POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST DIHYDROGRIESENIN, ATRAZINE AND HAEMOPHILUS PARAGALLINARUM, AND THEIR USE IN ENZYME LINKED IMMUNOSORBENT ASSAYS.

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A: atrazine

Ab: antibody

B cell: bone marrow derived cell

BSA: bovine serum albumin

CELIA: competitive enzyme-linked immunosorbent assay

CSIR: Council for Scientific and Industrial Research

c.v.: coefficient of variation

DCA-C-2-arm: 2-aminoacetic-acid-4-isopropylamino-6-ethylamino-s-triazine

DCA-C-6-arm: 2-aminocaproic-acid-4-isopropylamino-6-ethylamino-s-triazine

DEA-C-6-arm: 2-chloro-4-isopropylamino-6-aminocaproic-acid-s-triazine

DHG: dihydrogriesenin

DHG-cys: cysteinyldihydrogriesenin

DMEM: Dulbecco's modified Eagle's medium

DMEMx2: double concentrate of DMEM

DMEM-HS: DMEM with 10% horse serum

DMEM-HS-HT: DMEM-HS with hypoxanthine and thymidine

DMEM-HS-HAT: DMEM-HS with hypoxanthine, aminopterin and thymidine

DMSO: dimethylsulphoxide

DNP-: dinotrophenyl-

DPA-C-6-arm: 2-chloro-4-ethylamino-6-aminocaproic-acid-s-triazine

EDC: N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide

ELISA: enzyme-linked immunosorbent assay

EMBO: European Molecular Biology Organization

EMIT: enzyme-multiplied immunoassay technique

Fab: antigen binding fragment of immunoglobulin

Fc: crystallizing fragment of immunoglobulin

g: unit of gravitational force

h: hour

H: hapten

HAb: hapten-antibody complex

HGPRT: hypoxanthine: guanine phosphoribosyl transferase

Ig: immunoglobulin

Kass: intrinsic association constant

K'ass: functional association constant

M: mol per cubic decimeter

MoAb: monoclonal antibody

m.p.: melting point

P: propazine

PEG: polyethylene glycol

PBS: phosphate buffered saline

Rf: Chromatographic position of a chemical entity relative to frontal solvent movement

rpm: revolutions per minute

RT: room temperature

S: simazine

T cell: thymus derived cell

tlc: thin layer chromatography

Tris: tris(hydroxymethyl)aminomethane

TST: tris-saline-tween

CHAPTER 1: INTRODUCTION

Immunochemistry traditionally implied the use of antibodies (immunoglobulins) to identify, isolate, determine and characterize antigens *in vitro*. With the advent of the techniques of genetic engineering, this definition was recently broadened to cover *in vitro* manipulations of the antigens of the cellular immune response and their appropriate cellular receptors (Kohler, G., 1986).

The first study of specific precipitates of hemoglobin and its antibody was reported in 1927 by Wu *et al.*, and for the next three decades immunochemistry consisted mainly of qualitative and quantitative studies of precipitin and agglutination reactions of antibodies and antigens derived from hyperimmune serum (Kabat, 1980). During this period, Elvin A. Kabat became popularly known as the "father" of immunochemistry, due not only to his pioneering work in the field, but also to his textbook/laboratory manual, first published in 1948 (Kabat & Mayer, 1948), which covered the subject with authority and remains useful even today. Precipitin and agglutination techniques are currently still in use, but mainly for qualitative analyses. The sensitivity for detection of antigens by the quantitative precipitin reaction is in the order of only $1\text{-}10^{-3}$ g/dm^3, while incubation times of several days are required to accomplish complete equilibrium.

Radio-immunoassay, developed by Berson and Yalow (1959) changed the face of classical serology and added remarkable sensitivity (down to 10^{-9} - 10^{-12}M) to immunoassay. The principle of the assay relies on competitive

binding of radio-actively labelled and unlabelled antigen to antibody, the separation of bound from unbound or free antigen and the determination of the distribution of radio-activity between these two compartments. With this technique, the salient specificity of antibodies in whole antisera was demonstrated. For example, the presence or absence of methyl groups on small organic haptens like nicotine and N,N-dimethyltryptamine was shown to alter their binding to antibody by two to three orders of magnitude (Butler, 1977).

Although radio-immunoassay, by its sensitivity, laid the foundation for modern endocrinology, two major obstacles prevented it from gaining popularity as a general analytical technique. One was the problem of separating the antigen-antibody complexes from unbound antigen or antibody without significantly affecting the equilibrium. This could be accomplished by a variety of ingenious methods, but these were tedious and difficult to perform reproducibly. The other problem was the cost, restrictions, and hazard involved in the use of radio-isotopes. Both problems were overcome about two decades ago. Catt, Niall & Tregear (1967) introduced solid phase radio-immunoassay, where proteinaceous antigen or antibody was immobilized on polystyrene, polypropylene or polyvinyl by adsorption. Subsequent separation of free antigen or antibody could therefore be effected simply by washing. The ability to covalently conjugate an enzyme to an antibody, without significantly impairing their activities towards substrate conversion and antigen binding respectively, was accomplished by Avrameas (1969). This was soon followed by the development of solid phase enzyme-immunoassays (Engvall & Perlmann, 1971, Van Weemen & Schuurs, 1971 and Schuurs & Van Weemen, 1977) with almost the same sensitivity and specificity as classical radio-immunoassay.

The convenience of immunoassay using solid-phase immobilization of antigen or antibody and the high-sensitivity labelling of haptens, antigens or antibodies with enzymes was soon realized. The early seventies subsequently saw the launch of modern immunochemistry by numerous ingenious technical advances leading to various types of enzyme-immunoassays which were continuously improved upon and adapted to diverse fields of application. Enzyme-immunoassays were classified as being either homogeneous or heterogeneous. The homogeneous enzyme-immunoassay produces the measured enzyme signal in a homogeneous solution containing both antigen-antibody complexes and free antigen or antibody (Rubenstein et al., 1972). Heterogeneous enzyme-immunoassays have the antigen-antibody reaction taking place in one compartment, while the enzyme reaction is initiated in another compartment after the antigen-antibody complexes have been separated from unbound antigen and antibody. All solid phase enzyme-immunoassays, including enzyme-linked-immunosorbent-assay (ELISA), are heterogeneous assays (Engvall & Perlmann, 1971).

Homogeneous enzyme-immunoassay, also known as enzyme-multiplied immunoassay technique (EMIT), is based on the principle of modulation of enzyme activity upon binding of antibody to a hapten which is covalently bound near the active centre of the enzyme. It has found popular clinical use in the determination of small haptens such as opiates (Schneider et al., 1973), marijuana (Rodgers et al., 1978) and thyroxine (Jaklitsch et al., 1976), but is of little use in the assay of large antigens, such as proteins, and is less sensitive than the heterogeneous assays.

Heterogeneous enzyme-immunoassay, of which ELISA became the most popular, has found application in many areas of biology and medicine. Its sensitivity is comparable to that of radio-immunoassay, as long as care is

taken to maintain constant reaction conditions, exact incubation times and control of reaction temperatures (Muller-Esterl, 1986). It is possible that even higher sensitivity can be achieved by the recently developed substrate amplification system for enzyme-linked immunoassays (Carr et al., 1987), which utilises a binary enzyme cascade or cycle to amplify the specific ELISA-signal instead of the single enzyme that is conventionally used.

Different configurations of ELISA have been developed to suit particular purposes and these were extensively reviewed in recent literature (Masseyef, 1986; Blake & Gould, 1984; Butler et al.; 1987 and Tijssen, 1985). The two-site, or sandwich method of antigen assay became particularly popular, due to its higher sensitivity and lower background interference caused by non-specific interaction of analyte and indicator-reagents with the solid-phase support (Engvall & Perlmann, 1972). The method involves the capture of multivalent antigen from a sample solution by a capture antibody immobilized on the solid phase and its quantitative determination by an antigen-specific, enzyme-linked second antibody, which recognizes different determinants of the antigen. Prior affinity purification of the capture antibody is required to prevent loss of signal by dilution of the specific antibody with non-specific antibodies and proteins during immobilization. However, affinity purification often causes the loss of antibodies with the highest binding affinity due to the difficulty of their elution, thereby reducing the sensitivity of the assay (Masseyef, 1986). This problem was solved by the use of monoclonal antibodies produced in serum-free medium (Kawamoto et al., 1986) and purified with protein A (Ey et al., 1978).

The *in vitro* production of hybridoma cell lines producing monoclonal antibodies by Kohler and Milstein (1975), represented a major advance in immunochemistry. Monoclonal antibodies can distinguish single antigenic determinants on complex antigens. Thus, for example, the identification of single proteins on whole cell surfaces enabled the systematic study of the expression and function of histocompatibility antigens on mammalian cells (Lemke *et al.*, 1979) and cell surface differentiation markers on haematopoietic stem cells (Cooper *et al.*, 1986). The major advantages of monoclonal antibodies in enzyme-immunoassay are three-fold: First, they are available at virtually unlimited quantities as standardized reagents of known specificity and affinity. Second, being derived from cloned cell lines, monoclonal antibodies represent the ultimate in specificity that can be accomplished by immunoassay. Third, monoclonal antibodies have been produced against antigens ranging from single atoms (Reardan *et al.*, 1985) to cell specific markers, using whole mammalian cells as immunogens (Van Riet *et al.*, 1985). This clearly demonstrates the vast potential for the application of monoclonal antibodies in industry, agriculture and medicine.

In summary, speed, specificity, convenience, flexibility, sensitivity and the possibility towards automation are the attractive features of monoclonal antibody-based ELISA, which emerged as a powerful modern analytical tool from the discipline of immunochemistry. This warrants research into its value as a substitute for some conventional analytical techniques. In particular, three questions arise which may affect the universal applicability of ELISA and monoclonal antibodies:

- Do the standard methods which are currently in use for preparing antigen coated solid phases suit all kinds of analytes to be deter-

mined? Kemeny and Challacombe (1986) reviewed cases where distortion and disorientation of antigenic determinants of protein antigens upon adsorption to plastic surfaces occurred. Presentation of the antigen by antibodies which were previously immobilized on the matrix of the ELISA plate wells solved the problem. Pesce et al. (1986) observed that cationic proteins immobilized on plastic, produced unacceptable background signals with ELISA, due to the non-specific binding of serum proteins which are almost exclusively anionic at neutral pH. The problem could be solved by neutralization of the surface charge by the inclusion of polyanions like heparin in subsequently used incubation buffers. Sarkar and Mandal (1985), confronted by a loss of specific ELISA signal and high background values, developed a new solid phase system where protein antigen was covalently conjugated to polyester film, to good effect. Clearly, each individual antigen-antibody system may require unique ways of immobilization before it can be applied in ELISA.

- Is the antibody repertoire broad enough as to be able to induce antibodies to any chemical or biological analyte? Calculated from the possibilities of diversification of antibody genes, the answer would be affirmative, but practice showed this to be naive. The parameters that govern the expression of a particular pre-immunization antibody specificity repertoire from the vast possibilities of recombinatorial and junctional mechanisms of construction of V-region antibody genes are presently not understood (Manser et al., 1984). The mechanism of post-immunization clonal B-cell selection and the intricate regulation of expression of a particular set of antigen specific antibody producing cells are presently the subject of debate based on paradoxical, but well-substantiated data (Bretscher et al., 1986). The

probability of finding a particular antigen specificity is therefore dependent not only on the structure of the antigen, but also on the genetic and idiotypic construct of the immunized animal and the intentional or unintentional modulation of the immune response during the immunization program.

- Are the specificities of monoclonal antibodies sufficient to distinguish between closely related chemical structures? The answer to this question can be found in the closeness-of-fit of the antigen in the antigen combining site of the antibody. Pecht et al. (1982) described several examples of how different antigens and their chemical derivates fitted specific antibody combining sites in structural and kinetic terms. Electrostatic, electronic, steric and dipole interactions were shown to participate in determining the specificity of binding, but the relative contribution of each remained merely a calculated guess, until technology allowed the mapping of antigen combining sites by nuclear magnetic resonance spectroscopy (Anglister et al., 1985) and X-ray crystallography of antigen-antibody complexes (Amit et al., 1986). These recent achievements have opened up the possibilities of methodological chemical tailoring of haptens to optimize specificity of interaction between hapten and antibody within the constraints of immune regulation mentioned before. For the present, however, the degree of specificity that can be obtained against antigens by monoclonal antibodies remains to be determined by trial and error for each antigen-antibody system to be established (see for instance Ivanyi et al., 1985).

The purpose of this study was to extend the application of ELISA and monoclonal antibodies, or account for imperfections, in a selected number

of situations of interest to the field of agriculture, where problems were encountered either in generating the required antibodies, or in designing a suitable solid phase for enzyme-immunoassay:

- Dihydrogriesenin, a toxic sesquiterpene lactone from Geigeria plants affecting ruminant live-stock, exhibited immunorecessiveness upon conjugation to bovine serum albumin, which prevented the genesis of hapten-specific antibodies in immunized animals (Chapter 3).

- Atrazine, a herbicide requiring proper control of its concentration in soil in rotational crop farming practices, contains an exocyclic chlorine which serves as a convenient leaving group in the chemical reaction to produce a hapten-protein conjugate for use as immunogen and solid-phase hapten. Chapter 4 addresses the problem of the subsequent low affinity antibodies that were induced this way and the advantages and disadvantages of the hapten-protein conjugate as solid phase antigen in ELISA. The successful application of nylon as a macromolecular carrier for atrazine in the preparation of an ELISA solid phase is discussed in Chapter 5.

- A bacterial parasite of fowl, Haemophilus paragallinarum, induced fusogenic spleen cells upon immunization of mice, which impaired the proliferation of hybridoma cells in tissue culture. In Chapter 6, the solution to this problem is presented and its possibilities for application in cell fusion in general is discussed.

The investigations were all based on the application of principles, methods and techniques of fundamental significance to immunochemistry;

these are reviewed together in the next chapter in order to provide perspective for the experimental research reported subsequently.

CHAPTER 2: PRINCIPLES OF ELISA AND HYBRIDOMOLOGY

2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY

2.1.1 PROPERTIES OF ANTIBODIES

Only two of the five main classes of antibodies occur at sufficient concentrations in antiserum to be of use in the measurement of antigen or antibody. IgG is a protein of about 150 kD with a Y-shaped structure consisting of two identical antigen binding fragments (Fabs) and one fragment (Fc) that is responsible for mediating effector functions (Edelman, 1970). The other immunoglobulin class, IgM, appears as a pentameric IgG structure, although the monomers have their own characteristic, structural features. The five subunits are arranged in a closed circle with the Fc domains directed inwards and the Fab domains extending outwards (Chesebro et al., 1968). Electronmicroscopy of antibodies complexed with antigen or divalent haptens showed that both IgM (Feinstein & Munn, 1969) and IgG (Feinstein et al., 1971) are remarkably flexible and can bind to antigen in many configurations.

IgM is the earliest antibody to be formed in the primary response to antigen stimulation, while IgG typically occurs later. Affinity maturation of the antigen binding domains of antibodies takes place during an immunization program, which leads to an increased binding affinity of

antibodies for their particular antigens. The process apparently occurs by random somatic mutations in the antibody gene which are then selected according to affinity by antigen (Griffiths et al., 1984 and Berek et al., 1985). The appearance of high affinity antibody in the antiserum is therefore proportional to the duration of the immunization program. Due to the switching over from IgM to IgG early in the immune response, affinity maturation of IgM is restricted, which may explain why IgM has generally much lower binding affinity for antigen than IgG. Still, somatically mutated IgM antibodies produced by clonally related cells have been described although the phenomenon could not be correlated with increased affinity (Rudikoff et al., 1984).

When working with antisera, even from inbred strains of animals, it is important to realise that antibody heterogeneity exists on at least four levels (Steward and Steensgaard, 1983);

- they belong to different classes or subclasses,

- within a single antibody subclass, they react with different antigenic determinants,

- even when reacting with an individual antigenic determinant, they bind with different affinities and

- they exhibit a variety of different properties, like solubility, ability to form complexes, possible conformational changes upon antigen binding and cross-reactivity with related antigenic determinants.

Monoclonal antibody preparations are homogeneous in terms of class, specificity, affinity and physical and chemical properties. Interpretation of immunoassay data is therefore much simpler when using monoclonal antibodies as compared to antibodies in antisera.

2.1.2 ANTIGEN-ANTIBODY INTERACTIONS IN ELISA

A versatile and convenient type of ELISA is the direct binding type where antigen is immobilized on the matrix, antibody allowed to bind to it and the bound antibody indicated with a reporter antibody labelled with a suitable enzyme (Engvall, 1980). Enzyme-labelled anti- immunoglobulin is commercially available at a price and quality which discourage attempts at producing one's own on small scale. For qualitative antigen and/or antibody determinations, the only interaction of antibody with antigen occurs on the solid phase. Quantitative determination of antigen concentration can be done using the same system, but the antibody is allowed to interact with standard and unknown samples in solution before and/or during its incubation with immobilized antigen (Engvall, 1980). The amount of product produced by the enzyme is then inversely proportional to the amount of antigen in solution. This configuration of ELISA was termed CELIA (for Competitive Enzyme Linked Immunosorbent Assay) by Yorde et al. (1976).

Many improved versions of ELISA were designed to increase sensitivity, decrease background or suit particular experimental requirements. Fun-

damentally, two types of antigen-antibody interaction apply to most configurations of ELISA, viz. the reaction on the solid phase and the reaction in solution. The kinetics and thermodynamics of these differ completely and deserve separate consideration.

- Antigen-antibody interaction in solution

To express the interaction of antibody with its antigen in exact mathematical terms is an extremely difficult, if not impossible task. This is due to undefinable parameters like the heterogeneity of antibodies in antisera, the valence for binding of antigen and possible steric hindrance, the strain imposed on antibodies upon oligovalent binding of geometrically fixed antigenic determinants and different sizes of complexes that may form. In spite of this, equations have been formulated to describe antigen-antibody interactions, which have been successfully applied in the interpretation of data from immunoassays. These are lucidly discussed in the text by Steward and Steensgaard (1983). The following elements are relevant to the thesis research.

Standard affinity (A°) is defined as the energy (in kilojoules per mol) of association of antigen and antibody under standard conditions (temperature = 37°C and pressure = atmospheric)

$$A^\circ = -\Delta G^\circ = RT\ell nK_{ass} \quad - - - - - - 1$$

R = ideal gas constant = 8,314 joules.$mol^{-1}.K^{-1}$

T = absolute temperature = 310,15 K

K_{ass} = association constant

By substitution this approximates to

$$A^\circ = 6 \log K_{ass} (kJ.mol^{-1}) \text{ - - - - - - - -} \quad 2$$

In the case of divalent binding of antigenic determinants which occur as repeating determinants on the antigen, and assuming that the binding of one determinant does not influence the binding of the second, it follows that the energy of binding of the antibody is double that for monovalent binding. The functional association constant (K'_{ass}) is then the squared value of the so-called intrinsic association constant, K_{ass}, or

$$K'_{ass} = (K_{ass})^2 \text{ - - - - - - - - - - -} \quad 3$$

The simplest case of a divalent antibody like IgG (Ab) reacting with a monovalent hapten (H) can be described by the following equilibria:

$$H + Ab = HAb$$

and

$$H + HAb = H_2Ab$$

Since the antibody has two identical antigen binding sites, Ab and H_2Ab have chemical activities equal to twice their molar concentrations. The equation for the intrinsic association constant can then be written as

$$K_{ass} = \frac{[HAb]}{[H] \cdot 2[Ab]} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

and

$$K_{ass} = \frac{2[H_2Ab]}{[H][HAb]} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

Evidence exists that there may be some positive cooperativity when monovalent hapten binds to divalent antibody in solution, suggesting that even the simplest case of antigen-antibody interaction may deviate from ideal (Steensgaard et al., 1982).

