

BIOCHEMICAL AND IMMUNOLOGICAL
INVESTIGATIONS OF ANIMAL AND HUMAN
TUMOUR-ASSOCIATED ANTIGENS

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I hereby declare that the research described within
this thesis is based entirely upon my own work

Gerard Berns

Date _____

DEDICATION

I would like to dedicate this thesis to my
parents, John and Jean Berns, and my sister
Angela

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ABBREVIATIONS

| | |
|---------|---|
| Ab | Antibody |
| Ag | Antigen |
| AO/EB | Acridine orange/ethidium bromide |
| BSA | Bovine serum albumin |
| BsAb | Bispecific Ab |
| CNS | Central nervous system |
| DABA | 3,5-Diaminobenzoic acid |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| 2-DOR | 2-Deoxyribose |
| EBV | Epstein-Barr virus |
| ELISA | Enzyme-linked immunosorbent assay |
| FCS | Foetal calf serum |
| GA | Glutaraldehyde |
| HAMA | Human anti-mouse antibodies |
| HAT | Hypoxanthine, aminopterin and thymidine |
| HMAb | Human monoclonal antibody |
| HPLC | High performance liquid chromatography |
| Ig | Immunoglobulin |
| ip | Intra-peritoneal |
| LAT | Landschutz ascites tumour |
| LSA | Lipid-bound sialic acid |
| MAb | Monoclonal Ab |
| MHC | Major histocompatibility complex |
| NANA | N-Acetylneuraminic acid |
| NGNA | N-Glycolyl-neuraminic acid |
| O.D. | Optical density |
| PA-NANA | Protein-associated sialic acid |
| PBS | Phosphate buffered saline |
| PEG | Polyethylene glycol |
| PVC | Polyvinyl chloride |
| RAb | Recombinant Ab |
| TBA | Thiobarbituric acid |

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ABSTRACT

Total N-acetylneuraminic acid (NANA) and lipid-bound sialic acid (LSA) were evaluated as indicators of tumour burden in a range of human tumours and also an animal model system.

For human bladder carcinoma, both serum and urinary total NANA were found to lack the sensitivity required for use as markers for small tumour burden. However, serum NANA levels were significantly elevated in cases of advanced disease, thereby implying a role for this marker as a supplementary parameter in the clinical monitoring of high stage bladder carcinoma.

In the evaluation of total serum NANA as an indicator of human prostate tumour burden, NANA levels were monitored in 10 patients undergoing treatment for advanced disease over periods ranging from 3 - 6 months. Individual isolated NANA determinations did not always reflect tumour burden. This marker displayed characteristics of an acute-phase reactant, whereby a large incidence of false positive results indicated a deficiency in the specificity of NANA as an indicator of tumour burden. However, serial determination of NANA was found to be useful as a means of monitoring patient response to therapy.

Using an animal model system, both total serum NANA and serum LSA were evaluated as tumour markers for Landschutz ascites tumour (LAT). Prolonged monitoring of serum NANA and LSA levels showed that LSA more accurately reflected the extent of tumour burden.

A modified thiobarbituric acid micro-assay (VGR-Micro) was developed and used to determine total plasma NANA levels. The modifications introduced in the VGR-Micro assay, while increasing the safety factor and reducing the time required for analysis, did not adversely affect the performance characteristics of the original (Warren) assay.

A murine monoclonal antibody (A5F9) was produced against the EJ human bladder carcinoma cell line. This antibody recognises an antigen not present on either normal tonsillar lymphocytes or blood leucocytes. Analysis of A5F9 ascitic fluid (by ELISA) showed that this antibody can detect the presence of EJ cells at dilutions in excess of 1 : 7000.

AN OUTLINE OF THE AIMS OF THIS CURRENT PROJECT

The objective of this project was to evaluate the tumour marker potential of sialic acid, using biochemical and immunological investigations, in a range of tumours differing in nature and in species of origin.

Biochemical investigations involved thorough evaluation of sialic acid, both total NANA and LSA, as markers for animal and human tumours.

During the course of this research, significant problems were encountered with the Warren thiobarbituric acid (TBA) assay (the hitherto standard method of analysis for total NANA). These persistent problems inspired the development of a modified TBA assay. The modified assay was developed to achieve the following:

1. Reduction of interference from 2-Deoxyribose (2-DOR).
2. To replace cyclohexanone with a less toxic chromophore stabiliser, thereby making the modified assay more "operator friendly".
3. To include a degree of automation, thereby reduce the time required for analysis.
4. To replace dual wavelength analysis by single wavelength analysis (at 560 nm), enabling NANA determination from a standard curve.

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SECTION 1

INTRODUCTION

**The Clinical Potential of Sialic Acid
as a Tumour Marker.**

NOMENCLATURE, STRUCTURE AND DISTRIBUTION OF SIALIC ACIDS

Faillard (1989) stated that "although sialic acids are probably more than a million years old, they were discovered only half a century ago, and can therefore hardly be regarded as an object of historical research" Nevertheless, based on the special degree of interest shown in this group of sugars over the past 50 years, it is possible to state that sialic acids have been the subject of extensive research

The term "sialic acid" first appeared in the literature in 1952 (Blix et al) The standard method of nomenclature of sialic acids, adopted by consensus, was also proposed by Blix et al (1957), whereby neuraminic acid was designated as the unsubstituted parent structure (Figure 1) and sialic acid as the generic name for the family of related derivatives having an acyl group on the amino nitrogen and frequently O-substituents elsewhere The extreme diversity of this group of sugars is generated by the different types of O-substitutions (usually O-acetyl esters) at the 4-, 7-, 8- and 9-hydroxyl positions of the parent molecule (neuraminic acid) Prior to the introduction of the standard method of nomenclature, various sialic acids were previously referred to by other names, including hemataminic acid (Yamakawa and Suzuki, 1952) and gynaminic acid (Zilliken et al , 1955).

The most common of the naturally occurring sialic acids is N-acetylneuraminic acid (NANA), which has the systematic name 5-acetamido-3, 5-dideoxy-D-

glycero-D-galacto-nonulosonic acid (when named as an open-chain compound)

Another widely distributed sialic acid is N-glycolylneuraminic acid (NGNA). These two species, NANA and NGNA, comprise the structural backbone for the majority of known sialic acids. They occur in the pyranose ring form when glycosidically linked to other sugars, and this is also the preferred conformation in the free state (Figure 2, Ledeen and Yu, 1976)

The sialic acids are widely distributed in nature, either free or as components of homo- and heterosaccharides, glycoproteins and glycolipids (Tuppy and Gottschalk, 1972). They have been reported in viruses, bacteria, invertebrates, primitive chordates and vertebrates (including fishes, amphibians, reptiles, birds and mammals), with sialic acid being ubiquitous in tissues of all mammalian species studied to date (Ng and Dain, 1976). Within the eukaryotic cell, essentially all of the sialic acid is in a bound form incorporated in glycoproteins and glycolipids (sialoglycoconjugates), and these are, to a large extent, components of the cell membranes. Sialic acids are usually in terminal positions at the non-reducing ends of oligosaccharide moieties of sialoglycoconjugates. In general, the concentration is highest in the plasma membrane, lower in the smooth endoplasmic reticulum (and possibly Golgi apparatus), and lowest in the rough endoplasmic reticulum and nucleus (Warren, 1976).

BIOLOGICAL FUNCTIONS OF NANA

The sialic acids are an extremely versatile group of sugars, with numerous biological functions. The following section describes the principal functions of sialic acids and includes a discussion of their biological significance.

In 1962, Forrester et al performed micro-electrophoretic studies on clones of normal and polyoma-transformed hamster fibroblasts. Treatment of both cell types with neuraminidase, prior to electrophoresis, resulted in significant reductions in electrophoretic mobilities. These results indicated that approximately 35% of the surface charge (of both cell types) arose from surface sialic acid moieties. In further electrophoretic investigations of the same cell types, Forrester et al (1962) found that while untreated transformed cells showed greater electrophoretic mobility than their normal counterparts, neuraminidase treatment resulted in indistinguishable electrophoretic mobilities in an electric field. This observation demonstrated that the increased electrophoretic mobility of the transformed clone was due to the charge enhancing property resulting from increased concentration of surface NANA. Cook and Jacobson (1968), in a two year study, investigated the electrophoretic mobilities of normal and leukaemic murine cells so as to characterise and compare the chemical nature of their surface charges. Exposure of normal and leukaemic cells, to neuraminidase, lowered their electrophoretic mobilities by 27% and 49%, respectively. These results indicated that both cell types possessed significant amounts of NANA at their peripheries. Confirmation of reduced

surface NANA, as the principal cause of reduced electrophoretic mobilities, was demonstrated by the biochemical detection of liberated NANA in the supernatants of enzyme-treated cells. More specifically, since neuraminidase is an α -glycosidase, therefore unlikely to generate any cationic groups, the loss of electrophoretic mobility as a result of treatment with this enzyme was attributed to removal of the carboxyl groups of NANA. Kojima and Maekawa (1970) in a similar study, performed comparative studies on the effect of neuraminidase removal of NANA on the cellular electrokinetic charge of rat ascites hepatoma. While suggesting that NANA may not be the only factor responsible for the negative surface charge of cells (other factors not specified), results again indicated that observed reductions in electrophoretic mobility were caused by enzymatic removal of the carboxylic groups of cell surface NANA. Finally, Latner and Turner (1974), using electrophoresis, investigated surface modification of normal and transformed baby hamster kidney cells (BHK21 - C13 and BHK21 C13 Py6, respectively). Results from Py6 demonstrated that removal of surface NANA molecules did not expose an equal negative charge, thereby implying that NANA molecules constitute a significant proportion of the overall negative surface charge of the cell.

Other research has shown that the presence of terminal NANA residues can influence the lifetime of a range of circulating biomolecules. Morell et al (1971), using an animal model system, found that the viability of many serum glycoproteins and gonadotropic hormones depends on the presence of their normal complement of NANA residues. Removal of NANA results in exposure of terminal, non-

reducing galactosyl residues, thereby facilitating their recognition and removal by the liver Reuter et al (1980) suggested that O-acetylation of NANAs could also influence the lifetime of red blood cells

Vaheri et al (1972) were the first to demonstrate the stimulation of DNA synthesis and cell division of density-inhibited cells by exposure to neuraminidase (as evidenced by an increase in ³H-thymidine incorporation, mitotic index and cell counts), whereby enzyme levels of only 0.02 µg/ml were sufficient to cause a detectable degree of stimulation Neuraminidase treatment also resulted in increased sugar uptake These results provided direct evidence that control of cell growth is regulated by NANA residues on the cell surface Chandrakant and Bernacki (1975) suggested a possible role for NANA in the increased drug resistance of the murine leukaemic cell line, L1210, to guanyldrazone, due to competitive uptake of spermidine by NANA (acting as a receptor) Surface NANA was considered to be a contributing factor to increased drug resistance since its removal from the leukaemic cells (by exposure to neuraminidase), which greatly reduced spermidine uptake, was accompanied by increased growth inhibition (from guanyldrazone) Van Heyningen (1974) showed that LSAs act as receptors for certain toxins Tetanus toxin, which acts on the central nervous system (CNS), was shown to be found by at least three LSAs in nervous tissue, each differing from the other by NANA content The ability of LSAs to bind tetanus toxin, which increased with increasing NANA content, did not result in loss of toxicity In marked contrast, LSAs were found, not only to bind cholera

toxin, but also to inhibit its action in the small intestine and the skin

Berwick and Coman (1962) suggested that surface NANA affects specific physical properties of cells on finding that neuraminidase cleavage of NANA resulted in decreased "stickiness" of cells to siliconised glass Raz et al (1980), who found that removal of surface NANA reduced homotypic aggregation of B16 - F10 murine melanoma cells, suggested that peripheral NANA is also involved in cell aggregation. Similarly, Hara et al. (1980) demonstrated, from experiments involving neuraminidase, that surface NANA is the essential carbohydrate moiety required for the platelet - aggregating activity of tumour cells Since other research has indicated that platelets play an important role in the process of metastatic seeding, by causing cell adherence to vessel walls (Hilgrad, 1973), and also metastatic establishment, by secretion of growth factors (Hara et al , 1979), these observations concerning platelet aggregation imply a role for NANA in the promotion of metastatic growth

Extensive research has shown that NANA also may have the ability to mask specific recognition sites on molecules and cells, resulting in suppression of antigenicity The ability of NANA to "deamplify" antigenic expression can provide a means of immune response inhibition, thereby allowing certain types of sialylated cells (including cancer cells) to evade a host's defence mechanisms

Currie and Bagshawe (1968) attempted to elucidate the role of surface NANA in inhibiting the detection of the murine LAT by the immune system On finding that trypsin sensitive components of the antigenic

determinant group were protected from the active centre of the trypsin molecule by neuraminidase - susceptible NANAs, it was suggested that antigenic sites could be protected from immunocompetent cells of the host in a similar fashion. Suggested protection mechanisms were steric hindrance (due to the physical dimensions of the NANA molecule) and inhibition by the electrostatic effects of ionised carboxyl groups in the region of the sensitive site. Using a range of neuraminidase enzymes (with different sites of action on the NANA substrate), it was found that the masking ability of NANA required a specific type of linkage to the cell surface, with cleavage of $\alpha(2-6)$ -O-glycosidic linked NANA being essential for unmasking of antigenic sites. Urdal and Hakamori (1983) found that murine lymphoma cells treated with neuraminidase were 6-10 times more sensitive to complement-mediated lysis (by a specific monoclonal antibody) than were untreated cells, clearly indicating that tumour cell antigenicity is influenced by sialylation of membrane components. The importance of surface NANA as a modifier of the immune response was further demonstrated by Frohman and Cowing (1985), who examined the potential functional relevance of the differential glycosylation of Ia molecules (class 2 major histocompatibility complex, MHC, glycoproteins) on the antigen presenting function of resting B cells. Alteration of surface glycosylation, by treatment with neuraminidase, resulted in a 25-fold increase in B cell antigen presentation to T cells. Powell et al (1985) demonstrated that cell surface NANA influences tumour cell recognition, in that a B cell lymphoma (AKTB-1b) failed to stimulate thymic lymphocytes unless pretreated with neuraminidase. In agreement with the earlier work of Currie and Bagshawe, it was

found that neuraminidase-dependent stimulation of lymphocytes is due to the desialylation of specific membrane structures (as opposed to total desialylation), with removal of $\alpha(2-6)$ -linked NANA being a prerequisite for tumour recognition. Schauer (1985) proposed partial loss of NANA from the surface of cells, or molecules, as one of the causes of autoimmune disease. Sarna *et al* (1988) successfully used Cis-dichlorodiammine platinum 2 (cisplatin) in the treatment of mice with syngeneic transplantable fibrosarcoma. Cisplatin indirectly stimulates the immune response by increasing the antigenicity of tumour cells, thereby increasing their susceptibility to phagocytosis. Since incubation of tumour cells, with cisplatin, resulted in release of NANA, it was concluded that NANA plays a major role in the regulation of antigenic expression. Finally, Benoist *et al* (1988) demonstrated the importance of NANA in masking natural killer (NK) target recognition structures, whereby neuraminidase treatment of cells resulted in enhanced susceptibility to NK-mediated lysis. However, while the immunostimulatory effects of neuraminidase-treated cells and their role in rejection of established tumours is well authenticated, there has been some controversy as to whether neuraminidase affects transplantability. Smyth *et al*. (1977) investigated the effects of neuraminidase treatment on the transplantability of LAT cells (using 3 different mouse strains). While neuraminidase treatment reduced tumour transplantability at certain inoculum levels, no effect was observed, in any of the mouse strains, when greater than 10^6 LAT cells were inoculated. Nevertheless, in 2 of the 3 mouse strains tested, neuraminidase treatment was found to significantly reduce tumour transplantability when inoculum levels

of less than 10^6 cells were used, thereby implying that NANA removal resulted in increased LAT immunogenicity. Furthermore, the failure of NANA removal to inhibit LAT growth, on injecting large numbers of tumour cells, may have been due to the inability of the murine immune system to cope with the large tumour inoculum.

1 3

METHODS FOR DETERMINATION OF TOTAL NANA, PROTEIN-BOUND NANA & LSA

One of the earliest, and subsequently most commonly used, methods of NANA determination is the colorimetric thiobarbituric acid (TBA) assay of Warren (1959). The basis of this assay is the periodate oxidation of NANA to form β -formylpyruvate, following liberation of bound NANA using acid hydrolysis (by cleavage of O-ketosidic linkages), with chromophore formation and extraction using TBA and cyclohexanone, respectively. The major problem with this assay is interference from 2-deoxyribose (2-DOR), a constituent of DNA. Aminoff (1961) then developed an alternative TBA method for NANA determination. NANA was cleaved from sialoglycoconjugates using either acid hydrolysis or the enzyme neuraminidase. Deviations from the Warren assay included faster periodate oxidation at 37°C (as opposed to room temperature) and the replacing of cyclohexanone by acidified butan-1-ol (for chromophore extraction). Aminoff claimed that this modified TBA assay had greater sensitivity and also was less prone to interference when dealing with complex biological systems. Despite these claims, the method of choice for

colorimetric determination of NANA over the past 30 years has been the Warren TBA assay. However, the problem of interference by the ubiquitous 2-DOR persisted. Roboz *et al* (1981) developed a modified TBA assay whereby it was claimed that interference by 2-DOR was eliminated by pH-dependent extraction with cyclohexanone. The procedure involved prior removal of the 2-DOR chromophore by cyclohexanone extraction at pH 5.6 - 6.0. After readjusting the pH to 1.7 - 1.9, the NANA chromophore was then quantitatively extracted, again using cyclohexanone. Svennerholm & Svennerholm (1958) devised a quantitative method of NANA analysis using paper partition chromatography, incorporating a solvent system of n-butanol/n-propanol/0.1N HCl (1:2:1, v/v). After identification of NANA bands, using either the resorcinol or Ehrlich's reagent, followed by further extraction, NANA was analysed by the resorcinol reaction. This method of analysis, with an accuracy of $\pm 3\%$, was not extensively used, one of the reasons being the length of time required to perform the assay (the paper chromatography separation step alone required a minimum of 18 hours).

In more recent times, the development of sophisticated analytical techniques has resulted in a wide range of highly sensitive and highly specific methods for determination of NANA. Hammond & Papermaster (1975) developed a fluorometric microassay based on fluorescence of the TBA reaction product of periodate-oxidised NANA, using excitation and analysing emission wavelengths of 550 nm and 570 nm, respectively. This method, with its 500-fold increase in sensitivity over previously described colorimetric procedures, could detect picomolar (10^{-12} M) levels of NANA. Interference by 2-DOR was

also minimised since its major emission band is narrow and centred at 550 nm

High performance liquid chromatography (HPLC) has also been employed for NANA quantitation. Silver et al (1981) determined NANA levels using a rapid isocratic HPLC method, with separation achieved using an Aminex HPX-87 cation-exchange resin. This method is much more reliable than the conventional TBA assay and has a significantly improved maximum sensitivity of 0.8 nmol/ml. The rapid improvement in the analytical ability of HPLC, when applied to quantitation of NANA, was demonstrated when Shukla et al (1986) developed a method whereby it was possible, following separation on a strong anion-exchange resin, to determine a minimum amount of 0.6 pmol (200 pg) NANA. The low limit of detection allowed direct determination of NANA, thereby eliminating prior sample purification or concentration. An additional advantage was the time required for analysis (approximately 10 minutes per sample). The authors stated that this HPLC method is not subject to problems encountered when using colorimetric assays, whereby results (from the latter) may be influenced by saponification and migration of the O-acetyl groups due to the time and chemical conditions required for analysis.

Analysis of NANA was further improved by the introduction of chromatographic techniques, particularly gas chromatography (GC), coupled with mass spectrometry (MS). Sugawara et al (1983) determined NANA levels by GC-MS, using selected ion-monitoring technique, with a stable isotope (N-[²H₃]-ANA) as an internal standard. This highly specific and highly quantitative method of analysis, with its low limit of detection (several hundred

pg), has additional advantages of minute sample requirement (nL quantities), simplicity and speed of operation, with very accurate and reproducible measurements Sugiyama *et al* (1988) modified the method of Sugawara to determine levels of free NANA in human serum, using capillary GC-MS with selective ion monitoring and specific separation on a Sep Pak silica cartridge Using this method, the measurements were again specific, accurate and reproducible, with the mass fragmentogram of serum showing a good signal to noise ratio (indicative of low background interference) This method could also be used to determine free NANA levels in seminal fluid, cerebrospinal fluid, ascites and urine, thereby presenting a powerful analytical technique for routine clinical monitoring of free NANA levels in patients with disease (especially cancer) However, the use of these sophisticated analytical techniques, as routine methods of NANA determination, may be limited by the high cost of required instrumentation

Research, particularly over the past five years, has resulted in the production of a range of enzymatic assays for NANA determination, many of which are available commercially. Boehringer Mannheim have produced one such enzymatic kit This method incorporates a cascade reaction system, involving the enzymes neuraminidase, NANA-aldolase and pyruvate oxidase, ultimately resulting in the formation of hydrogen peroxidase (H_2O_2), the amount of the latter being equivalent to NANA content H_2O_2 is then converted to a dye (using peroxidase) and determined colorimetrically at 550 nm The main disadvantage of this method is that one of the series of reactions results in the production of pyruvic acid, a naturally occurring constituent of body fluids

For example, the concentration of pyruvate in serum, usually between 6 and 12 mg/L (Ogasahara *et al* , 1985), is equivalent to between 21 and 42 mg NANA/L, respectively, thereby resulting in a potentially significant source of interference in the assay. This particular method is also subject to interference from ascorbic acid (Vitamin C). Chien *et al* (1991) developed an enzyme system for NANA determination in serum and urine, involving the use of two immobilised enzyme pairs which were separately immobilised onto 1,12-diamino-dodecane agarose (a substance with high coupling capacity). While this method has the advantages of enhanced enzyme activity, reagent stability and overall operational stability, it is also subject to interference from pyruvate as a direct result of incorporating a pyruvate-forming step in its sequence of reactions. Ironically, a superior enzymatic assay for determination of NANA in serum was developed somewhat previously, by Teshima *et al* (1988). The principal advantage of this method was the removal of the pyruvate forming reaction with concomitant elimination of its interference. Additional advantages of this method included a highly sensitive endpoint, excellent linearity of the dilution curve (extending to approximately 3000 mg NANA/L) and elimination of ascorbic acid interference (by addition of ascorbate oxidase).

Protein-Bound NANA & LSA

A method utilising the acidic ninhydrin reaction, specifically designed to determine protein-bound NANA, was developed by Yao *et al* (1989). This simple direct method requires mixing of the sample solution with glacial acetic acid and acid ninhydrin.

reagent (2.5%, w/v, ninhydrin in acetic acid/HCl, 60:40, v/v). Following heating in a boiling waterbath (10 minutes) and rapid cooling, the O D s were recorded at 470 nm. Protein-bound NANA levels were determined from a standard curve (constructed using fetuin, a sialoglycoprotein), linear in the range 20 µg to 3 mg. The rapidity of this method makes it ideal when many samples are to be analysed simultaneously. A method used to determine LSA levels was developed by Katopodis & Stock (1980) and later modified (Katopodis & Stock, 1982). It involved ganglioside extraction using cold chloroform/methanol (2:1, v/v), followed by precipitation with phosphotungstic acid (1g/ml). After chromophore development, and extraction with butyl acetate/n-butanol, 85:15 (v/v), O D s were recorded at 580 nm. LSA levels were determined from a standard curve developed from a standard sample of NANA. Unquestionably, the procedure of Katopodis & Stock has been the method most extensively used for the determination of LSA levels in both serum and plasma. However, in recent years this method has been the subject of vociferous criticism. The main criticism of the method has been that the recommended extraction procedure results in extraction, not only of gangliosides, but also significant amounts of protein-bound NANA. Because of the discrepancy between amounts of extracted LSA (using the method of Katopodis & Stock) and the content of pure serum gangliosides, Voigtmann *et al* (1989) analysed the composition of extracted LSA, using immunochemical techniques. The "LSA" fraction was found to contain considerable quantities of sialoglycoproteins, including acid- α -1-glycoprotein, anti-trypsin, haptoglobin and anti-chymotrypsin. Furthermore, a strong correlation existed between elevated LSA levels and the content of acid- α -1-

glycoprotein in the sera of patients with malignancy. On the basis of their results, the authors suggested that the term LSA, as applied to the method of Katopodis and Stock, is misleading, since the major contributor to LSA elevations is, in fact, a sialoglycoprotein. Similarly, Berra et al (1986) stated that the procedure of Katopodis & Stock results in incomplete extraction of LSA from serum, followed by an overestimation of this fraction in the pellet, after phosphotungstic acid precipitation, because of the presence of glycopeptides (many containing NANA). Consequently, a modified procedure was developed, whereby LSA, extracted with chloroform/methanol (2:1, v/v), was partitioned in di-isopropylether, 1-butanol and 50mM NaCl (6:4:5, v/v), thereby facilitating improved separation of LSA and sialoglycopeptides. Tautu et al (1989), also critical of the aforementioned method, developed an alternative procedure for determination of serum LSA, which allegedly extracted all LSA while excluding sialoglycoproteins. This method involves prior removal of proteins and glycoproteins (including sialoglycoproteins) by precipitation (chloroform/methanol, 1:2 v/v) and centrifugation. The supernatants, containing LSA, are then dried under vacuum. Following resuspension in distilled water, LSA levels are determined using a modified method of the periodate-resorcinol procedure (Jourdain et al., 1971). Hess and Rolde (1964) devised a microfluorometric assay for the determination of LSA in human brain tissue, based on the reaction of LSA with 3,5-di-aminobenzoic acid (DABA) in hot dilute HCl (yielding a product with intense green fluorescence). Excitation and emission wavelengths were 405 nm and 510 nm, respectively. The use of these wavelengths

minimised interference from contaminating fluorochromes, with the main source of interference being hexoses (which gave 1.6% as much fluorescence as sialic acid on a molar basis) LSA levels were determined from a standard curve, linear over the range 0-5 nmoles LSA/100 μ l DABA

1.4

STRUCTURAL STUDIES AND CHARACTERISATION OF NANA

In addition to NANA determination, analytical techniques have also been employed in the study of NANA metabolism, with additional applications including identification and characterisation, thereby providing invaluable detailed analytical and clinical information concerning this important biomarker. For example, Reuter *et al* (1980) were the first to present unambiguous and unequivocal evidence, using GC-MS for structural analysis, that 9-O-acetyl-NANA is the almost exclusive NANA of Balb/c mouse erythrocytes. In a separate investigation, Reuter *et al* (1980), again using GC-MS for structural elucidation, also identified a new NANA (4-O-acetyl-9-O-lactyl-NANA) present in horse submandibular gland glycoproteins. Beau *et al* (1980) used thin-layer chromatography (TLC) to study the metabolism of 4-O-methyl-NANA (a synthetic NANA derivative containing a stable methoxyl substituent at C-4). Their results confirmed that substitution of the hydroxyl group at C-4 of NANA strongly hinders the degradation of sialoglycoconjugates, thereby enabling study of the biological influence of such modified NANAs when bound to glycoconjugates or cells. Hutchins *et al* (1988) demonstrated the presence of mono-, di- and tri-O-acetylated NANAs on human colon cells using radiochromatographic and

chemical techniques (including ion-exchange chromatography, paper chromatography and fast-atom bombardment mass spectrometry) Their investigation showed that aberrant expression of O-acetylated-NANAs was associated with adenocarcinoma of the colon, resulting in almost complete loss of di- and tri-O-acetylated NANAs It was suggested that the loss of O-acetylation in NANA molecules expressed on human colon cells could be used as a sensitive indicator of early malignancy change, thereby enabling a possible screening method for cancer of the colon

Monoclonal antibodies have also been used in characterisation studies of NANA . Their unique degree of target specificity enables distinction between closely related antigens, where the difference between antigenic epitopes may involve a single sugar residue or the type of sugar linkage Miyake et al (1988) generated two murine monoclonal antibodies against GM₂ gangliosides associated with lung carcinoma in order to identify the molecular species of the glycolipid Using these antibodies (MK2-34 and MK1-16, directed against NGNA and NANA, respectively), it was shown that significant amounts of NANA occurred in several types of lung cancer, whereas NGNA was not found in any glycolipid fractions from the same origins It was also found that the appearance of NANA on the GM₂ ganglioside correlated well with the degree of differentiation of the cancer cells in patients with squamous cell carcinoma and adeno-carcinoma of the lung Kannagi et al (1988), who performed both quantitative and qualitative characterisation of human cancer-associated serum glycoproteins (expressing closely related "sialoepitopes"), showed that subtle differences in the linkage of NANA residues (to

proteins) greatly affect the cancer specificity of carbohydrate antigens Cheresch *et al* (1984), also using a monoclonal antibody (01 1), were able to demonstrate the presence of an O-acylated NANA on human melanoma-associated gangliosides, occurring possibly as a result of aberrant O-acetylation caused by the malignancy

1 5

MECHANISMS SUGGESTING A ROLE FOR SIALIC ACID AS A TUMOUR MARKER

As previously stated (Section 1 1), NANA is mainly located at the cell surface (Warren, 1976). NANA aroused the interest of oncologists when it was found that levels were significantly elevated on the surfaces of many tumour cells (Warren *et al* , 1975, Van Beek *et al* , 1975, Yogeewaran *et al* , 1978) Black *et al* (1983) stated that, in tumour cells, shedding of highly sialylated glyconconjugates occurs continuously, into body fluids (such as blood or urine), in a disregulated way, with the turnover of peripheral sugars (including NANA) being significantly higher than that of core structures This finding implied a possible role for NANA as a marker for presence of tumour Feizi (1984), using monoclonal antibodies, revealed saccharide structures (including NANA residues) of glycoproteins and glycolipids as differentiation and tumour-associated antigens. Singhai and Hakomori (1990), in their review of molecular change in carbohydrate antigens associated with cancer, stated the abnormalities in the profiles of cell surface carbohydrates have been found in essentially all human cancers and that these include significant

conformational changes at the cell surface caused by the addition of NANA residues. The following results, from clinical research, provide direct evidence that increased surface NANA levels, of tumour cells, are due to altered rates of NANA biosynthesis and/or altered activity of the enzyme sialyltransferase (which attaches NANA to endogenous membrane receptors). Evans et al (1980) demonstrated that both serum and liver sialyltransferase activity increased dramatically after implantation of the R3230 AC rat mammary tumour. It was also shown that, when growth of the primary tumour was controlled by chemotherapy, no elevation in enzyme activity was detected, suggesting that size, or growth rate, of the tumour may be a determining factor in alterations of sialyltransferase activity. Dobrossy et al (1981), in their study of murine B16 melanoma, found that the degree of lung implantation (one of the steps in the metastatic process) correlated with the amount of tumour cell surface NANA accessible to neuraminidase and also tumour cell surface sialyltransferase (ectosialyltransferase) activity. Ivanov et al (1985), using [¹⁴C]-N-acetyl-mannosamine as the precursor of NANA, found that the biosynthesis rate of NANA was higher in chicken hepatoma (Mc-29) than in normal liver cells. Hakomori (1985) stated that aberrant glycosylation (including sialylation) of glycolipids is a ubiquitous property of cancer cell membranes. In a study of murine colon adenocarcinoma (Kijima-Suda et al, 1986), the metastatic potential of various sublines was found to correlate with neuraminidase-susceptible (surface) NANA and also sialyltransferase activity. Baker et al (1987), who studied the granulocytes of patients with chronic myelogenous leukemia (CML), found that

elevated activity of a specific sialyltransferase (2.8 times higher than that of normal cells) was responsible for the aberrant sialylation of O-linked membrane oligosaccharides in CML cells, suggesting that the resulting increase in sialylation could contribute to the pathophysiological behaviour of CML granulocytes

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TOTAL NANA AS A MARKER FOR TUMOUR BURDEN

The use of total NANA as an indicator of tumour burden has been a contentious issue for several decades, with numerous claims and counterclaims concerning its diagnostic and prognostic potential, as applied to oncology. Ryan *et al* (1980), who evaluated total serum NANA (using the Warren assay) and carcinoembryonic antigen (CEA, a glycoprotein of mol wt approx 200,000 containing 40 - 50% carbohydrate, Bates and Longo, 1987) as markers for human breast cancer, found significantly elevated NANA levels in cancer patients which correlated with tumour stage. CEA was found to be an unsuitable marker since, in all stages of disease, a lower proportion of patients had elevated CEA levels. Silver *et al* (1981) evaluated serum NANA and CEA as markers for carcinoma of the ovary, a disease difficult to clinically diagnose because of its diffuse intraperitoneal location. Whereas both markers were found to lack the specificity required for diagnosis, NANA was found to be a better indicator of tumour burden in patients with established disease, whereby NANA levels increased with increased tumour presence. Furthermore, in the majority of patients with progressing disease,

significant NANA elevations predated other clinical signs of increasing tumour burden by between 1 and 21 weeks, thereby implying a possible role for total NANA as a potentially valuable prognostic indicator for ovarian cancer. In their study of the total NANA content of both cancerous and healthy human liver tissue, Alhadeff and Holzinger (1982) found that NANA levels were increased (1.4 - 7.2 fold) in cases of metastacy when compared to their non-malignant counterparts. Silver et al (1983) employed a proportional hazards model to examine nine factors, including total serum NANA, as possible predictors of malignant melanoma recurrence. In this capacity, 5 of the 9 factors (age, sex, primary site, tumour diameter and tumour stage) were found to be unsuitable. Of the four factors giving positive results, only 2 were independent (measured tumour depth and total serum NANA). The combined use of these markers, as opposed to each one separately, greatly increased predictive ability of recurrence, whereby patients with lesions greater than 1.75 mm and total NANA greater than 2 $\mu\text{mol/ml}$ were found to have a 12-fold greater risk of recurrence, within 2 years, than those with lesions less than 1.75 mm and total NANA below 2 $\mu\text{mol/ml}$. Colli et al (1989) evaluated the diagnostic accuracy of total NANA in the diagnosis of malignant ascites. It was found that NANA could be regarded as an accurate marker for neoplastic involvement of the peritoneum, in that, a statistically significant difference ($P < 0.001$) existed, in NANA levels, between malignant and non-malignant ascites. The sensitivity, specificity and overall diagnostic accuracy of the test were found to be 70%, 90% and 82%, respectively.

