been found only in hydatid cyst fluid, worm extracts, liver flukes and fish roe [5, 25]. The presence of P + P_1 + P^k activity in mucoids could be due to *in vitro* shedding following the collection and storage of the blood in anticoagulants or to the *in vivo* shedding of the factors.

Our studies suggest that the *in vivo* shedding of P + P_1 + P^k and Ii determinants may take place in a variety of pathological conditions, such as lymphatic lymphomas, Hodgkin's disease and breast cancer. In contrast to the increase of P + P_1 + P^k and Ii determinants in patients with these illnesses, there was a significant decrease in T substance activity. The association between changes in blood group antigens and human cancers has been stressed by other investigators [10, 33, 37].

The ability of the seromucoid extract to prevent PHA-induced blast transformation is interesting in view of the elevation of seromucoid in cancer patients and the immunological anergy so often associated with the disease [9]. We suggest that this may influence tumour cell development by coating lymphocytes: because of the charged sialic acids present this may prevent their approaching tumour cells. Other authors have noted the requirement for sialic acid in immunosuppression by mouse sialated α-fetoprotein [38, 41].

Impairment of the immune response by sialic acid containing molecules attached to the cell surface of both tumour cell and immunological effector cell could explain the effect of neuraminidase on various immunological tests of lymphocyte activity *in vitro* [13, 32, 39].

Simmons and Perlman [31] have noted blood group activity associated with CEA. In our preparations we did not appear to have CEA as a component.

Further work is being undertaken at present to determine whether the seromucoid components are part of the tumour cell membrane. Also the mode of interference of seromucoid with PHA blast transformation is being investigated.

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Fig. 1. Elution profile of seromucoid on Sephadex G-200 chromatography; shaded area represents the void volume as determined by dextran blue.

Fig. 2. Immunoelectrophoresis: a Seromucoid from normal serum versus anti whole serum. Trough contains anti whole serum. Upper well contains seromucoid. Lower well contains whole serum. b Seromucoid from normal serum versus anti acid glycoprotein. Trough contains anti acid glycoprotein. Upper well contains seromucoid. Lower well contains whole serum.

Fig. 3. Polyacrylamide gel electrophoresis of seromucoid prepared from normal serum compared with whole serum on left-hand side.

Fig. 4. Inhibition of blast transformation of normal lymphocytes by seromucoid from patients with Hodgkin's lymphoma. Results are means of 5 tests with each preparation.

Fig. 5. Effect of seromucoid, from normal volunteers, on PHA induced blast transformation. Results are means of 5 tests with each preparation.

Fig. 6. Effect of increasing amounts of seromucoid on the PHA blast transformation of normal lymphocytes. Each point represents the mean ± SD of 5 results. Seromucoid was prepared from the serum of a patient with Hodgkin's lymphoma.

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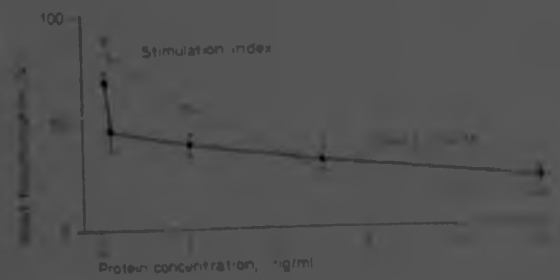
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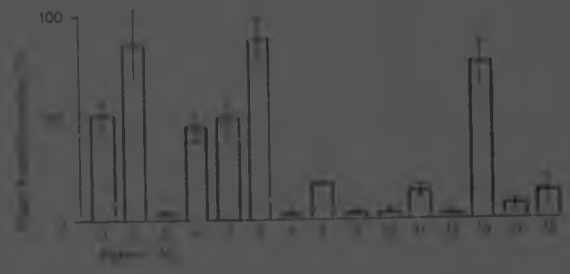
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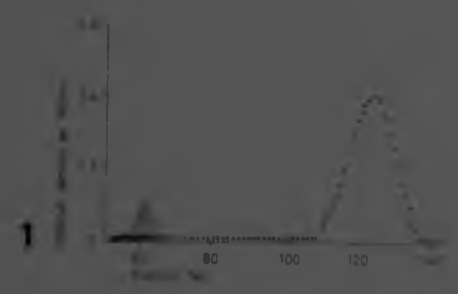
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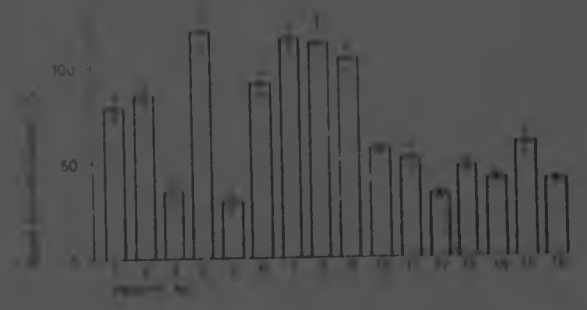
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THE PRESENCE OF TWO PROTEIN FRACTIONS IN THE SERUM OF RENAL ALLOGRAFT PATIENTS