This thesis addresses the quantitative determination of only low molecular mass haptens. Where this is done with monoclonal antibodies, as in the case for atrazine (Chapter 4), the above model applies. For the quantitative determination of proteins with immunoassay, the theoretical model becomes notably more complex. Affinity increases were observed when two monoclonal antibodies, directed against different determinants on a protein antigen, were used as a mixture in an antigen determination assay. Moyle et al. (1983) explained this phenomenon by proposing the formation of a circular antigen-antibody complex where the simultaneous binding of more than one antigenic determinant increases functional affinity without changing intrinsic affinity, according to the principle behind equations (2) and (3) above. These theoretical complexities do not, however, limit the

practical use of ELISA for quantifying antigen. The use of a range of standard concentrations of antigen to which an unknown sample can be compared in CELIA allows detection of hapten (Chapter 4) and protein (Engvall, 1980) alike with satisfactory sensitivity.

- Antigen-antibody interaction on the solid phase

Determination of the intrinsic affinity constant for several antigen-antibody systems have been described, using a variety of competitive type, non-solid phase immunochemical techniques (Steward & Steensgaard, 1983). Several attempts have been made to accomplish the same with direct binding ELISA (eg. Beatty et al., 1986), one even with a bold title announcing the authors' undoubted success (Friguet et al., 1985). However, all of these attempts suffer from the inability to determine the absolute concentration of immobilized antigen bound in an orientation which allows its unhindered association with antibody. Recently, the technique of ellipsometry was successfully applied to overcome this problem (Nygren et al., 1986).

Ellipsometry allows the accurate determination of extremely small differences of protein load on silica wafers (Stenberg & Nygren, 1982). An optical beam of linearly polarized light becomes elliptically polarized when it impinges on a reflecting surface. When a thin transparent film covers the surface, for instance by the immobilization of protein on the wafer, the vectors of the ellipse are altered as a function of the added mass. Because the signal is induced by an optical beam, the protein mass on the wafer is referred to as the optical mass. If antibody binds to an antigen which is

immobilized on the wafer, a mass increase occurs which can also be measured as added optical mass with great sensitivity. Likewise, the dissociation of antibody from the solid phase can be monitored continuously and can thus be described kinetically.

Using this technique, Nygren et al. (1986) noticed that the affinity of antibody for antigen was higher when the antigen was immobilized than when in solution. Moreover, the rate of both binding and dissociation was much slower on the solid phase. Following up their own work, Nygren et al. (1987) reported a more detailed investigation of the binding and dissociation rates of antibody with immobilized antigen and concluded as follows: First, initial binding of antibody to immobilized antigen is rapid and diffusion rate limited up to an antibody density of 1 pmol/cm^2, which approximates a saturated monolayer of immunoglobulin. Binding then continues at a much decreased rate for many hours, probably due to a reorganization of the layer of bound antibodies. Second, no dissociation of antibody in the absence of soluble antigen could be measured, implying that the results could not be interpreted simply according to the existence of an equilibrium at the surface. Finally, in the presence of antigen in solution, dissociation of antibodies from the solid phase did not follow a simple and identifiable rate constant. Assuming that the observations made on silica surfaces will also apply to polystyrene, it would appear that the thermodynamics and kinetics of antigen-antibody interactions at a solid phase cannot be described satisfactorily by the kinds of equilibria that describe antigen-antibody interactions in solution. The increased binding strength of antibodies due to their bivalence, though, may provide a partial expla-

nation for the increased stability of antigen-antibody complexes at a surface.

Other authors also noted the almost irreversible binding of anti-hapten antibody to immobilized haptenated protein (Kemp & Morgan, 1986), which could be abrogated either by using Fab fragments, or by drastically decreasing the hapten density on the haptenated protein immobilized on the ELISA plate, to prevent divalent associations. The much increased affinity of antibody for solid phase antigen due to multivalent binding has significance for IgM, which has the potential of decavalent binding to antigen. Hirayama et al. (1985) compared the behaviour in ELISA of monoclonal pentameric IgM, its derived monomeric IgM and a monoclonal IgG1, all with specificity against the same peptide determinant, with their common antigen, hen's egg white lysozyme, immobilized on the plate. In spite of a much lower intrinsic association constant characteristic of IgM, the pentameric molecule gave a stronger ELISA signal than the IgG1 and could be maintained over a much longer dilution range. In contrast, the monomeric IgM gave no signal at all. The low intrinsic affinity of IgM should cause its dissociation from the ELISA solid phase antigen during the washing procedures, unless it binds multivalently. According to the principle behind equation 3 mentioned earlier, the functional affinity of IgM for solid phase antigen becomes so high as to make its binding virtually irreversible, even in the presence of high concentrations of soluble antigen. Only exceptionally low antigen densities on the solid phase will allow inhibition curves to be obtained with IgM in CELIA (Kemp and Morgan, 1986).

The multivalent binding of antibody to solid phase antigen provides an explanation for the observed prozone or high-dose hook effect (Vos et al., 1987). Prozone occurs when, in an antibody dilution series, the ELISA signal is low at the highest antibody concentrations and increases to a maximum prior to the expected descent proportional to dilution. "Crowding" of the antibodies on the solid phase at high concentration prevents multivalent binding and therefore destabilizes the antigen-antibody complex at the surface, thereby causing the deflated ELISA signal in the prozone.

One may conclude that interpretation of binding data from ELISA in absolute mathematical terms is presently not feasible, due to the unpredictable nature of association and dissociation of antibody from immobilized antigen. This necessitates the use of standard curves in CELIA to enable quantitative determination of antigen or hapten.

2.1.3 IMMOBILIZATION OF ANTIGEN

In order to permit the assay of a large number of samples in a single run, 96-well microtitre plates, manufactured from polyvinylchloride or polystyrene, are commonly used as immobilization matrix for antigen. Systematic studies on the adsorptive behaviour of proteins on these materials have been done (Brash, 1977 and Cantarero et al., 1980), from which the following conclusions were drawn: First, binding is a linear function of the input protein concentration up to a limit of approximately 1µg/ml. At lower concentrations, proteins are said to bind in the region

of independence, i.e. the percentage of protein bound is independent of the input concentration. When protein concentration is expressed in molar terms rather than mass/volume, the limit of the region of independence was found to increase inversely proportional to the molecular mass of the protein, implying that saturation occurs when the adsorbed protein reaches a molecular monolayer on the plastic. Second, the binding was independent of the iso-electric point of the protein, indicating that mainly hydrophobic forces are involved in the adsorption. Third, binding was enhanced by longer incubation times and elevated temperatures. Fourth, binding beyond the region of independence occurred, although at a much decreased rate, but this led to protein-protein rather than protein-plastic interaction. This created a protein film of lesser stability which was measured as a decreased ELISA signal due to the washing away of antigen-antibody complexes. Finally, the presence of competing proteins has no effect on adsorption, as long as the total protein concentration of the mixture remained below the limit of the region of independence.

It follows that an optimal concentration of protein exists for the coating of microtitre plate wells. Beyond this concentration, gradual desorption of protein-antibody complexes during washing procedures cause variation of results and lower sensitivity. The same holds true when whole cells (either bacterial or mammalian) are used (Douillard & Hoffman, 1983). Too high cell densities cause formation of clumps that are detached during subsequent washing procedures, sometimes even causing false negative ELISA results. Partial denaturation of protein antigens is advantageous prior to their adsorption to polystyrene (Conradie et al., 1983), because the strength of the antigen-polystyrene interaction is higher for denatured antigens (Munoz et al., 1986). However, this may cause a loss of

antigenicity for sensitive proteins, leading to a weakening of the ELISA signal. The reduced antigenicity of substances upon adsorption to the solid phase can be avoided by the two-site, or sandwich method of ELISA, where antigen is captured by an antibody already immobilized on the plastic (Butler et al., 1987). As reduced antigenicity of hapten is not expected upon partial denaturation of the hapten-protein conjugate, the method of Conradie et al. (1983) provides a suitable method for the coating of ELISA plates with hapten-protein conjugates. Partial denaturation is effected by a ten minute exposure to low pH (glycine buffer, pH 2,5), followed by neutralization prior to distribution into the wells.

2.1.4 NON-SPECIFIC BINDING IN ELISA

The sensitivity of ELISA is often limited by high background signals arising from any of a variety of causes, e.g. incomplete saturation of the solid phase surface with antigen, non-specific protein-protein interactions and contamination with bacteria, which often are ubiquitous antigens for antibodies in sera. This problem is limited by inclusion of a detergent like Tween 20 in a blocking solution which is then incubated on the antigen coated microtitre plate prior to the antibody exposure steps (Kenna et al., 1985). Detergent is also generally used in the washing buffers for the plates between the steps. When the problem of non-specific binding persisted in spite of the use of detergents, proteins were arbitrarily tested and used to replace or complement detergent in

blocking and washing buffers. Bovine serum albumin became a popular choice for this purpose (Schonheyder and Andersen, 1984).

An investigation by Kenna et al. (1985) proved that casein was particularly well-suited as a protein for use in both blocking and washing and obviated the simultaneous use of detergents. In a more systematic approach by Vogt et al. (1987), nine proteins were tested for their effectivity as blocking agents in ELISA, showing casein and skimmed milk powder to be superior. Casein is a mixture of milk proteins varying in properties of size, glycosylation, sulphation and surface charge. This is in contrast with the homogeneity of bovine serum albumin and probably explains its success as blocking agent for diverse applications of ELISA. As milk powder was sometimes found to mask antigen from its interaction with antibody, casein is the more popular blocking and washing protein. It is considerably cheaper than other homogeneous proteins like bovine serum albumin, is effective at lower concentrations (0,5% in PBS) and is less prone to bacterial contamination.

2.1.5 STANDARDIZATION OF ELISA

The attainment of equilibrium in the reaction between antigen and solid phase, antigen and antibody and the enzyme reaction are time and temperature dependent processes. Standard conditions have been laid down by Muller-Esterl (1986) for these steps in ELISA, i.e. coating of microtitre plates with antigen overnight at 4°C, immune reaction for 3 hours at 37°C and enzyme reaction for 30 minutes at 37°C. However, these basic

parameters should be optimized for individual antigen-antibody systems and particular configurations of ELISAs. The incorporation of calibration standards or positive and negative controls in each run with which ELISA signals of unknown samples are compared, obviates the need to stringently adhere to these conditions. One may therefore shorten incubation times and work at ambient temperature without compromising the accuracy of the assay.

2.2 THE HYBRIDOMA TECHNIQUE

2.2.1 PRINCIPLE

If an antigen is injected into a mouse, lymphocytes of the B-cell lineage are selectively activated and home to the spleen where they differentiate into antibody producing plasma cells in an environment of antigen presenting cells and helper lymphocytes from the T-cell lineage. The selection of B-cells depend on the clonal occurrence of membrane bound antigen binding receptors which eventually are slightly modified, abundantly synthesized and secreted as antigen specific antibodies (Nossal, 1986b). Antibody producing cells are terminally differentiated and die in tissue culture within 3-6 days (Klinman & Press, 1975). When fused with a mouse plasmacytoma (myeloma) cell line, hybrid cells are generated which retain the ability of the spleen cell parent to produce and secrete antibody, but are now also capable of continuous growth and

proliferation, a property gained from the plasmacytoma parent (Kohler & Milstein, 1975). After cloning, such hybridoma cells produce monoclonal antibodies of defined specificity, affinity and isotype. The procedure is summarized in Fig. 2.1.

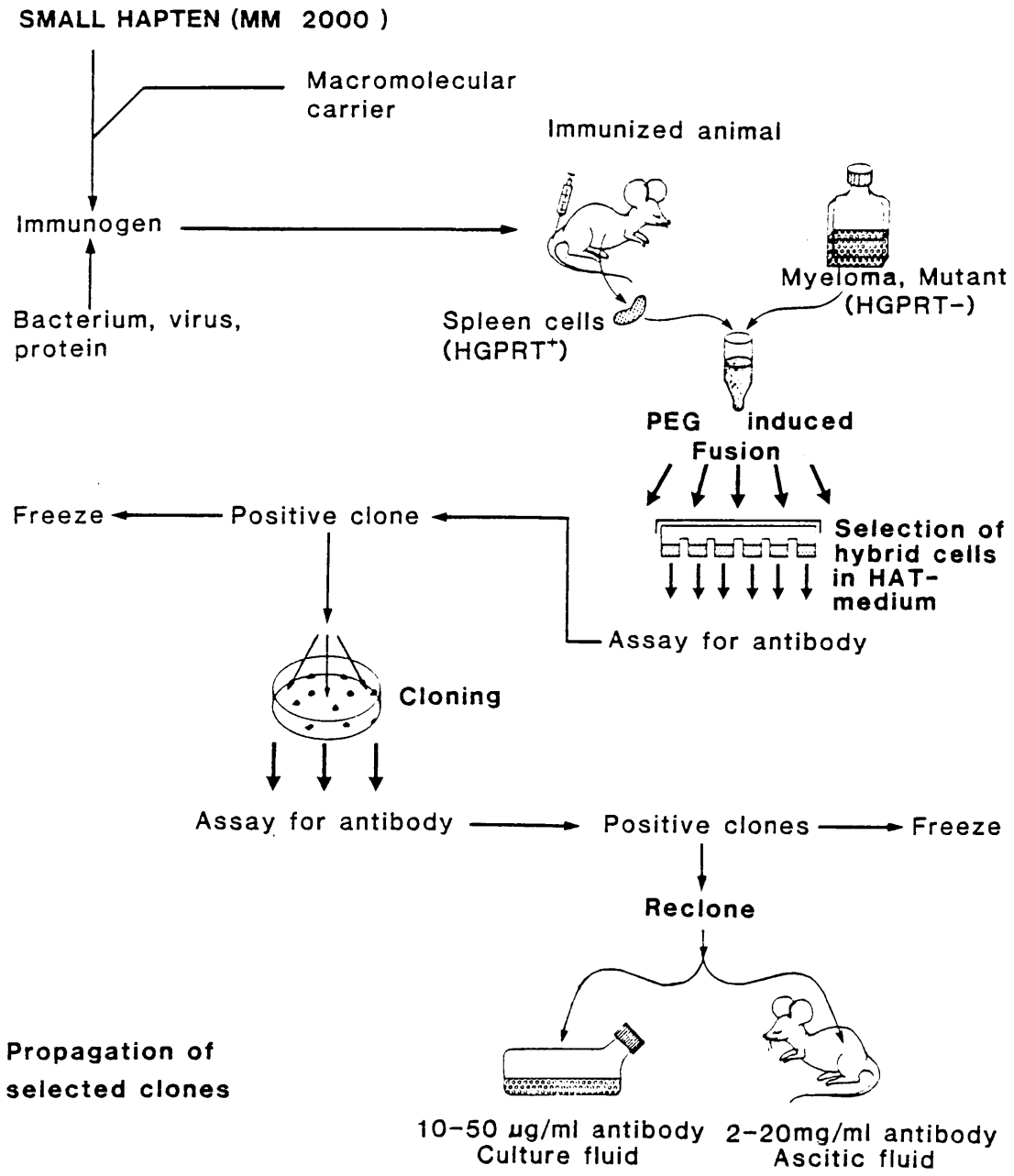

Figure 2.1. Protocol for the generation of hybridoma cells producing monoclonal antibodies. Adapted from Eshhar (1982).

In order to perform a cell fusion successfully, it is advantageous to be acquainted with the underlying mechanisms of each step of the protocol. For this purpose a variety of laboratory manuals (e.g. Galfre & Milstein, 1981) and books (e.g. Campbell, 1984) are available which give extensive coverage of the subject. Certain aspects, however, are discussed due to their relevance for the present study.

2.2.2 MECHANISM OF CELL FUSION

Fusion of somatic cells have been accomplished both with enveloped viruses and amphipathic reagents such as lysolecithin and polyethylene glycols (PEG). PEG has become the more popular fusogen due to its availability and wider variety of cell types for which it promotes fusion (Kennett, 1979). The mechanism whereby cell fusion occurs in the presence of PEG is not exactly known, but features of the process have been investigated and produced interesting results. In a review by Westerwoudt (1986), reference is made to studies which showed that fusion could not be induced by chemically pure PEG. Apparently, anti-oxidants and polymerization agents, present as additives in commercial PEGs, are essential synergistic components. Zimmerberg (1987) reported experimental data which indicated that the fusion of phospholipid membranes occurs in defined steps. Intimate contact between the two membranes that are to fuse is followed by dehydration of the inter-membrane space. The subsequent abrogation of hydrophobic forces allows molecular mixing and rearrangement, leading to a connection of the interiors of the two compartments by pore formation. This pore is then rapidly widened and fusion follows.

Swelling of the cells appeared to promote fusion, which may explain why PEG induces fusion preferentially between mitotic cells (Kennett, 1979). The reason for boosting mice with antigen only three to four days before fusion is to enrich the spleen with antigen specific mitotic cells which are then preferentially fused.

The temporary chromosomal instability of hybridomas after fusion was found to be difficult to define (Kennett, 1979). How rapidly stabilization is brought about, and the rate at which chromosome loss and rearrangement occurred, appeared to be functions of the mitotic synchronization of the fusion partners. Fusion between more than two cells simultaneously resulted in trinucleated or multinucleated hybrids which did not survive in tissue culture (Westerwoudt, 1986). It is therefore highly unlikely that hybridomas will occur which produce more than one kind of "monoclonal" antibody simultaneously.

2.2.3 FREEZING AND THAWING OF CELLS

Mammalian cells frozen in aqueous medium do not remain viable after thawing, due to their mechanical disruption by the formation of intracellular ice-crystals. This can be prevented by the inclusion of cryoprotective agents such as dimethylsulphoxide (DMSO), propylene or PEG in the freezing medium at relatively high concentrations. On the grounds of simplicity, rather than high efficiency of recovery of viable cells, Galfre and Milstein (1981) suggested the use of 10% DMSO, 20% serum and

70% growth medium as freezing liquid for hybridomas. DMSO rapidly permeates the cell membrane and allows supercooling of the cell interior and exterior, until the viscosity increases such that vitrification rather than crystallization occurs at the freezing point. This process was recently investigated by Rall and Fahy (1985) using mouse embryo cells. Cell viability of better than 80% could only be maintained if exposure of cells to DMSO at 4°C did not exceed 15 minutes, due to the toxicity of DMSO. This observation emphasizes the importance of speed and the maintenance of low cell temperatures during cell freezing. Vitrification proceeded well at moderate cooling rates, but thawing had to be very rapid to prevent crystallization and death of the cells.