Neoplastic transformation is not always accompanied by elevations in total NANA Ohta *et al* (1968) determined total NANA levels of normal and malignant cells, using the Warren assay (following liberation by either treatment with neuraminidase or acid hydrolysis) All the virally transformed cell lines were found to have slightly lower amounts of NANA than their respective normal counterparts. McClelland and Bridges (1973) determined the total NANA content of normal and lymphatic leukaemic lymphocytes (human origin) A highly statistically significant difference ($P < 0.001$) was found between the average total NANA content of the two groups, whereby normal levels were approximately ten times greater than those obtained for cancerous lymphocytes from chronic lymphatic leukaemic (CLL), with mean levels being $0.33 \mu\text{g}/10^6$ cells and $0.04 \mu\text{g}/10^6$ cells, respectively Furthermore, decreased lymphocyte total NANA levels, of the same magnitude as that observed in CLL patients, were also found in cases of acute lymphatic leukaemia (ALL) In a similar study, O'Kennedy *et al* (1982), who found a 60% reduction in the total NANA content of CLL cells (relative to normal peripheral lymphocytes) and also a diminution in surface (neuraminidase - susceptible) NANA, suggested that the observed reduction in NANA levels was probably due to the B cell nature of CLL cells

Other research has indicated that total NANA is unsuitable for use as an indicator of tumour burden. Hoon *et al* (1985) performed both *in vivo* and *in vitro* experiments, using a range of cell lines, to determine whether the release of bound NANA was related to mammary tumour metastasis The NANA was considered to be in bound form, since acid hydrolysis was required to release it for HPLC

analysis From in vivo experiments, where animals were injected with $1 - 5 \times 10^6$ cells, serum NANA levels were found to increase with tumour burden up to day 10, after which time, despite further tumour growth, levels began to plateau In vitro studies confirmed the findings of the animal experiments, namely, that the phenotypic expression of bound NANA shedding did not correlate well with the metastatic potential of the mammary tumour lines, suggesting that while NANA could possibly be used as a marker for the presence of tumour, it was an unsuitable indicator of tumour-burden Moran et al (1985) also suggested that the extent of tumour burden may not necessarily be reflected in total NANA levels A modified Warren assay was used to determine total serum NANA levels in patients with various types of malignant disease While statistically significant NANA elevations occurred in cases of CLL, no such elevations were found in serum NANA of patients with either primary or secondary breast cancer O'Kennedy et al (1991), in their comprehensive evaluation of NANA determinations in the diagnosis of malignancy, found that extent of tumour burden was not always reflected in total serum NANA levels, thereby limiting its application as a tumour marker

A commonly expressed reservation concerning the use of total NANA as a tumour marker arises as a result of the fact that, unlike lipid-bound NANA, significant elevations in total NANA occur in many non tumour-associated disorders, thereby implying NANA to be an acute-phase reactant (the latter being a substance which responds to various types of injury and inflammatory conditions by an increase in the rate of synthesis) This observation casts doubts on the clinical reliability of NANA determinations, since stimulators of an acute-phase

reaction include surgery, trauma and bacterial infection, the latter condition often associated with cancerous disease as a result of the impaired capability of immune system mechanisms in cancer patients. For example, Kiersnowska - Rogowska et al. (1978), who found elevated serum NANA levels in patients with acute micromyeloblastic leukaemias, readily admitted that the observed increases may have been partly due to accompanying inflammatory processes (caused by increased susceptibility to infection). Silver et al. (1979) found that total serum NANA was higher among melanoma patients than among aged and sex-matched normal controls, with levels tending to increase with tumour burden. However, when compared to normal controls, highly significant ($P < 0.001$) NANA elevations were also observed in patients with active rheumatoid arthritis. It was suggested that at least part of the observed increase in serum NANA levels was due to contributions from non-specific acute-phase reactants such as α_1 -acid-glycoprotein. Plucinsky et al. (1986) found significantly ($P < 0.001$) elevated NANA levels in patients with gastrointestinal and breast cancer, and also carcinoma of the pancreas and cholangiocarcinoma. While the sensitivity of the test was adequate to allow the use of NANA as a monitor of tumour burden (especially in cases of severe malignancy), the specificity was found to be lacking, since levels were also significantly elevated ($P < 0.01 - P < 0.001$) in non-tumour pathological controls, including intestinal, breast and regional enteritis. Shamberger (1984) also reported elevated total serum NANA levels in Crohn's disease (a chronic non-cancerous intestinal disorder) and psoriasis. Stefenelli et al. (1985) evaluated total serum NANA levels in patients with malignant tumours, bacterial

infections and chronic liver diseases From this extensive study, involving over 2,000 patients, total NANA levels were found to be of limited value as a diagnostic test for malignant neoplasms Inflammatory diseases (including tuberculosis) stimulated elevated NANA levels to such an extent that evaluation of simultaneously existing neoplastic processes was prevented Therefore, a prerequisite for accurate interpretation of test results, in the diagnosis, prognosis and follow-up of malignant tumours, is the ability to ensure the exclusion of bacterial infections or other acute inflammatory disorders (including tuberculosis and rheumatoid arthritis) as the cause of elevated NANA

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PROTEIN-BOUND NANA AS A TUMOUR MARKER

Protein-bound NANA (protein associated NANA, PA-NANA) has also been investigated as a possible tumour marker Macbeth and Bekesi (1962) evaluated plasma levels of several protein-bound carbohydrates, including hexoses (galactose and mannose), hexosamines (glucosamine and galactosamine), fucose and PA-NANA in patients with malignant and non-malignant disease. PA-NANA was found to be the most useful marker with significant plasma elevations occurring in all cases of malignant disease. Warren *et al.* (1975), who compared glycopeptides derived from surface and internal membrane glycoproteins of a murine melanoma with those of normal tissues (using double-label elution patterns from Sephadex G-50 columns), found a marked increase in NANA-rich, fucose-containing glycopeptides of melanotic cells These increases

in PA-NANA occurred both in malignant cells grown in vitro and in solid melanotic tumours in vivo. The contribution of PA-NANA to tumour glycoproteins was further verified by the significant reduction in size (of tumour glycopeptides) after treatment with neuraminidase. Van Beek et al (1975) performed molecular exclusion chromatographic analysis of tumour cell surface glycopeptides isolated from peripheral blood of patients with either active leukaemia or leukaemic transformation of lymphosarcoma. Whereas untreated tumour glycopeptides eluted ahead of the controls (normal peripheral blood lymphocytes), neuraminidase treatment (prior to chromatography) eliminated the difference between the elution profiles of normal and tumour samples, thereby indicating that the structural difference found in the fucose-containing glycopeptides of tumour cells could be attributed to an increased PA-NANA content. Van Beek et al (1973), again using molecular exclusion chromatography to compare a range of malignant cells with their normal counterparts (including lymphoblast baby hamster kidney cells and mouse fibroblasts), also found that neuraminidase treatment resulted in coinciding elution profiles, further confirming that the aberrant elution pattern of tumour cells was due to increased surface PA-NANA content. Bernaki and Kim (1977) found elevated serum PA-NANA levels in rats with spontaneously metastasizing mammary tumours, with no elevation occurring in non-malignant disease. Yogeewaran et al (1978) investigated the cell surface organisation of sialoglycoconjugates of mouse melanoma variant lines with either a high (F10) or low (F1) degree of lung implantation. F10 cells in culture showed an increase of 16.5%, in surface-exposed (neuraminidase susceptible) PA-NANA. Furthermore, studies of

short-term cultured in vivo tumour cells showed a quantitative increase in at least 4 major surface sialoglycoproteins in F10 cells relative to F1 cells. Therefore, these results suggested that highly tumourigenic and metastatic mouse melanoma cells are enriched with highly sialylated glycoproteins. Arakawa et al. (1989) using HPLC, determined the ratio of PA-NANA to PA-NGNA in glycopeptides isolated from rat colonic tumour and normal mucosa. The results showed that PA-NANA/PA-NGNA ratios were significantly elevated (approximately 55 fold) in cases of colonic tumour, with these elevations caused, to a large extent, by PA-NANA levels in tumour glycopeptides.

However, as in the case of total NANA, the clinical potential of PA-NANA, as a tumour marker, may be limited. Carroll (1988) stated that increased sialylation of membrane proteins is not unique to tumour cells. Rothbard et al. (1982) found that embryonic neural cellular adhesion molecules also contain large amounts of PA-NANA, in direct contrast to the adult forms of these proteins. Furthermore, other research has shown that elevated levels of protein-bound carbohydrates occur in cases of active metabolic and acute inflammatory diseases. For example, Waalkes et al. (1978) suggested, despite a 95% incidence of elevated serum PA-NANA levels in patients with metastatic breast cancer, that in relation to tumour burden, the guaranteed absence of other types of non-malignant disease is a prerequisite for accurate clinical interpretation of PA-NANA levels.

LSA AS A MARKER OF TUMOUR BURDEN

In more recent times, the emphasis has shifted from measuring total levels of NANA to determining the levels of the lipid-bound sub-fraction of NANA. Extensive research has been (and continues to be) carried out to evaluate lipid-bound sialic acid (LSA) as an indicator of tumour burden. Commonly used abbreviations for this marker include the afore-mentioned LSA, LASA (lipid-associated sialic acid, P-LASA and LASA-P (the final 2 abbreviations referring specifically to plasma levels of this marker) LSA levels are also referred to as levels of "gangliosides" (gangliosides being glycolipids containing NANA) The method most commonly used to determine serum or plasma levels is that of Katopodis and Stock (1980) and, unless otherwise indicated, all LSA determinations referred to in this section were obtained by this method

The evaluation of serum and plasma NANA as a tumour marker has encompassed a broad spectrum of cancers Horan (1982), who studied both total serum NANA and serum LSA levels in a range of malignancies (nature of malignancies not specified), found LSA to be a superior marker for the presence of disease (in terms of sensitivity and specificity) Elevations in serum LSA levels were found in cases of mild neoplastic disease (Stage 1), whereas no change occurred in total NANA. It was also found that overlapping occurred when total NANA levels of cancer-bearing and non-malignant disease patients were compared. However, on comparing serum LSA levels, for the same groups, no such overlapping occurred Musset *et al* (1986), in their study of advanced prostatic cancer, compared the tumour

marker potential of serum LSA with two other markers, CEA and PAP (prostatic acid phosphatase) The sensitivity of LSA was found to be 94.2%, whereas those of PAP and CEA were only 36.5% and 25.0%, respectively, thereby indicating that serum LSA may be a useful marker for malignancy Echenique et al (1988) evaluated serum LSA and CA-19-9 (a sialylated oligosaccharide found on cell surface glycoproteins) as potential tumour markers for renal cell carcinoma Whereas CA-19-9 was found to be unsuitable (all patients tested had values below the upper limit of normal), serum LSA was found to be a suitable marker for the presence of residual or metastatic disease, with a test sensitivity of 70% and a specificity of 82% However, poor correlation was found between serum LSA levels and staging of the disease, whereby no statistically significant differences were found when LSA levels of patients with varying stages of renal cell carcinoma were compared Mack et al (1985), using an animal model system, evaluated total serum NANA and serum LSA as markers for malignancies of the liver, colon and breast (chemically induced) Whereas total serum NANA was found to be an unreliable marker of tumour burden for all 3 malignancies investigated, very good correlation was found between serum LSA levels and occurrence of tumour Furthermore, LSA was found to be a very good marker for early stages of hepatic tumour Santamaria et al (1987) studied serum LSA as a means of early diagnosis and clinical monitoring of laryngeal cancers All incidences of cancer, including cases of small tumour burden, showed a positive correlation with elevated levels of LSA, with 100% specificity and 96% sensitivity The diagnostic potential of this tumour marker was reaffirmed in that, where clinical examination

suggested metastases, normal LSA levels would often predict negative results from histological tests Hirshaut et al (1985) found that plasma LSA levels reflected the clinical status of patients suffering from Hodgkin's disease Sawitsky et al (1985) found positive correlation between plasma LSA levels and tumour activity in patients with CLL, with increases in LSA levels often preceding clinically apparent progression of disease by 4 - 6 weeks

The potential use of LSA as a marker for gynaecological neoplasms has also been extensively studied Schwartz et al (1985, 1986 & 1987), who evaluated serum LSA as a marker for cancers of the ovary, cervix and uterus, found positive correlation between LSA levels and the clinical course of these diseases, and furthermore, LSA could be used to detect disease recurrence prior to surgical or clinical realisation Tseng et al (1989), in their study of uterine papillary serous carcinoma, found correlation between plasma LSA levels and extent of disease, with increasing LSA levels indicating disease progression and reductions in plasma levels indicating a more favourable prognosis (disease regression) Ovarian cancer is a disease which eventually develops in one out of every 70 newborn females (Cutler & Young, 1975) Patsner et al (1987) found that plasma LSA performed as well as CA-125 (an antigenic high molecular weight glycoprotein expressed by over 80% of non-mucinous ovarian adenocarcinomas) in terms of sensitivity, specificity and reflection of tumour burden It was also found that plasma LSA could be used to monitor patients suffering from invasive cancer of the ovary, as well as being able to predict the outcome of second look surgery In a similar study of advanced ovarian cancer, Vardi et al.(1989) also

found that serial determination of plasma LSA levels could be used as an additional clinical parameter to monitor the course of disease during treatment. Another finding was that, in many cases, rising LSA levels were the only sign of disease recurrence (prior to surgical investigation). However, in marked contrast to the positive findings of Vardi and co-workers, Stratton et al (1988) found serum LSA to be an unsuitable marker for gynecological malignancies. Whereas the specificity of serum LSA was very high, with few false positive reactions, the sensitivity was found to be lacking, in that the test failed to identify the majority of tumour-bearing patients. LSA levels reflected disease status in only 6 of 45 patients, representing a test sensitivity of 13%.

LSA has also been studied in relation to cancer of the breast, a disease which can be successfully treated if detected in the early stages, but has a low cure rate in cases of metastacy (Brinkley & Haybittle, 1984). In their study of breast cancer, Dnistrian et al (1982) suggested that while serial determinations of serum LSA could be of use as a means of assessing established disease progression, isolated LSA determinations lacked the sensitivity required for use as a method of screening, either of initial stages of the disease or early stages of a recurrence. The inability of LSA to distinguish between non-malignant and malignant disease, in certain cases, suggested that LSA also lacked the specificity required for use as a tumour marker. Rae et al (1987) also found serum LSA to be lacking in sensitivity when used as a marker for carcinoma of the breast. Whereas the magnitude of LSA elevations increased with tumour stage, the high degree of false negative results indicated that

normal levels of LSA could not be used to exclude carcinoma. However, LSA was found to be useful when used in conjunction with another clinical parameters for this particular cancer, whereby women with breast lumps and elevated serum LSA levels were found to be at very high risk for breast carcinoma, with the recommended action in such cases being immediate biopsy.

Bone marrow is a selective site for metastasis of many epithelial carcinomas, especially breast cancer. For example, Ginsbourg et al (1986) determined plasma LSA levels and the presence of heterotopic cells in the bone marrow using a monoclonal antibody-based assay. They found a strong statistical correlation ($P < 0.005$) between these distinct markers, thereby reaffirming a clinical role for LSA as a marker for metastatic breast cancer.

In addition to the positive findings implying a clinical role for both serum and plasma LSA as an autonomous tumour marker, other research has shown that diagnostic and prognostic accuracy can be increased when LSA is used in conjunction with other established tumour markers. Studies by Vardi et al (1989), who measured the levels of plasma LSA and serum CA-125 in patients with advanced ovarian cancer, showed that the ability to predict the clinical status of patients' was enhanced by combining the results obtained when both markers were determined. All patients who showed significantly elevated levels of both markers were subsequently found to have surgical evidence of disease. Furthermore, the predictive value for negative results (i.e. a normal level of tumour marker predicting absence of disease) also

increased, from 80% for plasma LSA alone, and 55% for CA-125 alone, to 89% for both markers combined

The use of LSA (in serum or plasma), as opposed to other tumour markers, was found to have additional advantages which suggested a greater clinical significance for LSA as an aid in the diagnosis and prognosis of neoplastic disease. Santamaria et al (1987) found that, unlike CEA, serum LSA levels were not correlated to age, sex, menstrual cycle, smoking habits or body weight. Katopodis (unpublished data) also found that LSA levels did not correlate with sex, age, stage of menstrual cycle or menopausal status. It was also found that, unlike total NANA or protein-bound NANA, LSA levels were in the normal range in patients with inflammatory disease such as rheumatoid arthritis (Katopodis and Stock, 1980), thereby indicating that LSA does not behave as an acute phase reactant.

It is therefore ironic that the major reservation concerning the use of LSA as a marker of tumour burden arises as a result of the problems (discussed in Section 1.3) associated with the principle method of LSA determination, namely, that of Katopodis & Stock.

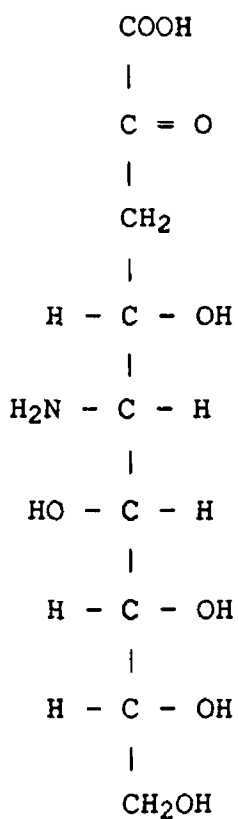


Figure 1 Structure of Neuraminic Acid

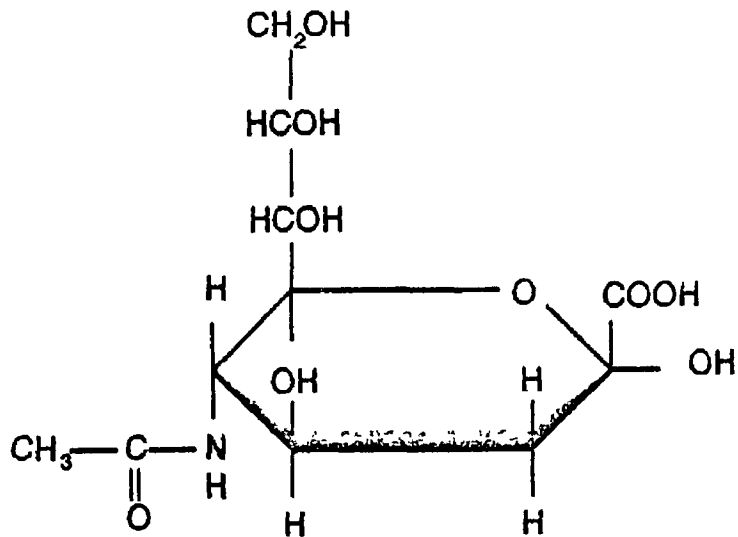


Figure 2 Structure of N-Acetylneuraminic Acid (NANA)

SECTION 2

Materials and Methods

2.1

MATERIALS FOR NANA EXPERIMENTS

2.1.1

REAGENTS AND CHEMICALS

NANA standard (type 8, 98% pure, from sheep submaxillary gland) and Pristane were purchased from Sigma Chemical Co., Poole, Dorset, England. Sodium arsenite (general purpose reagent, GPR), sodium periodate (analar), thiobarbituric acid (GPR), orthophosphoric acid (analar) and sodium sulphate (GPR) were purchased from BDH Chemicals, Poole, Dorset, England. Phosphotungstic acid, hydrochloric acid, sulphuric acid, chloroform, methanol (all reagents GPR) and cyclohexanone (pure) were purchased from Riedel-de Haen, Aktiengesellschaft, Wunstorfer Str. 40, D-3016 Seelze 1, Hanover, Germany. Ethanol (analar) was purchased from James Burrough, Fine Alcohols Division, 60 Montford Place, London SE11 5DF, England. PBS tablets, pH 7.3, were purchased from Oxoid Limited, Basingstoke, Hampshire, England. An enzymatic kit (Cat. No. 784192) for NANA determination was purchased from Boehringer Mannheim, Diagnostics and Biochemicals Ltd., Bell Lane, Lewes, East Sussex BN7 1LG, England.

2.1.2

LAT CELLS

LAT cells were obtained from Dr. Honor Smyth, Department of Biochemistry, University College, Dublin.

2 1.3

SAMPLES

Urine, serum and tissue samples from patients with either bladder or prostate cancer were obtained from Dr Aiden O'Brien, Department of Urology, The Meath Hospital, Heytesbury Street, Dublin. Human plasma samples (status of donors not specified) were obtained from St Vincent's Hospital and St. James's Hospitals, Dublin

2.2

MATERIALS FOR CELL CULTURE EXPERIMENTS

2.2 1

CELL LINES

The EJ human bladder cell line was obtained from Ms Mary Freshney, The Beatson Institute, Glasgow. All other cell lines were purchased from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U S.A.

2 2 2

ANIMALS

Balb/c and Schofield mice were obtained from Dublin City University

2 2 3

REAGENTS AND CELL CULTURE ACCESSORIES

All materials were purchased from Sigma Chemicals, Poole Dorset, England, unless otherwise indicated. All cell culture media and reagents were purchased from ICN-Flow, Irvine,

Scotland Culture flasks, plates and other disposable plastics were purchased from Sterilin Limited, Feltham, England. Sterile needles and syringes were purchased from Becton Dickinson, Dun Laoghaire, Co. Dublin. SDS was purchased from Aldrich Chemical Company, Gillingham, Dorset, England. Pipette tips were purchased from Monoject Scientific (Athy) and LIP (Galway). EDTA, TEMED, PEG (M.W. 1540), acetic acid, sodium carbonate, 2-Mercaptoethanol and bromophenol blue were purchased from Riedel-de Haen, Aktiengesellschaft, Wunstorfer Str. 40, D-3016 Seelze 1, Hanover, Germany. Sodium azide, TRIS, sucrose and ammonium persulphate were purchased from BDH Chemicals Ltd., Poole, Dorset, England.

2 3

SIALIC ACID METHODS

2.3.1

LAT CELLS

LAT cells were maintained by passage in Schofield mice as i.p. ascitic tumours. These tumours were established by i.p. injection of 1×10^7 (in 1.0ml PBS) into Schofield mice, aged between 12 and 15 weeks. After 7-10 days cells were harvested into 20mls of PBS and washed twice in PBS before use. Alternatively, LAT cells were stored in liquid nitrogen until required (Section 2.4.10).

2.3.2

TAIL BLEEDING OF MICE

At specified times blood samples were obtained

from mice by tail bleeding. A mouse was placed in a cage under a 300 watt bulb. This resulted in increased blood flow to the animal's extremities, including the tail. After five to eight minutes the mouse was transferred to a restrainer. A small incision was made in a major artery of the tail using a sterile scalpel blade. The blood was collected into a 1.5ml epindorph tube. Having obtained the sample (approx. 1.0ml) the tail was then swabbed in alcohol to prevent infection. Care was taken at every stage in this procedure to ensure that minimum distress was caused to the animal.

2.3.3

TREATMENT AND STORAGE OF SERUM SAMPLES

Blood samples from human patients or from mice, obtained by tail bleeding (Section 2 3.2), were allowed to clot at room temperature. Following centrifugation at 5000 r.p.m., for ten minutes, the serum was removed using a micropipette. 10ul aliquots were stored at -20°C , as recommended by O'Kennedy et al. (1991).

2.3.4

DETERMINATION OF TOTAL SERUM AND URINARY SIALIC ACID (NANA) LEVELS BY THE WARREN THIOBARBITURIC ACID ASSAY

The thiobarbituric acid method of Warren (1959) was used to determine total serum and urinary NANA levels.

Principle:

In the Warren colorimetric assay, NANA is first

subjected to periodate oxidation, resulting in the formation of β -formylpyruvic acid. This reacts with two molecules of thiobarbituric acid (TBA) to form a pink chromophore with maximum absorbance at 549nm. This chromophore is extracted into cyclohexanone, which results in greater colour stability and intensity.

2-Deoxyribose, a constituent of DNA, causes significant interference in this assay. To correct for this interference, Warren deduced a correction formula based on the extinction coefficients and involving an additional reading at 532nm:

umoles NANA per sample =

$$\text{O.D.}_{549\text{nm}} \times C_1 - \text{O.D.}_{532\text{nm}} \times C_2$$

The values for the constants (C_1 & C_2) were determined by the initial sample size:

| SAMPLE SIZE (ml) | C_1 | C_2 |
|------------------|-------|-------|
| 0.2 | 0.090 | 0.033 |
| 0.5 | 0.097 | 0.035 |

Sample Preparation:

Serum sample volumes were either 10ul or 50ul. Urine sample volumes were 50ul. The samples were diluted to a final volume of 0.2ml, for a 10ul sample, or to 0.5ml, for a 50ul sample, with 0.1N HCl.

Preparation of NANA standards

NANA standard solutions were prepared from a 1 umole/ml stock solution of NANA, preheated to 70⁰C, as follows.

| NANA STD. SOLUTIONS (nmoles) | Volume of standard (μ l) | Volume of 0.1N HCl (μ l) |
|------------------------------------|-------------------------------------|-------------------------------------|
| 0 (blank) | 0 | 200 |
| 10 | 10 | 190 |
| 20 | 20 | 180 |

Procedure:

The blank, NANA standards, and samples were hydrolysed in 0.1N HCl for one hour at 80⁰C to liberate bound-NANA from sialoglycoconjugates. Oxidation was carried out using 0.1ml periodate (0.2M sodium periodate in 9.0M orthophosphoric acid). This reaction was carried out, with constant shaking, at room temperature for 25 minutes. Oxidation was stopped by the addition of 1.0ml arsenite (10%, w/v, sodium arsenite in 0.5M sodium sulphate/0.1N H₂SO₄). Vigorous vortex mixing was required to ensure complete neutralisation of the periodate by the arsenite. Otherwise, precipitation occurred on addition of the TBA. Following addition of 3.0ml TBA (0.6%, w/v, TBA in 0.5M sodium sulphate: freshly prepared and preheated to 70⁰C), a pink chromophore was developed by heating in a boiling water-bath for 25 minutes. Immediately after heating, the chromophore was extracted into an equal volume of cyclohexanone. Centrifugation at 3000 r.p.m. for 10 minutes ensured complete separation of the aqueous and organic phases (cyclohexanone). The upper organic phase,

containing the chromophore, was then removed using a Pasteur pipette. To prevent the formation of cloudiness in the extracted chromophores it was necessary to place tubes, containing samples and standards, in a 37⁰C water-bath. A spectrophotometer (PU8625 uv/vis, Philips) was zeroed with cyclohexanone and the optical densities then recorded at 549nm and 532nm. Average values for corrected O.D.s (average sample reading minus average reading for the reagent blank) were then inserted into the correction formula to determine total NANA levels. The blank solution and the standards were assayed in duplicate, whereas samples (serum or urine) were assayed either in duplicate or in triplicate.

2.3.5

DETERMINATION OF LIPID-BOUND SERUM SIALIC ACID (LSA) USING THE METHOD OF KATOPODIS AND STOCK

The procedure of Katopodis and Stock (1980) was used to determine serum LSA levels. 50µl of serum were extracted on ice with 3.0ml ice-cold chloroform/methanol (2:1, v/v). Tubes were put on ice for five minutes. The lipid extract was partitioned with 0.5ml cold water (ultrapure). The ultrapure water was prepared by distillation, followed by filtration under pressure, using the Elgastat UHP system. The tubes were then spun at 1100 r.p.m. for ten minutes. Having removed the aqueous phase, containing the sialolipid fraction, LSA was precipitated with 50µl of phosphotungstic acid (1g/ml). After centrifugation, (3000 r.p.m. for 5 minutes), the supernatant was aspirated and the precipitate resuspended in 0.5ml HCl (0.1N). Following acid

hydrolysis at 80⁰C for 60 minutes, NANA in solution was determined by the Warren assay (Section 2.3.4).

2.3.6

DETERMINATION OF TOTAL PLASMA NANA BY A MODIFIED THIOBARBITURIC ACID ASSAY: THE VGR ASSAY

This method was developed at Dublin City University (DCU) and was named the VGR assay. Preliminary results have been published (Kinsella et al., 1991).

Principle:

As in the Warren TBA assay, NANA is oxidised with periodate and the oxidation product is reacted with TBA. Unlike the Warren assay, which requires the use of cyclohexanone, this modified assay uses a solution of 5% (v/v) HCl in ethanol to extract, intensify and stabilise the chromophore. A single wavelength (560nm) is used for O.D. measurements. At this wavelength there is considerable absorbance due to NANA, whereas that of 2-deoxyribose is reduced. Consequently, there is no need to use a correction formula and NANA levels are determined from a standard curve.

Sample Preparation:

Plasma samples (10 μ l) were brought to a final volume of 200 μ l with 0.1N HCl.

Preparation of NANA standard curve

A 1 μ mole/ml NANA solution, preheated to 70⁰C, was used to prepare a standard curve as follows:

| NANA STD. SOLUTIONS (nmoles) | Volume of NANA std (μ l) | Volume of 0.1N HCl (μ l) |
|------------------------------------|-------------------------------------|-------------------------------------|
| 0 | 0 | 200 |
| 10 | 10 | 190 |
| 20 | 20 | 180 |
| 30 | 30 | 170 |
| 40 | 40 | 160 |
| 50 | 50 | 150 |

Procedure:

The blank, NANA standards, and plasma samples were hydrolysed in 0.1N HCl for one hour at 80⁰C to liberate bound-NANA from sialoglycoconjugates. Oxidation was carried out using 0.1ml periodate (0.2M sodium periodate in 9.0M orthophosphoric acid). This reaction was carried out, with constant shaking, at room temperature for 25 minutes. Oxidation was stopped by the addition of 1.0ml arsenite (10%, (w/v), sodium arsenite in 0.5M sodium sulphate/ 0.1N H₂SO₄). Vigorous vortex mixing was required to ensure complete neutralisation of the periodate. Failure to do so resulted in precipitation on addition of TBA. Following addition of 3.0ml TBA (0.6%, w/v, TBA in 0.5M sodium sulphate: freshly prepared and preheated to 70⁰C), a pink chromophore was developed by heating in a boiling water-bath for 25 minutes. Immediately after colour development, tubes containing the blank and standard solutions each received 4.3mls of a solution containing 5% (v/v) HCl in ethanol. These tubes were placed in a 50⁰C water-bath, so as to prevent the formation of cloudiness in the

chromophore, until the O.D.s were recorded. After colour development, tubes containing the samples were centrifuged at 2200 r.p.m. for 10 minutes. This was necessary to remove precipitation which formed during colour development. After centrifugation 2ml of each (clear) supernatant were then added to an equal volume of a 5% (v/v) HCl in ethanol solution. The tubes were then placed in a 50⁰C water-bath (again to prevent cloudiness). The spectrophotometer (PU8625 uv/vis) was zeroed using the 5% (v/v) HCl in ethanol solution. The O.D.s of the blank the standards and the samples were recorded at 560nm. All O.D. readings were corrected for interference by subtracting the average reading for the reagent blank from all standard and sample readings. Plasma NANA levels were determined from the standard curve. The blank and standard solutions were assayed in duplicate, whereas the plasma samples were assayed either in duplicate or in triplicate.