Elevated levels of several protein species have been demonstrated in sera of patients with malignant disease (1–6). These include carcinoembryonic antigen (3), α-fetoprotein (1), β$_2$-microglobulin (5) and α$_1$-glycoproteins (6), all of which are found in normal mature human serum but in low concentrations.

Recently, Hoch *et al.* (7) described a unique DNA-binding protein in the serum of patients with various malignant neoplasms, which has a molecular weight of 36,000 daltons and is also found in cord blood. We (paper in press) have recently reported significantly raised concentrations of two further protein fractions in 26% of sera of a random series of patients with malignant neoplasms. These protein fractions have estimated molecular weights of 48,000 and 20,000 daltons respectively and have not been demonstrated in cord blood.

We report here similar studies of sera from our patients undergoing clinical renal transplantation. Sera were obtained from 57 patients 2 weeks to 84 months after transplantation and from 81 normal control subjects. Analysis of sera were done by using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). Aliquots of 0.1 ml of serum were diluted with 2 ml of solution containing 8 M urea, 1% sodium dodecyl sulphate, and 1% thioethanol. Diluted serum (10 ml) was

then added to polyacrylamide gels of 7.5% containing 1% SDS. Gel tubes were run for 1 hr at a current of 5 ma/tube. Bromophenol blue was used as a tracking dye. At the completion of the run the gels were placed in a solution containing 10% trichloroacetic acid for 1 hr and then stained for 1.5 hr in a solution of 0.1% Coomassie blue in 5% acetic acid and 10% methanol. Destaining was carried out for 48 hr in a solution of 5% acetic acid and 10% methanol. Gels were scanned on a Vitatron densitometer.