2.2.4 CLONING

Hybridoma cells can be cloned either on semi-solid support (agar) or by limiting dilution. The former is preferred as a consequence of its economy, accuracy, possibility towards simultaneous decontamination and the redundancy for feeder cells. There are instances, however, when limiting dilution is the only cloning option available. Immunization with bacterial antigens (Grooten, J. et al., 1980),but occasionally also with proteins (Mazurek, N. - personal communication), may lead to autoreactive antibody producing hybridomas which autolyze when their secreted antibody products are allowed to accumulate too much in the growth medium. Limiting dilution enables daily changes of growth medium, which prevents this from occurring.

The timing of cloning is an important consideration. Due to the chromosomal instability of recent hybridoma fusion products, the cells should be cloned as early as possible after fusion. However, cloning proceeds much more effectively at a later stage, albeit with decreased probability of finding the desired clone (Galfre and Milstein, 1981). A second cloning at a later stage is recommended to ensure the monoclonality of the hybridoma cell line under investigation, especially where limiting dilution is the cloning method. The belief that cloning should be performed as early as possible after cell fusion, due to the risk of non-producer hybridoma cells outgrowing the antibody producers, has been investigated by Westerwoudt (1986) and found to be exaggerated. This would enable one to maintain hybridoma cultures in microculture wells for several months, without loss of antibody production, provided that medium is refreshed periodically.

CHAPTER 3: SEMI-QUANTITATIVE DETECTION OF DIHYDROGRIESENIN

3.1 INTRODUCTION

Dihydrogriesenin (DHG) is a member of the group of toxic sesquiterpene lactones that are characterized by the presence of a reactive, exocyclic α-methylene group conjugated to a γ-lactone (Vermeulen et al., 1978). This property enables them to undergo Michael-type addition to sulphydryl bearing proteins. The subsequent inactivation or functional modulation of the alkylated proteins is thought to evoke the various disease symptoms caused by this group of compounds. In addition, it has been shown that the presence of the α-methylene-γ-lactone is a prerequisite for sesquiterpene lactones to cause allergic contact dermatitis in man (Mitchell, 1975). The effects of these interesting compounds, including DHG, on several glycolytic and tricarboxylic acid cycle enzymes and on the degranulation of mast cells have been reported recently (Gaspar, 1984; Gaspar et al., 1986; Gaspar et al., 1987 and Van Aswegen et al., 1982).

Dihydrogriesenin was isolated from the plant Geigeria africana (De Kock, Pachler & Wessels, 1968 and De Kock, Pachler, Ross, Wessels & Du Preez, 1968), one of several species of Geigeria causing vomiting disease in ruminants, especially sheep. The disease is characterized by myofibrillar degeneration in the oesophagal muscles, which prevents swallowing of regurgitated rumen contents and eventually causes death. Before the causative plants were identified, major economic losses were suffered by

farmers due to Geigeria poisoning of their live-stock, especially in Griqualand West (Grosskopf, 1964).

The development of an ELISA system to detect DHG was undertaken to determine the in vivo distribution of the toxin in tissues after ingestion of the causative plant. As it is expected that DHG will covalently attach itself to biological nucleophiles such as protein thiols soon after absorption, it was decided to use a carrier protein cysteinyl-DHG conjugate (see Fig. 3.1) as immunogen to obtain polyclonal specific antiserum. Subsequently, an ELISA for the detection of DHG was developed, as reported below and in an earlier publication (Verschoor et al., 1987).

3.2 MATERIALS AND METHODS

3.2.1 PREPARATION OF IMMUNOGEN

Dihydrogriesenin was isolated from G. aspera as previously described (Vermeulen et al., 1978). A solution of DHG (4,8 mmol) in methanol (25 ml) was stirred into a solution of cysteine (5,8 mmol) in 70% methanol (25ml) at room temperature. The reaction product solidified from the solution within 20 min. Methanol (50 ml) was added to the reaction mixture, which was then stirred for a further 2 h at 50°C. A product was obtained in 80% yield after filtration and washing with methanol, which appeared pure on tlc (Silica gel 60, Merck) developed with n-butanol:

acetic acid: water 3:2:1. A recrystallized sample with melting point 202 - 205°C was obtained from water and its chemical structure characterized. Elemental analysis, infrared and ultraviolet spectroscopy and nuclear magnetic resonance data were in agreement with the structural properties assigned to cysteinyl-DHG (Fig. 3.1).

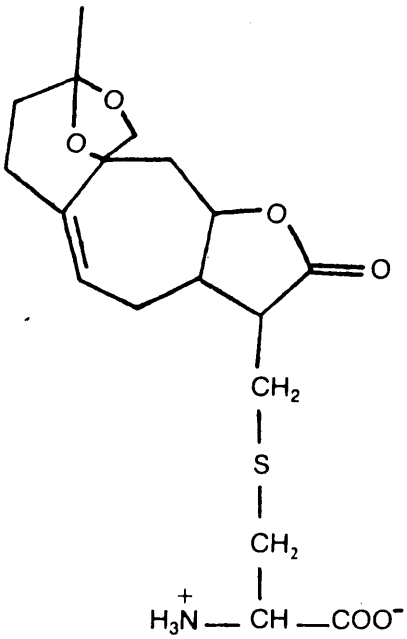

Figure 3.1 Structure of cysteinyl-dihydrogriesenin

A solution of N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide- HCℓ (EDC, 2,6 mmol) in water (2,5 ml) was added to a solution of cysteinyl-DHG (0,13 mmol) and serum albumin (100 mg) in water (5 ml). After stirring for 1 h at room temperature, the mixture was dialyzed and lyophilized to yield the immunogen. Epitope densities of the immunogens were determined by

difference spectrophotometry at 242 nm between the conjugate and the relevant serum albumin. The difference spectrum revealed a peak at 242 nm, which presumably relates to the absorbance at 238 nm of DHG for which a molar extinction coefficient of $1,5 \times 10^{3}$ M^{-1} cm^{-1} was determined. Conjugates with epitope densities, ranging between 10 and 20 mol DHG per mol carrier albumin, were used for this study.

3.2.2 IMMUNIZATION

Six-week-old Balb/c mice were immunized intradermally with DHG human serum albumin (60 µg) in Freund's complete adjuvant (Miles Laboratories,Inc.). DHG-dog serum albumin (30 µg) and DHG-human serum albumin (30 µg) were alternated in subsequent boosters, which were subcutaneously administered at 4-week intervals. The animals were bled 7 days after the last booster injection. A control group was immunized with saline and adjuvant.

3.2.3 TITRE DETERMINATION

DHG specific antibodies were detected by ELISA. The antigen solutions of DHG-rabbit serum albumin and unconjugated rabbit, dog and human serum albumins were each dissolved in 0,05M glycine - 0,1M NaCℓ, pH 2,5 at 1 - 10 µg/ml. The pH was adjusted to 7,0 with solid Tris (Conradie et al.,

1983). Aliquots of 100 µl of the antigen solutions were used to coat the wells on microtitre plates (Linbro Scientific, Hamden, Connecticut), the plates incubated at room temperature for 2 h with gentle shaking and washed with 0,05M Tris - 0,1M NaCℓ, 0,05% Tween 20, pH 8,0 (TST) and distilled water. Any remaining protein binding sites were blocked by incubation with 200 µl of 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at room temperature with gentle shaking. The antigen coated wells were dried under vacuum after the excess solution was removed. Aliquots (50 µl) of immune and control sera dilutions, prepared in 1% BSA/PBS, were transferred to the antigen coated wells, incubated for 1 h at room temperature, excess solution aspirated and the wells washed with TST and 1% BSA/PBS. A 1:1000 dilution of horse radish peroxidase conjugated rabbit anti-mouse IgG (Miles-Yeda, Israel) was prepared in 1% BSA/PBS, aliquots (50µl) were transferred to the wells and incubated at room temperature for 45 min. The wells were washed with 1% BSA/PBS, dried, and incubated with aliquots (50µl) of the substrate solution (o-phenylenediamine, 1 mg; hydrogenperoxide, 30%, 0,5 µl and citrate buffer 0,1M, pH 4,5, 1 ml). The enzyme reaction was monitored at 450 nm in a Titertek Multiskan MC (Flow Laboratories, Helsinki, Finland) after 20 min.

3.2.4 INHIBITION OF THE ANTIGEN-ANTIBODY REACTION

The ELISA procedure described above was also used to measure the inhibition of the binding of antibodies to antigen coated wells. Whole and affinity purified antisera were incubated with equal volumes of a

saturated solution of DHG-cys (0,043mM) in 1% BSA/PBS for half an hour before transferrance to the antigen coated wells.

3.2.5 IMMUNO-ADSORPTION OF ANTISERA

One gram of CH-Sepharose 4B (Pharmacia Biotechnology, Uppsala, Sweden) was washed with 0,5M NaCℓ on a sintered glass filter. The ligand solution - 0,3 µmol of equal amounts of human and canine serum albumin in distilled water (4 ml), pH 6,0 - was added to the gel and gently mixed. This was followed by drop-wise addition of an EDC solution (2 mmol in 4 ml of distilled water, pH 6,0) and gentle mixing for 18 h at room temperature. Unbound protein was removed by washing with carbonate buffer (0,1M, pH 8,3), acetate buffer (0,1M, pH 4,0) and distilled water. The immunosorbent thus prepared was equilibrated in PBS prior to use.

Removal of carrier-specific antibodies was done by adding one volume of immuno-adsorbent to 1,2 volumes of antiserum, gentle mixing for 1 h at 37°C and subsequent centrifugation at 8000g for 5 min.

3.3 RESULTS

3.3.1 SYNTHESIS OF IMMUNOGENS

Cysteinyl-DHG was synthetized in good yield with the structure, as shown in Fig. 3.1, determined by physical methods. It exhibited a lower Rf (0,64) on tlc than DHG (0,84), presumably because of the polar moieties of the cysteine. The use of cysteine as hapten spacer on the protein carrier had a dual advantage: firstly, surface charge on the carrier molecule remains unchanged upon conjugation with the hapten, thereby retaining important chemical properties of the carrier, such as isoelectric point, gross conformation and water solubility; secondly, the reactivity of the α-methylene group of DHG towards thiol groups on exposed tissue proteins is thought to be directly involved in incurring the observed tissue damage in poisoned animals, or indirectly by some immune mechanism (Pienaar et al., 1973). Antibodies against cys-DHG could therefore be used to determine the fate of DHG in poisoned animals, should it become bound to proteins and/or peptides.

3.3.2 PREPARATION OF ANTI-DHG SERUM

Anti-DHG serum was raised in animals by immunization with DHG conjugated to serum albumin. A preliminary investigation, using rabbits as exper-

imental animals, revealed a negligible anti-DHG response in comparison with the response against the carrier albumin. The ratio of anti-DHG to anti-carrier titre was significantly increased by alternating different serum albumins in the booster injections (D.C. Barnard, 1980, unpublished results). In that study, where Balb/C mice were used as experimental animals, anti-DHG ELISA titres of up to $2{,}2 \times 10^{4}$ were obtained against DHG-rabbit serum albumin as antigen after three booster injections (Fig. 3.2).

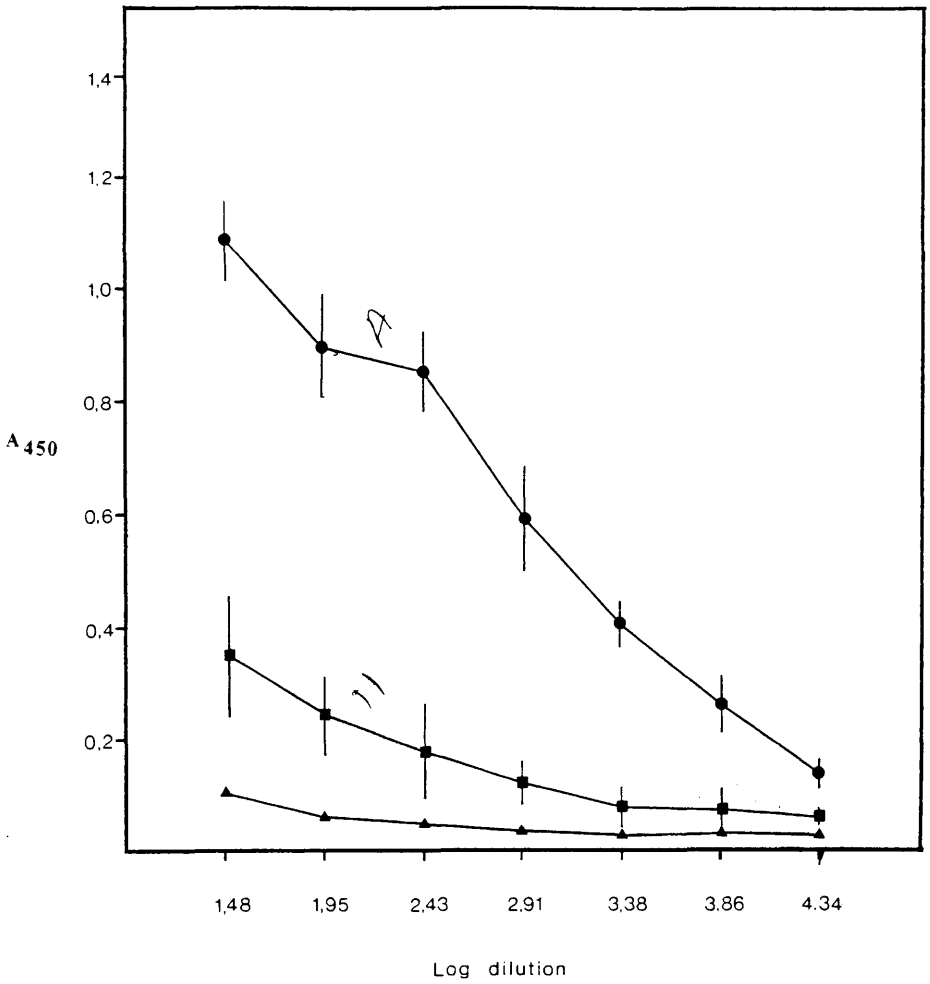

Figure 3.2. Mouse anti-DHG serum titre determination by ELISA

●——● Response (n = 5) against DHG-rabbit serum albumin

■——■ Response (n = 5) against rabbit serum albumin

▲——▲ Response of non-immune serum (n = 1) against DHG-rabbit serum albumin

3.3.3 INHIBITION STUDIES AND IMMUNO-ADSORPTION

As is evident from Fig. 3.2, an appreciable signal is generated from reaction of the immune serum to unconjugated rabbit serum albumin. Accordingly, inhibition of the ELISA- anti-DHG signal by prior incubation of antiserum with cys-DHG before incubation with DHG-rabbit serum albumin coated wells, could be effected to a maximum of only 50%, with optimal values in the lowest antiserum concentration range (Fig. 3.3). Purification of the antisera by immune adsorption with human and canine albumins, resulted in a significant increase in specificity of the purified antiserum, as almost 80% inhibition of the anti-DHG-rabbit serum albumin ELISA signal could be brought about by prior incubation of purified antiserum with cys-DHG (Fig. 3.4).

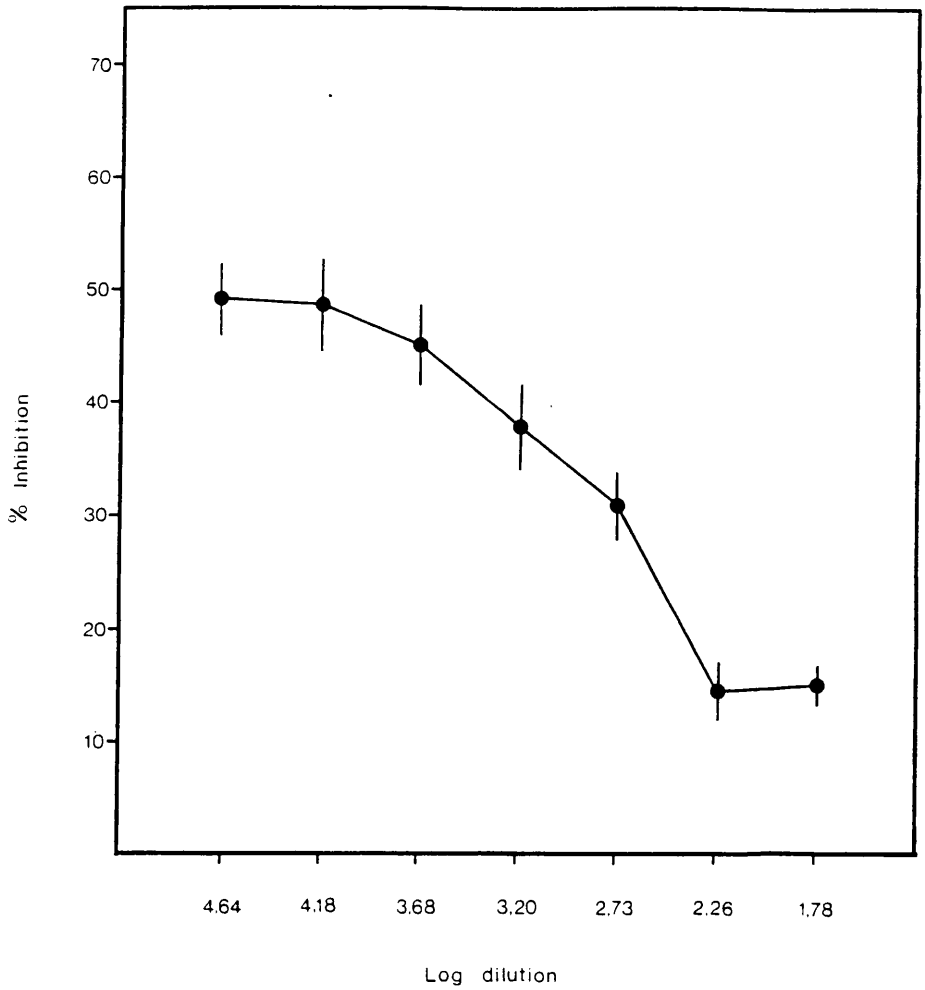

Figure 3.3. Inhibition of anti-DHG serum with cys-DHG. Each point represents triplicate values, using the antiserum of highest titre.