2.3.7

A MODIFIED VGR ASSAY, INCORPORATING A MICRO-SAMPLE DETECTION SYSTEM, FOR DETERMINATION OF TOTAL PLASMA NANA

Principle:

The principle of this assay is the same as that of the VGR assay. It was modified so as to reduce the time required for O.D. measurements, and involved recording O.D.s in 96-well microtitre plates.

Procedure:

The procedure was very similar to that described

in Section 2.3.6. However, it contained the following alterations:

Having stabilised the chromophores with the 5% (v/v) HCl in ethanol solution, 180 μ l samples from each of the tubes were placed in wells of a 96-well microtitre plate. The plate was placed in the 50⁰C incubator of the microtitre plate reader (Titertek Twinreader plus: type 381, ICN-Flow). Samples were allowed to equilibrate at the required temperature for 25 minutes. The spectrophotometer was zeroed at 560nm using a 5% (v/v) HCl in ethanol solution. O.D.s were then measured over a period of 30 seconds. All readings were corrected for interference by subtraction of the average value obtained for the blank. As in the VGR assay, plasma NANA levels were determined from an NANA standard curve.

2.4

CELL CULTURE METHODS

2.4.1

PREPARATION OF STOCK MEDIUM

Basic cell culture medium can be obtained in either concentrated or readily usable form. Concentrated medium (usually 10x), obtained as powder or liquid, is easier to prepare and much cheaper per unit than its 1x equivalent. The following method is for preparation of Dulbecco's Modified Eagle's (DME D5280) liquid medium. Ultra-pure water was used as diluent. 4.5L of water were dispensed into a 5L conical flask. The powdered DME medium (D5280) was added to the water, then gently stirred (without heating) until completely dissolved. The package was then rinsed with 250mls of water to remove all traces

of powder 475mls of DME medium were dispensed into ten 500ml Duran bottles, followed by autoclaving at 121⁰C for 20 minutes. Each bottle then received 15mls of sterile 7.5% (w/v) sodium bicarbonate in water, and 10mls of hepes buffer (1M solution). The latter reagent is an optional buffer system. The pH was adjusted to between 7.2 and 7.4, if necessary, with sterile acid (1M HCl) or base (1M NaOH). The stock medium was stored at 4⁰C until required.

2.4.2

PREPARATION OF MEDIUM FOR CULTURE

The following method was used to prepare a range of complete cell culture media ("complete" meaning that FCS had been added to the medium). They include.

1 Dulbecco's Modified Eagle's Medium

A. DME-D5280 (Sigma: Section 2.4.1)

B. DMEM 1X (ICN-Flow: Cat. No. 12-332-54)

2 Rosewell Park Memorial Institute Medium

(RPMI-1640 medium: 1x)

(ICN-Flow: Cat. No. 12-602-54)

3 Hams-F12 Nutrient Rich Medium

(Hams-F12 medium: 1x)

(ICN-Flow: Cat. No. 12-442-54)

99mls of 1x medium were dispensed aseptically into a sterile bottle. To this was added 1ml of l-glutamine (200mM), 2.0ml of hepes buffer (1M) and 1.0ml amphotericin B ("Fungizone" :250µg/ml). The following were also added if required: 2.0ml penstrep (penicillin; 5000 IU/ml and streptomycin; 5000 µg/ml) or 50µg

gentamicin (10mg/ml) Addition of antibiotics to culture medium is not advisable as it masks the presence of bacterial contamination. This could have more serious consequences since other less conspicuous forms of contamination, including Mycoplasma (Section 2.4.12), could also be present. In any case, proper aseptic technique eliminates the need for any antibiotics. 10mls of FCS (Mycoplasma screened) were then added and the pH adjusted to between 7.2 and 7.4, with sterile 1M NaOH or 1M HCl, if necessary. A sample of the culture medium was tested for sterility by incubation (5% CO₂, v/v, / 37⁰C) for at least 7 days. The medium was stored at 4⁰C until required L-glutamine and FCS were replenished every 12 days.

2.4.3

NSO MYELOMA CELLS

NSO cells were obtained from the ATCC. This is a Balb/c mouse myeloma cell line, developed by Milstein et al. (1981), which does not express IgG.

NSO cells were maintained in DMEM supplemented with 10% FCS. As this cell line is mildly adherent, harvesting and sub-culturing of these cells required procedures described in Section 2.4.6. NSO cells, and all other cell lines, were incubated in a humid 5% (v/v) CO₂ atmosphere at 37⁰C.

2.4.4

EJ BLADDER CELL LINE

EJ cells were kindly donated by Ms. Mary

Freshney, The Beatson Institute For Cancer Reseach, Glasgow, Scotland. The EJ cell line is a human bladder carcinoma, developed by Harris et al. (1983), and has an epithelial-like morphology.

As this bladder carcinoma is strongly adherent, harvesting and sub-culturing of these cells required procedures described in Section 2.4.7. EJ cells were also grown in large scale culture using a roller-bottle system (Section 2.4.8).

2.4.5

NRK CELLS

The NRK cell line was obtained from the ATCC. This is a normal rat kidney cell line and was first described by Duc-Nguyen et al. (1966).

This cell was maintained in DMEM supplemented with 10% (v/v) FCS. As a strongly adherent cell line it required trypsinisation (Section 2.4.7) for harvesting and sub-culturing of cells.

2.4.6

SUBCULTURING AND MAINTENANCE OF MILDLY ADHERENT CELL LINES

Many cells, when grown in culture, are mildly attached to the bottom of the culture container and can be removed by flushing with medium. Cells are usually harvested when they have grown to approximately 75% confluency.

All medium was removed from the culture container, thereby removing detached (dead) cells. Fresh serum-free medium was then added and the cells removed by flushing with medium,

with the aid of a sterile Pasteur pipette. The contents of the culture vessel were transferred to a sterile universal (30ml) and centrifuged at 2000 r.p.m. for five minutes. A cell count was taken (Section 2.4.9). The original culture was re-seeded at a concentration of 1×10^5 cells/ml, using 5mls of complete culture medium per 25cm^2 flask. Cells were allowed to adhere overnight at 37°C in 5% (v/v) CO_2 . An additional 5mls of complete culture medium were then added and the culture returned to the incubator (37°C / 5%, v/v, CO_2). The remaining harvested cells were used to initiate new cultures. Excess cells were stored in liquid nitrogen (Section 2.4.10).

2.4.7

SUBCULTURING AND MAINTENANCE OF STRONGLY ADHERENT CELL LINES

Unlike mildly adherent cells (Section 2.4.6) there are cells that firmly adhere to the bottom of the culture container and therefore cannot be removed by flushing with medium. To harvest and subculture cells of this type requires the use of trypsin or similar proteolytic agents.

All culture medium was removed from the culture flask. FCS, present in the medium, contains a trypsin inhibitor and, therefore, prevents enzymatic removal of adhered cells. Trypsin (2.5% w/v, heated to 37°C) was added to the flask, which was then placed in an incubator (5%, v/v, CO_2 / 37°C). After five to ten minutes the flask was returned to the laminar flow, and complete culture medium was added, thereby preventing further enzymatic digestion. The contents of the flask were transferred to a sterile universal (30ml) and centrifuged at

2000 r.p.m. for five minutes. The cells were seeded at $1-5 \times 10^5$ cells/ml, using 5mls complete culture medium per 25cm^2 flask.

2.4.8

LARGE SCALE CULTURE OF EJ CELLS

In order to obtain large cell numbers, large scale culture of the EJ bladder carcinoma cell line was carried out in roller-bottles.

EJ cells (Section 2.4.4) were obtained by either trypsinisation of an actively growing culture (Section 2.4.7) or recovery from liquid nitrogen (Section 2.4.11). A viable cell count was performed (Section 2.4.9). A roller-bottle was seeded with a total of 1×10^7 viable cells in 30mls of complete culture medium (DMEM with 10%, v/v, FCS and 2%, v/v, Hepes buffer) The culture vessel was placed in an incubator (37°C) on a roller-bottle apparatus (manufactured by Bellco) at one revolution per minute, and the cells were allowed to adhere overnight. An additional 70mls of DMEM were then added and the culture was returned to the incubator (37°C). When the culture was approximately 80% confluent the cells were harvested as follows. All medium was removed from the roller-bottle. Trypsin (2.5%, w/v, 15mls at 37°C) was added and the culture was returned to the incubator, on the roller-bottle apparatus, for 5-10 minutes. Complete culture medium was then added to prevent further enzymatic digestion. The contents of the roller-bottle were transferred to a sterile 50ml centrifuge tube. A sample was taken ($20\mu\text{l}$ - $30\mu\text{l}$) and a viable cell count was performed (Section

2.4.9). Cells were centrifuged at 3000r p m. for 10 minutes to obtain a cell pellet 1x10 viable cells were used to maintain the parent culture, and the remainder were stored in liquid nitrogen until required (Section 2.4.10).

2.4.9

CELL COUNTS AND VIABILITY STAINING

Cell counts were performed on an improved Neubauer haemocytometer slide. Two dye exclusion methods, with either trypan blue or acridine orange/ ethidium bromide, were routinely used. The viability of a cell suspension was determined as follows.

$$\% \text{ VIABILITY} = \frac{\text{NUMBER OF VIABLE CELLS}}{\text{TOTAL NUMBER OF CELLS}} \times 100\%$$

Acridine Orange/ Ethidium Bromide

The main advantage of this method is that colour contrast, between living and dead cells, is very good. However, it does require a fluorescence microscope, and care must be taken as both chemicals are carcinogens.

The stain solution (light sensitive) contained 0.1mg acridine orange and 0.1mg ethidium bromide in 100ml PBS. 20-30 μ l of a cell suspension were mixed with an equal volume of AO/EB in an epindorph tube. The cells were then examined by U.V. fluorescence microscopy. Live cells stained green while dead cells stained orange.

Trypan blue exclusion

The main advantage of this method over AO/EB is that it is cheaper (no fluorescence microscopy). However, as a result of using light microscopy the colour contrast is not as good as that obtained using AO/EB. This method is also hazardous as trypan blue is a teratogen and a possible carcinogen.

A volume of cell suspension (20-30 μ l) was mixed with an equal volume of trypan blue in an epindorph tube, followed by thorough vortexing. After 2-3 minutes the cells were counted under a light microscope. Live cells were clear while dead cells stained blue. In order to prevent uptake of the dye by healthy cells it was necessary that the count was taken within 5 minutes of mixing the trypan blue and cell suspension.

2.4.10

FREEZING CELLS IN LIQUID NITROGEN

The freezing mixture contained FCS supplemented with 5% (v/v) DMSO (a cryoprotectorant). It was prepared by dropwise addition of DMSO to ice-cold FCS, followed by sterile filtration (0.2 μ filter; Gelman). This reagent was stored at -20°C. When required it was thawed in a 37°C water-bath, then placed on ice for 30 minutes before being used.

Cells were removed from culture vessels either by flushing with medium (Section 2.4.6) or trypsinisation (Section 2.4.7). A viable cell count was performed (2.4.9). Washed cells were resuspended at 1-10x10⁶ cells/ml freezing

mixture. One ml aliquots were placed in sterile cryotubes. Cells were frozen at a rate of approximately 1°C per minute to -70°C on a freezing tray (Union Carbide) before being placed in liquid nitrogen.

2.4.11

RECOVERY OF CELLS FROM LIQUID NITROGEN

Successful recovery of cells from liquid nitrogen requires rapid removal of DMSO, as it can have adverse effects on the cells.

The contents of a cryotube were thawed, in a 37°C water-bath, until only approximately 5% of the cells remained frozen. They were transferred to a sterile universal (30ml) and 5mls of ice-cold serum free medium were added dropwise while gently shaking the universal. After centrifugation (2000 r.p.m for 5 minutes) the cells were given a further two washes. Culture medium was used when the cells were required for growth in culture, whereas cells for immunisation were washed in sterile PBS. Cells removed correctly from liquid nitrogen were approximately 90% viable.

2.4.12

MYCOPLASMA DETECTION: HOECHST-33258 FLUORESCENCE ASSAY

Hoechst 33258 fluorescence stain was used to detect the presence of Mycoplasma in the cellular cytoplasm. This method, employing the DNA intercalator bisbenzimid (Hoechst 33258), was first described by Chen (1977). The following

method is an adaptation of this procedure by M. Dooley and M. Clynes, Dublin City University (personal communication)

A 5×10^3 cells/ml suspension of Mycoplasma-free NRK cells (Section 2.4.5) was prepared. Sterile coverslips, placed in 10mm petri dishes, received 1.0ml of the cell suspension, and the cells were allowed to grow overnight. 1.0ml of culture supernatant from cells being tested (free of cells and debris) was then added to the NRK cells. A negative control (complete culture medium) was also included. NRK cells and supernatants were reincubated for a further 2-3 days, during which time the NRK cells continued to grow. The coverslips were then washed 3 times in PBS, followed by cell fixation for six minutes in a 1:1 (v/v) methanol:acetone solution (-20°C). After fixation, the coverslips were rinsed three times in PBS. The cells were stained for 10 minutes using Hoechst 33258 stain ($0.025\mu\text{l/ml}$) in PBS. Coverslips were washed twice in PBS, air-dried, and finally mounted on clean slides. The cells were stored in the dark until examined by fluorescence microscopy (40x or 100x with oil immersion). Defined spots of green fluorescence in the cell cytoplasm indicated a positive result (presence of Mycoplasma). If such a result was found in the negative control (culture medium) the assay was deemed invalid.

2.5

IMMUNIZATION OF BALB/C MICE FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES

For the production of monoclonal antibodies,

Balb/c mice were immunized with whole EJ cells. The human bladder carcinoma cells were freshly harvested from actively growing cultures by trypsinization (Section 2.4.7) and washed in PBS. A viable cell count was taken (Section 2.4.9). 1×10^7 cells in 1.0ml of PBS were administered by intraperitoneal injection (i.p.) into Balb/c mice aged between eight and twelve weeks. The routine immunization regimen involved i.p. injection of cells on days 0, 7, 14, 21, and a final boost on day 28. Three days after the final boost the splenocytes were harvested. The spleen cells were then either used in a fusion experiment on that day or stored in liquid nitrogen (Section 2.4.10) until required

2.6

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

A protocol for the setting up and performance of a solid-phase enzyme-linked immunosorbent assay (ELISA) is described for use with fixed cells as antigens.

2.6.1

CELL FIXATION FOR USE IN ELISA

Method 1:

In this method, cells to be fixed were obtained from actively growing cultures. This procedure was also used to fix normal human blood and tonsillar lymphocytes.

Flat bottomed flexible (PVC) plates (Cat. No.7717305, ICN-Flow) were washed 3 times in PBS, followed by treatment with poly-l-lysine (0.1%, w/v, in PBS: 100 μ l per

well). The latter reagent was re-used up to 10 times. After incubating for 1 hour at 37⁰C the poly-l-lysine was removed and the plates were again washed 3 times in PBS. 50μl of a 5x10⁵ cells/ml (PBS) suspension, of either washed cells obtained by trypsinisation (Section 2.4.7), or lymphocytes, were then added to each well. After one hour at 4⁰C, 150μl of glutaraldehyde (GA; 0.1%, v/v, in PBS) were added to each well to give a final concentration of 0.075% (v/v) GA. It was important, during GA addition, that the cell layer was not disturbed and that no air bubbles were present in any of the wells. After 10 minutes (max.) at 4⁰C, the plates were washed three times in PBS. Glycine (100mM in PBS; 100μl/well) was added to block any remaining GA. Having incubated the plates at 37⁰C for 30 minutes they were washed three times in PBS. 150μl of storage/blocking buffer (1% BSA, w/v, in PBS containing 0.1%, w/v, azide as preservative) were then added to each well. The plates were thoroughly sealed (to prevent evaporation of storage/blocking buffer) and then placed at 4⁰C. Under these conditions it was possible to store the plates for up to six months with no apparent loss of activity.

Method 2:

A second method of fixation was also used. This method was used exclusively to fix cells that grew in culture and was not used to fix lymphocytes. Cells were seeded in sterile 96-well tissue culture plates and then allowed to grow in complete culture medium. When the required density was reached the cells were fixed. One advantage of this method over method 1 is that it requires fewer cells, thus

increasing efficiency. The operator also has greater control over experimental conditions, in that it is possible to examine the cells under a microscope prior to fixation. A minor disadvantage of this method is that preliminary stages must be carried out in a laminar flow hood under aseptic conditions.

The procedure used was as follows: Cells were obtained by trypsinization (Section 2.4.7). A viable cell count was taken (Section 2.4.9). Pelleted cells were resuspended in complete culture medium at a concentration of 5×10^4 cells/ml. Each well received $100 \mu\text{l}$ of the suspension (5×10^3 cells). The plates were sealed, placed in an incubator (5%, v/v, CO_2 at 37°C) and examined daily. The time interval between seeding of cells and fixation depended on the growth rate of the cell line. Cells were usually ready for fixing after 2-3 days. Having reached approximately 80% confluency the cells were fixed as follows: plates were washed 3 times in PBS. $150 \mu\text{l}$ of GA (0.1%, v/v, in PBS) were added to each well to give a working concentration of 0.075% (v/v) GA. The remaining steps in this procedure were exactly the same as those of method 1 (previously described) regarding the addition of glutaraldehyde.

2.6.2

ELISA

In this indirect, non-competitive ELISA system the antigen is coated onto microtitre plates (Section 2.6.1) and antibody binding is detected using an enzyme-labelled second antibody.

Antigen coated plates were washed 3 times in 0.1% (v/v) Tween-20 in PBS. Non-specific binding sites were blocked with 1% (w/v) BSA in PBS with 0.1% (w/v) azide (100 μ l/well), for one hour at 37⁰C. Having re-washed the plates 3 times in PBS-Tween, 50 μ l of either control or test sample were added to specified wells. Each assay included a range of controls. The screening of hybridomas and clones against a target antigen required a positive control (serum from the mouse in which the antibodies were raised) and also a negative control (serum from a non-immunised mouse). To eliminate interference caused by FCS present in culture media a sample of serum-free medium was also tested. A PBS control was also included to ensure that non-specific binding of labelled second antibody did not occur. A monoclonal antibody (H12), obtained from Bernadette Lannon, Applied Biochemistry Group, Dublin City University), highly reactive with lymphocytes, was used as a positive control in the screening of hybridomas and clones against normal cells. Samples of serum free medium and PBS were also tested. Plates were incubated at 4⁰C for 30 minutes followed by one hour at 37⁰C. Unbound antibodies were removed by washing with PBS-Tween. 50 μ l of enzyme-labelled second antibody (1/500 dilution of β -galactosidase-linked sheep anti-mouse Ig, F(ab')₂ fragment) were added to each well. Plates were again incubated (30 minutes at 4⁰C followed by one hour at 37⁰C). Having washed the plates to remove unbound second antibody, 100 μ l of substrate solution (freshly prepared) were added to each well. This solution contained 3mM O-nitrophenyl- β -galactopyranoside (ONPG) in PBS

containing 10mM MgCl₂ and 0.1M 2-mercaptoethanol. The colour was developed at 37⁰C for 1-2 hours. The reaction was stopped with 1M sodium carbonate (50µl/well). This reagent also increased colour intensity. O.D.s were recorded at 405nm, against a PBS blank, using a microtitre plate reader.

2.7

SOMATIC CELL FUSIONS AND RELATED PROCEDURES

2.7.1

AZAGUANINE TEST FOR HAT SENSITIVITY OF MYELOMA CELLS

NSO myeloma cells were grown in complete DMEM (10%, v/v, FCS) for 4-5 days prior to harvesting (Section 2.4.6), washed twice in DMEM and counted (Section 2.4.9). They were resuspended at 2x10⁵ cell/ml of complete DMEM (10%, v/v, FCS) supplemented with 1x10⁻⁴M 8-azaguanine (2.0µg/ml). 75cm² flasks were each seeded with 2.5x10⁶ cells (12.5ml suspension per flask) and incubated for 3-5 days at 37⁰C in 5% CO₂. Only HAT-sensitive cells will grow in this medium. Therefore, cells failing to grow under these culture conditions were discarded and replaced with a known source of HAT-sensitive myeloma cells. NSO cells found to be HAT sensitive were washed in DMEM and then grown in 8-azaguanine-free complete DMEM (10%, v/v, FCS) for 4-5 days prior to use in cell fusions.

2.7.2

ISOLATION OF SPLEEN CELLS

Spleen cells are removed from immunised mice 3

days after the final boost with antigen (day 28). Ideally they are used immediately after removal. However, if the myeloma cells are found to be unsuitable for use in a fusion experiment (Section 2.7.5) on a particular day the spleen cells can be stored in liquid nitrogen (Section 2.4.10) until required. The main disadvantage of this methodology is that potentially valuable splenocytes may be lost during freezing and thawing of the cells.

The immunised Balb/c mouse was killed by cervical dislocation and placed in ethanol. It was then pinned onto a dissection board on its right side, placed in a laminar flow hood and sprayed with ethanol. The spleen was aseptically removed using sterile instruments and, after removal of fatty tissue, was placed in a petri-dish containing 10mls of serum-free medium (DMEM). Having dissected the spleen into small pieces, the cells were forced into the medium using the barrel of a sterile 10ml syringe. Other methods of obtaining splenocytes involve either the use of a sterile tissue dissociation sieve (Sigma) or else flushing the cells out of an intact spleen by injection of serum-free medium (5mls). The latter requires that the spleen is held using a sterile blunt forceps. Serum-free medium is injected into the upper end of the spleen. The medium forced out of the other end of the spleen will contain splenocytes. The resulting cell suspension was washed three times in serum free medium and then counted (Section 2.4.9). Spleen cells were either used in a fusion experiment (Section 2.7.5) or stored in liquid nitrogen (Section 2.4.10) for use at a later date. Viability

before freezing was usually >90% and each spleen yielded up to 1×10^8 splenocytes. A blood sample was taken from the immunised mouse and the resulting serum used as a positive control in screening of hybridoma supernatants by ELISA (Section 2.6.2).

2.7.3

PREPARATION OF 50% (W/V) POLYETHYLENE GLYCOL (PEG)

PEG was used as the fusogen in somatic cell fusion experiments (Section 2.7.5).

Solutions of 50% (w/v) PEG were prepared using both of the following methods.

A.

5g of PEG (Riedel-de Haen; molecular weight 1540) were fully dissolved in 3-4mls of serum-free DMEM (with heating) and the final volume adjusted to 10mls. The solution was sterilised using a sterile 0.22μ filter (Gelman) and stored in 2.0ml aliquots at -20°C .

B.

5g of PEG (M.W. 1540) were autoclaved at 121°C for 20 minutes to sterilise and dissolve the PEG. As it cooled, but before it solidified, (approx. 40°C), 5mls of sterile serum-free DMEM were added (i.e. a volume of medium equal to the number of grams of PEG autoclaved). As in the previous method, 2.0ml aliquots were stored at -20°C .

PREPARATION OF MOUSE MACROPHAGE FEEDER CELLS

Peritoneal macrophages were used as a source of feeder cells. The presence of these growth factor secreting cells greatly increases the ability of other cultured cells to grow at very low concentrations. Feeder cells are essential when plating out newly formed hybridomas (Section 2 7.5) and especially during the cloning of cells by limiting dilution (Section 2 7.7).

The method of preparation demands great care and attention as the macrophage feeder cells are very susceptible to contamination by intestinal bacteria during the extraction procedure. Therefore, the cells are usually prepared one day prior to the fusion experiment (Section 2.7 5) and are thoroughly examined for signs of bacterial contamination before being used.

Six Schofield were sacrificed by cervical dislocation and immersed in ethanol. A mouse was pinned onto a dissection board, sprayed with ethanol and placed in a laminar flow hood. The skin was then carefully cut away to expose the peritoneal membrane which was then sprayed with ethanol. The peritoneal membrane was lifted with a sterile blunt forceps (to avoid puncture) and 10mls of either serum-free DMEM or sterile PBS were injected into the peritoneal cavity, taking care not to pierce the gut. The abdomen was gently massaged to bring macrophage cells into suspension. 5mls of the cell suspension were then carefully withdrawn and dispensed into a sterile universal (30mls). This procedure was repeated for each mouse. As these mice were not

immunised, blood samples were taken and the resulting sera were used as negative controls in ELISA (Section 2.6.2) screening of hybridoma supernatants. Cells were pooled into three groups (cells from two mice per group), washed twice in serum-free medium and counted (Section 2.4.9). It is not advisable to pool all the cells together as one contaminated sample would result in the loss of all the feeder cells. They were resuspended to 1×10^5 cell/ml in complete DMEM (10%, v/v, FCS) and plated out in sterile 96-well tissue culture plates at a concentration of 1×10^4 cells/well. After incubation overnight (37°C in 5%, v/v, CO_2), the feeder cells were examined for signs of bacterial contamination before proceeding with the fusion experiment.

2.7.5

CELL FUSION PROCEDURE

This method, to be successful, requires delicate manual dexterity and rigorous attention to detail. The state of growth and viability of myeloma cells, the rate of addition and dilution of PEG and the fusion temperature are all critical factors in the procedure. For a successful fusion, hybridomas are usually visible as growing colonies after 7-14 days.

NSO cells, maintained in mid-log phase of growth for 7 days prior to fusion, and pre-tested for both HAT sensitivity (Section 2.7.1) and the presence of Mycoplasma (Section 2.4.12), were pooled, counted (Section 2.4.9) and washed three times in serum-free DMEM. Immunised spleen cells obtained either by splenectomy (Section 2.7.2) or recovered from liquid nitrogen (Section

2 4.11) were also counted and washed as above. Spleen cells and NSO cells, in the ratio of 2 spleen:1 NSO cells, were mixed and centrifuged at 3500 r.p.m. for ten minutes to yield a hard pellet. Ratios of 10:1 (spleen:NSO) have also been used successfully. The supernatant was fully removed. Cells were fused in 50% PEG (Section 2.7.3) at 37⁰C as follows: 1ml of PEG-1540 was added at a constant rate over one minute (0.1ml PEG/ 6.0 seconds) with gentle mixing. This was achieved by gently stirring the cell pellet with the tip of the pipette as the PEG was being added. The cell pellet and PEG were then stirred for one minute. The aim was to expose the cells to the PEG while maintaining as much cell contact as possible. PEG was diluted by dropwise addition of 3mls serum-free medium (DMEM at 37⁰C) over 3 minutes (1ml/minute), followed by a further 8mls of serum-free DMEM over 3 minutes (approx. 2.65mls/minute). The cells were gently spun at 1000 r.p.m. for 10 minutes to yield a soft pellet, then resuspended at 2x10⁵ cells/ml in enriched complete culture medium (DMEM with 20%, v/v, FCS) containing double strength HAT (Section 2.7.6). The cells were plated out at a concentration of 2x10⁴ cells/well in sterile 96-well tissue culture plates, precoated with macrophage feeder cells (Section 2.7.4), and incubated, undisturbed, at 37⁰C in 5% (v/v) CO₂ for 10 days. After this time hybridomas were fed at regular intervals (apprx. after every 2 days) by replacing 100μl of spent medium with 100μl of fresh medium. After approximately 14 days the cells were grown in complete DMEM (10%, v/v, FCS) containing HAT (Section 2.7.6). After a further 14 days the cells were weaned off HT and thereafter were

cultured in complete DMEM (with 10%, v/v, FCS).

2.7 6

HAT AND HT-SELECTIVE MEDIA

Single strength (1x) HAT medium consisted of complete DMEM (10%, v/v, FCS) supplemented with 100 μ M hypoxanthine (H), 1M aminopterin (A) and 16 μ M thymidine (T). Twice these amounts were used to prepare double strength (2x) HAT HT medium, used to wean cells off HAT, consisted of complete DMEM (10%, v/v, FCS) supplemented with 100 μ M hypoxanthine (H) and 16 μ M thymidine (T).

2 7 7

CLONING OF HYBRIDOMAS BY LIMITING DILUTION

A feeder layer of murine macrophage feeder cells (Section 2 7 4) was prepared one day prior to cloning. Cells producing antibodies of specific interest, as determined by ELISA (Section 2 6 2), were expanded from 96-well to 24-well sterile tissue culture plates as 1 0ml cultures. When confluent, the cells were harvested (Section 2.4.6) and counted (Section 2 4.9). As a precautionary measure a sample of cells was frozen for storage in liquid nitrogen (Section 2 4.10). The remainder of the cells were diluted in serum-free medium (DMEM), then plated in complete DMEM (10%, v/v, FCS) in sterile 96-well plates at concentrations of 10, 5 and 1 cell per well. The plates were incubated for seven days at 37⁰C in 5% (v/v) CO₂ and then examined for growth of clones. It is often the case that no growth occurs in the plates seeded with one cell/well. This can occur as a result of errors in the procedure. For example, an overestimation in the initial cell count would lead to excess

dilution of cells, the result being that many of the wells would receive no cells at all. Another possibility is that the macrophage feeder cells may not secrete sufficient amounts of growth factors required for early stages of cell growth. Therefore, it is essential that higher cell concentrations (5 and 10 cells per well) are seeded so as to prevent the loss of potentially valuable hybridomas. The most important plates, if growth occurs, are those with one cell per well, as they contain the least heterogeneous mixture of antibodies. Cells in these wells are expanded and rescreened for antibody production using ELISA (Section 2.6.2). Antibody secreting clones are recloned. Two limiting-dilution subclonings are usually adequate to produce a fully monoclonal antibody-producing hybridoma. However, it is advisable to reclone regularly in the first few months so as to test stability, expressed as continued ability to secrete antibody.

2 7.8

GROWTH OF CLONES AS ASCITIC TUMOURS IN MICE

To obtain large quantities of antibody, clones were grown as ascitic tumours in Schofield mice. Each animal was injected with 1×10^7 cells, having been primed 7 days earlier with 0.5ml of immunostimulant (Pristane or Freund's Adjuvant). The animals were observed daily for tumour growth. After 10-15 days the ascitic fluid was removed. The mice were sacrificed by cervical dislocation. The skin was cut away to reveal the peritoneal membrane. PBS (3-4mls) was injected into the peritoneal cavity and the stomach area

was gently massaged using a blunt forceps (to avoid puncturing the membrane). The purpose of the PBS injection was to dilute the viscous ascitic fluid, thereby facilitating its removal. The fluid was carefully withdrawn (using a sterile needle and syringe) and then centrifuged at 3000 r.p.m. for 5 minutes to pellet the cells. Having removed the supernatant (rich in antibodies), thimerosal (a preservative) was added to a concentration of 0.001% (v/v). The ascitic fluid was dispensed into epindorph tubes and stored at 4⁰C until required.

2 8

METHODS OF STATISTICAL ANALYSIS

2 8.1

TWO-SAMPLE STUDENT'S T-TEST

Statistical analysis using this test required use of Student's two-sample t-statistic. To test a null hypothesis (H_0) which states that there is no difference between the two sample means requires the following form of the statistic.

$$T = \frac{X_1 - X_2}{S_p (1/n_1 + 1/n_2)^{0.5}}$$

where X_1 and X_2 are the sample means,
 n_1 and n_2 the sample sizes and
 S_p the pooled standard deviation

$$s_p^2 = \frac{(n_1-1) \times s_1^2 + (n_2-1) \times s_2^2}{n_1+n_2-2}$$

S_1 and S_2 are the standard deviations

The rule is to reject H_0 if the absolute value of t is greater than or equal to C , where C (the critical value) is obtained from Student's t -table for a two sided test and $V = n_1+n_2-2$ degrees of freedom, thereby accepting the alternative hypothesis (H_1), which states that a difference does exist between the two sample means.