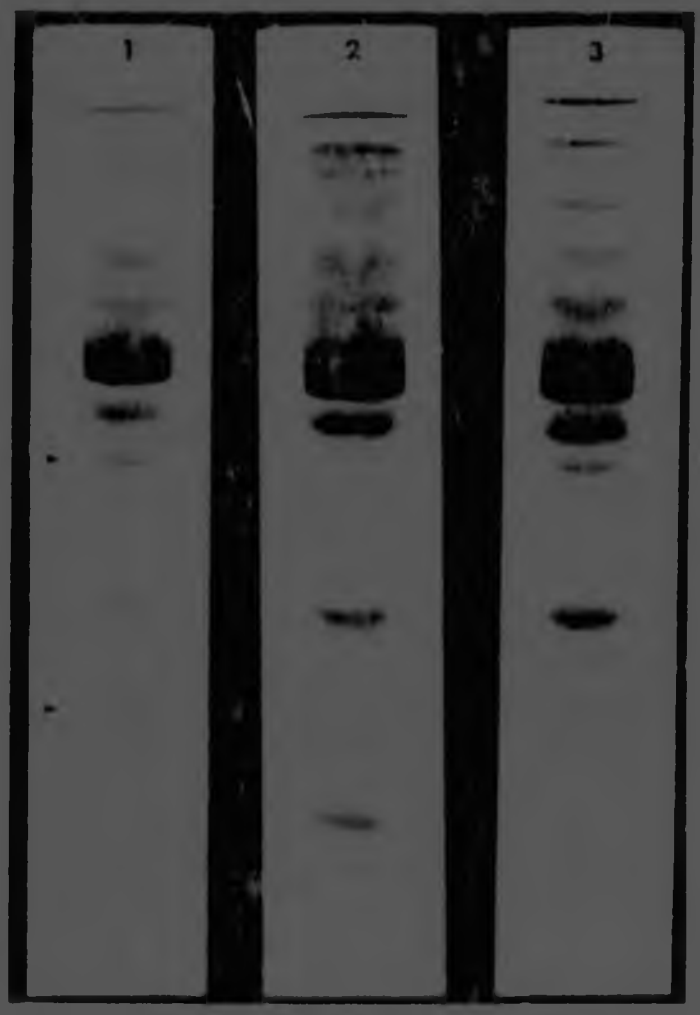

FIG. 1. Polyacrylamide electrophoresis patterns obtained from a transplant patient (1), a normal control (2), and a patient with a malignant tumour (3). The A and B bands are arrowed. The dark band at the bottom of the centre gel (2) is haemoglobin.

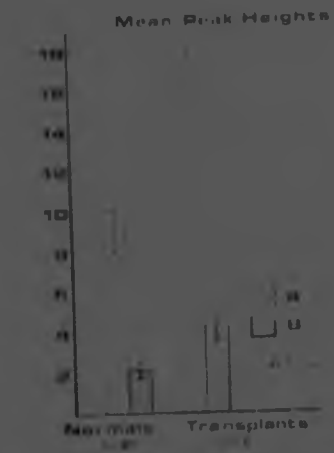

FIG. 2. Mean peak heights on densitometric scanning of sera from 81 normal controls and 65 renal allograft patients. Mean peak height for A band in controls = 9.4 (±SD 1.4) and for the B band = 4.8 (±SD 1.4). The mean peak height for the A band in transplant patients is 14.0 (±SD 3.2) and for the B band 12.1 (±SD 6.1). Both A and B bands differ significantly in the two groups ($P < 0.0001$).

Figure 1 shows representative examples of SDS PAGE specimens of sera from a normal control, a patient with a renal transplant and a patient with a malignant neoplasm. Raised concentrations for the upper (A band) and the lower (B band) protein fractions are apparent in the SDS PAGE specimens of both the transplant patient and the patient with malignant disease.

Figure 2 shows the mean peak heights plus S.E.M. obtained on densitometric readings of SDS PAGE specimens in post transplant patients and normal controls. Mean peak heights of both A and B bands are significantly higher in the post transplant patients ($P < 0.0001$).

The post-transplant patients were subdivided into two groups (Fig. 3). Group 1 ("non rejectors") consists of 45 patients with good graft function (serum creatinine 1.2 mg/100 ml or less) no clinical evidence of active rejection. Group 2 ("rejectors") consists of 32 patients (3 of whom had lost their grafts from rejection and 4 patients with poor and slowly deteriorating graft function (serum creatinine 4 mg/100 ml). The mean peak heights of both A and B bands are greater in group 2 than in group 1. The differences are not significant for the A band but are highly significant for the B band ($P < 0.0001$).

The upper limit for the B band in controls is 6.8 (mean + 2 SD). A rise above 6.8 for the B band was demonstrated in group 2 ("rejectors") in 27 out of 32 patients (84%). A similar rise of the B band was seen in 6 of 45 patients (13%) in group 1 ("non rejectors"). The significance of this finding is at present unclear as is the identity and origin of these two protein fractions.

The possibility of these two proteins being products of the cell membrane glycoproteins is highly speculative. The tendency of the low molecular weight protein (B band) to be increased in the "rejecting" group of patients may have immunological and diagnostic significance or may be the result of nonspecific cell damage.

Isolation, identification and immunological significance of these protein fractions will be investigated.

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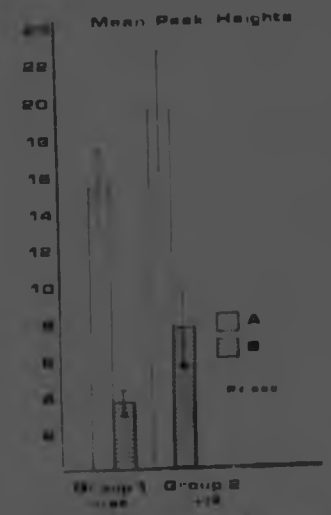

FIG. 3. Mean peak heights and ± S.E. of the mean of A and B protein bands in group 1 (non-rejecting) and group 2 (rejected) patients. There is no statistical difference between A bands, but the B bands differ significantly ($P < 0.001$).

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