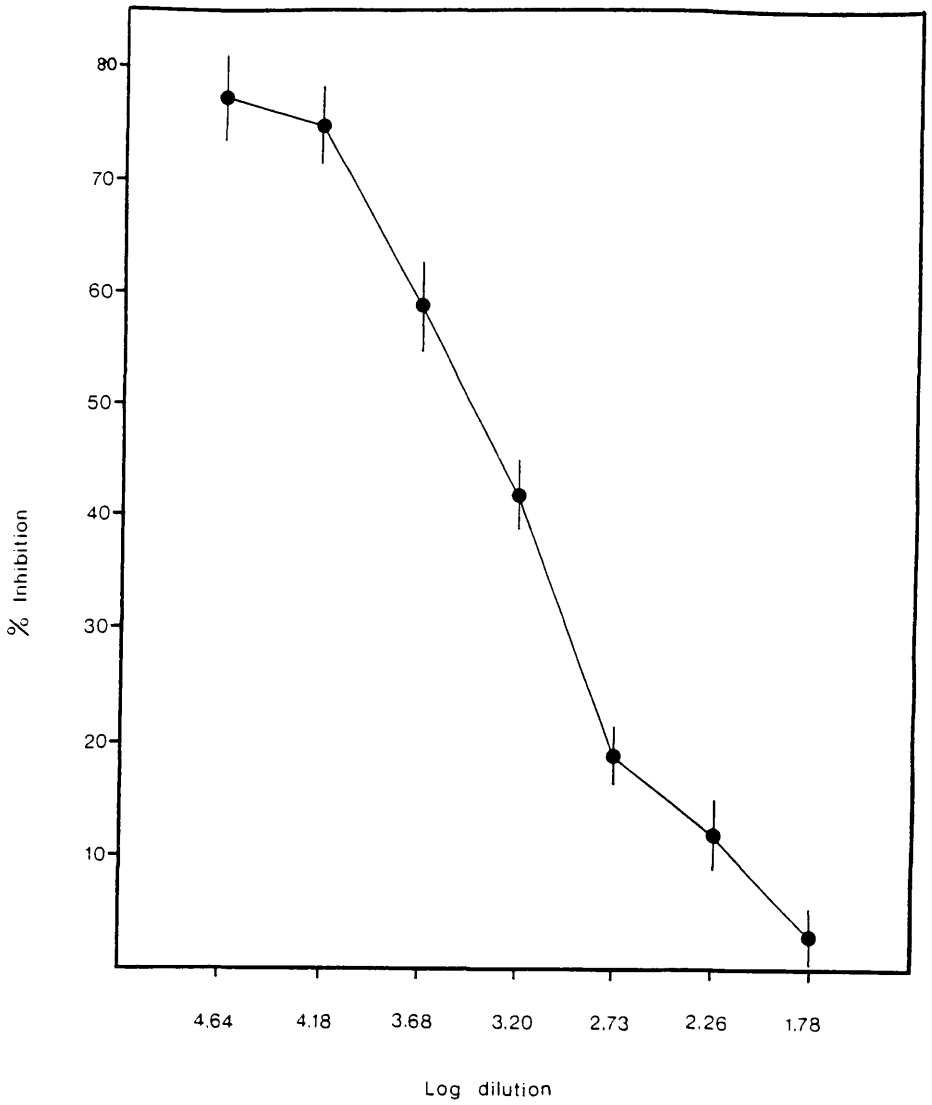

Figure 3.4. Inhibition of affinity purified anti-DHG serum with cys-DHG. Each point represents triplicate values, using the antiserum of highest titre.

3.4 DISCUSSION

The negligible anti-DHG response observed upon immunizing rabbits with DHG-rabbit serum albumin conjugates, is consistent with previous obser-

vations that carrier molecules need to be immunogenic for their hapten conjugates to stimulate the production of hapten specific antibodies (Benacerraf et al., 1970). However, even homologous protein carriers may become immunogenic and elicit an antibody response, as reported by Onica et al. (1978), who observed that new antigenic determinants were generated by the treatment of homologous proteins with cross-linking agents such as glutaraldehyde and carbodiimide.

Upon immunization with a heterologous serum albumin conjugate of DHG, only a weak anti-DHG response was measured, indicating the relative immunorecessiveness of the hapten. Success was eventually achieved by alternating the source of albumin used as carrier in primary and booster immunizations. Affinity maturation of the anti- carrier response was thus retarded, while the continued presence of DHG allowed unhindered selection of affinity matured anti-DHG antibody producing lymphocytes (see Chapter 2, Griffiths et al., 1984 and Berek et al., 1985). A significant increase in the resulting anti-DHG response resulted, while the contribution of the carrier to the generation of the ELISA signal diminished. Since an ELISA signal selectively reflects the presence of antibodies of the highest functional affinity (Steward & Steensgaard, 1983), the result implies that high affinity, hapten-specific antibodies have been produced in the immunized animals.

Polyclonal antiserum against hapten-protein conjugates contain antibodies with three classes of specificity (Benacerraf et al., 1970). Some are directed against carrier determinants, others against the hapten and some against determinants that arise from the covalent interaction of the carrier and the hapten. In the response against DHG-serum albumins these different specificities were distinguishable. Inhibition of antiserum by

incubation with cysteinyl-DHG resulted in only a 50% decrease of ELISA signal (Fig.3.3), while removal of anti-carrier antibodies by affinity purification increased the effectiveness of inhibition by cysteinyl-DHG to almost 80%. The remaining antibody activity that could not be inhibited, probably reflects low affinity antibodies of the first and third classes mentioned above, for which 0,043mM hapten solution is insufficient for 100% inhibition.

In conclusion, although a specific anti-DHG antiserum has been successfully prepared, the required hapten specificity could only be achieved after affinity purification. The hybridoma technique for producing monoclonal antibodies allows selection of clones of hybridoma cells producing antibody of the required specificity. This eliminates the requirement for affinity purification of antibody preparations and offers an alternative approach for studying antibody-hapten interactions in a more quantitative fashion. This is described in the next chapter, where the herbicide atrazine was used as hapten.

CHAPTER 4: MONOCLONAL ANTIBODIES AGAINST ATRAZINE

4.1 INTRODUCTION:

Atrazine (A, Fig. 4.3) is the preferred member of the chloro-s-triazine herbicides for use with maize (Gast, 1970). Depending on factors such as rainfall, temperature and soil pH, the compound is gradually hydrolyzed to inactive hydroxy compounds (Weber, 1970). After atrazine has been used, analysis of environmental samples for their residual atrazine content is a prerequisite for the correct selection of subsequent cultivation procedures such as crop rotation on the same land. The analytical technique should be able to distinguish between atrazine and its inactive degradation products. Although this has been successfully achieved with both gas chromatography (Muir and Baker, 1978) and high performance liquid chromatography (Vermeulen et al., 1982), it could not be applied widely in practice, due to the variability between soil samples requiring a large number of random samples to obtain a proper statistical mean, the tediousness and cost of the prior extraction of atrazine and the filtering of sample solutions and, in the case of gas chromatography, the chemical derivatization required to volatilize atrazine and its metabolites. Any alternative analytical technique should ideally have minimal requirements for prior extraction and purification, the specificity to distinguish between atrazine and closely related metabolites, the capacity to analyze many samples in a short time, and the sensitivity to detect the analytes at 0,1-10 parts per million.

The ELISA-technique provides a fast, safe, sensitive and reliable immuno-assay for large scale screening of samples. A competition enzyme linked immuno-assay (CELIA) is the simplest and most convenient adaptation of the direct binding ELISA for quantitative determination of antigens, including low molecular weight haptens like atrazine (Yorde et al., 1976). By using monoclonal antibodies, the required specificity can be achieved (Kohler and Milstein, 1975). The development of a monoclonal antibody based CELIA for an atrazine derivate and its use in the affinity assessment of a range of different s-triazines related to atrazine is reported below.

4.2 MATERIALS AND METHODS

4.2.1 HAPTENS

All haptens, except hydroxy-atrazine, and hapten-protein conjugates were synthetized by N.M.J. Vermeulen. Structures appear in Fig. 4.3. The protocols are here briefly described.

Atrazine (2-chloro-4-isopropylamino-6-ethylamino-s-triazine, abbreviated A), propazine (2-chloro-4,6-bis-isopropylamino-s-triazine, abbreviated P), simazine (2-chloro-4,6-bis-ethylamino-s-triazine, abbreviated S), depropylamino-aminocaproic acid atrazine (2-chloro-4-ethylamino-6-aminocaproic-acid-s-triazine, abbreviated DPA-C_6-arm) and de-ethylamino-

aminocaproic acid atrazine (2-chloro-4-isopropylamino-6-aminocaproic-acid s-triazine, abbreviated DEA-C_6-arm) were synthetized according to Thurston, J.T. et al. (1951).

Dechloro-aminocaproic acid atrazine (2-aminocaproic-acid-4-isopropylamino 6-ethylamino-s-triazine, abbreviated DCA-C_6-arm) and dechloro-amino-acetic acid atrazine (2-amino-acetic-acid-4-isopropylamino-6-ethylamino s-triazine, abbreviated DCA-C_2-arm) were synthetized according to a modified method of Kaiser, D.W. et al. (1951):

Atrazine (20 mmol) and either ε-amino-caproic acid or glycine (20 mmol), for the synthesis respectively of DCA-C_6-arm and DCA-C_2-arm, were dissolved in methoxy-ethanol (50 ml). This solution was reacted under reflux (approx. temp. = 125°C) for 1,5 hours while the pH was maintained at the inflection point of phenolphthalein with 10% aqueous NaOH solution. The product was evaporated to dryness under vacuum.

The DCA-C_6-arm preparation was redissolved in ethanol (50 ml), the pH adjusted to 12 and impurities filtered off. An equal volume of water was added, the pH was adjusted to 6,7 with dilute HCℓ and the product left to crystallize. The DCA-C_2-arm preparation was dissolved in water (75 ml) and crystallization was initiated by adjusting the pH to 4,1 with dilute HCℓ. Crystals were washed twice with water before being dried under high vacuum.

The purities of the products were indicated by single spots on tlc and confirmed by melting points determined at 173 - 8°C (A), 213 - 5°C (P), 222 - 7°C (S), 201 - 3°C (DCA-C_2-arm), 164 - 5°C (DCA-C_6-arm), 143 - 4°C (DEA-C_6-arm) and 164 - 5°C (DPA-C_6-arm). Structural characterization of

the compounds by mass spectroscopy and proton magnetic resonance confirmed the structures depicted in Fig. 4.3.

Hydroxy-atrazine, abbreviated HA, was kindly donated by Ciba-Geigy (Johannesburg, South Africa).

4.2.2 HAPTEN-PROTEIN CONJUGATES

DCA-C_6-arm, DPA-C_6-arm and DEA-C_6arm were coupled separately to bovine serum albumin according to Davis & Preston (1981) with a modification to facilitate the solubilization of the atrazine derivatives. Bovine serum albumin (66,5 mg, research grade, Serva Biochemicals, Heidelberg, West Germany) was dissolved in 0,3 M sodium phosphate buffer pH8,0 (5 ml) and methanol (1 ml). About 60 mg of the relevant hapten (2mmol) was suspended in methanol (3,5 ml) and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC, 192 mg) and 0,025M sodium phosphate buffer (0,5 ml) added. After a few minutes stirring, a fine suspension was obtained to which the solution of BSA was added dropwise. The reaction was left overnight at 4°C, after which it was dialyzed exhaustively against distilled water and freeze dried. Hapten densities for the DPA-C_6-arm- and DEA-C_6-arm-conjugates were obtained from the difference spectra between conjugated and unconjugated BSA. The molar extinction coefficient for atrazine at 260nm was determined as $3{,}6.10^3 M^{-1} cm^{-1}$ and the shoulders observed at 260 nm in the difference spectra indicated hapten densities of more than 60 mol/mol BSA. The substitution of chlorine with aminocaproic acid in DCA-C_6-arm re-

sulted in the disappearance of absorptivity at 260 nm, which prevented the determination of the hapten density of the DCA-C_6-arm-BSA conjugate.

4.2.3 IMMUNIZATION OF MICE

DCA-C_6-arm was selected as hapten as it resembles atrazine in respect of the substitutions at the 4 and 6 positions of the s-triazine ring, while position 2 remained antigenically masked. It was hoped that the antibodies produced with this hapten would react with all the active atrazines, irrespective of whether a chloro-, methylthio- or methoxy-group resided at position 2.

Any inbred line of mice would have served the purposes of this investigation as long as they produced proper antibody titres against the relevant antigens. C57 Black/6 mice were selected merely because of their availability at the H.A. Grove Research Centre in Pretoria. Freund's Complete Adjuvant (Freund, 1956) was used to elicit the anti-hapten responses in mice. To date, its effectiveness as a secondary immune response stimulator for the production of antigen specific IgG remains unchallenged (Leclerc & Vogel, 1986). The mice were immunized repeatedly with 50µg doses of DCA-C_6-arm-BSA as follows: In 2:1 Freund's Complete Adjuvant emulsion on day 0 intradermally in the footpads and subcutaneously on day 14. In saline on days 42, 43, 70 and 71, alternating intravenously and intraperitoneally. The immune status of the animals was evaluated by ELISA with antisera drawn from the immunized animals on day 21. Cell fusion was performed on day 73.

4.2.4 CELL FUSION

The Sp2/0 myeloma cell line derived from Balb/c mice (Shulman et al., 1978) was used for two reasons: First, these cells synthetize neither heavy nor light immunoglobulin chains. Hybridomas derived from these cells will therefore only produce immunoglobulin dictated by the spleen cell parent. Second, Sp2/0 tend to adhere to the plastic of tissue culture containers which simplifies medium changes. As Sp2/0 cells are already hybrid, one would have expected lesser chromosomal stability after fusion, but this has not been observed.

Monoclonal antibody producing hybridoma cell lines were established according to the method of Galfre and Milstein (1981). Cells obtained from the homogenized spleen of an immunized mouse were fused in 40% PEG (Whittaker Bioproducts, Walkersville) to Sp2/0 myeloma cells in a ratio of 5:1. The fusion product was suspended in HAT medium and distributed into 96-well tissue culture plates (Nunc, Denmark). HAT medium consisted of Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Ayrshire, Scotland) supplemented with $NaHCO_3$ (3,7g/ℓ), sodium pyruvate (1mM) fungizone (1µg/ml), penicillin-streptomycin mixture (10 IU/ml and 10µg/ml respectively), hypoxanthine (100µM), aminopterin (0,4µM), thymidine (10µM) and 10% donor horse serum (Highveld Biological (Pty) Ltd., Kelvin, Johannesburg). Cultures were fed with fresh medium every third day. After one week, aminopterin was excluded from the medium and after the second week hypoxanthine and thymidine were omitted as well. Culture supernatants were screened by ELISA (see 4.2.5), and positive cultures transferred to 24 well multidishes. Confluent cultures from the multidish

were cloned and recloned on soft agar before being frozen away in 10% DMSO, 20% horse serum and 70% DMEM.

The observation by Westerwoudt (see 2.2.4) that non-producer hybridomas do not necessarily outgrow antibody producing hybridomas, was verified by my own experience. Hybridomas could be maintained in their initial 96-well microculture plates for several months as long as medium was refreshed every three days. This strategy was adopted whenever screening or subsequent cloning of positive cultures did not immediately give the desired result. It also allowed a more convenient schedule to maintain the cultures.

4.2.5 CLONING ON SOFT AGAR

DMEMx2 was made up by dissolving 0,82 g DMEM powder in 30 ml sterile water. Sodium bicarbonate (0,22 g) was added and the pH adjusted to 7,1 to 7,2 with 1N HCℓ. The following components were added to the final concentrations indicated in parentheses; sodium pyruvate (2mM), penicillin-streptomycin mixture (20 IU/ml and 20µg/ml respectively) and Fungizone (2 µg/ml). DMEM was made up by diluting 9 ml DMEMx2 with 9 ml sterile water. To the DMEM was added 15 ml DMEMx2 and 12 ml donor horse serum. The resulting mixture was filter sterilized and placed in a water bath at 45 ^{0}C. Agar (0,5 g) was suspended in 25 ml sterile water and autoclaved. The agar solution was added to the filtered medium to yield a 0,5% agar solution, which was maintained at 45 ^{0}C to prevent the agar from solidifying.

Some of the 0,5% agar solution was placed in each of three Petri dishes (15 ml per dish) and allowed to solidify at room temperature while the remaining agar solution was placed in each of three sterile, capped test tubes (1,2 ml per tube) and which were placed in a water bath at 45 ^{0}C.

Hybridoma cells were harvested from their growth flasks and counted using the trypan blue exclusion method (Hoskin et al., 1956). The cells were centrifuged to form a pellet which was suspended to a cell density of $1x10^{5}$ cells/ml with DMEM-HS. Aliquots of this suspension were further diluted with DMEM-HS to yield densities of $5x10^{4}$ and $1x10^{4}$ cells/ml. These three cell suspensions were then individually added to each of the three test tubes containing the agar solution (0,8 ml per tube) and the tubes vortexed gently. The cell-agar suspensions were poured on to each of the three Petri dishes (marked with the appropriate cell density) and the agar allowed to solidify. Once the second layer of agar had solidified, the Petri dishes were placed in the incubator. The Petri dishes were regularly monitored for the presence of growing clones. Once clones were just visible to the naked eye, they were picked from the plates using drawn out long form Pasteur pipettes and individually placed in the 96-well microculture plates.

4.2.6 FREEZING OF CELLS

With the research of Rall and Fahy (1985) kept in mind (see 2.2.3), the procedure that was adopted in our laboratory was as follows.

Freezing medium: DMEM powder (0,48 g) was dissolved in 35 ml sterile water and 0,13 g $NaHCO_3$ added. The pH was adjusted to 7,1 to 7,2 with 1N HCℓ. To this was added 5 ml DMSO and 10 ml donor horse serum.

Cells were centrifuged and all culture medium was removed. The cells were suspended in freezing medium to approximately 10^6 cells/ml and the cell suspension transferred to cryotubes which were wrapped in cheesecloth and placed in a polystyrene container at -70 ^{0}C. The rate of cooling of the cells in this container was approximately 1 ^{0} per minute. The cells were kept in the container for 24 hours after which they were transferred to cryoboxes and returned to -70 ^{0}C.

4.2.7 ELISA

Preparation of antigen coated ELISA-plates was done according to Conradie et al. (1983). Protein-hapten conjugate was dissolved in 0,05M glycine, 0,1M NaCℓ buffer pH2,5 to a concentration of about 10µg/ml. The solution was maintained at this low pH for 10 minutes to effect a partial protein denaturation which improves adsorption to the plastic. After neutralization to pH7,0 with solid Tris, the solution was distributed over 96 well microtiter plates (Cooke Microtiter System M29A, Sterilin, Middlesex, U.K.) at 100µl per well. Overnight incubation at room temperature was followed by flicking out the contents of the plate and washing with distilled water (500 ml) under a specially designed shower (Conradie et al., 1981). Antigen coated plates were found to have limited shelf-life and had to be used within 4 weeks when stored dry at 4°C. Non-

specific binding sites were blocked with 0,5% casein (according to Hammersten, Merck, Darmstadt, West Germany) in PBS pH7,4 (200µl/well) for 2 hours at room temperature, flicked out, aspirated to dryness under vacuum and incubated for 1 hour with hybridoma culture supernatant (50µl/ well). For CELIA, hybridoma culture supernatant was diluted 1:1 with solutions of either A, P, S, DCA-C_6-arm, DPA-C_6-arm, DEA-C_6-arm, DCA-C_6-arm or HA in 0,5% casein/PBS over a suitable concentration range on a separate plate and incubated for half an hour before transfer to the antigen coated plate at 50µl/well. The weak solubility in water of the chlorinated atrazines limited the inhibitor concentrations that could be used. In uninhibited controls, culture supernatant was diluted 1:1 with 0,5% casein/PBS. All subsequent incubations were done in volumes of 50µl/well for half an hour and washing between each step was done with 0,5% casein/PBS distributed from a syringe. For screening of hybridoma culture supernatants and CELIA analysis, the plates were developed with goat anti mouse IgG(H+L) peroxidase (Cappel, Worthington) at 1:1000 dilution in 0,5% casein/PBS followed by substrate solution consisting of o-phenylenediamine (10 mg) and urea-hydrogenperoxide (8 mg) in 0,1 M citrate buffer pH4,5 (10 ml). Colour development was monitored at 450nm with a Titertek Multiskan MC (Flow Laboratories, Inc., Helsinki, Finland), using the dual wavelength option to correct for indeterminate light dispersion.