2.8.2

STUDENT'S T-TEST

A form of Student's t -Test was used to compare the magnitude of the change (usually an increase) in total serum NANA levels observed in the tumour group (LAT) with those of the PBS and Pristane control groups. This was based on the comparison between the average changes in serum NANA levels between day -1 (one day prior to injection) and either day 3 or day 6 (3 or 6 days after injection).

The null hypothesis (H_0) stated that no difference existed between the average change observed in serum from the LAT-bearing mice and those of the control groups. The alternative hypothesis (H_1) stated that a difference did exist. Testing of this hypothesis required the following form of the t -statistic.

$$t = \frac{X_1 - X_2}{S_p (1/n_1 + 1/n_2)^{0.5}}$$

where X_1 and X_2 are the average changes in total serum NANA levels over a specified time interval, n_1 and n_2 are the sample sizes and S_p the pooled mean square estimate of the standard deviation.

$$S_p^2 = \frac{\sum X_{11}^2 - [(\sum X_{11})^2/n_1] + \sum X_{21}^2 - [(\sum X_{21})^2/n_2]}{n_1 + n_2 - 2}$$

X_{11} and X_{21} are the changes in total serum NANA in individual mice over a specified time interval.

The rule is to reject H_0 if the absolute value of t is greater than or equal to C , where C (the critical value) is obtained from Student's t -table for a two-sided test and $V = n_1 + n_2 - 2$ degrees of freedom

SECTION 3

**The Evaluation of Total Serum Sialic Acid
as an Indicator of Tumour Burden in Human Patients
with Cancer of the Prostate.**

3.1

INTRODUCTION

The aim of this research was to evaluate total serum NANA as a tumour marker for cancer of the prostate. An ideal tumour marker is a substance, the concentration of which varies solely as a result of changes in the extent of tumour burden. It must be highly sensitive (i.e. give a low % of false negative results), and highly specific (i.e. give a low % of false positive results). Another desirable quality is that the marker can be determined both rapidly and economically. An ideal tumour marker has yet to be found. However, extensive research is constantly in progress to find a substance which best satisfies the above requirements.

NANA has been repeatedly investigated with regard to its potential as a tumour marker. Elevations in serum NANA have been found not to be specific for any one particular tumour. Lodha et al (1979) found elevated NANA levels in a wide range of tumours, with the most significant elevations occurring in cases of metastatic disease. Erbil et al. (1986) found that NANA lacked the specificity required for early detection of prostatic cancer, but suggested its possible use as a marker of tumour activity, during follow-up under treatment in cases of advanced disease. Khanderia et al. (1983, 1988), who found poor correlation between isolated NANA levels and stage of neoplastic disease, suggested that serial determination of serum NANA could be a more useful monitor of response to therapy in a

range of neoplasms, including metastatic embryonal cell carcinoma, transitional cell carcinoma of the bladder, cancer of the colon and hepatocellular adenocarcinoma.

The reasons for this caution are well founded. O'Kennedy et al. (1991) stated that while significant elevations in serum NANA can occur in tumour-bearing individuals, the reason for these elevations is not solely tumour derived. There are substances known as acute-phase reactants, the concentrations of which are altered (usually in the form of an increase) in response to any of the following. tissue injury, infection, trauma or inflammation. Bradley et al. (1977) stated that the increase occurs within 12-36 hours. NANA itself is an acute phase reactant since NANA levels are also elevated by the above mentioned stimuli. For example, Berra et al. (1986) discovered that total serum NANA levels were greatly modified by different manipulations of patients. When blood samples were drawn after an operation, NANA was specifically increased. Shamberger et al. (1987) found elevated levels of NANA in several inflammatory diseases, including osteoarthritis, Crohn's disease and mucosal ulcerative colitis. Hogan-Ryan et al. (1981), in their study of serum NANA concentrations in malignant melanoma, purposely omitted patients from the study if they had infection, so as to exclude the non-tumour specific acute-phase production of NANA. These findings implied that NANA lacked the specificity required for use as a tumour marker because of the possible incidence of false positive results.

In this evaluation of total serum NANA as a

tumour marker for cancer of the prostate, the following questions were posed.

1.

Is the tumour burden of a patient suffering from cancer of the prostate, at a specific time, reflected in an isolated determination of total serum NANA?

2

Could serial determination of total serum NANA be used to monitor patient response to therapy?

3

Does total serum NANA demonstrate properties of an acute-phase reactant when used as an indicator of tumour burden for cancer of the prostate?

3.2

EXPERIMENTAL DESIGN

Ten patients suffering from cancer of the prostate were examined. All patients initially had advanced disease, with some also suffering from metastases. Following initial diagnosis and therapy each patient regularly attended the Meath Hospital as part of routine follow-up and clinical monitoring. At each visit to the hospital a sample was taken. Total serum NANA levels were determined using the thiobarbituric acid method of Warren (Section 2.3.4). The duration of NANA monitoring varied for each patient as follows:

| PATIENT [*] NAME | DURATION OF STUDY (weeks) |
|------------------------------|------------------------------|
| P1 | 25 |
| P2 | 36 |
| P3 | 31 |
| P4 | 27 |
| P5 | 30 |
| P6 | 25 |
| P7 | 23 |
| P8 | 18 |
| P9 | 13 |
| P10 | 11 |

* : To preserve patient anonymity, real names were not used. Each of the ten patients were therefore given a coded name (P1-P10), where for example, P1 = prostate cancer patient No.1.

3.3

ANALYSIS OF RESULTS

Total serum NANA levels for patients P1-P10 are tabulated in the appendix at the end of this section (Tables 3.1-3.10, respectively).

Results for P1 are shown in Figure 3.1.

This patient presented with advanced disease, but later responded well to therapy and was diagnosed as being free of disease at the end of the study (week 25).

Total serum NANA levels fluctuated very little over the entire 25 week period, ranging from 1700 nmoles/ml to 1900 nmoles/ml. Therefore, the readings were quite constant, and grouped around the centre of the normal range for total serum NANA (1117 nmoles/ml-2525 nmoles/ml). In marked contrast, the state of health of the patient varied considerably, from advanced disease initially, to total absence of disease after only six weeks. This dramatic decrease in tumour burden was not reflected in total serum NANA levels. No dramatic decrease in serum NANA was observed. Serial determination of NANA would have provided no indication of tumour regression.

Results for P2 are shown in Figure 3.2.

This patient presented with advanced disease, but later responded well to therapy and was diagnosed as being free of disease at the time of the last clinical examination (week 36).

Initial analysis of total serum NANA gave a value of 1950 nmoles/ml. The level progressively decreased over the following 9 weeks. Between weeks 10 and 14 there was a slight increase in total serum NANA levels, giving a maximum value of 1800 nmoles/ml at week 14. From that point until the end of the study serum NANA levels continued to decrease, with the exception of a very slight elevation at week 32, to give a final level of 1150 nmoles total serum NANA per ml of serum at week 36.

In this case there was very good correlation between tumour burden and total serum NANA. The

general trend in NANA levels was downwards, coinciding with progressively decreasing tumour burden. The level of total serum NANA at week 36, the time at which the patient was pronounced free of disease, was only 1150 nmoles/ml, that is, towards the lower end of the normal range. Very slight increases were observed between weeks 10 and 14 and also between weeks 28 and 32 (increases observed were 300 nmoles/ml and 200 nmoles/ml, respectively). These elevations were probably the result of an acute-phase response, which could have occurred as a result of any of the aforementioned causes of such a reaction.

One indication of these results is that isolated NANA determinations must be interpreted with caution. For example, week 1 gave a total serum NANA level of 1950 nmoles/ml, a value well within the normal range, at a time when the patient was known to have advanced disease with metastasis. However, a possible use for serial determination of total serum NANA as a monitor of response to therapy is indicated as there was a definite decrease in NANA levels as therapy successfully reduced the tumour burden.

Results for P3 are shown in Figure 3.3.

In this case, advanced disease was followed by a positive response to therapy and gradual tumour regression.

A cursory examination of Figure 3.3 shows that serial determination of total serum NANA is in no way indicative of the extent of prostatic cancer. Dramatic decreases in total serum NANA levels were observed between weeks 1-6, 12-20 and 22-31.

However, dramatic increases also occurred between weeks 8-12 and 20-22. These increases were obviously not tumour-associated as the tumour was actually regressing during these times. Results showed no particular trend, with total serum NANA levels fluctuating, within the normal range, between 1250 nmoles/ml and 2250 nmoles/ml.

Results for P4 are shown in Figure 3.4.

This patient initially had advanced disease with metastasis. At first he responded well to therapy, but later had a relapse and died.

Serum NANA levels fluctuated erratically, within the normal range, during the course of the seven month study. There was no consistent pattern or trend. Decreases in NANA levels were observed between weeks 1-5, 15-19 and 23-27, whereas levels were seen to increase between weeks 5-15 and 19-23. A gradual increase in NANA levels would have been expected, to coincide with progression of the disease. This did not occur. In fact, towards the end of the study, serum NANA levels were approaching, albeit erratically, values towards the lower end of the normal range. This was at a time when the state of health of the patient was rapidly deteriorating. Serial determination of total serum NANA gave no indication or warning of impending death.

Results for P5 are shown in Figure 3.5.

In this case the patient, who initially had advanced disease, later responded very well to therapy.

Between weeks 1 and 8 there was a considerable decrease observed in total serum NANA levels, from 1950 nmoles/ml to 1450 nmoles/ml. The levels then increased to a level of 2000 nmoles/ml after 18 weeks. After this time, total serum NANA levels gradually decreased. At week 30 (final sample), a value of 1800 nmoles total NANA per ml of serum was obtained. At no point during the 30 week investigation did total serum NANA levels go outside the normal range. There was no decrease in NANA to coincide with the improvement in the state of health and well-being of the patient. The levels actually increased over a 10 week period (weeks 8-18) despite an improvement in the patient's status during that time. As tumour burden was actually decreasing between weeks 8 and 18 it is possible to state with certainty that the observed increase in total serum NANA was not tumour-associated. Therefore, for this patient total serum NANA levels were not indicative of the extent of tumour burden.

Results for P6 are shown in Figure 3.6.

This patient suffered initially from advanced disease but later responded to therapy and improved.

Between weeks one and seven there was a dramatic decrease in total serum NANA levels, from 2400 nmoles/ml to 1500 nmoles/ml. There was only a slight increase in NANA levels between weeks 7 and 17. The serum NANA content then increased by 500 nmoles/ml between weeks 17 and 21. Four weeks later (week 25:final sample) a value of

1950 nmoles/ml was obtained

The presence of advanced disease was reflected in a high serum NANA level (2400 nmoles/ml). However, it must be noted that this value is still within the normal range (upper limit is 2525 nmoles/ml). As the patient improved there was a dramatic decrease in total serum NANA levels up to week 7. The patient continued to improve from week 7 until the end of the observation period (week 25). However, this further improvement in the health of the patient was not reflected in total serum NANA levels, which were actually seen to increase.

Results for P7 are shown in Figure 3.7.

This patient initially had advanced disease with distant metastasis. There was no response to therapy and the unfortunate patient died later.

The initial level of total serum NANA was extremely high (3550 nmoles/ml at week 1). This value was over 1000 nmoles/ml higher than the upper limit of the normal range (2525 nmoles/ml). After 3 weeks the value increased still further to 4200 nmoles NANA per ml of serum. While there was a decrease in NANA levels between weeks 3 and 9, the values were still extremely high (week 5: 4000 nmoles/ml, week 9: 3950 nmoles/ml). From week 9 until the end of the study (week 23) there was a gradual increase in total serum NANA levels. The value obtained for week 23, the final sample, was 4500 nmoles/ml.

In this case there was very good correlation between tumour burden and total serum NANA

levels. All values obtained were very high. There was also an increase in NANA levels as the patient's condition deteriorated (weeks 9-23). Therefore, for this patient, total serum NANA was a very good indicator of tumour burden.

Results for P8 are shown in Figure 3.8.

This patient initially suffered from advanced disease. He later responded to therapy and his clinical status improved significantly. At the last examination (week 18) the patient was found to be completely disease free.

Between weeks 1 and 5, total serum NANA levels were elevated, but constant (week 1: 2650 nmoles/ml, week 2: 2600 nmoles/ml, week 5: 2650 nmoles/ml). Between weeks 5 and 10, total serum NANA levels decreased dramatically from 2650 nmoles/ml to 1950 nmoles/ml. NANA levels continued to decrease, giving a value of 1450 nmoles/ml at the end of the study (week 18: patient free of disease).

For this patient, total serum NANA was found to be an excellent indicator of tumour burden. Initially the levels were high (above the upper limit of the normal range), coinciding with serious advanced cancer of the prostate. As the clinical status of the patient improved the levels of serum NANA decreased dramatically. By the time the patient was diagnosed as being completely free of disease, total serum NANA levels were approaching the lower limit of the normal range.

Results for P9 are shown in Figure 3.9.

This patient initially had advanced disease with metastasis, but later responded well to therapy. However, by the end of the study (week 13) the patient was no longer responding to therapy.

Weeks 1 and 3 gave total serum NANA levels of 1150 nmoles/ml and 1050 nmoles/ml, respectively. Weeks 5, 7 and 9 gave higher levels, ranging from 1350 nmoles/ml to 1550 nmoles/ml. Between weeks 9 and 11 total serum NANA levels dropped dramatically, but were found to be on the increase again at week 13 (end of study).

Total serum NANA levels did not correlate well with tumour burden. Levels approaching (and going below) the lower limit of the normal range (1117 nmoles/ml) were obtained for weeks 1 and 3, at a time when the patient had advanced disease. Total serum NANA levels, while within the normal range, were actually elevated at a time when the patient was responding to therapy. Only towards the end of the study was there any degree of correlation between tumour burden and NANA levels. A dramatic decrease was observed between week 9 (1450 nmoles/ml) and week 11 (800 nmoles/ml), coinciding with a further improvement in the state of health of the patient. By the end of the study the patient was showing symptoms of therapy failure. This was reflected in increased serum NANA levels.

The extent of disease varied considerably from advanced cancer initially, to reduced tumour burden, and finally to symptoms of a return to a more serious condition. This clinical progression was not reflected in total serum NANA

levels. These results especially demonstrate the unsuitability of using an isolated, individual determination of serum NANA as an indicator of tumour burden. The low levels of NANA obtained for weeks 1 and 3 would indicate that the patient was not seriously ill, whereas other clinical parameters had shown the presence of advanced disease with metastasis.

Results for P10 are shown in Figure 3.10.

This patient initially suffered from advanced cancer of the prostate with metastasis. The disease later stabilised and had not progressed at the time of the last examination (week 11).

Total serum NANA levels were extremely high for weeks 1 and 3 (3550 nmoles/ml and 3800 nmoles/ml, respectively), when compared with the upper limit of the normal range (2525 nmoles/ml). The levels decreased somewhat for week 5 (3000 nmoles/ml) and week 8 (3150 nmoles/ml), but were still considerably elevated. Week 10 showed a dramatic decrease in serum NANA, giving a value of 1950 nmoles/ml (a drop of 1200 nmoles/ml).

In this case there was some correlation between tumour burden and total serum NANA, as advanced metastatic disease coincided with highly elevated NANA levels. It was only towards the end of the study that NANA levels were within the normal range (week 10: 1950 nmoles/ml, week 11: 2200 nmoles/ml). However, these results must be interpreted with extreme caution. The patient was diagnosed as having advanced disease with metastasis, which later stabilised and showed no signs of any further progression. Total serum NANA levels, if used as an indicator of tumour

burden, would have indicated a more optimistic, and in this case incorrect, prognosis. NANA levels fell dramatically, from values well above the upper limit of the normal range, to values within that range, at a time when the disease had merely stabilised. Total serum NANA obtained would suggest that the disease had not merely stabilised, but significantly regressed.

3.4

DISCUSSION

Table 3.11 shows the degree of correlation between total serum NANA levels and extent of prostatic cancer tumour burden for all the patients studied.

This experiment, namely, the evaluation of total serum NANA as a marker for cancer of the prostate, produced conflicting results. It was not possible to give a definite answer concerning its clinical significance.

Patients P1, P2, and P8 all had identical disease profiles. All initially had advanced disease. Similarly, they all later responded well to therapy and were deemed to be completely free of disease at the last clinical examination. In the case of patient P1, total serum NANA levels hardly changed at all, despite dramatic changes in the extent of disease. However, patients P2 and P8 both showed very good correlation between serial NANA determination and response to therapy. In both cases there was a definite downward trend observed in serum NANA levels as the state of health of the patients improved, thus suggesting that NANA could be

of use as a monitor of response to therapy. Patients P3, P5 and P6 also had similar clinical histories, with good response to therapy resulting in gradual disease regression. Patient P5 showed no downward trend in total serum NANA levels to coincide with tumour regression. The levels were actually seen to increase consistently, over a ten week period, during which time the patient was actually improving. This trend, of elevated serum NANA levels coinciding with decreasing tumour burden, was also observed in patient P6. For patient P3, total serum NANA levels fluctuated, within the normal range, and showed very poor correlation with extent of disease.

Patients P4 and P7 could also be grouped together as both eventually succumbed to the disease. Results obtained for P7 strongly indicated that total serum NANA was a very good indicator of tumour burden. NANA levels were extremely high from the start and continued to rise still further as the disease progressed. These findings were totally contradicted by the results obtained for patient P4, where NANA levels fluctuated erratically within normal range values. There were no elevated NANA levels to coincide with the initial severely advanced disease. Neither was there a consistent increase in serum NANA levels as the patient suffered a relapse and approached death. NANA levels indicated that this patient was, at the very least, stable.

Patient P10 stood alone in that he suffered from metastatic disease which remained stable, that is, there was no further progression. However,

total serum NANA levels indicated a completely different scenario, namely that the patient was undergoing a dramatic improvement. Whereas the extent of tumour burden remained constant, the levels of total serum NANA fell dramatically.

Despite these contradictory results, this experiment did produce some definite findings. One such finding is that individual isolated total serum NANA levels must be interpreted with extreme caution. A possible role for NANA in relation to prostatic cancer is the use of serial determination of total serum NANA levels as a tenuous indicator of response to therapy, and only if used in conjunction with other clinical parameters. No clinical or therapeutic decisions should be made solely on the strength of a sialic acid determination. NANA lacks the sensitivity required for screening purposes, that is, the detection of early stages of tumour development. It is also clearly lacking in specificity, as results obtained for patients P3, P5, P6 and P9 were indicative of a typical acute-phase reaction, where elevations in total serum NANA levels could not be attributed to the extent of prostatic tumour burden. Another consistent trend observed in this experiment was that elevated serum NANA levels were always present in cases of severely advanced disease.

Finally, the results showed one other interesting trend which may be of clinical significance. In 6 of the 7 patients (86%) where initial NANA levels were within the normal range, there was very poor correlation between serial NANA determination and prostate tumour burden (P1, P3, P4, P5, P6 and P7), with only one patient (P2) showing good correlation. However, in the 3

cases (P7, P8 and P10) where initial NANA levels exceeded the upper limit of the normal range, patients P7 and P8 (67%) were found to have excellent correlation between tumour burden and NANA levels.

3.5

CONCLUDING COMMENTS

Based on the results obtained in this experiment, it is possible to state the following:

Individual isolated NANA determinations do not necessarily reflect the extent of tumour burden in patients with cancer of the prostate.

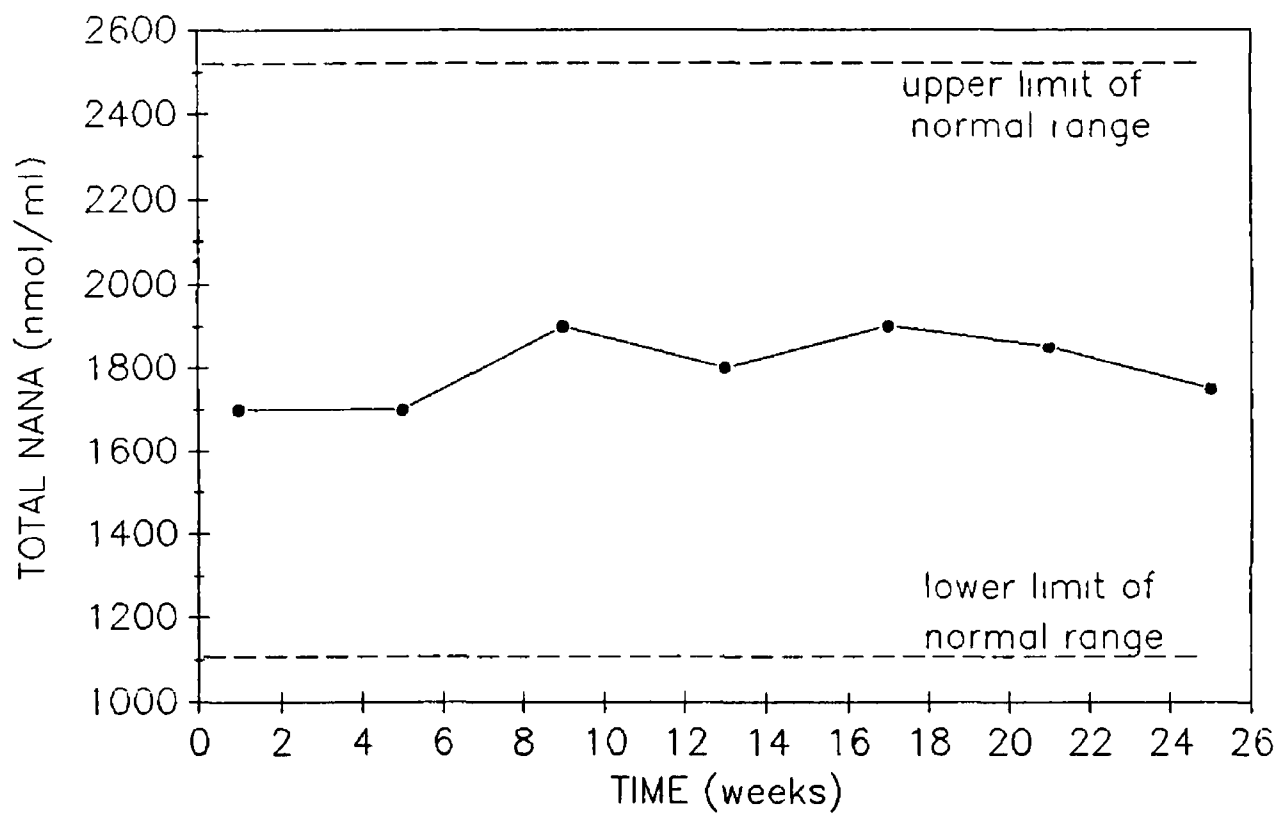
Total serum NANA levels are dramatically elevated in cases of advanced metastatic disease.

Total serum NANA lacks the specificity necessary for its use as a marker of tumour burden, and shows properties of an acute-phase reactant. Elevated serum NANA levels are observed in cases where the cause (or causes) are clearly not directly associated with prostatic tumour burden.

Total serum NANA lacks the sensitivity required for use as a screening assay for cancer of the prostate.

NANA, as an indicator of tumour burden, is at its most useful when initial NANA levels exceed the upper limit of the normal range. This suggests a possible role for serial determination of total serum NANA levels as a means of monitoring patient response to therapy, if used in conjunction with other recognised clinical parameters.