For isotype determination of the monoclonal antibodies, incubation with culture supernatant was followed by successive incubations with heavy chain specific rabbit- anti-mouse immunoglobulins (Miles Research Products, Indiana, USA) at 1:1000, goat-anti-rabbit IgG(H+L)-alkaline phosphatase (Bio-Yeda, Israel) at 1:1000 and substrate solution consisting of p-nitrophenyl phosphate (35 mg) in 2-amino-2-methylpropane-1,3-diol

(0,3M) pH10,25 containing 2mM $MgC\ell_2$. Colour development was monitored at 405nm.

4.3 RESULTS

4.3.1 PRODUCTION OF MONOCLONAL ANTIBODIES

DCA-C_6-arm appeared to be a strong hapten determinant, and no need existed for alternating the carrier serum albumin as was done in the case of dihydrogriesenin in Chapter 3. The cell fusion with spleen cells from a mouse immunized with DCA-C_6-arm-BSA resulted in 368 primary hybridoma cultures of which 31% produced antibodies against the immunogen, as determined with ELISA using DCA-C_6-arm-BSA as coating antigen. Prior incubation of culture supernatants with equal volumes of DCA-C_6-arm (10^{-3}M) resulted in complete inhibition of the ELISA signal of 7% of all the cultures, as is shown in Fig. 4.1 for a set of typical ELISA results obtained on 48 culture supernatants. These also illustrate the hapten specificity and non-IgM nature of the monoclonal antibodies, since Kemp and Morgan (1986) had demonstrated that multivalent binding by an IgM antibody to an ELISA plate prevents proper ELISA-inhibition assays with soluble monovalent antigen from being carried out. The non-inhibited A7 (A1) and H12 (H6) wells represent hybridoma clones which either produced anti-hapten IgM, or anti-BSA immunoglobulin.

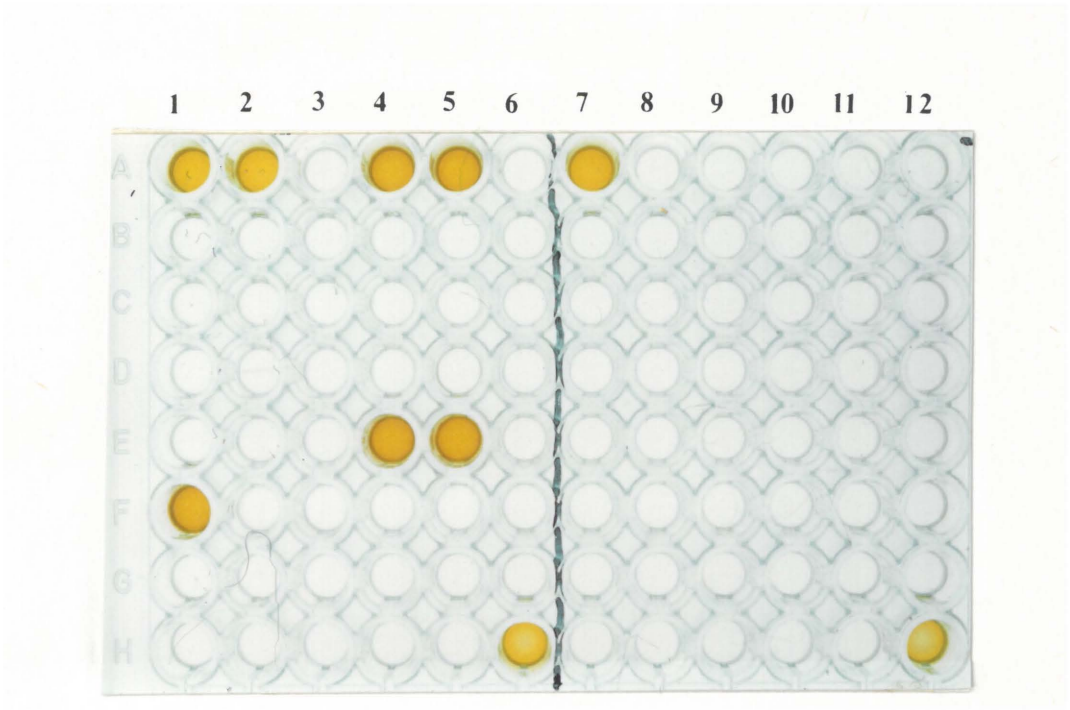

Figure 4.1. Results of an ELISA screening of hybridoma culture supernatants with (columns 7 - 12) and without (columns 1 - 6) prior inhibition with the hapten, DCA-C_6-arm (10^{-3}M).

Two clones of inhibitable antibody producing hybridomas, designated DCA-9 and DCA-10, were selected to be used for affinity and specificity studies towards hapten. Their isotypes, determined directly by ELISA, were found to be IgG_{2a} and IgG_1, respectively. The clone represented by well A7(A1) in Fig. 4.1 was found to produce IgG1 antibodies against BSA, but H12(H6) was not investigated further.

4.3.2 SENSITIVITY DETERMINATION BY CELIA

A serial twofold dilution range of DCA-C_6-arm ($2x10^{-5}$M) was preincubated with a dilution of culture supernatant which gave an ELISA-signal between 0,2 and 0,3 at zero inhibition after half an hour. From this a concentration range for DCA-C_6-arm was derived which was used to accurately determine the 50% inhibition point. The inhibition curve for antibody DCA-10, at 100-fold dilution of mature hybridoma culture supernatant, is shown in Fig. 4.2. The titre of $5,6.10^{-9}$M relates to a sensitivity for detection of DCA-C_6-arm of 0,002 parts per million. Antibody DCA-9 was used at a 25-fold dilution of mature culture supernatant and gave an almost identical titre for DCA-C_6-arm ($2,5.10^{-9}$M). The different dilutions of culture supernatant required to produce similar ELISA-signals are assumed to be a function of the respective concentrations of antibody in the culture supernatants.

4.3.3 SPECIFICITY OF ANTIBODY DCA-10

The apparent affinity constant (aK), which is a function of the true equilibrium constant, can be calculated from the titre ($[H]_{50}$) after correction for the two-fold dilution of hapten with culture supernatant (Nieto et al., 1984), assuming that the amount of solid-phase hapten bound to antibody is negligible with respect to total available hapten, and that free hapten at 50% binding to the solid phase therefore approximates the

total hapten concentration at 50% binding. The aK(DCA-C_6-arm) for antibody DCA-10 amounts to $3,6.10^8 M^{-1}$ (Fig. 4.2).

The specificity of antibody DCA-10 was determined by comparing the aK values obtained by CELIA titrations (Fig. 4.3) for the DCA-C_6-arm, DCA-C_2-arm, DPA-C_6-arm, DEA-C_6-arm, P, HA, A and S. The exact point of 50% inhibition of A and S could not be obtained due to their limited solubility in water.

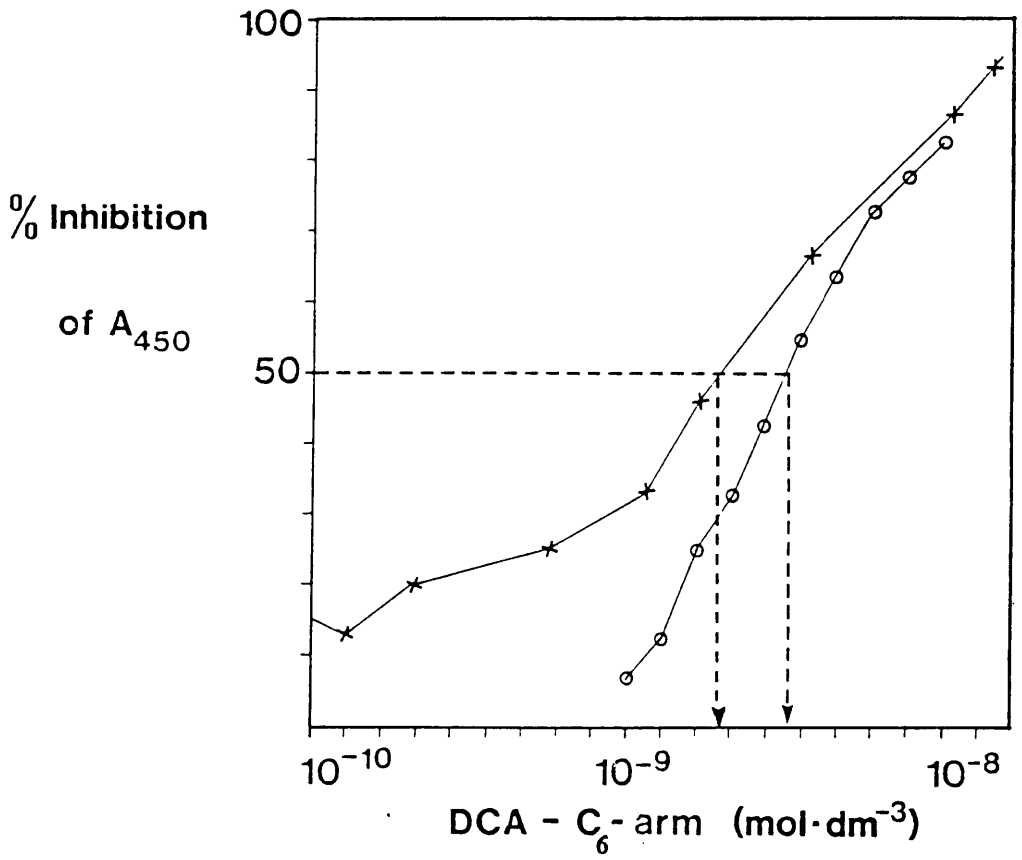

Figure 4.2. Inhibition curve of antibody DCA-10 in CELIA with soluble hapten DCA-C_6-arm. Each point represents the statistical mean of quadruplicate values (c.v. < 9%) obtained from plates coated with either DCA-C_6-arm-BSA (0----0), or DEA-C_6-arm-BSA (X----X).

Although a more than 1000-fold difference in relative binding affinity between DCA-C_6-arm and DPA-C_6-arm/DEA-C_6-arm was observed in CELIA with DCA-C_6-arm-BSA coated ELISA-plates (Fig. 4.3), comparable aK(DCA-C_6-arm) values were obtained when either DPA-C_6-arm-BSA or DEA-C_6-arm-BSA was used as the solid phase (see Fig. 4.2).

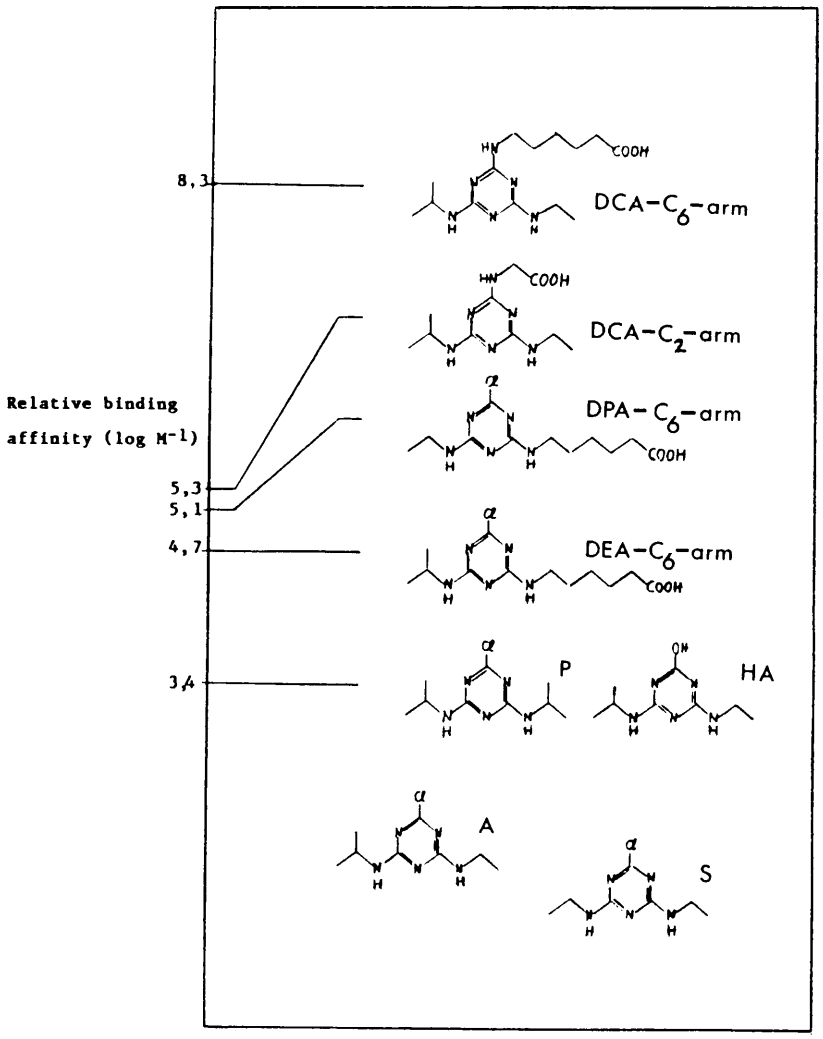

Figure 4.3. Relative binding affinities of several atrazine derivatives with monoclonal antibody DCA-10. The plates were coated with DEA-C_6-arm-BSA.

4.4 DISCUSSION

The poor solubility of the atrazines in water presented a difficulty in the design of a method for their conjugation with serum albumin. The procedure described in Materials and Methods is not easily reproduced and the products showed loss of activity after several months of dry storage at 4°C. The work reported here considers parameters of importance in the design of chemical derivates of atrazine as immunogens and solid phase antigens for the development of a sensitive enzyme-immunoassay for atrazine.

It is evident from Fig. 4.2 that the antibody DCA-10 can be applied in CELIA with adequate sensitivity and reproducibility for quantitative determination of the atrazine derivative, DCA-C_6-arm. Atrazine however, shows weak affinity for binding to the antibody ($aK<4.10^3 M^{-1}$, Fig. 4.3). Consideration of the DCA-C_6-arm and DEA-C_6-arm, whose major structural difference is the presence of a substituted chlorine or ethylamine group on the aromatic ring, shows an almost 4000-fold difference in binding affinity (Fig. 4.3) with antibody DCA-10. This suggests that the degree of electronic saturation of the ring in atrazine derivatives, which is affected differently by the two substituents, may be the main contributing factor to the observed differences in binding specificity. Pecht (1982) came to a similar conclusion when he compared the binding kinetics of multiple substituted nitrotoluenes with a monoclonal antibody. The less than 3-fold difference in binding affinity between DEA-C_6-arm and DPA-C_6-arm agrees with this since they only display minor structural differences in the groups substituted at position 4 of the ring. The same

interpretation regarding the importance of exocyclic substituents can be made by comparing the relative aK's of DCA-C_2-arm with HA or A.

The anticipation that antibodies would universally bind atrazines with different substitutions at position 2, but identical groups on positions 4 and 6 upon immunization with DCA-C_6-arm-BSA was therefore misfounded, due to the effect of exocyclic groups other than chlorine on the antigenicity of the triazine ring of atrazine.

By contrast, the relatively low binding affinity of DCA-C_2-arm compared to that of DCA-C_6-arm does not fit this proposed model for binding specificity, as the exocyclic atoms are identical for these haptens. An alternative explanation may be that the caproic acid arm of the immunogen forms an integral part of the antigenic determinant that is being recognized by the antibody. Simple molecular modelling allows interaction between the caproic acid carbonyl oxygen and 6-amino hydrogen on DCA-C_6-arm (see Fig. 4.4). It is postulated to form a three-dimensional haptenic determinant in this manner, with the hydrogen bond screened off from the aqueous environment either by molecular hapten stacking, or by the proximal association of the hapten with side-groups on the albumin carrier and/or immunoglobulin antigen binding domains. The lower aK of DEA-C_6-arm compared to DPA-C_6-arm can then be explained by the steric obstruction towards hydrogen bonding caused by the more bulky 4-isopropylamine on the former.

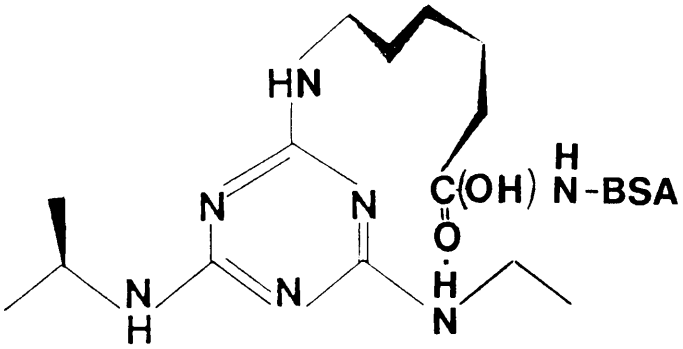

Figure 4.4. Model of hydrogen-bonded haptenic determinant of DCA-C_6-arm.

When the ability for hydrogen bonding of a C_6-arm carbonyl is not involved, the binding of hapten to antibody is actually enhanced by the isopropyl side groups (e.g. when P and A are compared in Fig. 4.3), indicating improved hydrophobic forces due to larger interacting surfaces of the hapten and antigen combining site of the antibody. Similar work was reported by Pecht (1982), who studied the kinetics of interaction between a monoclonal myeloma immunoglobulin (Protein 315) and a homologous series of DNP- alkylamines and DNP derivatives of branched, cyclic and aromatic side-chains. The energy of association between hapten and antibody was found to increase with increased length of unbranched side-chain up to four carbon atoms. This increase was more pronounced with branched side-chains, with t-butylated haptens (K_{ass} = 6,1.10^{+6}) binding stronger than isopropylated (K_{ass} = 3,4.10^{+6}) and methylated (K_{ass} = 0,94.10^{+6}) haptens.

The similar inhibition curves for DCA-C_6-arm observed in CELIA on solid phases coated with either weak or strong binding hapten-conjugates (Fig. 4.2), deserve some comment. Apparently the intrinsic binding force of antibody with solid phase antigen played no significant part in deter-

mining the aK's for different haptens in solution. An explanation may be found in the fact that antibodies exhibit increased binding strength for solid phase antigen due to their bivalence (Vos et al., 1987). The dissociation of antibody from the solid phase antigen was reported to occur at a much slower rate than from antigen in solution (Nygren et al., 1986) and did not follow a simple and identifiable rate constant; it could in practice only be effected in the presence of soluble antigen (Nygren et al., 1987). Inhibition of antibody with hapten concentrations up to the inflection point may therefore only be able to prevent bivalent association with the solid phase. Assuming that antibody exists in excess over solid phase antigen, relatively large differences in intrinsic affinities of antibody with solid phase haptens will only register as small differences in the amount of antibody bound to it. Subsequent removal of soluble hapten during washing will then allow the stabilization of antibody to the solid phase by divalent binding and effect the similar ELISA signals presented in Fig. 4.2.

One may conclude that several parameters govern the requirements for immunogens and solid phase antigens to be used in a competitive enzyme-linked immunosorbent assay for atrazine. For the immunogen, a strict conservation of exocyclic atoms is required, while the solid phase antigen appears less sensitive to these positional changes. This allows the use of the conveniently synthetized DCA-C_6-arm for continuous use as solid phase hapten in CELIA, while the more difficult synthesis of DEA-C_6-arm is required only for the preparation of immunogen in the development of a suitable monoclonal antibody producing hybridoma.

The ELISA system described above for the determination of atrazine derivatives provided reproducible data for the comparison of affinities of

the monoclonal antibodies towards different but related structures of atrazine. The problems encountered in the synthesis and solubilization of the hapten-carrier conjugates as well as their stability were, however, not addressed.

Atrazine and its derivatives have extremely limited solubility in water, which is a property essential for a herbicide to prevent its premature leaching from soil. Accordingly, no homogeneous hapten solution could be obtained during the conjugation of these haptens to protein in an aqueous environment. As a result, the hapten density of the conjugate could not be predicted or properly controlled by reaction conditions. Hapten densities of up to 158 were measured, although only 10 lysines are available for coupling per molecule of bovine serum albumin. Probably, considerable non-specific binding of hapten to BSA occurred during the coupling reaction. Alternatively, cross-linking of hapten via its carboxyl and secondary amines could have occurred in the presence of the large excess of carbodiimide that was used, analogous to the process of cross-linking proline in the presence of dicyclohexylcarbodiimide (Miyoshi et al., 1970). The high concentration of EDC required to drive the reaction also led to extensive protein-protein cross-linking with subsequent weak solubility of the conjugate. Large amounts of conjugate had to be used for solubilization to achieve an eventual concentration of 10µg/ml of clear, filtered solution of the antigen for coating ELISA microtitre plates. The conjugates were only stable for a few months after synthesis while stored dry in the cold (4°C), either as lyophilized powder or immobilized on ELISA-plates. Because of this, the synthesis of nylon-atrazine conjugates and their immobilization on polystyrene ELISA plates were next investigated in an attempt to establish a more reliable screening assay for atrazine specific monoclonal antibodies.

CHAPTER 5: NYLON-ATRAZINE AS ELISA SOLID PHASE

5.1 INTRODUCTION

Small haptens are usually covalently conjugated to a protein prior to adsorption to ELISA plates (Coleman et al., 1986; Verschoor et al., 1988). This has several drawbacks: the protein is subject to microbial degradation, necessitating storage in the cold to prolong the limited shelf life of the coated plates, protease activity in antigen samples may destroy the immobilized protein antigens (Viscidy et al., 1984), it is difficult to avoid some cross-linking of the protein during the coupling reaction, which often results in a lowered solubility of the conjugate, and haptens which are only sparingly soluble in the aqueous medium required for conjugation with the protein cannot be used at the hapten excess which is required for proper reaction to occur. An example of the latter was reported by Chase et al. (1976), who developed a radio immunoassay for the detection of cannabinoids. Addition of a polar moiety to tetrahydrocannabinol was required to render it soluble in water for its subsequent coupling to a protein carrier molecule.

Atrazine is a typical example of a hapten which has limited solubility in water, i.e. 33 parts per million (Jordan, 1970) and is not easily coupled to a protein with a water-soluble carbodiimide in an aqueous environment. Although a derivative of atrazine, 2-aminocaproic-acid-4 isopropylamino-6-ethylamino-s-triazine (I, see Fig. 5.1), could be conjugated to bovine serum albumin for use as immunogen to produce monoclonal

antibodies (see Chapter 4), the conjugate was extensively cross-linked, unstable and of such limited solubility that it could, in the long run, not be effectively used for the coating of ELISA-plates.

Due to the incompatibility of the atrazines and water, a macromolecular carrier was sought that dissolves well in organic solvents which are also effective solubilizers of the atrazines. Such a macromolecular substance was found in poly-amide 6 or nylon. It dissolves readily in m-cresol or concentrated acids. m-Cresol apparently competes for hydrogen bonding between the poly-amide chains, thereby dissolving it. This may also explain its solubilization of atrazines, with their carboxyl- and secondary amino groups.

Nylon beads had previously been used as a matrix for immobilization of antigen or antibody in ELISA (Hendry & Herrmann,1980), but its conjugation in solution and subsequent adsorption to polystyrene has not been reported on.

A satisfactory coating antigen was developed by coupling 2-chloro-4 isopropylamino-6-aminocaproic-acid-s-triazine (II, see Fig. 5.1) to solubilized nylon. What follows, is how this conjugate, prepared in a cresol-dichloromethane solution with DCC, could be reproducibly adsorbed to polystyrene plates and used successfully for the assay of atrazine specific antibodies in hybridoma culture supernatants.

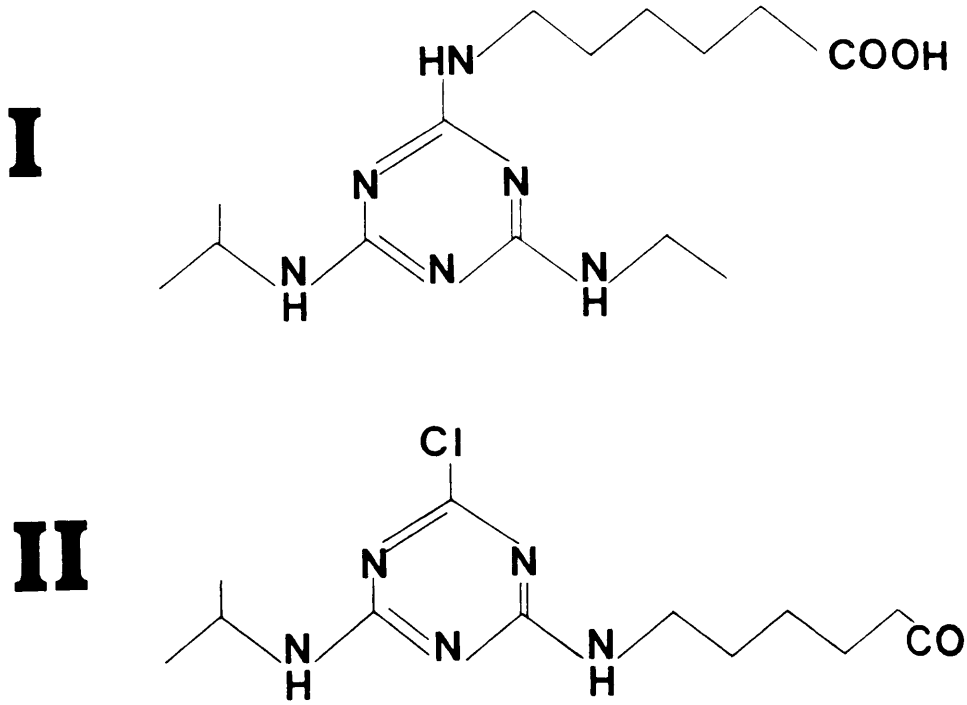

Figure 5.1. Structure of atrazine derivates. I = 2-aminocaproic-acid-4-isopropylamino-6-ethylamino-s-triazine, II = 2-chloro-4-isopropylamino-6-aminocaproic-acid-s-triazine.

5.2 MATERIALS AND METHODS

5.2.1 REAGENTS

The haptens, 2 - aminocaproic - acid - 4 - isopropylamino - 6 - ethylamino - s triazine(I) and 2 - chloro - 4 - isopropylamino - 6 - aminocaproic - acid - s triazine(II) were synthetized (see 4.2.1). Polyamide powder (Woelm, West Germany, for column chromatography) was purchased from SAARCHEM, South Africa. Dicyclohexylcarbodiimide (DCC), m-cresol, ethanol (96%), dimethylformamide, dichloromethane, casein (according to Hammersten) and buffer chemicals were general purpose reagents from BDH or Merck.

Polystyrene microtitre plates for ELISA (Cooke Microtiter System M29A, Sterilin products, Middlesex, England) were purchased from Sterilab Services, South Africa.

MoAb DCA-10 culture supernatant (see Chapter 4) was derived from a confluent hybridoma culture, matured for 3 days in Dulbecco's modified Eagle's medium (Flow Laboratories, Ayrshire, Scotland) supplemented with $NaHCO_3$ (3,7g/ℓ), sodium pyruvate (1mM), Fungizone (1µg/ml), penicillin-streptomycin mixture (10 IU/ml) and 10µg/ml respectively) and 10% donor horse serum (Highveld Biological (Pty) Ltd., South Africa).

Goat anti-mouse-immunoglobulin G (H+L)-peroxidase conjugate was obtained from Boehringer Mannheim (West Germany).

5.2.2 SYNTHESIS OF HAPTENATED NYLON

Polyamide 6 (100mg) was dissolved in m-cresol (0,7ml) and diluted with dichloromethane (0,7ml). Hapten II (optimized at 1mg, see Fig. 5.3) was dissolved in 50% m-cresol-dichloromethane (1ml) and added to the nylon solution. DCC (optimized at 200mg) was dissolved in dichloromethane (1ml), and added dropwise to the stirred solution of nylon and hapten. After shaking for 2 hours at room temperature, the reaction mixture was added dropwise to a vigorously stirred volume (50ml) of 20% dimethylformamide in dichloromethane. After half an hour, the insoluble conjugate was collected by filtration on hard filter paper (Whatman no 50), washed once with a volume of 20% dimethylformamide/dichloromethane, and desiccated overnight under vacuum. Hapten conjugation was qualitatively assessed by the ability of the product, immobilized on polystyrene microtitre wells, to produce an ELISA signal with monoclonal antibody DCA-10.

5.2.3 COATING OF ELISA-PLATES

Haptenated nylon (4mg) was dissolved in m-cresol (0,4 ml) and a total volume of 3,6ml of 67% aqueous ethanol (see 5.3.2) was added to the nylon solution. After the addition of about 2 ml of aqueous ethanol, some turbidity developed in the mixture, which was left standing for a further half hour after ethanol addition. The milky suspension was then dispensed at 100µl/well in the microtitre plate. After 1 hour incubation at room

temperature, unadsorbed solution was flicked out and the plate washed with tap water (500ml) under a specially designed shower (Conradie et al., 1981). Plates coated this way were air dried and stored in a cupboard at room temperature, and showed no loss in activity for at least 6 months. Plates coated with unhaptenated nylon served as negative controls.

5.2.4 ELISA

Haptenated or unhaptenated nylon-coated ELISA-plates were incubated for one hour at room temperature with a blocking/washing buffer consisting of 0,5% casein in phosphate buffered saline (PBS) at 200µl/well (Vogt et al., 1987). After flicking out the contents, the plates were aspirated to dryness under vacuum and incubated with hybridoma culture supernatant (50µl/well) for 45 min. at room temperature. For negative control, either culture medium or non relevant hybridoma culture supernatant was used. The plates were washed three times with 0,5% casein/PBS, evenly dispensed with a syringe and 20G needle. Thorough washing was found to be particularly important to ensure reproducibly low background signals in wells coated with unhaptenated nylon. Incubation for 30 min. with goat anti-mouse IgG (H+L)-peroxidase (Boehringer Mannheim, West Germany) at the recommended dilution, was followed by washing. After aspiration of the wells to dryness, substrate solution consisting of o-phenylenediamine (Sigma, St. Louis, USA, 10mg) and urea-hydrogenperoxide (8mg) in 0,1M citrate-buffer pH 4,5 (10ml) was added at 50µl/well. Colour development was monitored after one hour in a Titertek Multiskan colorimeter at 450nm.

5.3 RESULTS

5.3.1 DCC, HAPTEN AND NYLON STOICHIOMETRY

The results in Fig.5.2 were obtained when reaction mixtures containing 100mg nylon and 1mg hapten II were coupled with different amounts of DCC ranging from 0 to 400mg, the products (4mg) dissolved in m-cresol (0,4ml), titrated with 67% ethanol to turbidity and used for coating of an ELISA-plate. The ELISA signal increased proportionally with the amount of DCC used up to 50mg, at which point it levelled off. Amounts of DCC in excess of 200mg again showed a proportional increase in ELISA signal, probably due to a DCC driven side-reaction. Moreover, the reaction mixtures tended to solidify with time at the higher concentrations, so that 200mg DCC per 100mg nylon was selected as the upper limit for conjugation purposes. Subsequently, solutions of 0,125 to 15mg of hapten II in 50% m-cresol/dichloromethane (1ml) were added to the nylon solutions (100mg/ml), reacted with 200mg DCC, the conjugates coated and their ELISA signals compared (Fig. 5.3). The results showed that under these reaction conditions the optimum coupling concentration of hapten was 1mg or more.

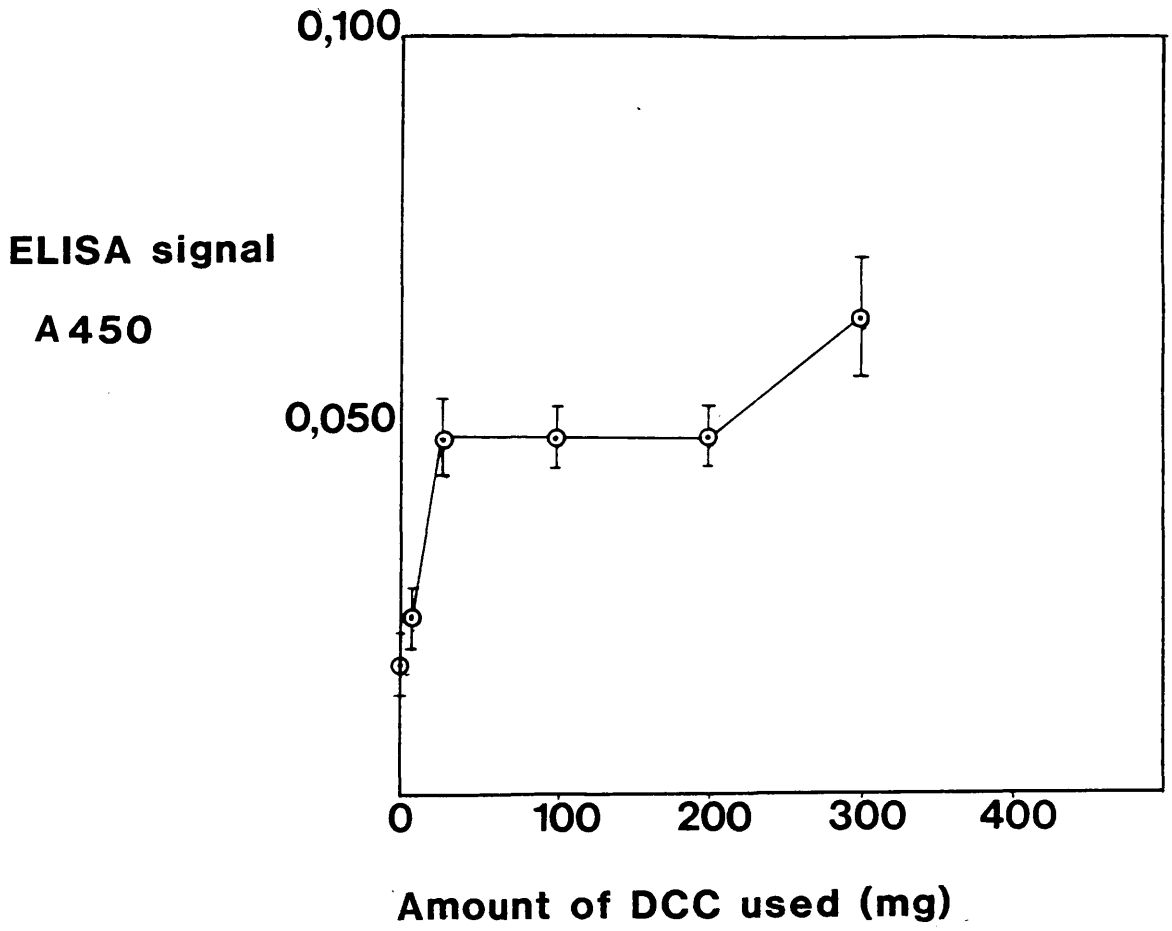

Figure 5.2. Effect of DCC-concentration in the synthesis of haptenated nylon for use in ELISA (n = 8).

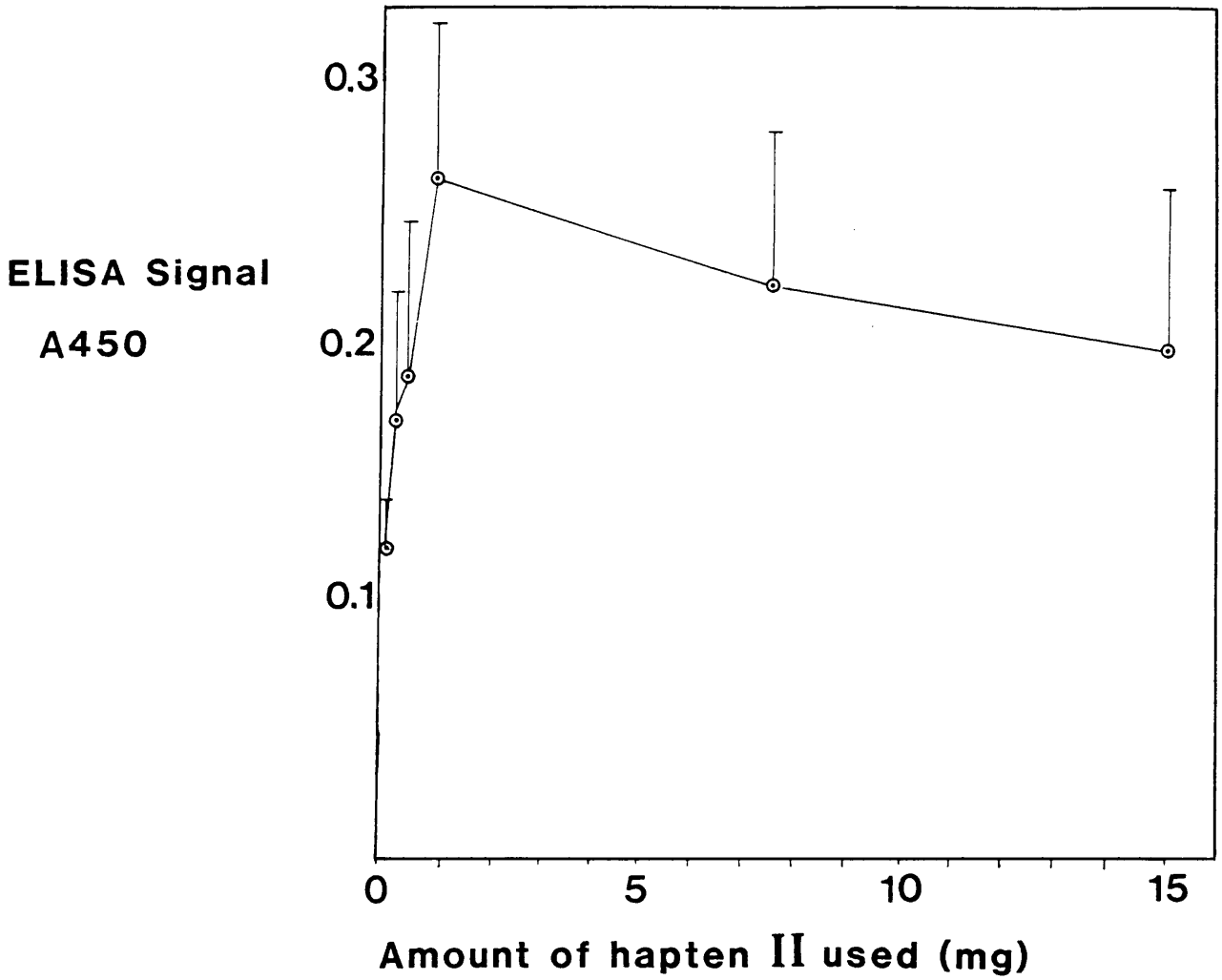

Figure 5.3. Optimization curve for the amount of hapten II required in the synthesis of haptenated nylon (n = 8).