FIGURE 3 1
CHANGES IN TOTAL SERUM NANA WITH TIME



W 43-17
+

FIGURE 3 2
CHANGES IN TOTAL SERUM NANA WITH TIME

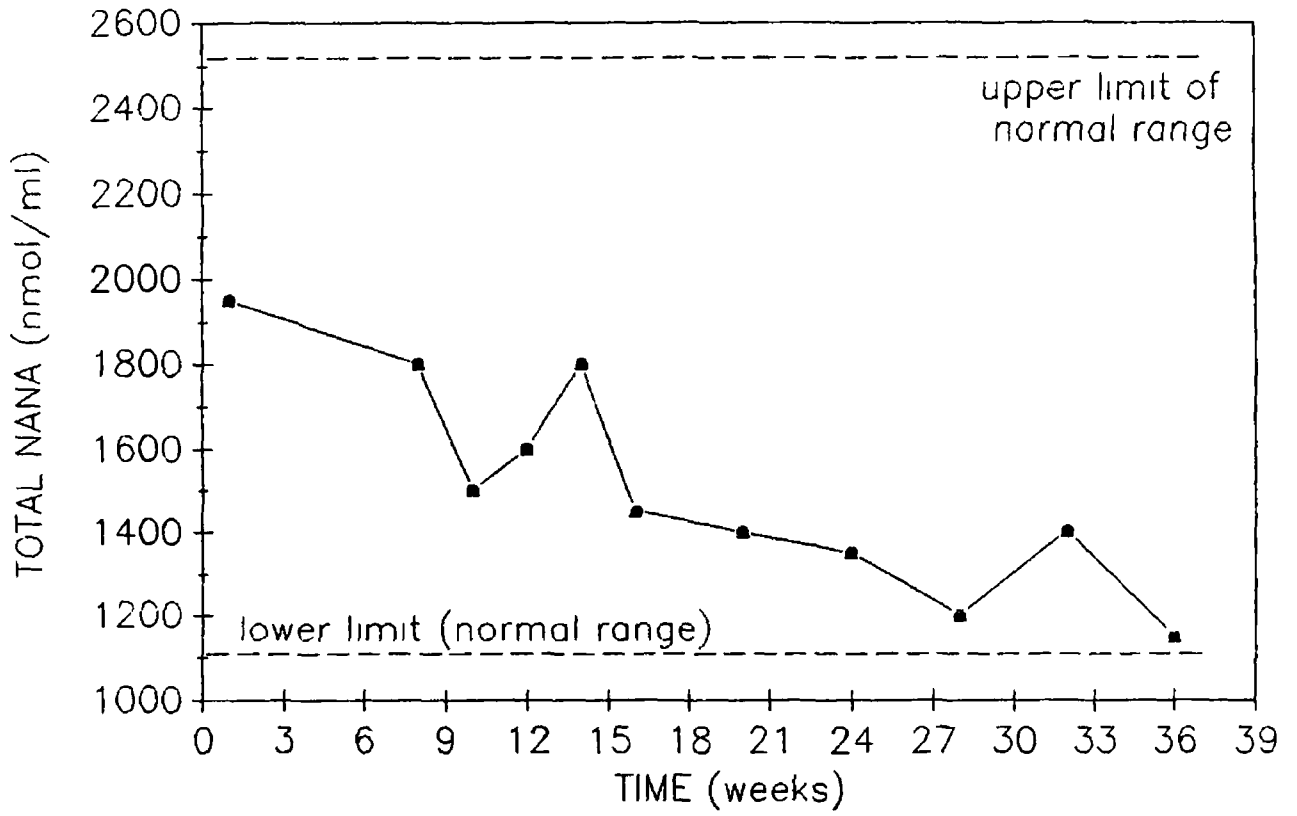


FIGURE 3 3
CHANGES IN TOTAL SERUM NANA WITH TIME

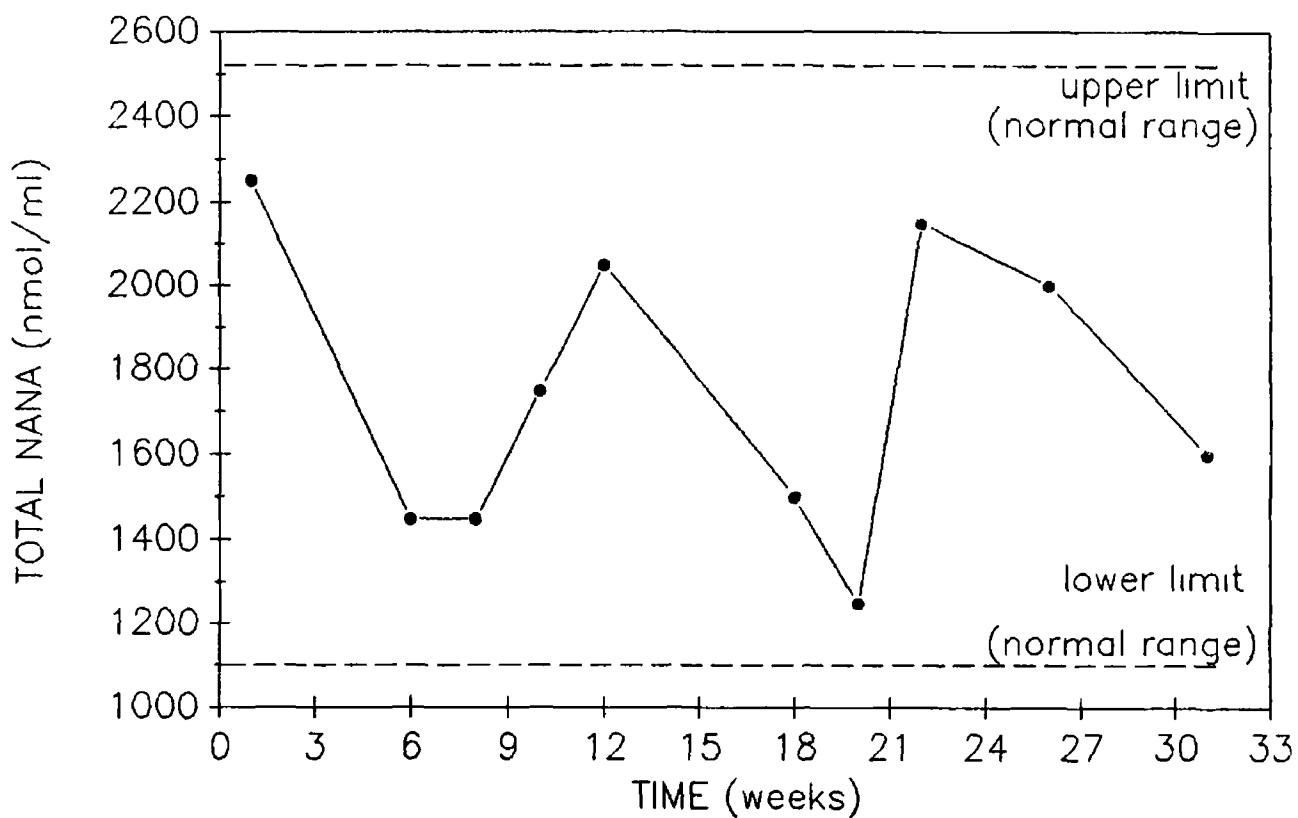


FIGURE 3 4

CHANGES IN TOTAL SERUM NANA WITH TIME

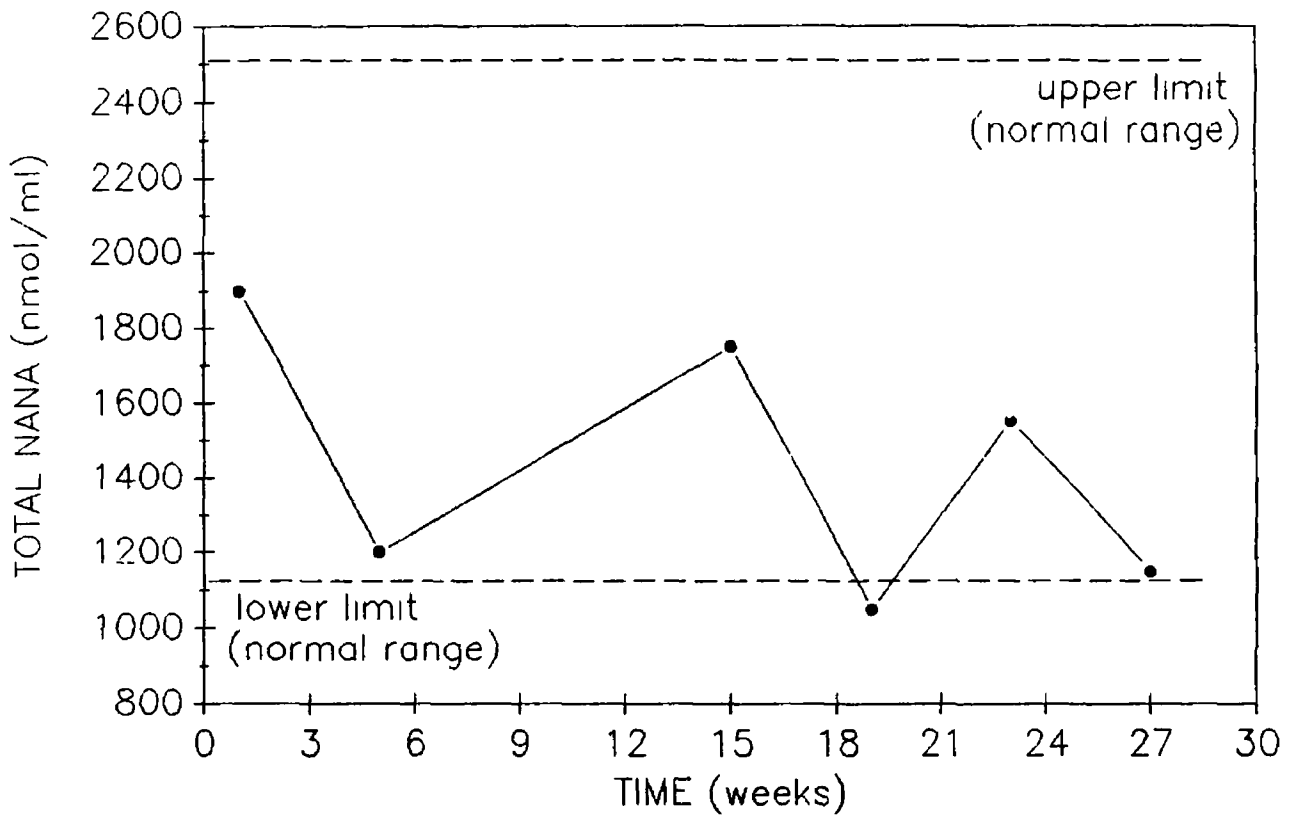


FIGURE 3 5

CHANGES IN TOTAL SERUM NANA WITH TIME

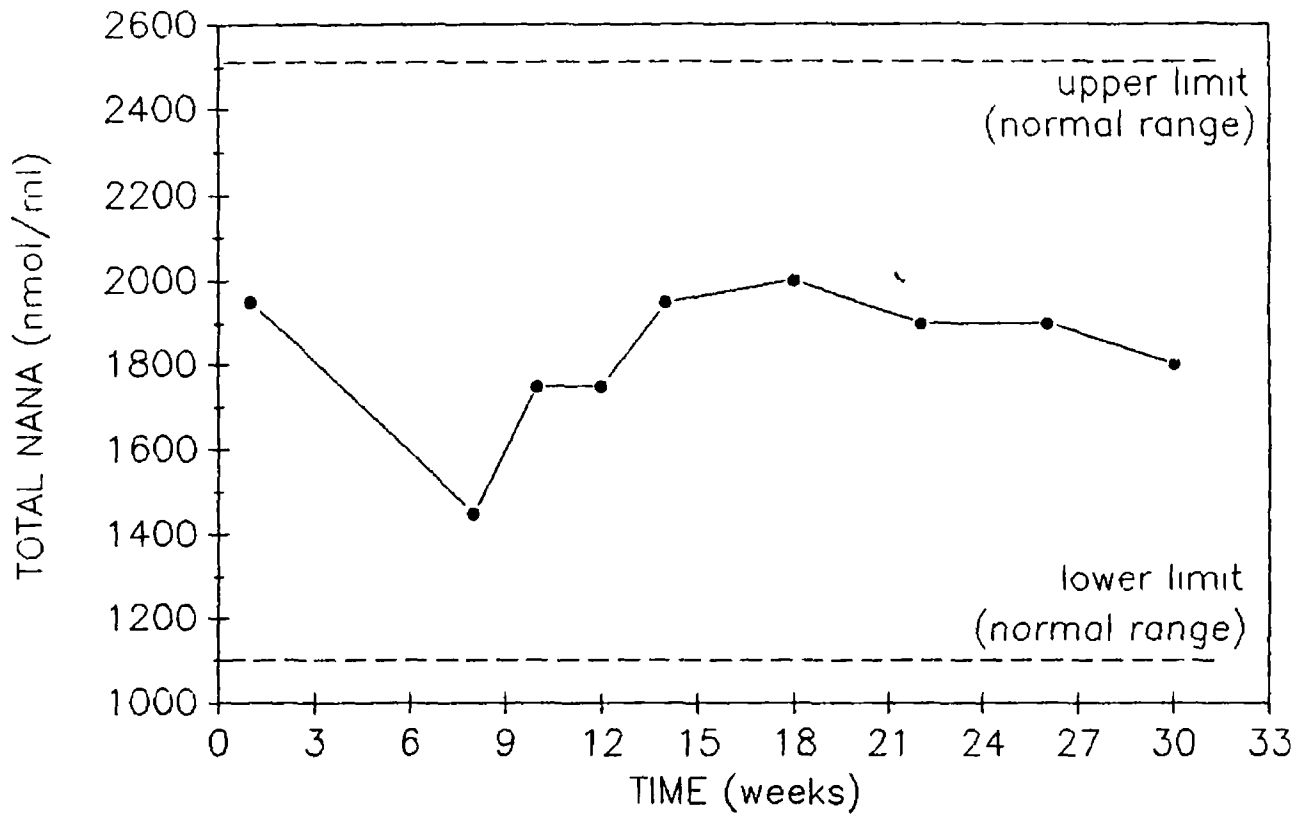


FIGURE 3 6
CHANGES IN TOTAL SERUM NANA WITH TIME

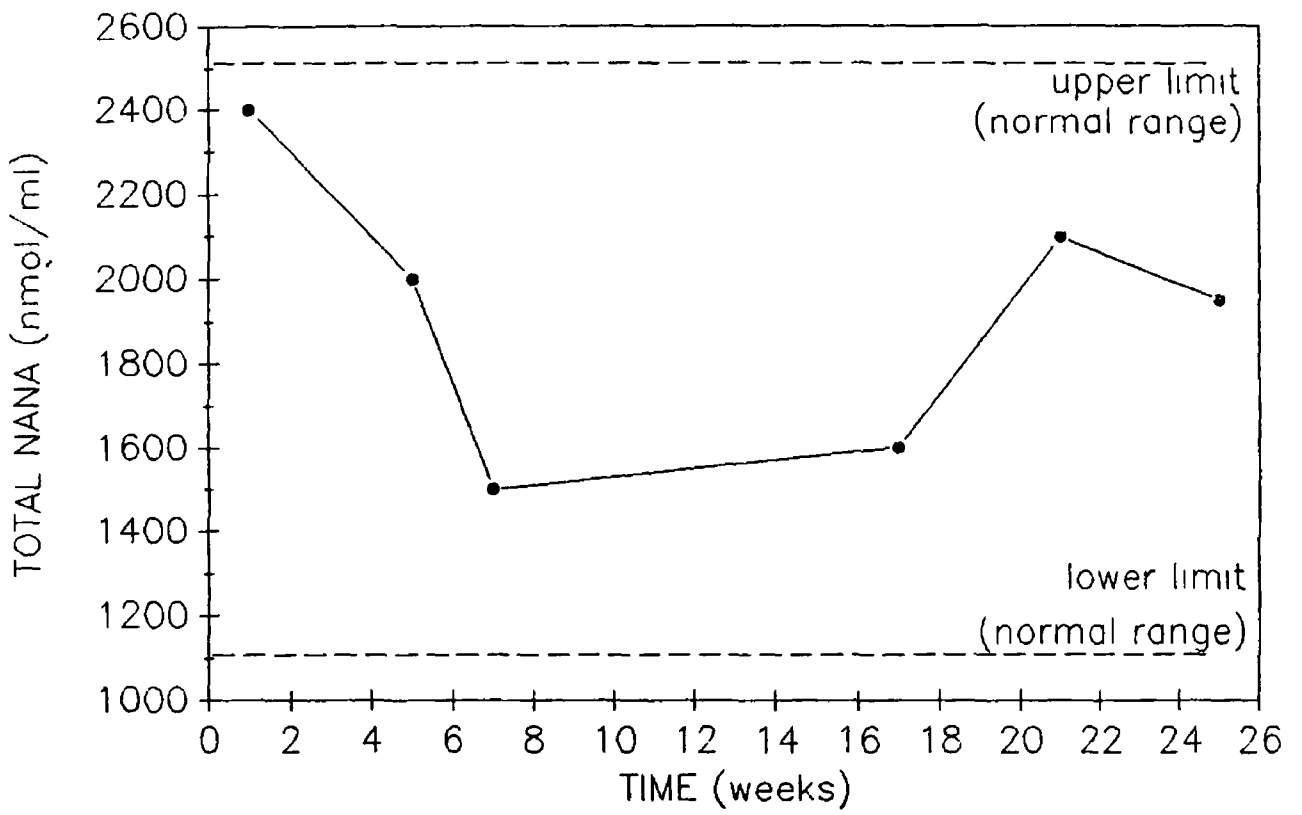


TABLE 3 7

CHANGES IN TOTAL SERUM NANA WITH TIME

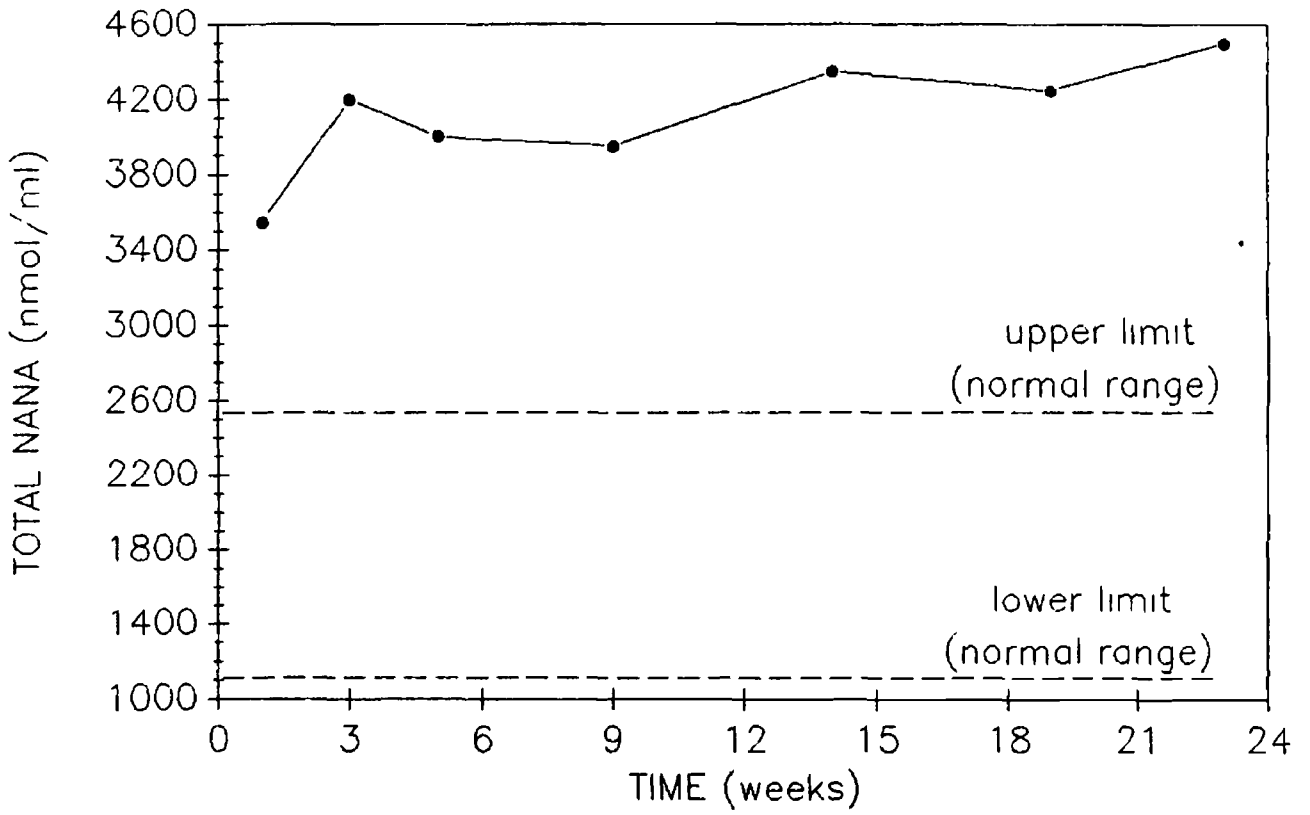


FIGURE 3 8

CHANGES IN TOTAL SERUM NANA WITH TIME

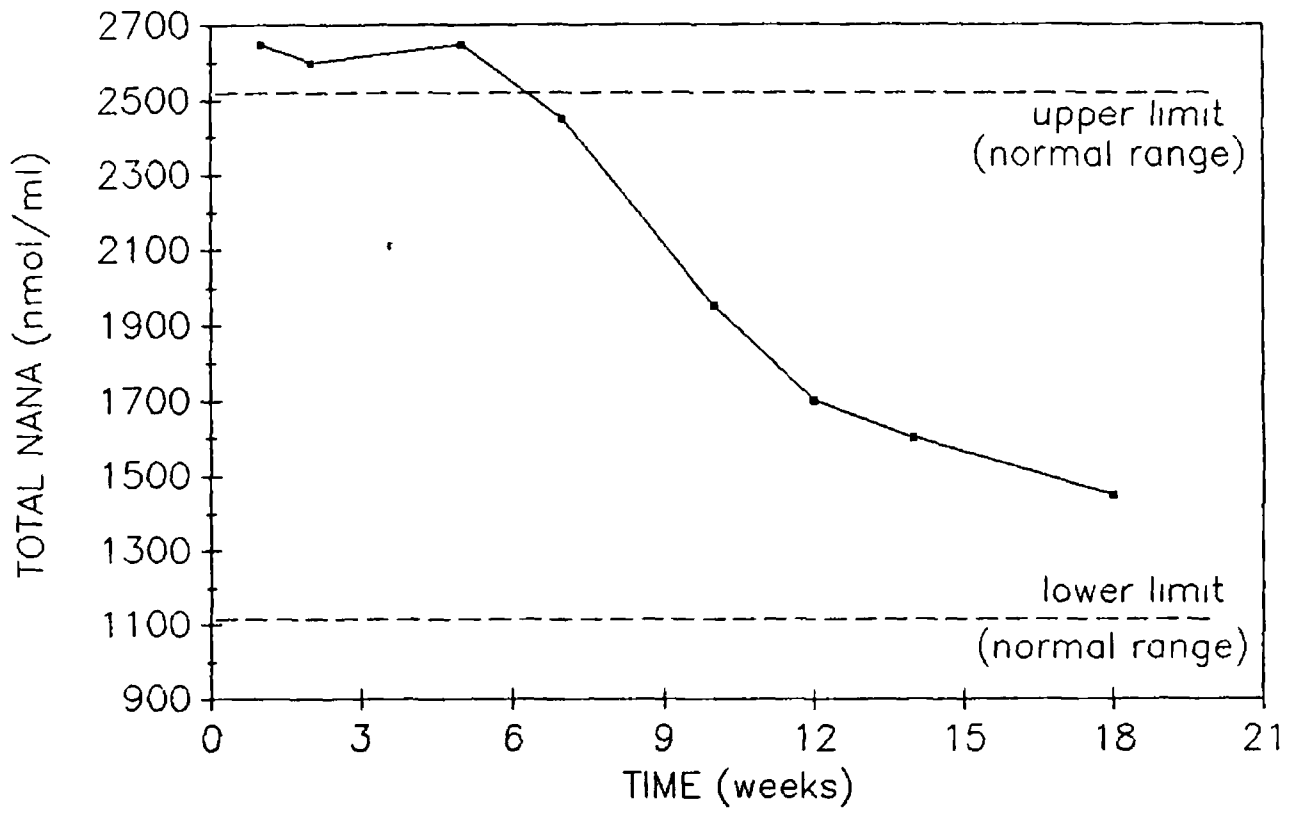


FIGURE 3 9

CHANGES IN TOTAL SERUM NANA WITH TIME

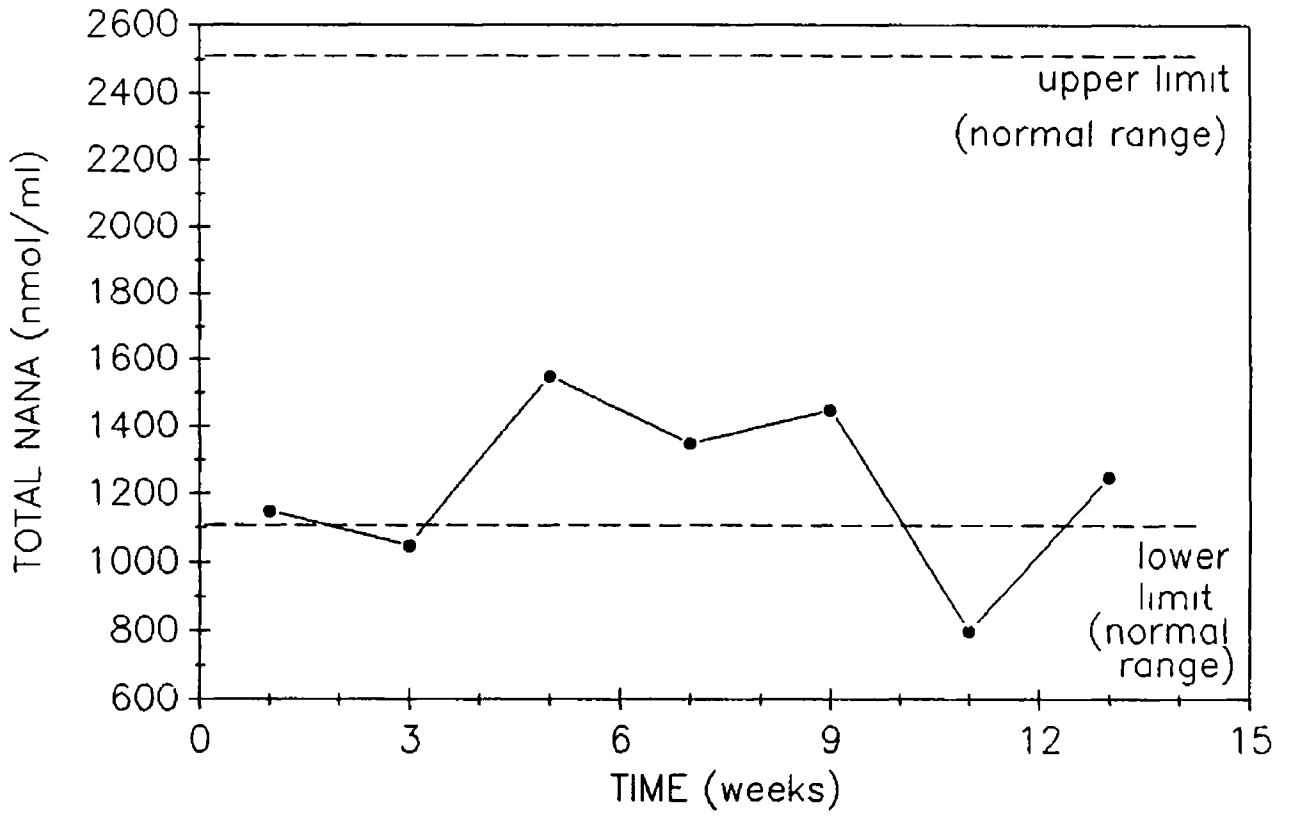
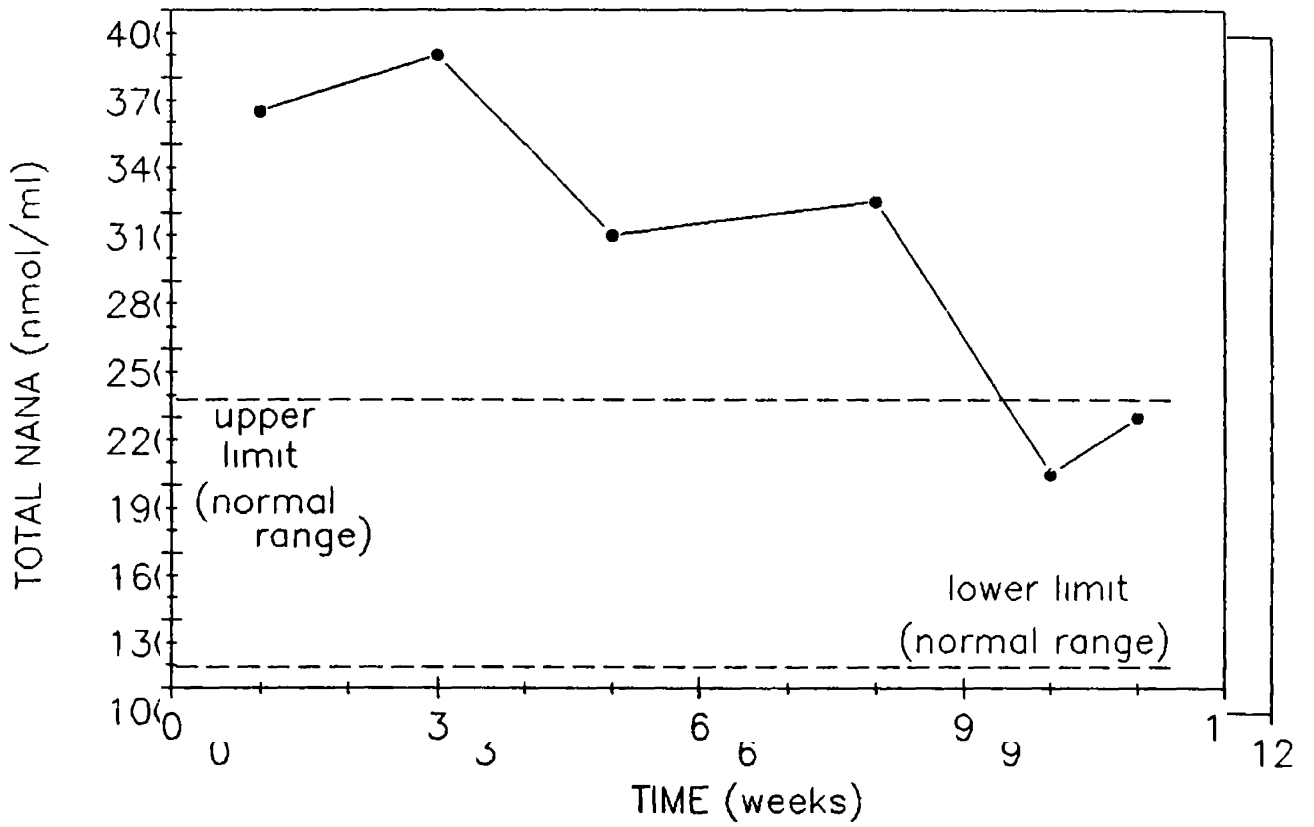


FIGURE 3 10

CHANGES IN TOTAL SERUM NANA WITH TIME



SECTION 3

APPENDIX

TABLES 3.1 - 3.11

TABLE 3 1

PATIENT

P1

CLINICAL HISTORY : Patient responded well to therapy. Disease free at last clinical examination (week 25)

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> nmoles/ml |
|------------------------|--------------------------------------|
| 1 | 1700 |
| 5 | 1700 |
| 9 | 1900 |
| 13 | 1800 |
| 17 | 1900 |
| 21 | 1850 |
| 25 | 1750 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 2

PATIENT

P2

CLINICAL HISTORY .Patient responded well to therapy. Disease free at last clinical examination (week 36).

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 1950 |
| 8 | 1800 |
| 10 | 1500 |
| 12 | 1600 |
| 14 | 1800 |
| 16 | 1450 |
| 20 | 1400 |
| 24 | 1350 |
| 28 | 1200 |
| 32 | 1400 |
| 36 | 1150 |

SERUM NANA NORMAL RANGE.

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 3

PATIENT : P3

CLINICAL HISTORY: Patient responded well to therapy and showed gradual disease regression.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 2250 |
| 6 | 1450 |
| 8 | 1450 |
| 10 | 1750 |
| 12 | 2050 |
| 18 | 1500 |
| 20 | 1250 |
| 22 | 2150 |
| 26 | 2000 |
| 31 | 1600 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 4

PATIENT

P4

CLINICAL HISTORY . Patient initially responded well to therapy, but later had a relapse and died.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 1900 |
| 5 | 1200 |
| 15 | 1750 |
| 19 | 1050 |
| 23 | 1550 |
| 27 | 1150 |

SERUM NANA NORMAL RANGE:

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 5

PATIENT

P5

CLINICAL HISTORY Patient responded very well to therapy.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 1950 |
| 8 | 1450 |
| 10 | 1750 |
| 12 | 1750 |
| 14 | 1950 |
| 18 | 2000 |
| 22 | 1900 |
| 26 | 1900 |
| 30 | 1800 |

SERUM NANA NORMAL RANGE:

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 6

PATIENT . P6

CLINICAL HISTORY: Patient initially had very bad metastatic disease, but later improved

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> <u>(weeks)</u> | <u>TOTAL SERUM NANA</u> <u>(nmoles/ml)</u> |
|-------------------------------|---|
| 1 | 2400 |
| 5 | 2000 |
| 7 | 1500 |
| 17 | 1600 |
| 21 | 2100 |
| 25 | 1950 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525nmoles/ml

TABLE 3.7

PATIENT

P7

CLINICAL HISTORY

Severe metastatic disease

No response to therapy

Patient died.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 3550 |
| 3 | 4200 |
| 5 | 4000 |
| 9 | 3950 |
| 14 | 4350 |
| 17 | 4250 |
| 23 | 4500 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525 nmoles/ml

TABLE 3.8

PATIENT

P8

CLINICAL STATUS Patient initially suffered from severe metastatic disease, but later responded to therapy. Disease free at time of last examination.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 2650 |
| 2 | 2600 |
| 5 | 2650 |
| 7 | 2450 |
| 10 | 1950 |
| 12 | 1700 |
| 14 | 1600 |
| 18 | 1450 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 9

PATIENT

.P9

CLINICAL STATUS

:Severe metastatic disease initially. Later responded well to therapy. Patient was not responding to therapy at end of study.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 1150 |
| 3 | 1050 |
| 5 | 1550 |
| 7 | 1350 |
| 9 | 1450 |
| 11 | 800 |
| 13 | 1250 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 10

PATIENT .P10

CLINICAL STATUS : Patient initially had severe advanced disease but later stabilised and had not progressed at time of final examination.

SERIAL DETERMINATION OF TOTAL SERUM NANA

| <u>TIME</u> (weeks) | <u>TOTAL SERUM NANA</u> (nmoles/ml) |
|------------------------|--|
| 1 | 3550 |
| 3 | 3800 |
| 5 | 3000 |
| 8 | 3150 |
| 10 | 1950 |
| 11 | 2200 |

SERUM NANA NORMAL RANGE

1117 nmoles/ml-2525 nmoles/ml

TABLE 3 11

DEGREE OF CORRELATION BETWEEN TOTAL SERUM NANA AND TUMOUR BURDEN

| PATIENT | DEGREE OF CORRELATION |
|---------|-----------------------|
| P1 | * |
| P2 | * * * * |
| P3 | * |
| P4 | * |
| P5 | * |
| P6 | * |
| P7 | * * * * * |
| P8 | * * * * * |
| P9 | * |
| P10 | * * |

Symbols used to represent degree of correlation represent the following

| SYMBOL | DEGREE OF CORRELATION |
|-----------|-----------------------|
| * | POOR |
| * * | REASONABLE |
| * * * | GOOD |
| * * * * | VERY GOOD |
| * * * * * | EXCELLENT |

SECTION 4

**A Critical Evaluation of Serum and Urinary Sialic Acid as
Indicators of Tumour Burden in Human Patients with
Cancer of the Bladder.**

4.1

INTRODUCTION

The aim of this experiment was to evaluate serum and urinary total NANA as indicators of tumour burden in human patients with bladder cancer, an aggressive disease which, in the United States of America alone, can claim as many as ten thousand lives each year (Javadpour et al., 1984). One of the first symptoms of bladder cancer is blood in the urine, a condition known as haematuria. Unfortunately, in many cases the disease will be significantly advanced before haematuria occurs. This factor, combined with the limitations of other available clinical, roentgenographic and laboratory methods in the diagnosis of urological cancers, or in the determination of the precise extent of disease, have lead to the search for alternative tumour markers. Ideally the tumour marker, present in serum or urine, should be capable of detecting early stages of tumour development, thus allowing its use as a method of cancer screening. As elevated serum NANA levels have been associated with other types of cancer it was decided that serum and urinary NANA should be evaluated as possible markers for bladder carcinoma.

It had been found that elevated NANA levels are not specific for any one type of cancer. Lodha et al. (1979) found highly significant elevations in serum NANA levels in a variety of malignant neoplastic conditions. Erbil et al. (1986), in their study of genitourinary malignancies (including bladder cancer), found that serum protein-bound NANA and LSA levels correlated very

well with stage and grade in patients with advanced disease, and therefore had potential as indicators of response to therapy. However, the suitability of NANA for use as a method of screening has been questioned. Erbil et al. (1985), who evaluated serum NANA and LSA levels as markers for colorectal cancer, found that they both lacked the sensitivity and specificity required for screening purposes. Silver et al. (1978), who studied serum NANA levels in malignant melanoma patients, also found that NANA lacked the sensitivity required for cancer screening, reflected in a high incidence (36%) of false negative results.

In this evaluation of serum and urinary total NANA as markers for bladder cancer, the following questions were being asked:

1

Is the tumour burden of a patient with bladder cancer, at a specific time, reflected in an isolated determination of total serum or urinary NANA?

2

Is the presence of advanced bladder cancer accompanied by significantly elevated levels of serum and urinary NANA levels?

3

Can total serum or urinary NANA be used as a marker for the presence of small tumour volume, thus allowing their use as methods of bladder cancer screening?

4.2

EXPERIMENTAL DESIGN

Five healthy volunteers were used as controls. Three subject groups were studied. The first group contained 13 patients who previously had bladder cancer but had no clinical or endoscopic evidence of disease at the time of study. The second group consisted of 7 patients who previously had bladder cancer and had small volume, superficial tumour recurrence amenable to transurethral fulguration or resection at the time of study. The third group contained 6 patients with newly diagnosed tumours that were at least invasive of muscle. Samples of blood and urine were collected preoperatively. NANA levels were determined by the Warren assay (Section 2.3.4).

4.3

STASTICAL ANALYSIS

Normal levels of serum and urinary total NANA were defined using the mean levels obtained from 5 healthy volunteers. The two-sample Student's t-Test (Section 2.8.1) was used to compare normal levels of both serum and urinary NANA with the mean values of each of the three tumour groups.

4.4

DISCUSSION

Results are shown in Table 4.1.

No significant differences were found in either serum or urinary mean NANA levels between the control group and those who previously had cancer but who were free of disease at the time of study (tumour group 1). In the second tumour group,

where patients previously had bladder cancer and now had small volume, superficial (non-muscle invasive) disease, it was again found that no significant elevation in serum or urinary NANA occurred, when compared to normal levels. Even in cases of advanced muscle invasive disease (final tumour group), it was only in serum NANA levels that significant elevations occurred ($P < 0.02$). While urinary NANA levels were also elevated above normal levels, the observed increases were not statistically significant ($P < 0.2$).

4.5

CONCLUDING COMMENTS

Based on the results obtained in this experiment it is possible to state the following:

1

Tumour burden is not reflected in either serum or urinary NANA levels in cases of small volume, superficial bladder carcinoma.

2

Serum and urinary NANA levels are elevated, when compared to normal levels, in cases of advanced bladder carcinoma, with significant increases occurring only in serum levels.

3

As both both serum and urinary NANA levels lack the sensitivity required to detect small tumour burden, both markers are therefore unsuitable for use either as a method of cancer screening, or as a method for detecting small volume, recurrent tumours.

4

Significantly elevated levels of serum total NANA, in cases of advanced disease, suggest that this marker may be suitable as a supplementary parameter in the follow-up of high stage bladder cancer, particularly with regard to the assessment of response to, and efficacy of, therapeutic procedures.

TABLE 4.1

SERUM AND URINARY MEAN NANA LEVELS IN NORMAL CONTROLS AND PATIENTS WITH CANCER OF THE BLADDER

| TUMOUR MARKER | EXP. GROUP | SAMPLE SIZE | NANA (nmoles/ml) MEAN +/- S.D. | CRITICAL VALUE | (V) |
|---------------|------------|-------------|--------------------------------|----------------|------|
| SERUM NANA | CONTROL | 5 | 1670.0 +/- 375.2 | (-) | (-) |
| | TUMOUR (1) | 13 | 1857.7 +/- 314.1 | 1.07 | (16) |
| | TUMOUR (2) | 7 | 1914.3 +/- 587.2 | 0.81 | (10) |
| | TUMOUR (3) | 5 | 2400.0 +/- 316.2 | 3.34* | (8) |
| URINARY NANA | CONTROL | 5 | 184.0 +/- 98.9 | (-) | (-) |
| | TUMOUR (1) | 13 | 213.8 +/- 71.1 | 0.71 | (16) |
| | TUMOUR (2) | 7 | 191.4 +/- 76.0 | 0.15 | (10) |
| | TUMOUR (3) | 6 | 326.7 +/- 147.7 | 1.82 | (9) |

S.D. = Standard Deviation

V represents the number of degrees of freedom for a particular t-Test and is determined by the sample size, where $V = n_1 + n_2 - 2$.

Only one significant difference found.

* : significant difference ; $P < 0.02$

SECTION 5

**The Evaluation of Total Serum Sialic Acid
and Lipid-Bound Serum Sialic Acid as
Indicators of Tumour Burden in Mice
Bearing the Landschutz Ascites Tumour.**

5.1

INTRODUCTION

The aim of this investigation was to evaluate the potential use of total serum NANA and serum LSA as indicators of tumour burden in LAT-bearing mice. To achieve this, four separate experiments were performed. Three involved the measurement of total serum NANA levels while one measured serum LSA levels.

Erbil et al. (1985) suggested that increased levels of total serum NANA found in the sera of patients with tumours are due to an increased release and breakdown of sialoglycoconjugates from tumour cell membranes. Debray et al. (1986) showed that there is an increase in highly branched oligosaccharides in transformed human tumour cells. Miller et al. (1963), who compared the NANA content of the mouse erythrocyte with that of the murine Ehrlich ascites tumour (EAT) cell, found that the density of NANA on the EAT cell surface was approximately four times that on the erythrocyte. It was our aim to discover if a similar phenomenon occurred in the LAT cell, that is, increased synthesis of surface NANA, reflected in elevated levels of total serum NANA.

It has been suggested that serum LSA might be a more useful and discriminating marker for tumour detection than total serum NANA. The potential use of LSA as an indicator of tumour burden has been evaluated in a range of animal model systems. Skipski et al. (1975) studied serum LSA levels in rats bearing Morris hepatoma. The presence of tumour was accompanied by

qualitative and quantitative changes in ganglioside patterns. Poli et al. (1986), who evaluated serum LSA as an indicator of tumour burden in dogs, found good correlation between effectiveness of therapy and LSA measurements. However, elevated serum LSA levels were also associated with chronic inflammatory and degenerative disease, thus implicating LSA as a possible acute-phase reactant. Kloppel et al. (1981) studied serum LSA levels in horses with neoplasms. Their results showed that LSA could be used to detect neoplastic growth and also to monitor response to treatment. The specificity (% true negatives) of the test was high (91%), with false positive results occurring only in cases of acute infections. Kloppel et al. (1977) also studied serum LSA levels in mice bearing transplantable mammary carcinomas. The presence of tumour was associated with elevated NANA levels, with the elevations occurring principally in the ganglioside fraction. Increases in serum LSA levels were often seen to predate visual signs of tumour presence. Surgical removal of tumours was also accompanied by dramatic reductions in serum LSA levels.

Tumour development is not always accompanied by increases in NANA levels. Hakim (1980) studied levels of serum protein-bound, perchloric acid soluble NANA (PA-NANA), in rats bearing a mammary adenocarcinoma. In general, PA-NANA levels increased with tumour burden, and the magnitude of the increase varied with the number of implanted tumour cells. However, they also found that a maximum level of PA-NANA was obtained twenty days after implantation of 10^5 tumour cells, and that levels progressively decreased in animals that lived for longer periods. Saha et

al. (1988) measured LSA levels in the plasma of mice bearing EAT. Plasma LSA gradually increased up to day 6 (6 days after intraperitoneal injection of tumour), but then rapidly declined, to values below normal levels, by day 10. The authors suggested that the observed decrease in LSA could be due to simplification in ganglioside synthesis, combined with a decrease in metabolic activity of the tumour.

5.2

EXPERIMENTAL DESIGN

Four separate experiments, three measuring total serum NANA and one measuring serum LSA, were performed in order to evaluate total NANA and LSA as indicators of LAT burden in an animal model system. The experiments had the following steps in common. Male Schofield mice were used. LAT cells (Section 2.3.1), recovered from liquid nitrogen (Section 2.4.11), were counted (Section 2.4.9) prior to injection. Mice in the tumour-bearing groups received either 1×10^7 or 1×10^5 viable LAT cells (both in 1.0ml PBS). Control groups received 1.0ml of either PBS or Pristane (2, 6, 10, 14-tetramethylpentadecane; an immunostimulant). All injections were intraperitoneal. Blood samples, obtained by tail-bleeding (Section 2.3.2), were taken before and after injection of LAT cells, PBS or Pristane. Total serum NANA levels were determined (experiments 1-3) using the Warren thiobarbituric acid method (Section 2.3.4). The Warren assay was also used to determine serum LSA levels (experiment 4), following extraction of gangliosides by the procedure of Katopodis and

Stock (Section 2.3.5).

The details of each individual experiment can be summarised as follows.

| SERUM MARKER | EXP. NO. | EXPERIMENTAL GROUPS | MATERIAL INJECTED |
|--------------|----------|---|-------------------------------------|
| NANA | 1 | LAT GROUP CONTROL GROUP (1) CONTROL GROUP (2) MICE PER GROUP (6) | 10^7 CELLS PBS PRISTANE |
| NANA | 2 | LAT GROUP (1) LAT GROUP (2) CONTROL GROUP MICE PER GROUP (4) | 10^7 CELLS 10^5 CELLS PBS |
| NANA | 3 | LAT GROUP CONTROL GROUP MICE PER GROUP (5) | 10^7 CELLS PBS |
| LSA | 4 | LAT GROUP CONTROL GROUP MICE PER GROUP (4) | 10^7 CELLS PBS |

5.3

STATISTICAL ANALYSIS

The magnitude of average increases in total serum NANA levels observed in LAT-bearing mice were compared to those of control groups using Student's t-Test (Section 2.8.2). This method of analysis required the monitoring of serum NANA elevations in each mouse.

The two-sample Student's t-Test (Section 2.8.1) was also used to compare normal serum NANA and LSA levels with those obtained after 6 days of LAT growth. Normal levels of NANA and LSA were defined using the mean values obtained from pre-injection samples.

5.4

ANALYSIS OF RESULTS

5.4.1

EXPERIMENT 1

Results are shown in Figure 5.1.

The large number of injected LAT cells (10^7) caused a rapid increase in tumour mass. Therefore, to minimise the distress caused to the animals, the maximum period of NANA monitoring was 11 days. However, even over this short period, it was obvious that NANA levels showed no correlation with tumour burden.

In the LAT tumour-bearing mice, mean NANA levels rose sharply from 1192 nmoles/ml to 1967 nmoles/ml between day -1 (one day before injection of LAT cells) and day 3 (three days after injection). This represents a 65% increase in serum NANA on resting (pre-injection) levels. Initial elevations were also observed in the PBS and Pristane control groups, after 3 days. Statistical analysis of the results showed that highly significant differences existed between the average increase in NANA levels observed in LAT-bearing mice and those which

occurred in both the PBS and Pristane control groups (Table 5.1).

The results obtained in this experiment are typical of those expected for an acute-phase reaction. The initial elevation in total serum NANA, in the tumour group was not caused by the tumour itself since further increases in tumour burden were not accompanied by further elevations in serum NANA. Between day 3 and day 7 there was an obvious increase in tumour burden whereas the level of total serum NANA actually decreased. The initial dramatic peak, observed in LAT-bearing mice, would appear to have been caused by the trauma of the injection process.

5.4.2

EXPERIMENT 2

Results are shown in Figure 5.2.

The results obtained for the first tumour group, where each mouse received 1×10^7 cells, were very similar to those obtained in experiment 1. There was no correlation between total serum NANA and tumour burden. A dramatic increase in mean total serum NANA was again obvious by day 3. The levels rose from 1538 nmoles/ml (day -1) to 2225 nmoles/ml (day 3), representing a 45% increase on resting levels. This increase was followed by an even greater decrease over the next 2 days. The value obtained for day 5 was 1525 nmoles/ml total serum NANA. Therefore, after 5 days of tumour growth, the level of NANA was actually less than that obtained prior to injection of tumour. An initial peak, although small, was also observed in the PBS control group. Analysis of the results showed that a statistically

significant difference did exist between the average increase in serum NANA observed in the LAT group and that which occurred in the PBS control (Table 5.1).

In the second tumour group each mouse received only 1×10^5 cells. The advantage of injecting fewer LAT cells is that tumour burden and associated serum NANA levels can be monitored for almost 4 weeks. As in the previous LAT group, mean serum NANA levels peaked after 3 days, but then fell dramatically after a further 2 days of tumour growth. Mean total serum NANA levels rose from 1338 nmoles/ml (day -1) to 2083 nmoles/ml (day 3), an increase of 56% on resting levels, but then fell to 1688 nmoles/ml by day 5, a decrease of 19% compared to the level of day 3. The increase in serum NANA levels of LAT-bearing mice, between day -1 and day 3, was not significantly different from that which occurred in the control group over the same period (Table 5.1).

A second peak in total serum NANA occurred in the LAT-bearing mice on day 14. However, a similar increase was also observed in the PBS control group on the same day, thus showing that the increase in NANA levels was not directly due to the increased tumour burden.

5.4.3

EXPERIMENT 3

Results are shown in Figure 5.3.

In the tumour-bearing group, mean total serum NANA levels increased from 1760 nmoles/ml (day -1) to 2140 nmoles/ml (day 6), representing an increase of 21.6% over resting levels. However,

an increase of 17.2% in NANA levels was also observed in the PBS control group over the same period. Therefore, the difference in the percentage increases in mean total serum NANA levels from day -1 to day 6, between the two groups, was 4.2%. This means that, in the LAT group, only 4.2% of the observed increase in mean total serum NANA levels can be attributed solely to the extent of tumour burden, with the remaining 95.8% being due to non-tumour associated effects. Statistical analysis, based on the comparison between the average increases in serum NANA, confirmed that no difference existed between the two groups (Table 5.1). However, when the mean level of serum NANA, prior to injection (i.e. normal level), was compared to the mean value after six days of tumour growth, a highly significant difference ($P < 0.001$) was found to exist (Table 5.2).

5.4.4

EXPERIMENT 4

Results are shown in Figure 5.4.

In the tumour-bearing group, mean serum LSA levels rose from 513 nmoles/ml on day -1 to 757 nmoles/ml on day 6. This represents a dramatic increase of 47.6% in mean LSA levels when compared to resting levels, whereas the observed increase in the control group over the same period was only 20.5% (484 nmoles/ml on day -1 to 583 nmoles/ml on day 6). Statistical analysis, based on the comparison between the average increases in LSA levels between day -1 and day 6, showed that no difference existed between the two

groups (Table 5.1), whereas comparison of the mean levels on those days did show a small, but significant increase ($P < 0.02$) in LSA levels (Table 5.2).

No further increases in serum LSA levels were observed in the tumour-bearing mice after day 6, despite an obvious visual increase in tumour burden. A value of 691 nmoles/ml was obtained on day 9, representing a decrease of 8.7% in LSA levels, when compared to the value of day 6. After an additional 2 days of tumour growth the mean LSA level was found to be 690 nmoles/ml, further confirming that serum LSA was not increasing proportionally with tumour burden.

Between days 6 and 9, at a time when mean LSA levels were decreasing in the tumour-bearing mice, the levels were seen to increase in the PBS control group, from 583 nmoles/ml to 625 nmoles/ml, a rise of 7.2% for the three day period. A negligible change in LSA concentration (3 nmoles/ml) was observed in the control group between days 9 and 13.

5.5

DISCUSSION

The principal aim of these experiments was to evaluate serum NANA and serum LSA as indicators of tumour burden in mice bearing LAT. Berra et al. (1986) found that serum NANA concentration was modulated by hormonal conditions, therefore all mice used in these experiments were male. LSA has been extensively studied with regard to its tumour marker potential in a variety of animal model systems. The main emphasis in this investigation was therefore placed on the

evaluation of total serum NANA as an indicator of LAT tumour burden.

The first of the three experiments used to evaluate total serum NANA contained one tumour group (10^7 LAT cells per mouse), and two control groups, PBS and Pristane. The latter (Pristane) is not a true negative control as it occasionally results in tumour formation. Total serum NANA showed no correlation with tumour burden. The rapid rise and fall in NANA levels observed in the LAT group suggested that NANA is an acute-phase reactant, with the dramatic elevation occurring as a result of the actual injection of tumour cells. Bradley et al. (1977) stated that levels of acute-phase reactants rise within 12-36 hours in response to various stimulants, including tissue injury and trauma.

The second experiment used two tumour-bearing groups and one PBS control group. Results for the LAT group, where each mouse was injected with 1×10^7 cells, confirmed the findings of the previous experiment. No correlation was found between total serum NANA and LAT burden. Injection of LAT cells again resulted in a dramatic short-term increase in serum NANA levels. As a result of injecting 1×10^5 cells into each mouse (the second tumour group) it was possible to monitor NANA levels for a longer period (26 days), thus allowing a more thorough evaluation of this possible tumour marker. In marked contrast to the previous group, no difference existed between the increase in NANA levels of LAT-bearing mice and that observed in the control group, the likely cause being that, in this group, fewer LAT cells were injected (10^5 as opposed to 10^7). Further alterations in serum

NANA levels in the LAT group were accompanied by very similar changes in the PBS control group, indicating once again that tumour burden was not being reflected in total serum NANA levels. Finally, after 26 days of LAT growth the mean level of total serum NANA was found to be almost identical to that obtained prior to injection of the tumour.

It was obvious that serum NANA levels were being affected by the injection process, as reflected by dramatic elevations in NANA levels after three days. Consequently, the design of the third (and final) experiment involving total serum NANA was slightly modified. The day three sample was omitted. Mice were injected with either PBS or LAT cells (1×10^7 cells per mouse) and then left undisturbed for six days, at which time serum samples were taken for NANA analysis. After six days of tumour growth, elevation in mean total serum NANA was found to be highly significant ($P < 0.001$), indicating that mean NANA levels are elevated as a result of LAT growth during the early stages of tumour development.

In the experiment used to evaluate serum LSA as a marker for LAT, the day 3 sample was also omitted. Initial results were encouraging, with mean serum LSA levels significantly elevated ($P < 0.02$) after six days of tumour growth. However, this increase was not as dramatic as that observed in serum NANA. Furthermore, LSA levels decreased between days 6 and 9 despite a further increase in tumour burden. This trend in serum LSA levels has been observed elsewhere. Pennington et al. (1988), who studied serum LSA levels in human cancer patients (nature of cancer not specified), found that patients with

exceptionally heavy tumour burdens tended to show declining levels in LSA in the final weeks preceeding death. Therefore, while it has been shown that serum LSA does not increase linearly with tumour burden, it is possible that serial determination of LSA could be of use in monitoring the clinical status of a patient, whereby a reduction in serum LSA levels may be indicative of significantly increased tumour burden.

It might be argued that the observed decreases in mean NANA levels, observed between days 3 and 6 (experiments 1 and 2), were caused to a certain extent by the taking of blood samples, whereby the loss of blood resulted in decreased levels of circulating NANA. However, it is unlikely that the process of tail-bleeding significantly affected NANA (or LSA) levels. For example, in experiment 2, using 10^5 injected LAT cells (Fig. 5.2), total serum NANA levels increased between days 7-11 and 11-14, despite the taking of blood samples on days 7 and 11, respectively. Furthermore, in experiment 4 (Fig. 5.4), the taking of a blood sample on day 9 did not result in decreased serum LSA levels on day 11 (only two days later). Finally, the assays used in these experiments determined serum NANA and LSA concentrations. A reduction in the volume of blood in a mouse (caused by tail-bleeding), while decreasing the total amounts of NANA and LSA present, should not adversely affect their concentrations (the amount of NANA/LSA per unit volume of blood). However, it is possible that tail-bleeding could have a minor effect, whereby the process of regenerating the removed blood may have a short term dilution effect on circulating

levels of NANA and LSA. Therefore, since the overall effect (if any) of the tail-bleeding process on serum NANA and LSA levels is minimal, it is possible to conclude that serum NANA and LSA levels, as determined by the aforementioned assays, accurately reflect the true serum levels of these markers.

5.6

CONCLUDING COMMENTS

Based on the results obtained in these experiments it is possible to state the following:

1.

The rapid increase in total serum NANA in LAT-bearing mice strongly suggests that NANA is produced by the host in response to the tumour's presence in the form of an acute-phase reaction, caused by the trauma of the injection process.

2.

The magnitude of the acute-phase reaction increases with increasing numbers of injected LAT cells.

3.

Despite highly significant elevations in mean serum NANA levels during the early stages of tumour development, prolonged monitoring shows that NANA levels fluctuate erratically with further increases in tumour burden, thus making it unsuitable for use as a marker for LAT growth.

4.

During early stages of growth serum LSA increases proportionally with LAT burden, with mean LSA levels significantly elevated when compared to normal levels. In marked contrast, later stages of LAT growth can be accompanied by decreasing LSA levels.

5.

Serial determination of serum LSA could be used to monitor response to therapy in LAT-bearing mice, thereby providing a means by which newly developed anti-tumour agents (immunological, radiometric or chemotherapeutic in nature), could be assessed in an animal model system.

6.

While elevations in mean serum NANA levels were more significant than those of serum LSA during the early stages of LAT development, LSA was found to be a more reliable marker in cases of prolonged tumour growth.

7.

The results of this investigation provide further evidence to suggest that serum LSA could be a more useful and discriminating indicator of tumour burden than total NANA concentration, and therefore may have a clinical role in the routine follow-up and monitoring of patients suffering from neoplastic disease.

TABLE 5.1

AVERAGE CHANGES IN SERUM NANA AND LSA LEVELS IN
NORMAL CONTROLS AND LAT-BEARING MICE

| TUMOUR MARKER | EXP NO | EXP GROUP | AVERAGE CHANGE IN NANA (nmoles/ml) | CRITICAL VALUE |
|------------------|-----------|----------------------------------|---|---------------------|
| SERUM NANA | (1) | PBS [*] | 266.6 | (-) |
| | | PRISTANE ⁺ | 90.0 | (-) |
| | | 10 ⁷ LAT [*] | 775.0 | (3.91) ¹ |
| | | 10 ⁷ LAT ⁺ | 775.0 | (5.91) ² |
| SERUM NANA | (2) | PBS | -50.0 | (-) |
| | | 10 ⁷ LAT | 750.0 | (3.42) ³ |
| | | 10 ⁵ LAT | 687.5 | (2.56) |
| SERUM NANA | (3) | PBS | 337.5 | (-) |
| | | 10 ⁷ LAT | 380.0 | (0.317) |
| SERUM LSA | (4) | PBS | 91.3 | (-) |
| | | 10 ⁷ LAT | 306.0 | (1.21) |

The following P values represent the significance of average changes in total serum NANA and serum LSA levels relative to those of PBS (*) and Pristane (+) controls

- 3 significant differences were found
 1 : significant difference ; P<0.005
 2 : significant difference ; P<0.001
 3 : significant difference , P<0.020

TABLE 5.2

MEAN LEVELS OF SERUM NANA AND SERUM LSA IN
NORMAL CONTROLS AND LAT-BEARING MICE

| TUMOUR MARKER | EXP NO | EXP. GROUP | MEAN +/- S.D (nmoles/ml) | CRITICAL VALUE |
|---------------|--------|----------------------------|--------------------------------------|-----------------------------|
| SERUM NANA | (3) | PBS 10 ⁷ LAT | 1705.6 +/- 146.7 2140.0 +/- 232.9 | (-) (4.325) [*] |
| SERUM LSA | (4) | PBS 10 ⁷ LAT | 496.3 +/- 62.0 757.3 +/- 205.1 | (-) (3.268) [#] |

The following P values represent the significance of elevations in mean total serum NANA and serum LSA levels relative to PBS controls levels.

* : significant difference ; P<0.001

: significant difference ; P<0.020

FIGURE 5 1
THE VARIATION OF TOTAL SERUM NANA (nmoles/ml) FOR
CONTROL AND TUMOUR-BEARING MICE WITH TIME (days)

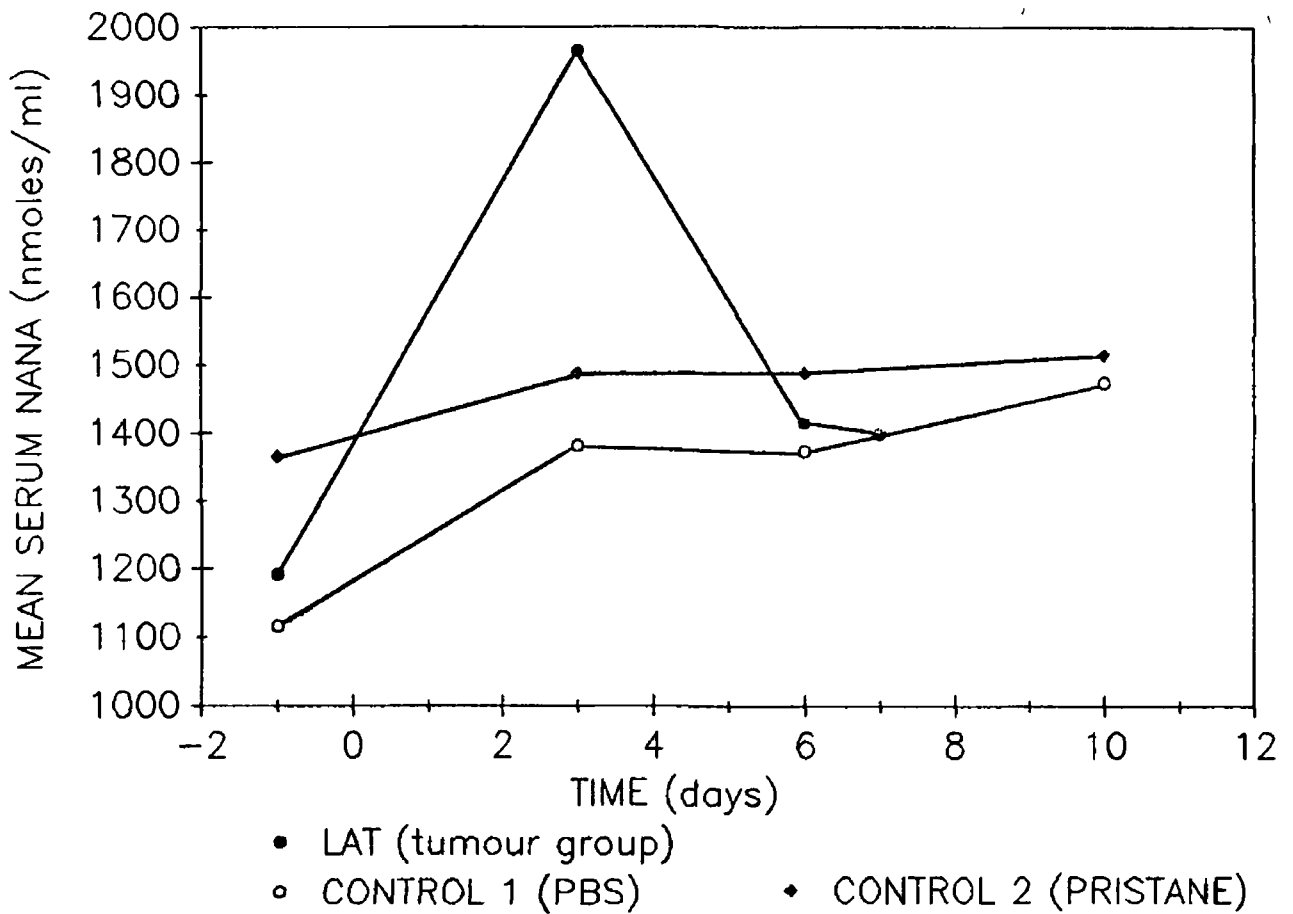


FIGURE 5 2
THE VARIATION IN TOTAL SERUM NANA (nmoles/ml) FOR
CONTROL AND TUMOUR BEARING MICE WITH TIME (days)

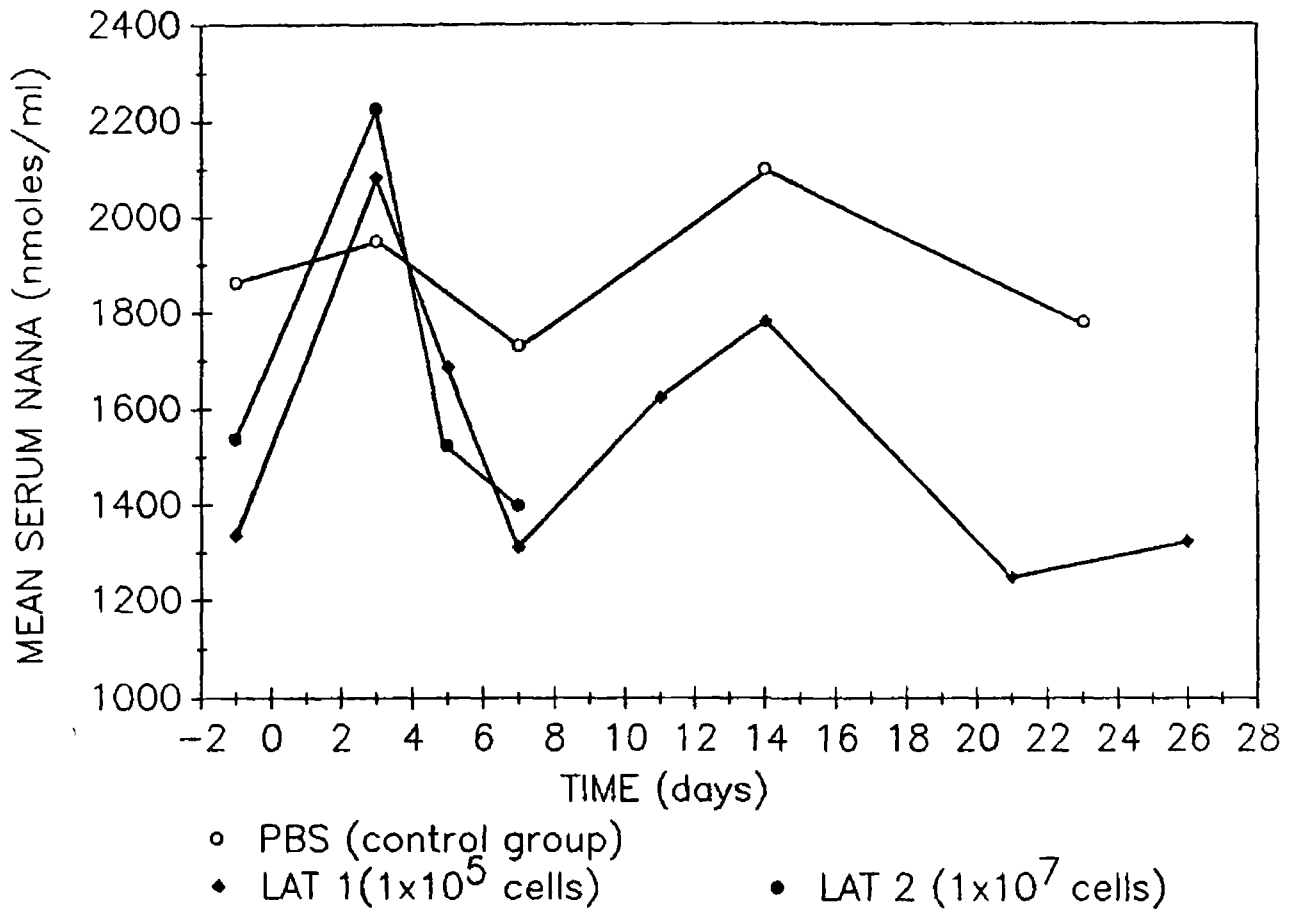


FIGURE 5 3
THE VARIATION OF TOTAL SERUM NANA (nmoles/ml) FOR
CONTROL AND TUMOUR-BEARING MICE WITH TIME (days)

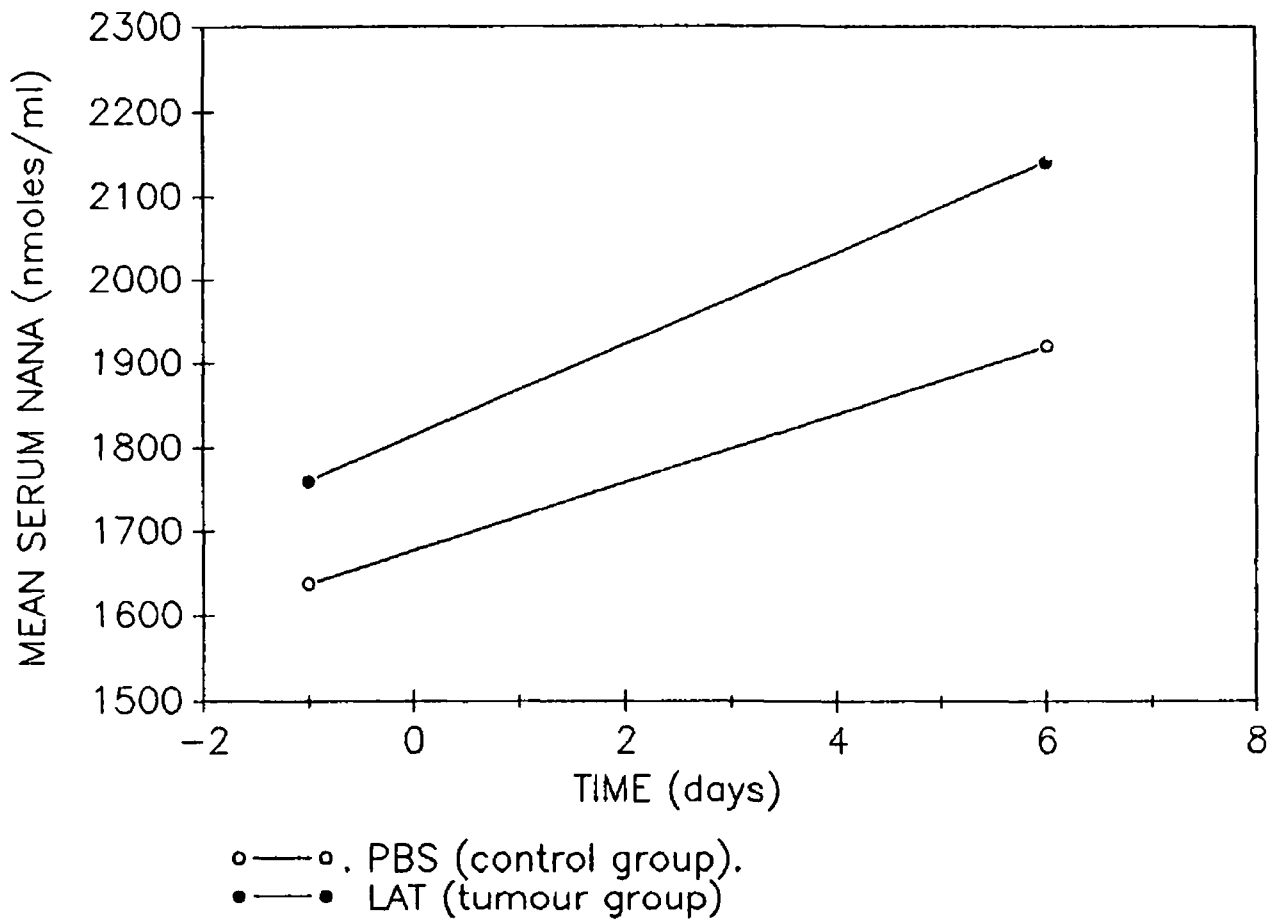
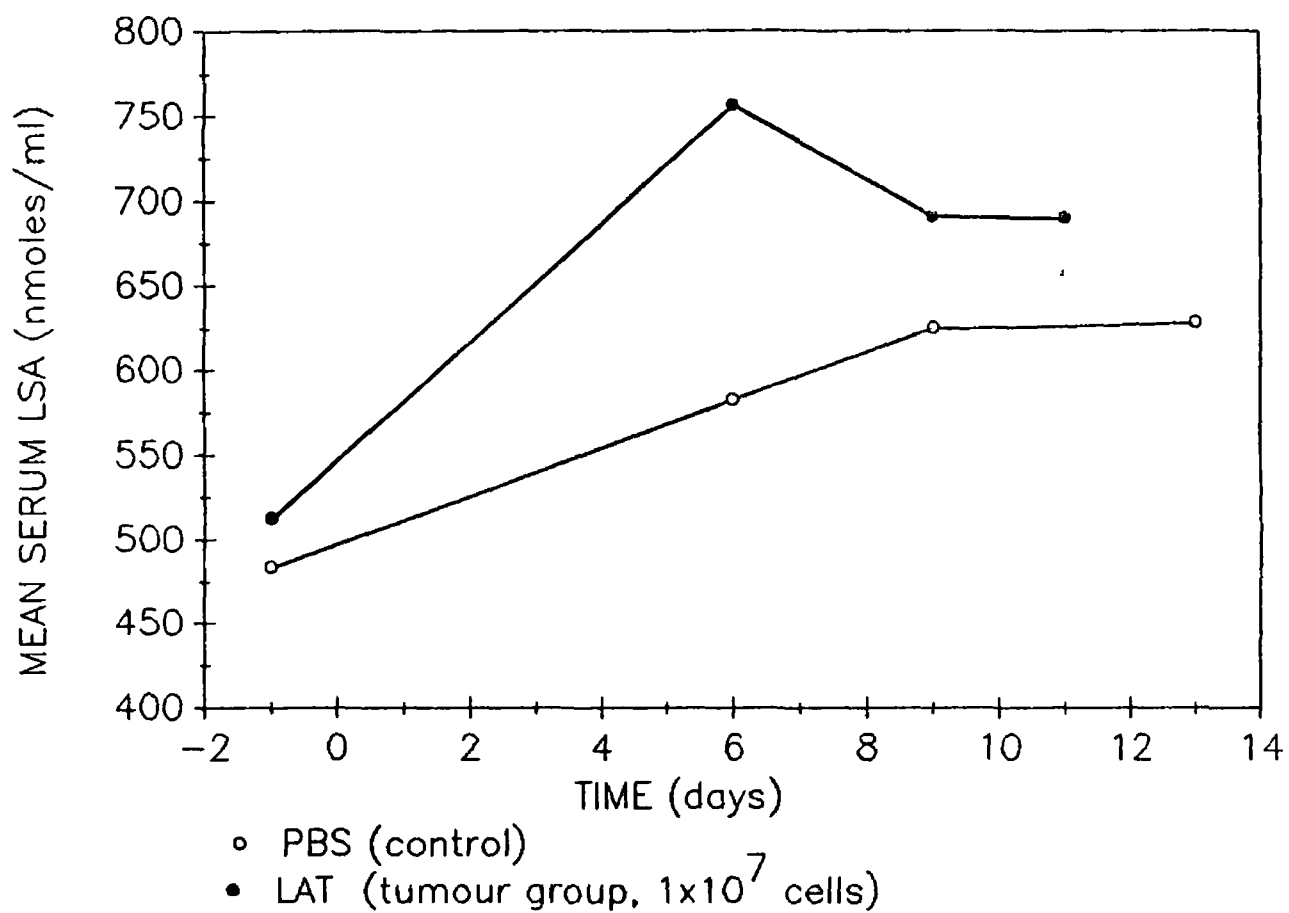


FIGURE 5 4
THE VARIATION OF SERUM LSA (nmoles/ml) FOR CONTROL
AND TUMOUR-BEARING MICE WITH TIME (days)



SECTION 6

**The Development of the VGR Assay, a
Modified Thiobarbituric Acid Method, for
the Determination of Total Plasma NANA.**

INTRODUCTION

In 1959, Leonard Warren developed the thiobarbituric acid (TBA) method for the determination of free (unbound) NANA (Warren, 1959). Despite modifications by other researchers, including Aminoff (1961) and O'Kennedy (1979), the original method, as devised by Warren, has been extensively used over the last 32 years.