5.3.2 INFLUENCE OF 67% ETHANOL ON COATING EFFICIENCY

Increased efficiency of coupling of the haptenated nylon to polystyrene was observed upon diluting the cresolic antigen solutions with 67% ethanol. This effect is demonstrated in Fig. 5.4, where solutions of haptenated nylon in m-cresol, diluted with increasing volumes of 67% ethanol, were used as coating solutions in ELISA. The best ELISA signals

were obtained when 2,6 to 4,6ml of 67% ethanol had been added to a 0,4ml cresolic solution of haptenated nylon (10mg/ml) before coating of the polystyrene plates.

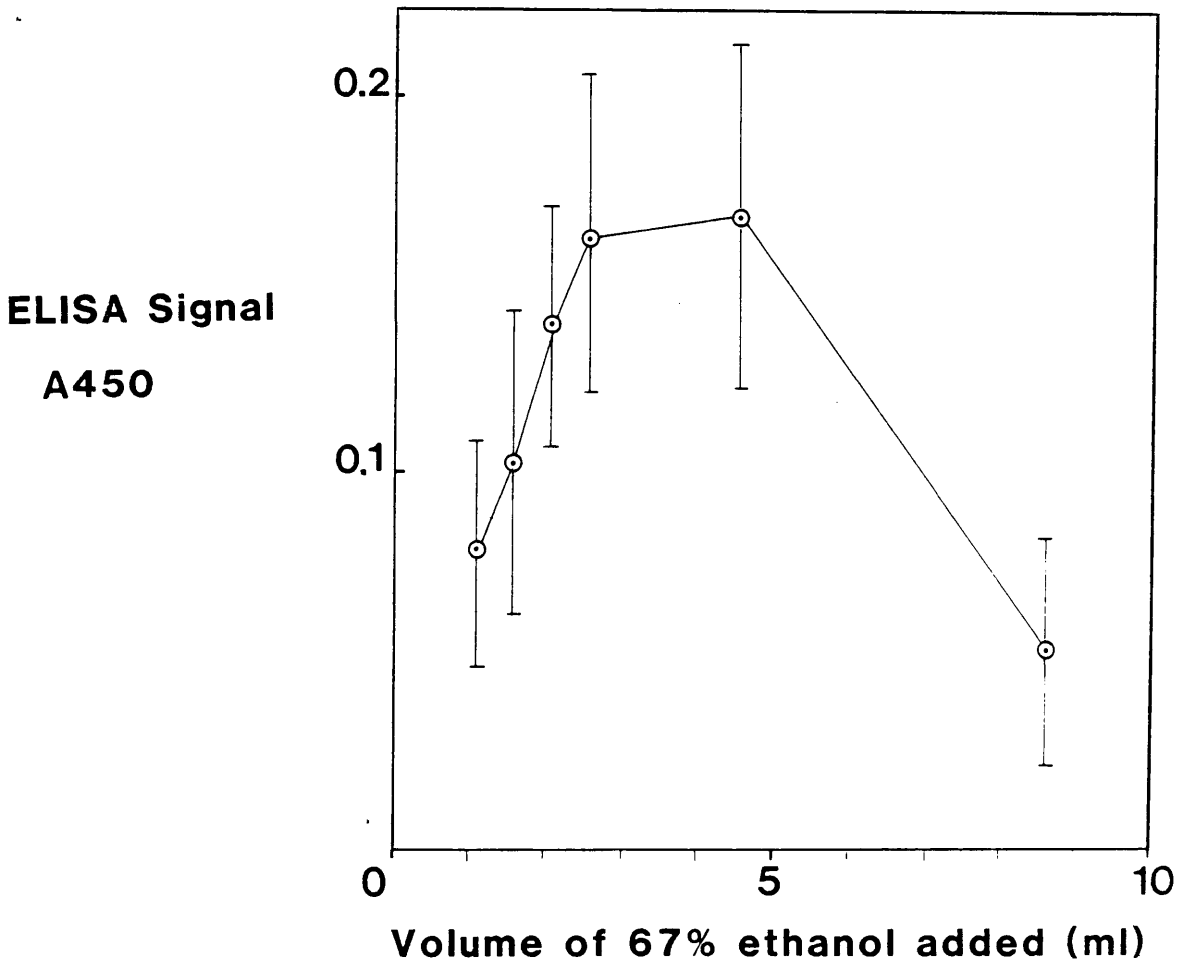

Figure 5.4. Optimization curve for the volume of 67% ethanol to be added to m-cresol solutions (0,4 ml) of haptenated nylon (10 mg/ml) required for coating of an ELISA plate (n = 8).

5.3.3 ADSORPTION ISOTHERM OF HAPTENATED NYLON TO POLYSTYRENE

An adsorption isotherm was obtained by titrating a range of haptenated nylon concentrations (0 to 40 mg/ml in m-cresol) before coating of the plates with 67% ethanol until the first appearance of turbidity. The subsequent adsorption curve obtained in Fig. 5.5 reached a plateau value at a final haptenated nylon concentration of about 3 mg/ml of coating solution, or 300µg/well. It resembles the adsorption of protein antigens on polystyrene, suggesting that hydrophobic forces are also involved in the adsorption of nylon to polystyrene (Butler, 1981).

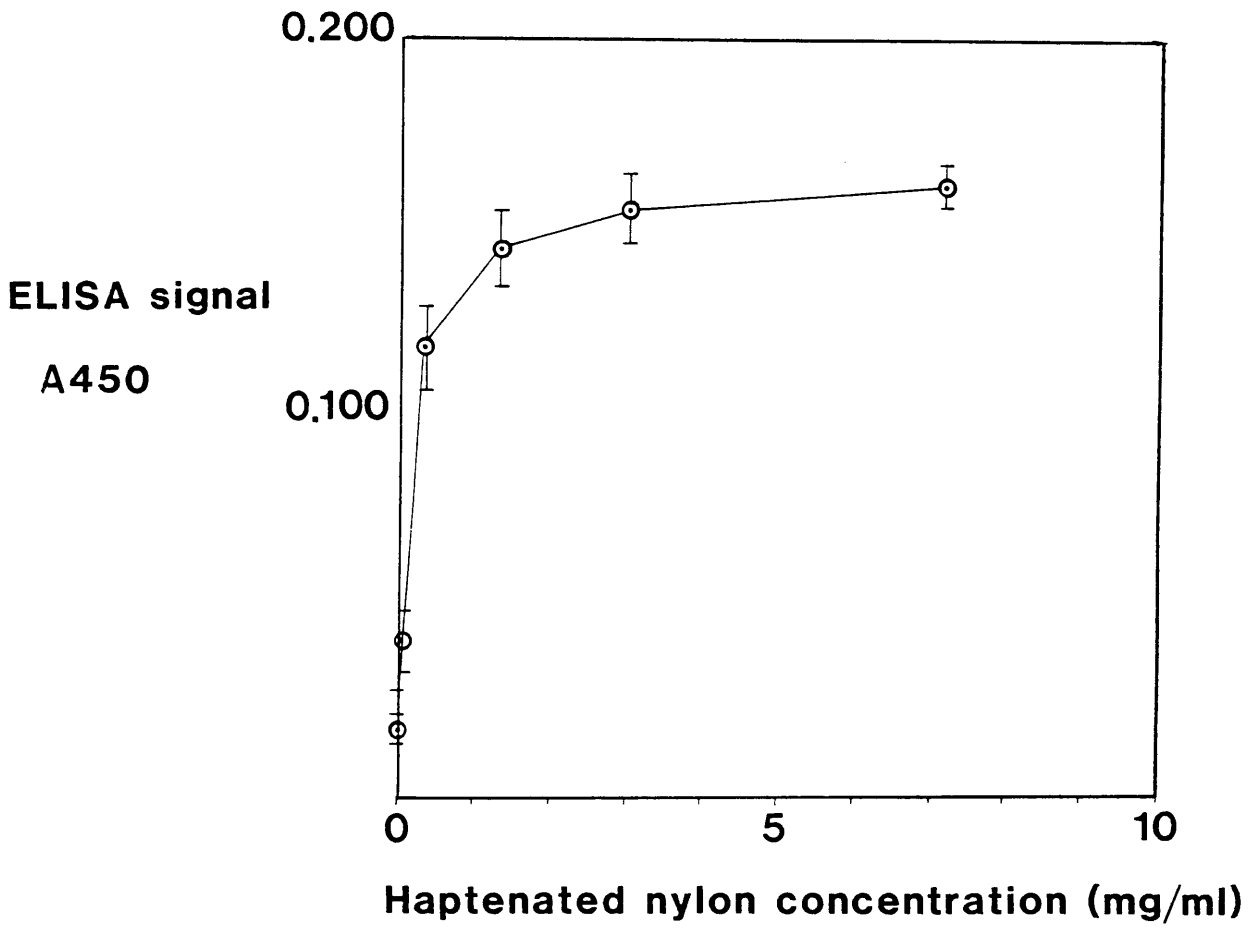

Figure 5.5. Adsorption isotherm of haptenated nylon to polystyrene (n = 8).

5.4 DISCUSSION

The use of solutions of nylon-hapten conjugates to coat microtitre plates with solid-phase hapten in ELISA represents a new approach in screening for hapten specific antibodies in antisera or hybridoma culture supernatants. It complements other techniques of immobilization of haptens or other antigens for solid phase immunoassays.

The method of Hendry and Herrmann (1980) for covalent conjugation of protein antigens to nylon balls, is relatively simple and provides a more efficient coupling of antigen than non-covalent adsorption to polystyrene, but sacrifices the advantages gained by doing the complete assay in microtitre plates. Direct covalent linkage of haptens possessing free amino groups to polystyrene plates has been attempted to good avail (Weigand et al., 1982 and Suter, 1982), but applies only to water soluble antigens. Many haptens, like atrazine, are only weakly soluble in water and require organic solvents for their conjugation to macromolecules. Nylon is a macromolecule with well-defined structure, and is soluble in ethanol-cresol mixtures. It consists of polymerized ε-aminocaproic acid monomers of which the methylene backbone can form hydrophobic interactions with polystyrene, while the terminal amino and carboxyl groups are available for covalent conjugation to haptens with suitable agents like DCC. Nylon 6 has a polymer length of approximately 200 units of ε-aminocaproic acid (The Merck Index, 1983), giving it a unit molecular mass of approximately 20 000. Optimal synthesis of haptenated nylon, as determined for the hapten used in this investigation, occurred at molar ratios of nylon:hapten:DCC = 1.5:1:300, i.e. about one hapten group per nylon molecule. Hapten density may probably be improved by partial

hydrolysis of the nylon with hydrochloric acid prior to the hapten coupling step (Hendry and Herrmann, 1980), but this was not investigated further. Cresol apparently causes a side reaction with DCC, but this can be compensated for by using large excesses of the reagent in the coupling reaction.

Two considerations were taken into account in the design of the procedure to adsorb the haptenated nylon to polystyrene. First, for hydrophobic adsorption of the haptenated nylon to the plate to occur, water had to be included in the coating solution. Second, cresol etches polystyrene and should be amply diluted before application to the wells of a microtitre plate. Addition of water caused immediate precipitation of nylon from a cresolic solution, while addition of ethanol was tolerated to a much greater extent. Aqueous ethanol (67%) allowed ample dilution of the cresol to prevent etching of the polystyrene, while the water content of the diluent was sufficient for the satisfactory adsorption of the haptenated nylon to polystyrene.

The haptenated nylon product was applied in a direct, heterogeneous ELISA for the detection of anti-hapten monoclonal antibodies in a hybridoma culture supernatant, using horse radish peroxidase conjugated goat-anti-mouse IgG (H+L) as indicator reagent. An ELISA signal to background value of better than 10 was obtained (c.v. < 12%), using unhaptenated nylon, coated under similar concentration and conditions, as background control. The method proved to be very useful in the screening of hybridoma cultures for anti-atrazine monoclonal antibodies, and may be expected to have application in other hapten-antibody systems.

Haptens, like atrazine and dihydrogriesenin, have the advantage of being well defined in terms of chemical and physical properties, which allow their experimental manipulation for use as immunogens, or for synthetizing suitable solid phases for immunoassays. In contrast, bacteria, the most abundant natural target for the immune response, are complex variable antigens, often armed with immune evasion/modulation mechanisms and difficult to use as reagents in immunology. In the next chapter, some innate problems of the immunochemistry of bacteria are addressed.

CHAPTER 6: MONOCLONAL ANTIBODIES AGAINST H. PARAGALLINARUM

6.1 INTRODUCTION

Generally speaking, bacteria are the most immunogenic of all natural antigens and provide the major challenge to the immune response due to their abundant natural presence in the mammalian digestive tract. In addition, pathogenic bacterial infections are the most common cause of disease with which the immune response has to cope. Accordingly, the immune response is particularly endowed with the ability to react against bacterial antigens (Nossal, 1986a). Multiple repeating epitopes on bacterial surfaces often evoke T-cell independent humoral responses, bacterial endotoxins are capable of polyclonal, non-specific B-cell activation, bacteria are rapidly opsonized by macrophages and various bacterial determinants activate the alternative pathway of complement, providing a rapid clearance mechanism to rid the animal of bacterial intruders soon after infection. The rapid proliferation of bacteria and their concomitant rate of mutation poses a particular problem to the cells of the immune system which divide 12-hourly at best, thereby necessitating mechanisms other than mutation rate to provide the body with a dynamic repertoire of antigen binding T- and B-cells. For this purpose, somatic gene translocation, point mutation and combinatorial association occur in lymphoid cells to equip antigen binding T- and B-cell receptors with the necessary diversification mechanisms to statistically keep abreast with new bacterial mutants in the environment (Goverman et al., 1986).

The immunomodulatory effects of various bacterial antigens have been well documented. Some examples are, for instance, adjuvanticity of Mycobacteria, which was exploited by its inclusion in what is popularly known as Freund's Complete Adjuvant (Freund & McDermott, 1942) and is today used as probably the most powerful elicitor of experimental antibody production in animals; Mycobacterium leprae, which can retard the cellular immune response against other bacterial antigens (Orme, 1987), and Micrococcus luteus, which was shown to evoke autoreactive antibodies against murine lymphoid cells (Grooten et al., 1980), causing interesting immunomodulatory effects in vivo.

The problem that presented itself for investigation by ELISA with monoclonal antibodies, was the serotyping of vaccine and field strains of Haemophilus paragallinarum.

H. paragallinarum infects the upper respiratory tracts of fowls, causing the disease known as infectious coryza (Sawata et al., 1985). It exists in an encapsulated or non-encapsulated state of which mainly the former causes the typical symptoms. The infected mucosa soon becomes infiltrated by mast cells, causing inflammatory oedema with concomitant exudate, persisting for at least one week. Due to the infectiousness of the disease, major economic losses are frequently suffered as a result of reduced egg production in laying flocks (Piechulla et al., 1985).

H. paragallinarum represents a species of pathogenic, gram-negative bacteria related to, but biochemically distinguishable from Actinobacillus spp. included under the family Pasteurellaceae (Piechulla et al., 1985). Several serotypes exist of which three have been serologically classified. Page (1962) originally defined three serotypes, A, B and C, from

isolates collected in California. Japanese isolates of the organism were classified as serotypes 1 and 2 which, respectively, were found to correlate with Page's serotypes A and C (Sawata et al., 1978 and Sawata et al., 1984). These studies were based on agglutination techniques and serum bactericidal reactions, using conventional antisera as agglutinating or bactericidal reagents. A number of other workers have likewise isolated and classified isolates in various parts of the world and so-called non-typable strains were often encountered. This indicates the need for reliable, constant, well-defined serotyping reagents which can be applied to unambiguously characterize new isolates.

In recent years bacterins have been widely used to immunise chickens against infectious coryza caused by H. paragallinarum. Several investigators reported on the early development and use of such products (Clark and Godfrey, 1961; Page et al., 1963 and Davis et al., 1976). Information on the production and evaluation of the oil emulsion vaccine in laying birds used in this investigation was published by Coetzee et al. (1983). Strains 083 (type A) and 0222 (type B), were used in the vaccine. Minimum standards for the bacterial cultures used in the production of this formalin inactivated bacterin were defined. The oil used was Marcol 52 and the emulgents were Arlacel 83 and Tween 80. The quality specifications of the emulsion were defined and the medium term immunity assays indicated that satisfactory protection of egg producing fowl can be expected for at least 30 weeks following one intramuscular injection of one millilitre of vaccine at 13 weeks of age. Nearly 40 million doses of this vaccine have been successfully used since 1981. During the period 1984 to 1987, a few outbreaks of infectious coryza occurred in South African flocks of vaccinated laying hens, resulting in typical symptoms of the disease, as well as reductions of egg production varying between 20 and

30 percent. Isolates of two of these outbreaks, designated M85 and SB86, were isolated in 1985 and 1986 respectively.

Several controlled laboratory studies were undertaken to evaluate the causes of incidental vaccine failure. Davis et al. (1976) reported that thimerosal-treated bacterin potentiated with $Al(OH)_3$ gave improved protection as a vaccine compared to a formalin-treated and mineral oil based vaccine comparable to that which we used. This conclusion was confirmed recently by Blackall and Reid in experiments which were more rigorously controlled in terms of experimental conditions and statistical processing (Reid and Blackall, 1987 and Blackall and Reid,1987). Earlier work by Coetzee et al. (1982) and unpublished results, produced results indicating the opposite and provided the impetus to undertake this study in an attempt to establish a system whereby the antigenic outer surface of vaccine and field strains of H. paragallinarum can be compared more accurately. The hybridoma technique for producing monoclonal antibodies provides the required technology, and has proven its value to this end for various bacterial systems (Macario and De Macario, 1985).

The development of a panel of monoclonal antibody producing hybridomas derived from four groups of mice separately immunized with H. paragallinarum strains A, B, M85 and SB86 is reported on below.

6.2 MATERIALS AND METHODS

6.2.1 BACTERIAL STRAINS

The H. Paragallinarum field isolates M85 and SB86, produced typical disease symptoms in hens vaccinated with strains 083 (type A) and 0222 (type B), the latter which were acquired from the Onderstepoort Veterinary Institute, Republic of South Africa. Significantly, no disease could be induced when similar groups of vaccinated hens were infected with the vaccine strains, A and B. All strains and isolates were stored in SPF egg yolk at -30°C as seed stock. Modified Casman's medium (Rimler et al., 1975) was used to culture the bacteria in glass containers. The pH of the medium was adjusted to 7,5 and NAD was substituted by 5% inactivated filtered chicken serum. Bacterial growth took place at 37°C while the medium was shaken for 18 hours. Samples of the cultures were tested for purity and colony morphology, as well as monitored for pH and colony forming units (CFU) per millilitre of the cultures. A minimum of 10^7 CFU/ml were obtained for each of the four strains. After inactivation of the bacteria by adding formalin (0,25% v/v) and incubating at 10°C for 24 hours, the bacterial cells were washed four times in PBS (pH 7,2) by centrifugation. Sedimented cells from strains A, B, M85 and SB86 were used to immunize mice.