The importance of NANA as a biological marker has resulted in the development of a range of highly specific and highly sensitive methods of quantitation, including spectrofluorometric (Hammond & Papermaster, 1976), chromatographic (Silver & Karim, 1981) and enzymatic methods of analysis (Teshima *et al*, 1988). These more recently developed methods have several advantages over the Warren assay. Chromatographic and spectrofluorometric methods are highly automated and are capable of detecting picomolar (10^{-12} M) levels of NANA. Enzymatic analysis, one of the most recent developments, has greatly reduced assay time. However, these methods also have disadvantages. For example, the instrumentation required for chromatographic and spectrofluorometric analysis can be very expensive, as are many of the enzyme kits currently available. Additional problems associated with enzymatic methods include reagent instability, light sensitivity (exposure to light can increase blank O.D. readings) and interference from naturally occurring substituents of body fluids (especially pyruvate and ascorbic acid). Therefore, despite the genesis of these elaborate and more sophisticated methods, the Warren assay, which is economical (does

not require expensive instrumentation), sensitive (can detect nmolar quantities of NANA) and reproducible (10% inter-assay variability), is still used as a routine method for the determination of NANA. However, the Warren assay itself has several disadvantages and limitations. It is time consuming and laborious, lacks any degree of automation and requires the use of toxic reagents, including sodium arsenite (human poison and carcinogen, Sax and Lewis, 1989) and cyclohexanone (an organic solvent capable of dissolving plastic, thereby requiring the use of quartz cuvettes). 2-deoxyribose (2-DOR) interferes in the Warren assay to such an extent that a correction formula is required to negate its effect.

The purpose of this research was to develop a new method for the determination of NANA, using the principles and procedures of the Warren assay as a foundation. It was hoped that the modified assay would have the following characteristics:

1. Reduced assay time
2. Reduced interference due to 2-DOR
3. A degree of automation
4. A greater element of safety.

The main prerequisite for acceptance of a particular modification was that it did not interfere with, or hinder in any way, the performance characteristics of the assay, namely its sensitivity and specificity. It was crucial that the modified assay maintained the analytical "integrity" of the parent assay.

EXPERIMENTAL DESIGN

Total plasma NANA levels were determined by the VGR assay (Section 2 3 6), referred to as "VGR-Macro" (since a large sample volume, 1.0 ml, was required for O D measurements) The stability of the VGR-Macro chromophore was determined by monitoring the O.D of a plasma sample over an 85 minute period. NANA levels were also measured using a modified VGR assay (Section 2 3 7) known as "VGR-Micro" (the method incorporated a micro-sample detection system whereby the O D s of samples, 180 μ l, were recorded in 96-well microtitre plates) When using the VGR-Micro assay, 2 methods of incubation (50°C) were employed The first method required that the microtitre plates were placed in the incubator of the plate reader prior to O D measurements, whereas in the second method, microtitre plates were incubated in a conventional oven incubator and then transferred to the plate reader, where O D s were recorded The standard incubation time was 25 minutes However, to determine chromophore stability, 50 minute incubations prior to O D. measurements, using both methods of incubation, were also employed Finally, in order to validate the VGR-Macro and VGR-Micro assays, total plasma levels were also determined using a commercially available enzymatic kit for NANA (Boehringer Mannheim, Cat No 784192) The principle of this enzymatic assay has already been discussed (Section 1 3)

6 3

STATISTICAL ANALYSIS

The two-sample Student's t-Test (Section 2 8 1) was used to compare sample means, between experimental groups. Where the t-Test (two sided) was employed, samples were assayed in triplicate ($N_1 = N_2 = 3$). Therefore, all critical t-values were based on 4 degrees of freedom (V), where $V = N_1 + N_2 - 2$.

6 4

ANALYSIS OF RESULTS

The ability to determine plasma NANA levels using the VGR-Macro and VGR-Micro assays is made possible due to the linear relationship between NANA concentration (over a range of 0 - 50 nmoles/sample) and O D at 560 nm. Typical standard curves obtained, using both methods, are shown in figures 6 1 and 6 2. The strength of this relationship is reflected in consistently high correlation coefficients (R values) for the standard curves, with these values in the range 0.995 - 0.999.

Since cyclohexanone, used in the Warren assay, had been replaced by a solution of 5% (v/v) HCl in ethanol, it was necessary to investigate the stability of both the VGR-Macro and VGR-Micro chromophores. Tables 6 1 shows O D values, obtained from a sample assayed by VGR-Macro, over an observation period of 85 minutes. The initial reading (0.178) increased to 0.183 after 85 minutes, representing a rise in O.D. of 2.8%. During the

same period, the difference between the highest and lowest O D values (0.183 after 85 minutes and 0.176 after 18 minutes) was 4.0%. These results confirmed that addition of the 5% (v/v) HCl in ethanol solution resulted in a stable VGR-Macro chromophore, whereby small variations in O.D. values occurred over an 85 minute period. For the VGR-Micro chromophore, O D s were recorded at regular intervals, during a 40 minute incubation period (in the incubator of the microplate reader). The results (Table 6.2) show decreasing O D. values, in all samples, during the first 15 minutes of incubation, whereas the values stabilised after 20 minutes. These results are consistent with the following explanation. The samples were turbid during early stages of incubation as a result of sample temperatures being less than the required temperature of 50°C. As the samples approached 50°C the turbidity decreased, resulting in decreased O D readings. O D stabilisation indicated that a 20 minute incubation period was necessary for sample equilibration at 50°C. Therefore, based on these results, an incubation period of 25 minutes, prior to recording of O D s, was chosen as the standard incubation period for the VGR-Micro assay, thereby ensuring sample equilibration at the required temperature.

Having confirmed chromophore stability in both VGR-Macro and VGR-Micro assay systems, it was then necessary to quantify inter-assay variation. Total plasma NANA levels were determined in 13 plasma samples (using both methods) and mean levels were then compared. The results (Table 6.3) showed that, in 12 of the 13 samples (92%), no statistically significant differences were found between mean levels of plasma NANA. In one sample (No. 11), a

small, though statistically significant, difference was found to exist. However, closer examination of the results for the sample shows that, in this particular instance, the calculated t-value was exaggerated due to the small value obtained for S_p (one of the terms in the denominator of the two-sided Student's t-Test, Section 2.8.1). This in turn was a result of a standard deviation value of zero (a rare occurrence) obtained from the VGR-Micro determination.

To further assess the stability of the VGR-Micro chromophore, the following experiment was performed. Having determined plasma NANA levels, in 4 samples, using the standard VGR-Micro assay (incubation at 50°C for 25 minutes), the samples were incubated for a further 25 minutes (total of 50 minutes), after which time the O.D.'s were again recorded. Plasma NANA levels were then determined using the new O.D. readings. Therefore, 2 values for mean plasma NANA were obtained for each sample, the first value determined after a 25 minute incubation period, the second after 50 minutes. Analysis of the results (Table 6.4) showed no statistically significant differences between mean plasma NANA levels.

Since the majority of ELISA plate readers do not have an inbuilt incubator, the possibility of using a conventional oven incubator, in the VGR-Micro assay, was also investigated. Total plasma NANA levels were determined, in 5 samples, using two variations of the VGR-Micro assay, with both methods incorporating an incubation period of 25 minutes. The standard VGR-Micro assay was used, whereby samples were incubated in the microplate reader. The second method used a conventional oven incubator, with the plates being transferred to the

microplate reader, after incubation, for immediate recording of O D 's. In 4 out of 5 samples tested (80%), no statistically significant differences were found between mean levels of plasma NANA, as determined by the VGR-Micro assays (Table 6 5). However, since a significant difference between mean plasma NANA levels was found in one of the samples, a second experiment (using new samples) was performed, in which samples were allowed a period of 50 minutes in the conventional incubator prior to transfer and recording of O D 's in the microplate reader. The purpose of the longer incubation step was to facilitate sample equilibration at 50°C. Mean plasma NANA levels determined by this modified assay were compared to those obtained using the standard VGR-Micro assay. The results (Table 6 6) showed statistically significant differences in 2 of the 5 samples (40%). Furthermore, while no difference was found between NANA mean levels for Sample 1 ($P < 0.5$), it must be noted that the standard deviation obtained for that sample, using the modified VGR-Micro assay (conventional incubator for 50 minutes), was very large ($\pm 29\%$), thereby preventing reliable statistical comparison of results.

Finally, to validate the modified assay, total plasma NANA levels were determined in 16 samples, using the standard VGR-Micro assay and a commercially available enzymatic assay for NANA. The results (Table 6 7) showed no statistically significant difference, between mean plasma NANA levels, in 13 of the 16 samples (81%). However, statistically significant differences were found in the remaining 3 samples.

DISCUSSION

The purpose of these experiments was to modify the Warren thiobarbituric acid assay. Major deviations from the original method included an attempt to determine NANA levels from a standard curve and also the inclusion of a new solution for chromophore stabilisation. For the VGR-Macro assay, correlation coefficients for standard curves (0 - 50 nmoles NANA / std) were consistently in the range 0.995 - 0.998. For the same assay, it was shown that the use of a 5% (v/v) HCl in ethanol solution, in place of cyclohexanone, did not adversely affect chromophore stability, with variations in O.D. readings of only 4.0% occurring over an 85 minute period. The next stage was to miniaturise the VGR-Macro assay by using a microplate reader for recording of O.D.s. The VGR-Micro assay consistently produced standard curves with correlation coefficients in the range 0.996 - 0.999. Furthermore, once the samples had equilibrated at the required temperature (50°C), the reproducibility of the VGR-Micro chromophore was also found to be superior to that of the VGR-Macro assay, with negligible changes in O.D. readings over a period of 20 minutes (sufficiently adequate since the O.D.s of an entire 96-well plate could be recorded in 30 seconds). When plasma samples were assayed for total NANA, using both methods (VGR-Macro and VGR-Micro), statistical analysis showed no difference between mean plasma NANA levels. Therefore, subsequent experiments were designed solely to assess the VGR-Micro assay, since its automated O.D. recording ability, coupled with its superior performance characteristics (high quality standard curves and highly stable chromophore), made

it the preferred method for NANA analysis To further evaluate the VGR-Micro assay, plasma NANA levels were determined in 4 samples, using O D s recorded after both 25 and 50 minute incubation periods Statistical analysis showed no difference between mean plasma NANA levels, thereby reaffirming the high degree of stability in the VGR-Micro chromophore The possibility of incorporating a conventional oven incubator into the VGR-Micro assay (a cost reducing step) was then investigated. Plasma NANA determinations, following an incubation period of 25 minutes, suggested a possible role for a conventional incubator However, when the incubation period was increased to 50 minutes (to allow samples a longer "warm up" time), mean plasma NANA determinations were significantly altered Therefore, the method of incubation, prior to recording O D s, has a definite effect on mean plasma NANA levels, as determined by the VGR-Micro assay The heater in the microplate reader is a heating plate, upon which the microplate rests This is an efficient method of heating since the plate and the source of heat are in close contact, thereby reducing the time required for sample equilibration at 50°C A heating plate is also a good source of homogeneous heat, thereby ensuring a minimal temperature differential across the plate (a crucial requirement for the VGR-Micro assay where variation in O D with temperature is significant) In contrast, the conventional incubator uses circulating heated air as the method of heating It is possible that temperature homogeneity in the oven incubator is inferior to that of the microplate reader heating plate apparatus, thereby resulting in fluctuating O D values Another possible cause of the inferior results obtained using the conventional incubator is that the 25 minute incubation period

may have been inadequate to allow proper sample equilibration at 50°C. Increasing the incubation period to 50 minutes caused additional problems whereby O D reproducibility, in several cases, deteriorated significantly (possibly as a result of sample evaporation). Finally, when using the incubator of the microplate reader, the O D s were recorded "in situ", with no disturbance of samples, whereas use of the conventional oven incubator required transfer of the plates to the microplate reader, thereby making samples vulnerable to problems of spillage and cooling.

In the final experiment of this investigation, plasma NANA levels were determined by the VGR-Micro assay and a commercially available enzyme kit. Statistical analysis showed no difference, between mean plasma NANA levels, in 81% of samples studied. However, the existence of significant differences in the remaining 19% of samples indicated that the observed differences were caused by interference in either the enzymatic assay (from plasma pyruvate and ascorbic acid) or the modified TBA assay (from 2-DOR). These results suggested that further examination of 2-DOR interference was necessary. Later research at D.C.U. led to refinements in the VGR-Micro assay. As a result of definite interference (by 2-DOR) it was necessary to re-introduce a correction formula (with O.D s recorded at two wavelengths). However, the use of the microplate reader, which is capable of reading the O D s of a 96-well plate at two wavelengths in less than 5 minutes, coupled with a computer programme designed to compute NANA levels from input O D readings, more than compensates for the introduction of the correction formula, thereby making the VGR-

Micro assay a very viable method for analysis of plasma NANA

6 6

CONCLUSIONS

Based on the results obtained in these experiments, it is possible to draw the following conclusions.

- 1 The use of a 5% (v/v) HCl in ethanol solution (as opposed to cyclohexanone), in the modified assay, results in the formation of a highly stable chromophore
- 2 The elimination of cyclohexanone from the TBA assay has the following advantages
 - a) Cyclohexanone is moderately toxic by ingestion and inhalation. It is also a skin and severe eye irritant (Sax & Lewis, 1989). Human systemic effects by inhalation includes changes in sense of smell and conjunctiva irritation, therefore requiring the use of a laminar flow hood. The replacing of cyclohexanone, with a less toxic solution, therefore results in a more "operator friendly" assay, while also eliminating the need for a laminar flow hood. This is very important in view of recent developments in legislation concerning safety at work.
 - b) The use of cyclohexanone (organic phase) in the original assay, as a chromophore stabiliser, required a centrifugation step,

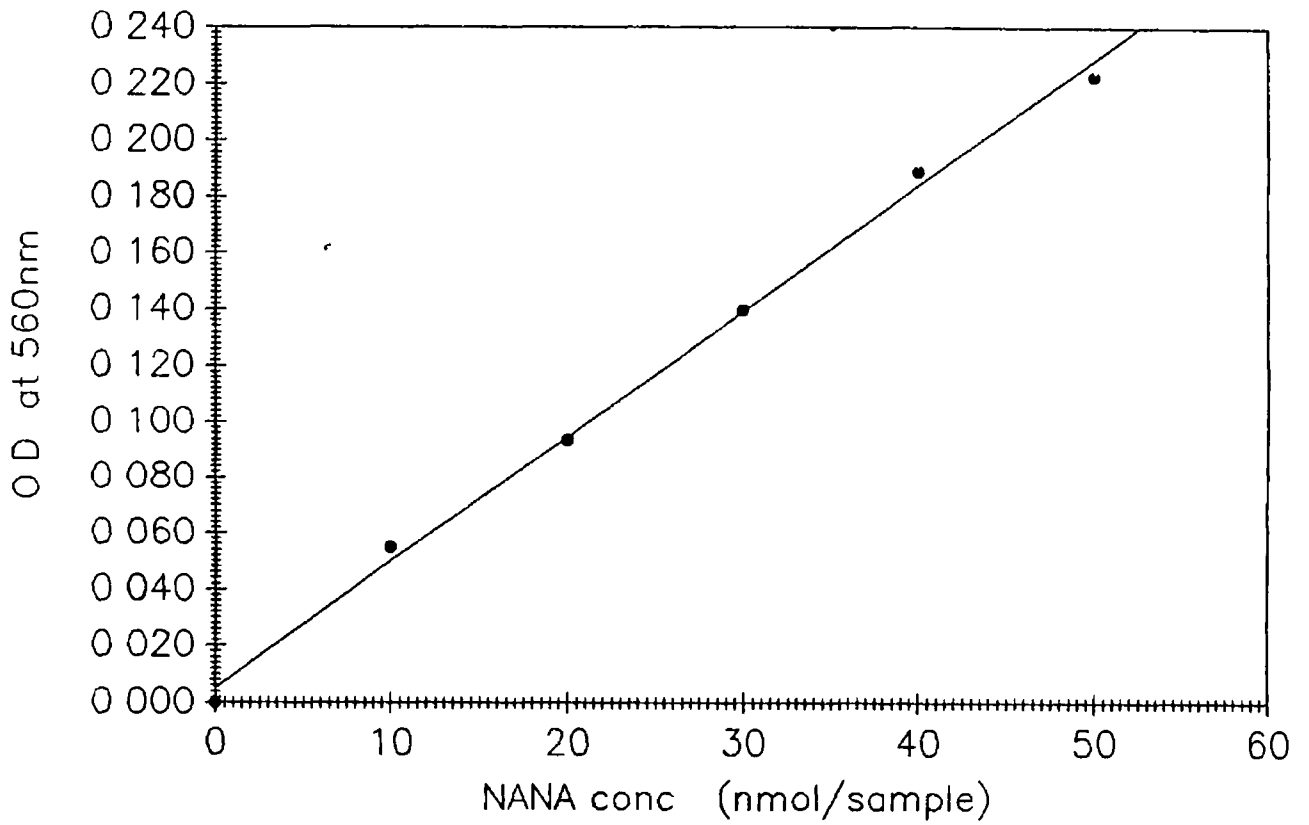
followed by removal of the organic phase (containing the chromophore) using a Pasteur pipette. By replacing the cyclohexanone with a solution of 5% (v/v) HCl in ethanol (aqueous), thereby resulting in the formation of a one phase system, these laborious and time consuming steps are no longer necessary.

- c) The elimination of cyclohexanone from the modified assay introduced a cost saving step, since the 5% (v/v) HCl in ethanol solution (unlike cyclohexanone) does not dissolve plastics, thereby eliminating the need for the use of quartz cuvettes
- 3) It has been shown by statistical analysis that, in the modified TBA assay, the method employed for recording of O D s does not effect NANA determinations, whether it be a conventional spectrophotometer, as in the VGR-Macro assay, or a microplate reader, as in the VGR-Micro assay
- 4) For accurate work, the VGR-Micro assay requires a microplate reader with an inbuilt incubator, thereby allowing the recording of O D s "in situ". The use of a conventional oven incubator is not advisable as NANA determinations, using this method of incubation, are less reliable
- 5) If a microplate reader with an incubator is not available, it is advisable to use the VGR-Macro assay, where O D.s are recorded in a conventional spectrophotometer with temperature control attachments

- 6 The rapid O D recording ability of the microplate reader greatly reduces the time required to perform the assay. It also reduces fluctuations in O.D s, a problem often associated with instrumental drift (e.g. changes in light source emission intensity)
- 7 Results obtained during validation of the VGR assay indicated interference by 2-DOR. However, this problem may be eliminated by dual wavelength analysis of results.
- 8 While further evaluation of the VGR assay is required, initial results are very promising, with a definite possibility that this modified TBA assay could become a routine method of NANA analysis.
- 9 If further validation of the VGR assay continues to give positive results, another possible future application of this assay could be in the determination of plasma LSA levels, whereby the VGR assay would be coupled to a method for gangliosidic extraction.

FIGURE 6 1

A TYPICAL STANDARD CURVE USING THE VGR MACRO ASSAY



note correlation coefficient (R) =0.998

FIGURE 6 2
A TYPICAL STANDARD CURVE USING THE VGR MICRO ASSAY

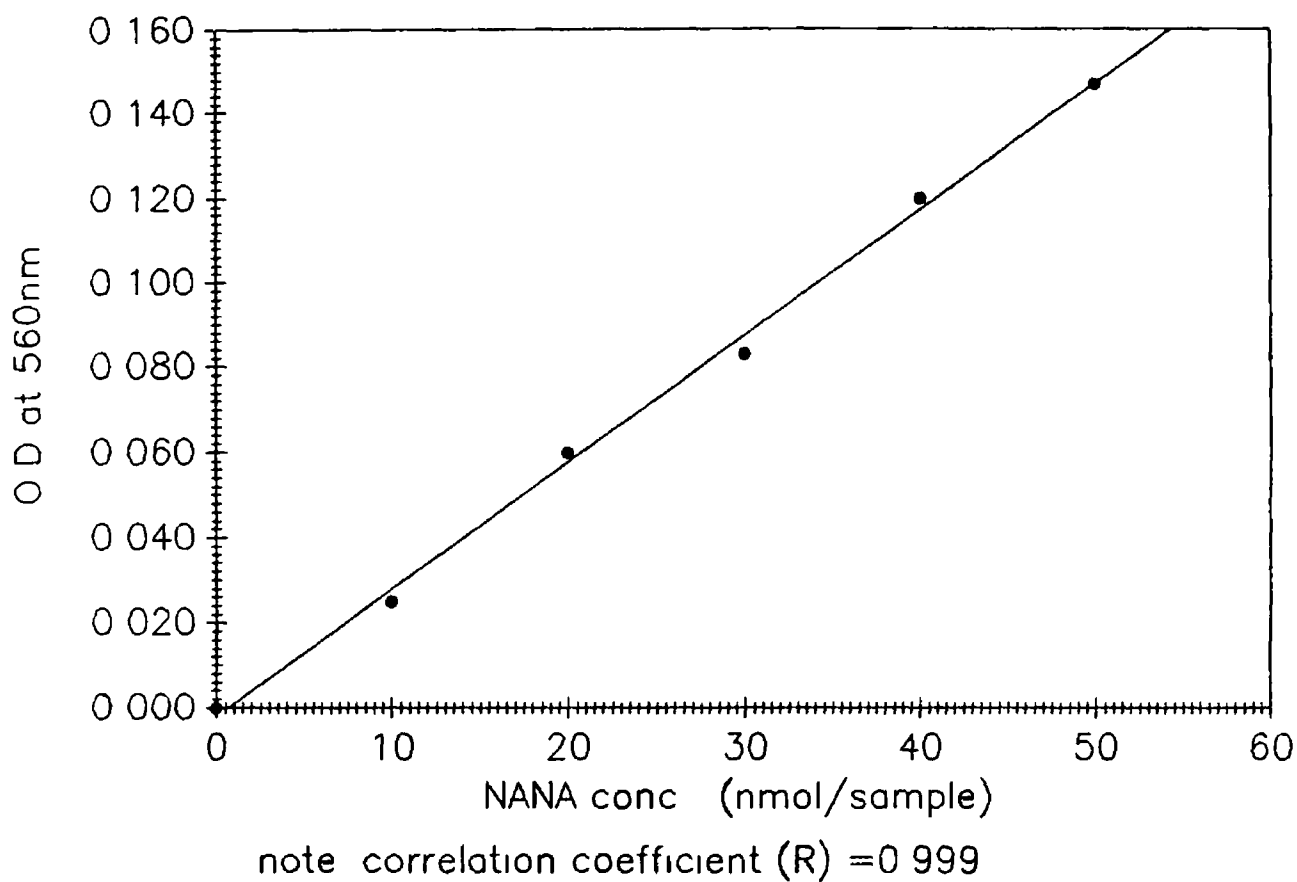


Table 6 1 Variations in O D readings (at 560 nm)
with time in the VGR-Macro Chromophore

| TIME (MINUTES) | O D |
|----------------|-------|
| 0 | 0 178 |
| 12 | 0 180 |
| 18 | 0 176 |
| 23 | 0 177 |
| 35 | 0 179 |
| 45 | 0 184 |
| 60 | 0 182 |
| 85 | 0 183 |

O D s are the mean values of 3 determinations

Table 6 2 Variations in O D Readings (at 560 nm)
with Time in the VGR-Micro Chromophore

| SAMPLE (NANA) (NMOLES/SAMPLE) | AVERAGE O D (560 nm) AFTER SPECIFIED INCUBATION PERIODS (MINUTES) | | | | |
|-------------------------------------|--|-------|-------|-------|-------|
| | 5 | 15 | 20 | 30 | 40 |
| 0 + | 0 049 | 0 044 | 0 045 | 0 045 | 0 045 |
| 10 ++ | 0 066 | 0 060 | 0 061 | 0 061 | 0 061 |
| 20 ++ | 0 096 | 0 091 | 0 091 | 0 091 | 0 093 |
| 30 ++ | 0 122 | 0 115 | 0 117 | 0 116 | 0 117 |
| 40 ++ | 0 158 | 0 155 | 0 153 | 0 152 | 0 153 |
| 50 ++ | 0 191 | 0 185 | 0 186 | 0 186 | 0 187 |

+ O D s are the mean values of 8 determinations

++ O D s are the mean values of 4 determinations

Table 6 3 A comparison of total plasma NANA levels determined by the VGR-Macro and VGR-Micro Assays

| SAMPLE NUMBER | VGR-MACRO TOTAL NANA (nmoles/ml) MEAN ± S D | VGR-MICRO TOTAL NANA (nmoles/ml) MEAN ± S D/ | CRITICAL t-VALUE |
|---------------|--|---|---------------------|
| 1 | 1500 0 ± 482 2 | 1516 7 ± 378 6 | 0 047 |
| 2 | 1875 0 ± 90 1 | 1933 3 ± 57 7 | 0 944 |
| 3 | 1316 7 ± 101 0 | 1400 0 ± 114 6 | 0 945 |
| 4 | 2666 7 ± 354 7 | 2708 3 ± 232 3 | 0 170 |
| 5 | 1566 7 ± 260 2 | 1641 7 ± 252 9 | 0 358 |
| 6 | 850 0 ± 175 0 | 908 3 ± 115 7 | 0 482 |
| 7 | 766 7 ± 62 9 | 675 0 ± 82 6 | 1 530 |
| 8 | 2316 7 ± 80 4 | 2300 0 ± 109 0 | 0 213 |
| 9 | 1383 3 ± 125 8 | 1508 3 ± 104 1 | 1 327 |
| 10 | 2391 7 ± 62 9 | 2266 7 ± 175 6 | 1 161 |
| 11 | 2741 7 ± 152 8 | 2475 0 ± 0 0 | 3 026* |
| 12 | 2433 3 ± 52 0 | 2258 3 ± 115 5 | 2 395 |
| 13 | 2250 0 ± 150 0 | 2208 3 ± 175 6 | 0 313 |

S D = Standard Deviation

Only one significant difference was found

* significant difference, P < 0 05

Table 6 4 Total plasma NANA levels determined by VGR-Micro assays incorporating incubation periods of 25 and 50 minutes

| SAMPLE NUMBER | TOTAL NANA (nmoles/ml) | TOTAL NANA (nmoles/ml) | CRITICAL t-Value |
|---------------|--------------------------|--------------------------|------------------|
| | 25 Minutes MEAN ± S D | 50 Minutes MEAN ± S D | |
| 1 | 1508 3 ± 104 1 | 1508 3 ± 275 4 | 0 000 |
| 2 | 2266 7 ± 175 6 | 2133 3 ± 128 3 | 1 063 |
| 3 | 2258 3 ± 115 5 | 2116 7 ± 80 4 | 1 745 |
| 4 | 2208 3 ± 175 6 | 2075 0 ± 139 2 | 1 031 |

S D = Standard Deviation

No significant differences found

Table 6 5 Total plasma NANA levels determined by the standard VGR-Micro assay and a modified VGR-Micro assay incorporating a conventional oven incubation period of 25 minutes prior to recording of O D.s.

| SAMPLE NUMBER | TOTAL NANA (nmoles/ml) Standard Assay Mean \pm S D | TOTAL NANA (nmoles/ml) Mod Assay Mean \pm S D | CRITICAL t-Value |
|---------------|--|---|------------------|
| 1 | 1641 7 \pm 252 9 | 1550 0 \pm 152 1 | 0 538 |
| 2 | 908 3 \pm 115 7 | 983 3 \pm 104 1 | 0 835 |
| 3 | 675 0 \pm 82 6 | 591 7 \pm 28 9 | 1 652 |
| 4 | 2300 0 \pm 109 0 | 2041 7 \pm 38 2 | 3 877* |
| 5 | 2858 3 \pm 137 7 | 2866 7 \pm 125 8 | 0 077 |

S D = Standard Deviation

Only one significant difference was found

* significant difference, P < 0 02

Table 6 6 Total plasma NANA levels determined by the standard VGR-Micro assay and a modified VGR-Micro assay incorporating a conventional oven incubation period of 50 minutes prior to recording of O D s

| SAMPLE NUMBER | TOTAL NANA (nmoles/ml) Standard Assay Mean \pm S D | TOTAL NANA (nmoles/ml) Mod Assay Mean \pm S D | CRITICAL t-Value |
|---------------|--|---|------------------|
| 1 | 1508 3 \pm 104 1 | 1475 0 \pm 420 6 | 0 133 |
| 2 | 2266 7 \pm 175 6 | 2175 0 \pm 204 6 | 0 589 |
| 3 | 2475 0 \pm 0 0 | 2291 7 \pm 94 6 | 3 357* |
| 4 | 2258 3 \pm 115 5 | 2041 7 \pm 62 9 | 2 856** |
| 5 | 2208 3 \pm 175 6 | 2083 3 \pm 125 8 | 1 003 |

S D = Standard Deviation

Only two significant differences were found

* significant difference, P < 0 05

** . significant difference, P < 0 05

Table 6 7 A comparison of total plasma NANA levels determined by VGR-Micro and a commercially available enzymatic assay

| SAMPLE NUMBER | TOTAL NANA (nmoles/ml) | TOTAL NANA (nmoles/ml) | CRITICAL t-VALUE |
|---------------|------------------------|-------------------------|------------------|
| | VGR-MICRO MEAN ± S D | ENZYME ASSAY MEAN ± S D | |
| 1 | 2193 5 ± 246 6 | 2310 5 ± 27 2 | 0 817 |
| 2 | 2183 3 ± 246 0 | 2036 4 ± 54 9 | 1 010 |
| 3 | 3177 5 ± 180 2 | 3054 1 ± 61 1 | 1 124 |
| 4 | 3146 8 ± 138 7 | 2919 2 ± 63 8 | 2 584 |
| 5 | 3464 5 ± 309 5 | 3500 3 ± 19 8 | 0 200 |
| 6 | 3034 0 ± 116 4 | 3189 0 ± 98 4 | 1 763 |
| 7 | 3495 3 ± 185 4 | 3769 6 ± 27 5 | 2 538 |
| 8 | 2870 0 ± 216 0 | 2898 5 ± 53 7 | 0 222 |
| 9 | 2255 0 ± 209 3 | 1939 8 ± 133 8 | 2 199 |
| 10 | 1558 0 ± 154 8 | 1469 1 ± 21 2 | 0 987 |
| 11 | 2265 3 ± 197 7 | 2428 6 ± 25 9 | 1 420 |
| 12 | 2378 0 ± 128 0 | 2651 4 ± 28 4 | 3 905* |
| 13 | 3515 8 ± 200 1 | 3702 4 ± 34 7 | 1 592 |
| 14 | 2429 3 ± 171 2 | 2787 7 ± 54 7 | 3 456** |
| 15 | 3177 5 ± 209 3 | 3369 4 ± 75 0 | 1 489 |
| 16 | 2890 5 ± 215 3 | 3295 4 ± 65 6 | 3 117*** |

S D = Standard Deviation

3 significant differences were found

* significant difference, P < 0 02

** significant difference, P < 0 05

*** significant difference, P < 0 05

SECTION 7

The Production of Monoclonal Antibodies against Human Bladder Tumour-Associated Antigens

INTRODUCTION

Kohler and Milstein were the first to successfully fuse myeloma and antibody-producing cells (B lymphocytes), resulting in a set of single cells (hybridomas) that could be grown in large numbers and could secrete antibodies in large quantities (Kohler and Milstein, 1975). Each cloned cell secreted one antibody (Ab) type with a single binding affinity and specificity, hence the name "monoclonal". This first successful fusion of two cell types counts as one of the most significant experiments in the history of scientific research. The genesis of monoclonal antibody (MAb) technology provided clinical researchers with an invaluable diagnostic and therapeutic tool, due to the unique specificity of these Abs. It enabled clinical oncologists to investigate the surface components of tumour cells with an unprecedented degree of precision. Due to the enormous impact of MAbs, Kohler and Milstein were awarded a Nobel prize in 1984.

Kohler (1985), in the Nobel lecture, compared MAbs to those obtained from conventional polyclonal antisera. The main disadvantages of the latter were stated to be the following

- 1 Low Ab titres.
- 2 Polyclonal antisera contain a heterogeneous population of Abs, with different affinities and avidities for the target antigen (Ag)
3. Ab supply is limited
- 4 Reproduction of identical Ab populations, using another animal, is impossible.

In marked contrast, MAbs have the following advantages

- 1 Single specificity · each hybrid produces one Ab
2. Unlimited Ab supply the fusion procedure results in hybrid immortalisation.
- 3 Pure Ab reagents can be obtained from impure Ags.
- 4 Enrichment of specific hybridomas, whereby specific B cells are enriched 10 - 100 fold in hybridoma populations (relative to corresponding spleen cell populations)

Early MAbs were produced using animal model systems (usually rodents), with the most common fusogen being polyethylene glycol (PEG) The past 16 years has seen the development of novel methodologies, resulting in improved methods of MAb production and the development of new types of MAbs, including human MAbs (HMAbs) The attempt to produce HMAbs arose as a result of the limitations of rodent MAbs, coupled with the realisation that, where Abs were to be used in prophylaxis or therapy, those derived from human sources would be desirable (especially if repeated administration was involved) One of the main limitations of rodent MAbs is that, in certain situations, mice and rats might not recognise the relevant Ags against a background of immunodominant epitopes (Carlsson and Glad, 1989) Another problem which interferes with the in vivo use of rodent MAbs is that they can elicit an immune response when used in vivo in humans, resulting in the production of human anti-mouse antibodies, known as HAMA (Colnaghi, 1991) Problems associated with HAMA production include rapid removal of MAbs, immunocomplex formation and liver accumulation

HMAbs have been produced using the innovative technique of electric field-induced cell fusion. The process of electrofusion is based on the observation that close membrane contact can be established between two cells in an alternating electric field. Following cell alignment (which can be visualised using a microscope), application of high-intensity, short-duration pulses causes reversible increases in membrane permeability and fluidity which, if locally restricted, then leads to both electropermeabilisation and cell electrofusion (Zimmermann and Unovitz, 1987). Refinements of the basic procedure have resulted in further improvements in both the quality and quantity of Abs. Lo *et al* (1984), using electrofusion, devised a method for selective fusion of B-cells secreting Abs of interest, based on the findings that B-cells express, on their surface, Ag receptor immunoglobulin (Ig) of the same specificity as the secreted Abs. In this method the Ag, covalently conjugated to avidin, binds to the surface Igs on B-cells. The complex (B-cell-Ag-avidin) binds to biotin covalently attached to the surface of myeloma cells, thereby resulting in close alignment of myeloma cells and B-cells secreting Abs directed against the target Ag. Fusion of these cells resulted in a population of hybridomas with a high proportion of highly Ag-specific Abs. This method has also been used to increase the yield of Ag-specific Ab-secreting hybrids in PEG fusion experiments. Reason *et al* (1987) used the avidin-biotin method of bridging specific B-cells to myeloma cells to increase the frequency of azobenzene arsonate-specific hybrids. Fong *et al* (1989) produced a panel of HMAbs to human cytomegalovirus (by electrofusion), using as few as 1×10^6 Epstein-Barr virus (EBV) activated B-cells.

Despite these positive developments, the majority of HMABs produced to date have been disappointing, particularly those directed against tumour cells. There have also been serious problems associated with the actual production of these Abs. The ability to capitalise on the practical opportunities afforded by HMAB technology has been limited mainly as a result of difficulties experienced in generating stable human hybridomas secreting adequate amounts of Ab. The availability of suitable sources of immune lymphocytes is limited, as are suitable fusion partners (James, 1989). Furthermore, HMABs are usually of the IgM isotype and, therefore, show relatively low affinity for the primary epitope. In the case of anti-tumour Abs it has been found that many HMABs react or cross-react with intracellular cytoskeletal proteins or nuclear Ags rather than membrane components (tumour-specific or otherwise), thereby limiting their clinical potential (Campbell et al , 1987).

In an attempt to overcome these difficulties, recombinant DNA techniques have recently been employed to generate new types of MABs. Using protoplast fusion and electroporation (using a series of pSV2 vectors), Nakatani et al. (1989) produced transfected-derived recombinant antibodies (RABs) by introducing human cloned Ig genomic DNA into Ig non-producing mouse myeloma cells. The genetically engineered Abs, and those secreted by the origin cells (transformed human B-cells), did not differ in either binding specificity or affinity for the target Ag (Pseudomonas aeruginosa exotoxin A). Bruggemann et al (1989) successfully established stable hybridomas, from transgenic mice, following introduction of human Ig gene segments (in the unrearranged configuration). Gillies et

al (1989) obtained stable clones that secrete HMABs, following the introduction of DNA expression vectors (obtained from a mouse-human heterohybridoma secreting anti-tetanus toxin Ab) into a non-Ig producing murine hybridoma cell line. Glick (1990) stated that this radical approach, whereby MABs can be isolated without pre-production of hybridomas, has numerous advantages over conventional hybridoma producing methodologies. For example, bacteria cells (used as a source of MABs) can be grown to a high cell density in 1 - 2 days (using readily available fermentation vessels), whereas hybridoma cells, in comparison, require several weeks of growth to achieve only moderate cell densities. A major advancement, using recombinant DNA techniques, is that it may no longer be necessary to immunise animals in order to obtain MABs directed against a particular Ag. This will be a welcome development to many researchers, including McGuil and Rowan (1989), who expressed concern over the levels of stress (and distress) caused to animals by conventional hybridoma-forming methods of MAB production.

Recent developments in modifying Abs, using genetic engineering or fusion techniques, have also resulted in new types of reagents such as chimaeric Abs and quadromas (Reuveny and Lazar, 1989). Chimaerics are Abs consisting of mouse variable regions with the human constant region, thereby suitable for use in humans since they combine mouse Ab specificity with low immunogenicity of the human fraction. Quadromas are bifunctional Abs with two different active sites generated by the fusion of two different hybridomas. One potentially invaluable application of bifunctional Abs is in the treatment of cancer, where one of the "arms" of the Ab could bind to a

tumour marker and the other to a drug, toxin or cytotoxic cell. One of the most significant advantages of bifunctional Abs is that the Ab molecule is active in its native state and requires no chemical alteration in order to bind either the target cell or the functional agent (Nolan and O'Kennedy, 1990). Fanger *et al.* (1991) stated that results from recent clinical trials, using bifunctional Abs which bind to target cells and trigger molecules (such as T-cell receptors), demonstrated the potential of these reagents to redirect and focus natural immune mechanisms in the treatment of tumours and infectious disease without the side effects of drugs, radioisotopes or toxins.

MAb technology, being all of 16 years old, is still in its infancy, with its full potential still to be realised. The significant advances which have been made since the genesis of this technology, coupled with the extensive research presently in progress, indicate that MAbs will soon enter the frantic and fruitful phase of adolescence.

Extensive research has shown that alterations in the cell surface components of bladder tumour cells greatly affects tumour behaviour (Richie *et al.*, 1982). For example, the presence or absence of blood-group Ags (A, B and H) can be used to predict the biological behaviour of transitional cell carcinoma of the bladder (D'Elia *et al.*, 1982). The consensus among clinical urologists is that the loss of blood-group Ags from the surface of neoplastic epithelial cells is correlated with aggressive tumour behaviour, whereas bladders with morphologically similar tumours, which retain these Ags, do not develop invasive disease (Vallancien *et*

al , 1983, Wiley et al , 1982, Lange et al , 1978, Bergman and Javadpour, 1978)

Methods used to demonstrate the presence of these blood-group Ags have included immunofluorescence, using polyclonal antisera (Hammou et al , 1982), and the specific red cell adherence (SRCA) test (Coombes et al., 1956) MAbs have also been used to detect these Ags Stephenson et al (1985) produced a panel of MAbS directed against A, B and H Ags, thereby enabling greater sensitivity and specificity in the detection of these prognostic markers It has also been reported that expression of the normally cryptic blood group-related Thomsen-Friedenreich Ag (T-Ag) in bladder carcinoma, also heralds an unfavourable prognosis (Coon et al , 1982) Summers et al (1983) found that bladder tumours, in which the T-Ag is undetectable after neuraminidase treatment, were also highly aggressive This important result provided direct evidence that surface NANA plays a major role in the determination of the metastatic potential of bladder tumour cells, whereby removal of NANA significantly alters the surface expression of blood-group Ags

Numerous attempts have been made to produce MAbS against tumour Ags that are unique for cancer of the bladder (Grossman, 1983, Gozzo et al 1977, Trejdosiewicz et al , 1985). However, the results have been disappointing In fact, no significant marker of human bladder cancer has been consistently demonstrated (Huben, 1984). At best, MAbS detect differentiation Ags with various degrees of restricted expression (Lange and Limas, 1986). A tumour specific Ag, unique to human bladder cancer, has yet to be found (Steffens et al., 1985) Elucidation of the mechanisms involved in neoplastic

transformation of the bladder remains a formidable challenge. George R. Prout Jr , an eminent clinical urologist, stated that, "there is more to learn about bladder carcinoma now than there was twenty five years ago" (Prout, 1982) Ogasawara et al (1988) further implied a role for surface NANA in the antigenic properties of human tumour cells, whereby pretreatment of immunised human gastric cancer cells with neuraminidase, enhanced binding of tumour-specific MAbs

Based on our previous biochemical investigation of total NANA content in the sera of patients with cancer of the bladder, and the aforementioned work of Summers et al (1983) and Ogasawara et al (1988), the aims of our immunogloclial investigations were as follows

- 1 To produce a panel of murine MAbs directed against human bladder tumour (EJ) cells, in the hope of finding a tumour-associated Ag unique for this particular cancer
- 2 To investigate the biochemical nature of any such Ag, with particular regard to any structural contribution made by NANA.

7.2

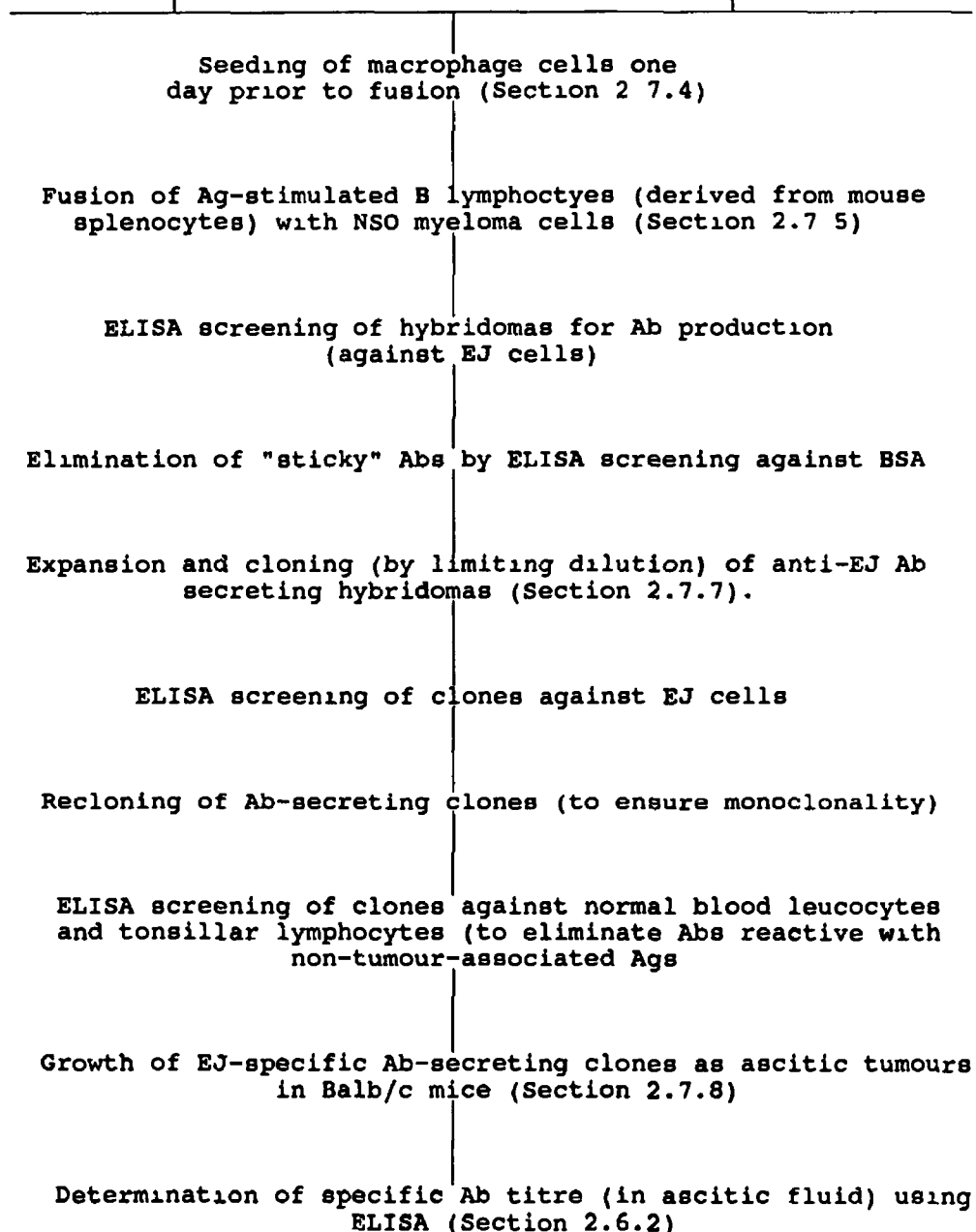
EXPERIMENTAL DESIGN

The sequence of methodologies used to generate MAbs against human bladder (EJ) cells were as follows:

Immunization of Balb/c mice with whole EJ cells (Section 2.5)

NSO myeloma cells tested for HAT sensitivity (Section 2.7.1) and presence of Mycoplasma (Section 2.4.12)

ELISA screening of mice sera for Ab production (Section 2.6.2)



RESULTS

A total of 15 fusions were performed during the course of this immunological investigation. The first 13 fusions were unsuccessful, resulting in no hybridoma formation. The most likely causes of these failures were technical errors during the difficult fusion procedure, whereby the slightest deviation from the recommended protocol results in unsuccessful fusing of the parent cells (Fazekas de St Groth and Scheidegger, 1980)

The first successful fusion (No. 14) yielded hybridomas. However, the Ab-secreting abilities of these hybridomas were found to be poor. A total of 16 hybridomas secreted Ab, albeit weakly, when initially screened against EJ cells (in 96-well plates). Expansion of these hybridomas, into 24-well plates, was followed by rescreening. No hybridoma was found to secrete Ab levels which merited further investigation. In fact, many hybridomas ceased to secrete Ab altogether. This is a common problem encountered in MAb production, whereby unstable hybridomas lose the ability to secrete Ab during initial stages of expansion (Goding, 1980). The results obtained for ELISA screening of these hybridomas (in 24-well plates) are provided in Table 7.1.

Thankfully, the final fusion (No. 15) produced interesting hybridomas. In this fusion, hybridomas were seeded in four 96-well plates. After ten days, growth was observed in 62-wells, representing a fusion success rate of 16%. The first ELISA screening, after 11 days, showed Ab production in 28 wells (45%). Following expansion (into 24-well

plates) and rescreening (using ELISA) Ab production was found in 16 wells (57%) Due to time restrictions, it was decided to concentrate on the strongest reactor, 2AC2 (i e. the hybridoma showing the strongest reactivity with EJ cells, based on ELISA results). The subsequent progress of this hybridoma is summarised in Table 7.2.

Examination of Table 7 2 shows that, ultimately, only two clones were generated which showed Ab reactivity with human bladder cancer (EJ) Ags One of these potentially useful clones, A5F9, was grown as an ascitic tumour in Schofield mice Following removal of the ascitic fluid, the Ab titre was determined to be in excess of 1 7,000 (Table 7 3), based on ELISA screening of a range of ascitic fluid dilutions (Figure 7 1 and Table 7 4)

7 4

DISCUSSION

The production of MAbs is a long and arduous task (Galfré and Milstein, 1981, Hurrell, 1982) The prerequisites for a successful experiment include

- 1 Correct Ag presentation and immunisation regimen
- 2 An efficient method of screening for Ab production
- 3 Good aseptic and cell culture techniques
4. Efficient safeguard procedures.

The first stage in MAb production requires stimulating the immune system of a specific host with the Ag of interest (e.g. tumour derived Ags), resulting in Ab production. Ags of very low molecular weight (M.W., less than 1000), are generally very poorly immunogenic, but may evoke strong responses when coupled to larger (more immunogenic) carrier molecules, such as BSA or ovalbumin (Goding, 1983). Ags of higher M.W. do not require conjugation.

The immune system of the host can also be primed by injection of immunostimulants (Sikora and Smedley, 1984). The most commonly used immunostimulant is Freund's complete adjuvant (Herbert, 1973). Use of this adjuvant requires the mixing of equal portions of a diluent (such as saline), in which the Ag is dissolved or suspended, with an emulsifier-mineral oil mixture (containing Mycobacteria). However, the use of Freund's complete adjuvant (potentially hazardous) requires caution. Accidental injections into humans can cause severe, chronic inflammatory reactions (Mishell and Shiigi, 1980). With cell surface Ags (as was the case in our experiments), adjuvant is not necessary since these Ags are usually highly immunogenic when presented on intact (e.g. EJ) cells (Goding, 1980). Having decided on an immunisation regimen, it is of critical importance that injections are performed on the specified days and that splenocytes are collected 3 days after the final Ag boost, so as to ensure that B-cell clones of interest are maximally stimulated (Milstein, 1982). As already stated, no tumour-specific Ags have been found for cancer of the bladder. Consequently, if such Ags exist on the EJ cell, they are unlikely to be very immunogenic. Furthermore, our search for MAbs, directed against

bladder tumour associated Ags, required the injection of whole EJ cells. The problem with this approach is that there are immunodominant components in the Ag (whole EJ cell), namely that of normal Ags (present on the surface of the EJ cells) over the weakly immunogenic tumour-associated Ags (Mayer and Walker, 1987)

Therefore, it was necessary to have a sensitive and specific ELISA, capable of detecting a weak immune response (directed against tumour-associated Ags) against a high background of normal Abs

The ELISA assay plays a pivotal role in the process of MAb production, with its principal functions being the following

- 1 Evaluation of an immunisation regimen by testing serum from an immunised mouse, prior to a somatic cell fusion experiment, for Ab production
- 2 Initial screening of all hybridomas to test for Ab production
- 3 Elimination of "sticky" Abs by screening against BSA
- 4 Elimination of non-tumour-associated Abs by screening against normal cells.
- 5 The routine monitoring of clones for continued Ab secretion

It is, therefore, essential that the assay is fully functional prior to the commencing of a fusion experiment, as a large number of samples may require screening over a limited time period (Pruslin et al , 1991)

As the process of MAb production is a lengthy one, a prerequisite for success is good aseptic technique. Care must be taken at all times to minimise the occurrence of microbial contamination. It is also advisable that all of the relevant cell culture methodologies are thoroughly practised, prior to the starting of an actual fusion experiment. Finally, it is important that safeguard procedures are in place (to protect valuable clones), namely, back-up generators for all incubators, and efficient auxillary CO₂ supply

Overall, the results of this immunological investigation were extremely disappointing. There was a very considerable input of effort before anything was gained. The high failure rate for the fusions (86%) meant that when hybridomas were eventually obtained, there was very little time to do any clinically significant evaluation, since it was necessary to stop experimenting at a time when the really interesting work was only beginning. Hybridoma 2AC2 was cloned twice before its large progeny were screened against normal cells. It could be argued that this screening should have been performed at a much earlier stage, thereby eliminating a large number of uninteresting clones. However, since antibodies were raised against a whole tumour cell (containing many normal antigens), it was necessary to ensure monoclonality, prior to screening against normal cells, so as to prevent the loss of potentially valuable clones. One such clone

(A5F9), not reactive with normal cells, yielded a good titre, whereby it could detect the presence of EJ cells when diluted in excess of 1 7000

The major problem with all but two of the Abs obtained was that they reacted with normal Ags present on the surface of the EJ cell. Furthermore, while the remaining two Abs, reactive with EJ Ags, were found not to react with normal Ags (present on either tonsillar lymphocytes or blood leucocytes), it would be necessary to carry out further extensive characterisation studies, by screening against a panel of both normal and tumour cell types, before they could be truly designaed as recognising uniquely bladder tumour-associated Ags

Since the existence of bladder tumour-associated Ags has yet to be proven (as discussed in Section 7 1), it is possible that no uniquely bladder-specific tumour Ags exist on the surface of the EJ cell. Nevertheless, this does not mean that the Abs are of no use. It is not an essential prerequisite that an Ab should exhibit absolute specificity for a tumour, as against normal cells, in order for Ab to be of use in the detection of cancer (O'Carroll, 1988). Abs demonstrating only very low levels of reactivity with normal cells (as opposed to tumour cells) can be of use in the detection and in vivo localisation of a tumour. In other words, an Ab need only be "operationally specific" for the tumour cell (Feder et al , 1983). In relation to the bladder tumour Ag, it is not stictly necessary that the specific marker is exclusive of the tumour. It is sufficient that it is specific in the given situation (Cimino et al , 1987). For example, an exclusively embryonic Ag may be an absolute marker for tumour if found outside its normal situation.

CONCLUDING COMENTS

A monoclonal antibody (A5F9) was produced which reacted with the EJ human bladder carcinoma cell line, yet showed no reaction with either normal tonsillar lymphocytes or blood leucocytes. Analysis of ascitic fluid showed a capability for EJ detection at dilutions in excess of 1 : 7000. These results suggest that further evaluation of this antibody may be warranted to assess its clinical potential.

Table 7 1 Screening of Hybridomas against EJ Cells (Fusion 14)

| HYBRIDOMA | AVERAGE O.D AT 405 nm |
|-----------|-----------------------|
| BB2 | N A.D. |
| BB4 | 0.100 |
| BBC4 | 0 073 |
| DB4 | N A D |
| AB4 | 0.130 |
| AB2 | 0 125 |
| DDB2 | 0 140 |
| DDB4 | 0 170 |
| EB4 | N A D |
| EC2 | 0 120 |
| CCB2 | 0 120 |
| CB2 | N A D |
| BAB2 | N A D |
| BAB4 | N A D |
| DB3 | 0 060 |
| DB4 | 0 080 |

Positive Control* O D (av) = 0 621

Negative Control** O D (av.) = 0 120

* Serum from EJ immunised mouse (neat)

** Serum from non-immunised mouse (neat)

N A D : No Ab Detected

Table 7 2 Results obtained from Immunological Investigation of Hybridoma 2AC2

| | |
|--|--|
| Initial Reactivity of 2AC2 with EJ Cells | Average O D of 2AC2 = 0 364 Average O D of neg control = 0 077 $\frac{2AC2}{neg. control} = 4 7$ |
| Wells with growth after first cloning of 2AC2 | $\frac{288}{480}$ (60%) |
| Wells secreting Ab against EJ cells | $\frac{75}{288}$ (26%) |
| Wells secreting Ab against EJ cells following expansion | $\frac{61}{75}$ (81%) |
| Wells secreting "sticky" Ab | $\frac{16}{61}$ (26%) |
| Loss of clones (incubator shutdown) | $\frac{15}{45}$ (33%) |
| Wells with growth after second cloning of B5B2 (a clone of 2AC2) | $\frac{282}{480}$ (59%) |
| Clones secreting Ab against EJ cells | $\frac{19}{282}$ (7%) |
| Clones found to secrete Ab against normal Ags | $\frac{17}{19}$ (89 5%) |
| Number of second-generation clones secreting antibodies against EJ tumour-associated Ags | $\frac{2}{19}$ (10 5%) (A5F9 and B1F7) |

Table 7 3 Calculation of Ab Titre for Clone A5F9

| | |
|---|----------|
| Cut-off O.D. Value | 0.231 * |
| Corresponding Dilution of Ascitic Fluid | 1 : 4255 |
| Dilution of Fluid (by PBS) during removal | 1 1.75 |
| Actual Ab Titre | 1 · 7446 |

* The cut-off O D value, used to determine Ab titre, was defined as the O D value 0 1 units greater than that of the control (PBS, 0 131)

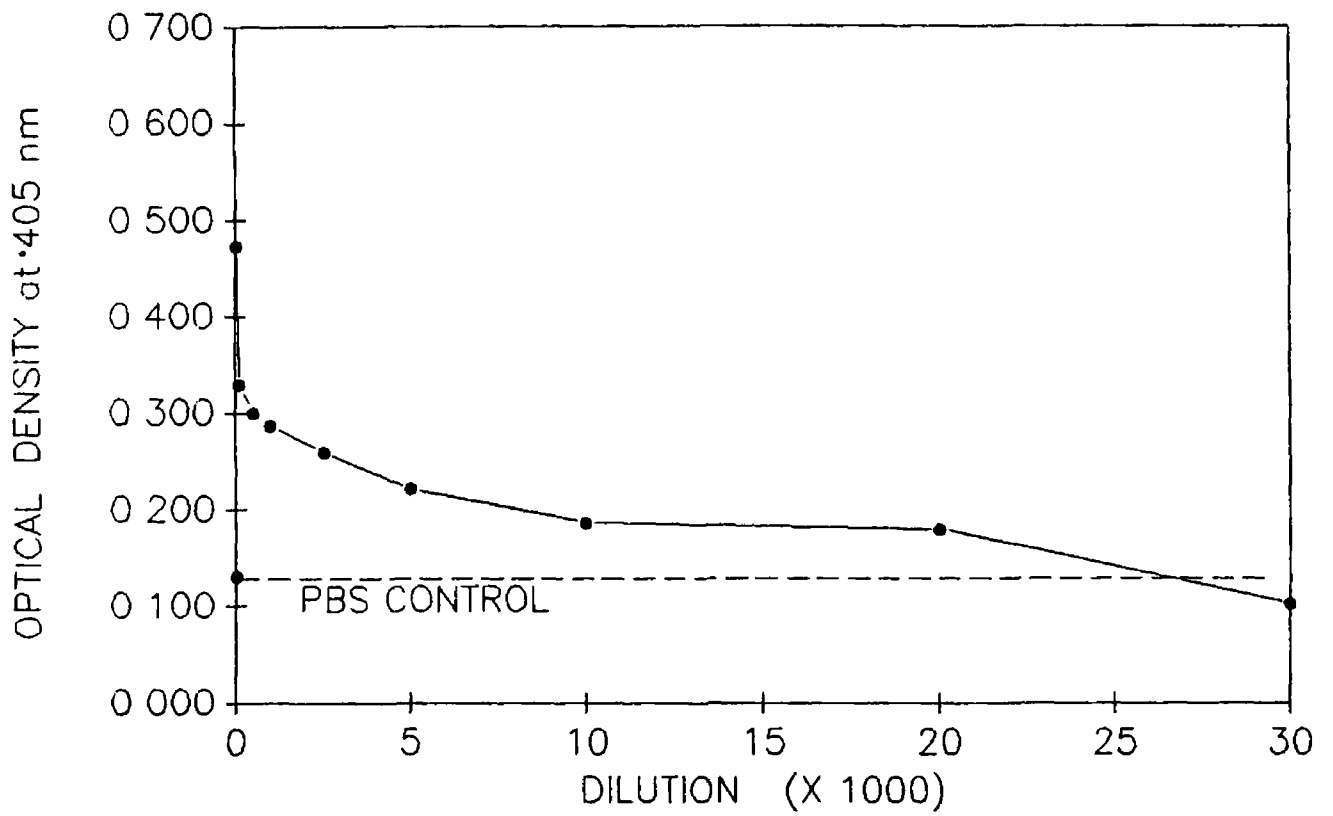
Table 7 4 O D Readings for Determination of Ab
Titre for Clone A5F9

| ASCITIC FLUID DILUTION | AVERAGE O D AT 450 nm |
|---------------------------|-----------------------|
| 1 10 | 0 473 |
| 1 100 | 0 329 |
| 1 500 | 0 300 |
| 1 1000 | 0 287 |
| 1 2500 | 0 259 |
| 1 5000 | 0 221 |
| 1 10000 | 0 187 |
| 1 20000 | 0 180 |
| 1 30000 | 0 103 |

Ascitic Fluid diluent

PBS

FIGURE 7 1
Titre of Monoclonal Antibody A5F9



SECTION 8

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