6.2.2 IMMUNIZATION OF MICE

C57Bl/6 mice were obtained from the H.A. Grove Research Centre, Pretoria, South Africa. Each of the four H. paragallinarum strains were used to separately immunize 4 groups of mice. Primary and booster immunizations were all administered intraperitoneally at 10^7 cells in 1 ml of saline. Cells of the 4 strains were counted once with a hemocytometer and the count correlated with absorbance at 540 nm. Concentrations of 10^8 cells/ml exhibited an absorbance of 0,10, irrespective of strain. Mice were immunized on days 0, 14 and 59, the latter being 3 days before cell fusion was performed. Polyclonal immune sera were prepared from blood drawn from the 4 groups of mice on day 20.

6.2.3 PRODUCTION OF HYBRIDOMAS

Parental Sp2/0 myeloma cells, developed by Shulman et al. (1978), were obtained from the Onderstepoort Veterinary Institute, Pretoria, South Africa. Cell fusion between immune spleen cells and the HGPRT deficient, non- secreting mouse myeloma cells (Sp2/0) was performed according to the method of Galfre & Milstein (1981). Spleens from each of the 4 groups of mice were homogenised and combined into one tube. Half of the cells thus obtained were fused 5:1 to myeloma cells using 40% (w/v) PEG (Whittaker, M.A. Bioproducts, Walkersville, USA). Spleen cells not used for fusion were added to the fused cell suspension in HAT-medium just before distribution into 96-well tissue culture plates (Nunc, Roskilde, Denmark)

to serve as feeder cells. HAT-medium consisted of DMEM (Flow Laboratories, Ayrshire, Scotland) supplemented with $NaHCO_3$(3,7g/ℓ), sodium pyruvate (1mM), Fungizone (1 µg/ml), penicillin-streptomycin mixture (10 IU/ml and 10 µg/ml respectively), hypoxanthine (100 µM), aminopterin (0,4µM), thymidine (16 µM) and 10% donor horse serum, all of which were purchased from Highveld Biological (Pty) Ltd., Kelvin, South Africa. Cultures were fed with fresh medium every third day. After one week, aminopterin was excluded from the medium and after the second week hypoxanthine and thymidine were omitted as well. Screening of the culture supernatants for the presence of specific antibody was done when the cultures approached confluency. Positive cultures were transferred to 24-well multidish plates and cloned on soft agar upon reaching confluency and after determination of the type specificity of the secreted antibody against all 4 bacterial strains. Hybridomas could be maintained in the multidish plates for at least 6 months, without expansion or dilution of the cells. Cloned, positive hybridoma cultures were subcloned on agar before being frozen away in 10% DMSO, 20% horse serum, 70% DMEM. Culture supernatants drawn for screening and antibody characterization were from confluent cultures matured for at least 3 days.

6.2.4 ANTIBODY SCREENING AND SPECIFICITY ASSAY

Both the screening of the hybridoma culture supernatants and the determination of the specificity of the monoclonal antibodies obtained, were done by ELISA (Engvall, 1980), using whole bacteria as solid phase antigen.

For screening, equal volumes of 10^7 cells/ml saline from each of the 4 strains of H. paragallinarum were mixed and used for coating 96-well microtitre plates (Cooke Microtiter system M29A, Sterilin products, Middlesex, England) at 100µl per well. The bacteria were dried for 1 hour on the plate under a heating lamp and laboratory fan and were fixed with 70% methanol (200µl/well) for 10 minutes. After flicking out the solvent, the plates were dried under the heating lamp and fan and could be stored at 4°C for at least 3 months with no significant loss of ELISA-signal.

For specificity determination, four duplicate rows of a microtiter plate were covered individually with the different strains of H. paragallinarum at 10^7 cells/ml and treated as described above.

Blocking and washing buffer consisted of 0,5% casein in PBS pH7,4. Casein (according to Hammersten) was purchased from Merck, Darmstadt, West Germany. After blocking the antigen coated wells with casein buffer (200µl) for one hour at room temperature and washing, the wells received undiluted hybridoma culture supernatant (50 µl) for screening and specificity determination, in the latter case dispensing each culture supernatant in duplicate columns over each of the 4 coated H. paragallinarum strains. After washing, the plates were developed with 50 µl goat anti-mouse immunoglobulin G(H+L)-peroxidase conjugate from various commercial sources at the recommended dilution. After final washing, 50 µl of substrate solution consisting of o-phenylene diamine (10 mg) and urea-hyrogen peroxide (8 mg) in 10 ml citrate buffer (0.1M, pH4.5) was added. Only o-phenylene diamine obtained from Sigma (St. Louis, USA) was found suitable for this purpose. Colour development was measured at 450nm on a Titertek Multiscan MC colorimeter (Flow Laboratories, Helsinki, Finland).

6.2.5 DETERMINATION OF IMMUNOGLOBULIN CLASS BY ELISA

Hybridoma culture supernatants were dispensed in duplicate rows of the appropriate antigen coated wells at the usual volume (50µl) and probed, after washing, with a $1/_{500}$ dilution of heavy chain specific rabbit anti-mouse immunoglobulin serum (Miles Research Products Division, Indiana, USA), dispensed in duplicate columns over the plate. The plate was developed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G(H+L) (Bio-Yeda, Rehovoth, Israel) and substrate solution consisting of p-nitrophenylphosphate (35 mg) in 2-amino 2 methylpropane 1,3-diol buffer (0.3M, pH10.25) containing 2mM $MgC\ell_2$. Colour development was measured at 405nm using the Titertek Multiskan MC.

6.3 RESULTS

6.3.1 SPECIFICITY ASSAY OF POLYCLONAL ANTISERA

Antisera were obtained from 4 groups of mice, each immunized with one of the 4 strains of H. paragallinarum. Each antiserum was assayed for its specificity by ELISA against each of the 4 bacterial strains. A background signal was obtained by using normal neutral serum on the antigen coated wells. Signal to background values are presented in Figure 6.1.

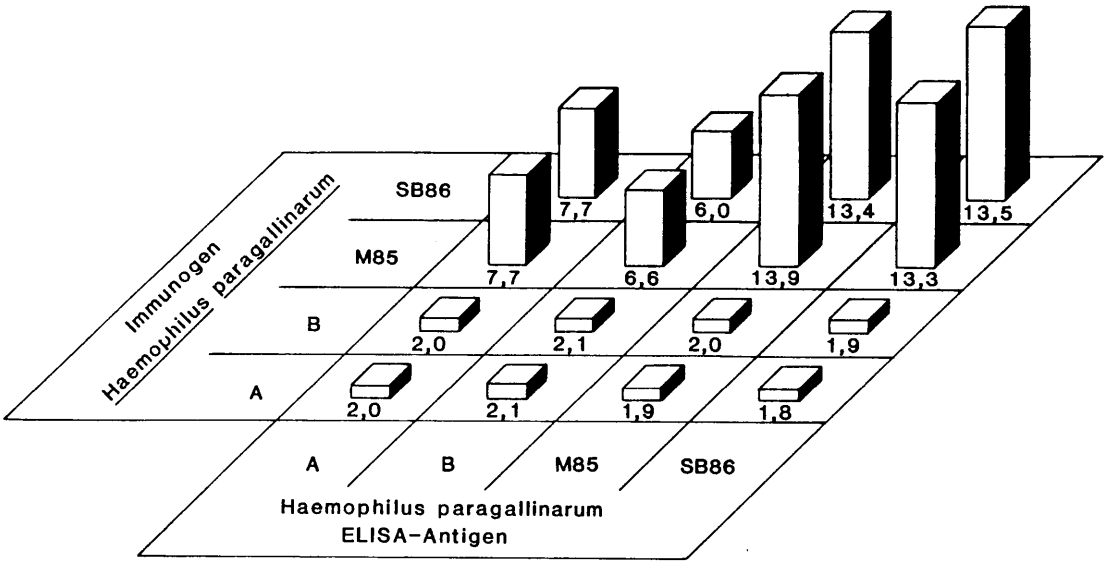

Figure 6.1. ELISA signal to background values of polyclonal antisera against 4 isolates of H. paragallinarum. Coefficients of variation for absorbance values of between 1 and 10% were observed for all the data presented (n = 4).

Two observations can be made regarding the specificity of the polyclonal antisera: Firstly, the two field isolates, M85 and SB86, can clearly be distinguished from the vaccine strains A and B by an overall increase in ELISA signal using antisera obtained from mice immunized with the field isolates. Secondly, antibodies from mice immunized with strain M85 or SB86 gave stronger ELISA signals against strain A or B antigens than when the latter strains themselves were used as immunogens. No distinction could be made between the two vaccine strains or between the two field isolates by this polyclonal specificity assay.

6.3.2 PROPERTIES OF MONOCLONAL ANTIBODIES

A mixture of spleen cells derived from 4 mice, each immunised with one of the 4 different isolates of H. paragallinarum, was fused with Sp2/0 myeloma cells. The experiment yielded 334 viable oligoclonal hybridoma cultures of which 77 tested positive for antibody production against the antigens. Specificity assays by ELISA against all 4 isolates of H. paragallinarum grouped these hybridoma cultures into 5 different specificities. How these specificities differ from each other is depicted in Fig. 6.2. The frequency at which these specificities occurred among the 77 positive oligoclonal cultures is also indicated. The signal to background values were determined with supernatants from selected, cloned cultures, but represent all of the various specificities that were exhibited by the oligoclonal hybridoma cultures. The nomenclature of the different specificities was designed to indicate the specificity for vaccine (V) strains, field (F) isolates or cross-reactivity (VF) by capital letters followed by serial numbers to indicate monoclonal antibodies of different reactivities towards the different antigen strains or isolates.

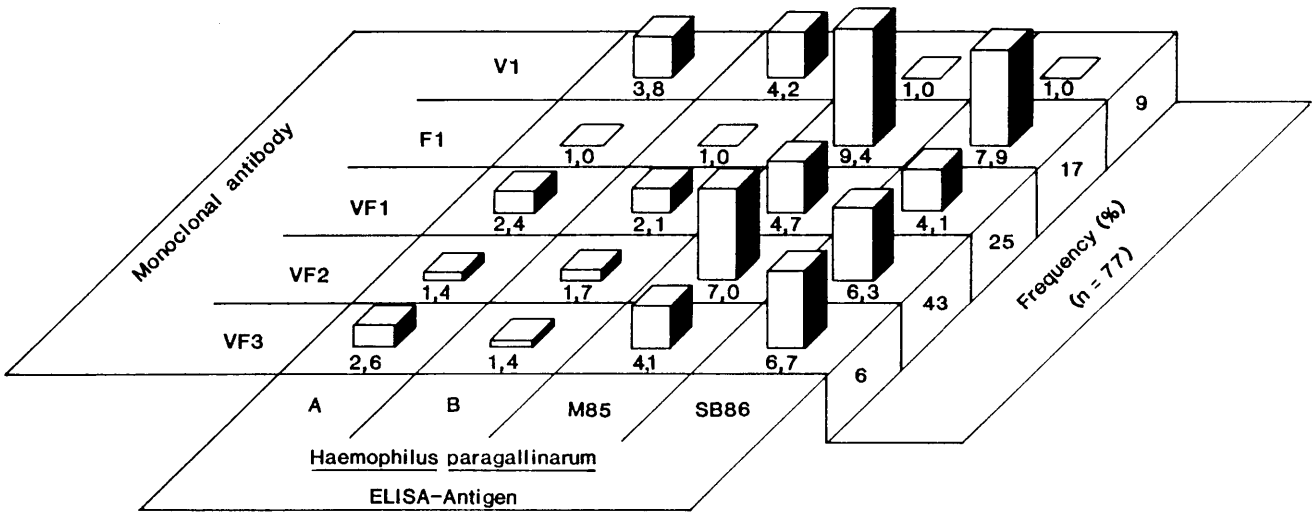

Figure 6.2. ELISA signal to background values of monoclonal hybridoma culture supernatants against 4 isolates of H. paragallinarum. Coefficients of variation for absorbance values of between 1 and 5% were observed for all the data presented (n = 4). The values were reproducible for different plates coated with the same batches of antigens.

Class determination of the immunoglobulins secreted by various hybridoma clones of the 5 different specificities were determined by ELISA. The results are summarized in Table 5.1.

Table 5.1 Immunoglobulin class determination of different hybridoma clones of various specificities against 4 isolates of Haemophilus paragallinarum

MoAb	Number of clones tested	Immunoglobulin classes found
VF_3	2	IgG2a, IgG2b
VF_2	1	IgG2b
VF_1	1	IgG2b
F_1	1	IgG2a
V_1	2	IgG3

An IgG3 antibody, (MoAb V1) was obtained which showed absolute specificity for the vaccine strains A and B, while an IgG2a antibody (MoAb F1) reacted only towards the M85 and SB86 isolates. Two monoclonal antibodies of different isotypes, IgG2a and IgG2b (MoAb VF3) had the capacity to resolve strains A from B ($P > 99,9\%$) and M85 from SB86 ($P > 99.9\%$). This panel provided a unique fingerprint for each of the 4 strains of H. paragallinarum. Of the remaining two antibody specificity classes, especially MoAb VF1 (IgG2b) was found useful as a control to test for reproducible adsorption of the different strains of bacteria to the ELISA plate, as it cross-reacted universally.

6.3.3 STABILITY OF EPITOPES

Before the system could be used as a field test for the characterization of new immune evasive field isolates of H. paragallinarum, the stability of the antigens of the strains of bacteria that were used on the solid phase had to be determined. Bacteria of the two field isolates were grown up and concentrated as mentioned in 6.2.1 and compared with the batches used for the production and screening of the monoclonal antibodies. Figure 6.3 shows the result for new batches of H. paragallinarum isolates M85 and SB86.

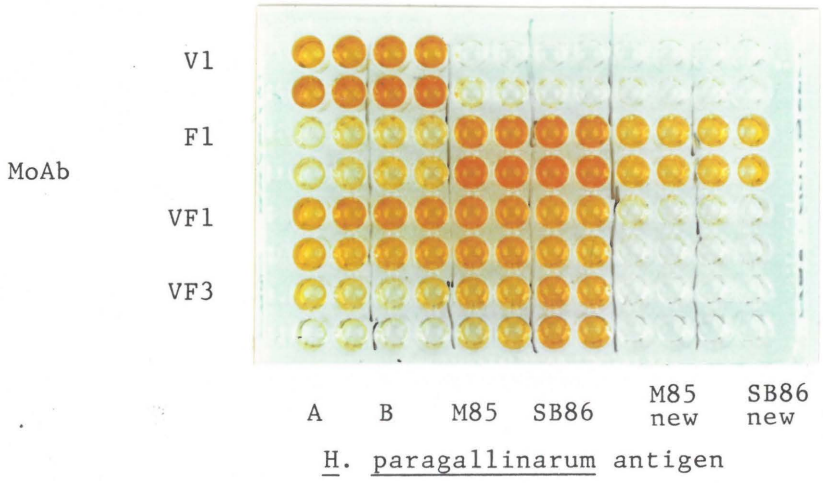

Figure 6.3. Comparison of new batches of isolates M85 and SB86 of H. paragallinarum with the reference set of strains used for production and screening of monoclonal antibodies.

It is evident that the fully cross-reactive antigen(s) recognized by MoAb's VF1 and VF3 are not stable and may appear or disappear depending on the growth media or culturing conditions. Variations in the chicken sera used as medium supplement may be the most logical factor to induce changes in the surface structure of the pathogen, e.g. by their variable content of undetectable antibodies against H. paragallinarum (Sawata et al., 1984).

6.3.4 SUICIDE HYBRIDOMAS

A very interesting phenomenon occurred in the cultures of hybridomas against strains of H. paragallinarum during the first few weeks after fusion: After initial proliferation of the hybridomas up to about half the bottom surface of the micro tissue culture plate wells, the supernatants were screened for their antibody content directed against H. paragallinarum. All tested positive. Normally, only about 10% of all wells test positive. The cultures then suddenly regressed and within five days only a few viable cells could be observed amongst a mass of dead cell debris. This apparent "suicide" was followed by gradual recovery of individual clones of cells over several weeks. These recovered cells did not re-enter suicidal cycles and screening was done again. This time 55% of all the clones tested positive and were used for further characterization of isotype and specificity as explained in 6.2.4. The course of events is summarized in Fig. 6.4.

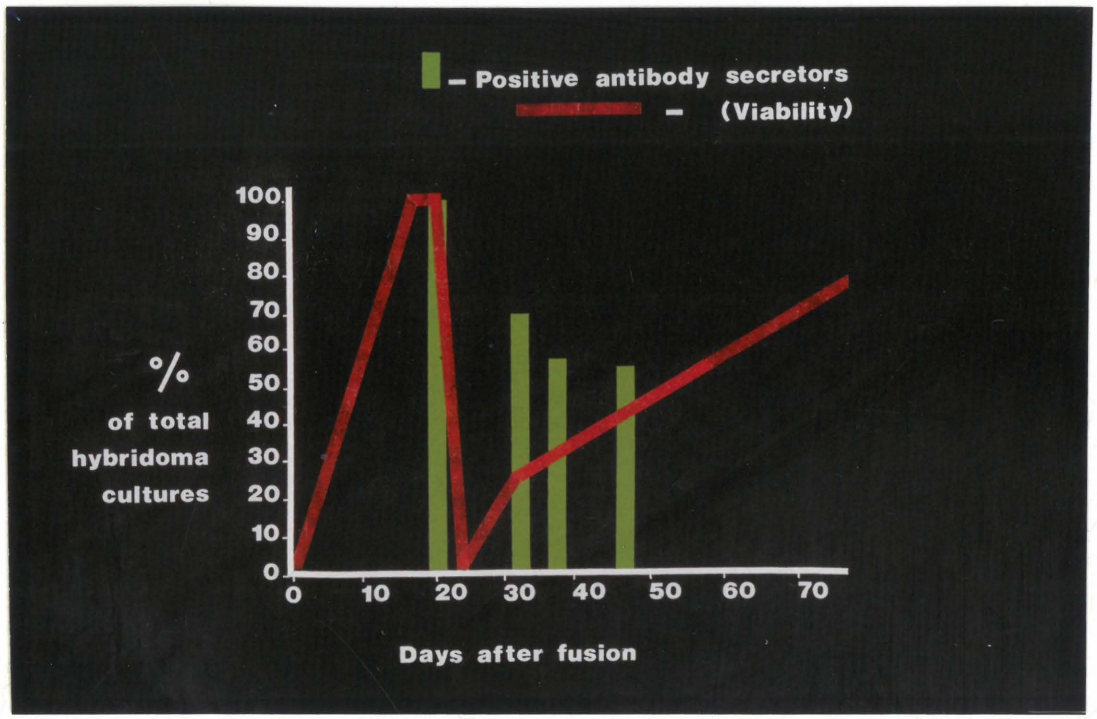

Figure 6.4. Rise and fall of hybridoma cell growth and specificity of the produced antibodies against H. paragallinarum

The phenomenon was experimentally investigated further by sacrificing a mouse immunized with H. paragallinarum strain SB86 and performing a "fusion" on the spleen cells as usual, but omitting the PEG fusogen. A control was done likewise, but using a non-immune mouse. After nine days hybridoma growth occurred in all 40 wells of the experiment, while only three out of 42 wells exhibited growth in the control experiment. On day 11, the supernatants were screened for antibody production against the immunogen. The result is shown in Fig. 6.5. All the wells of the experiment tested positive, but no signal was detected for the control wells.

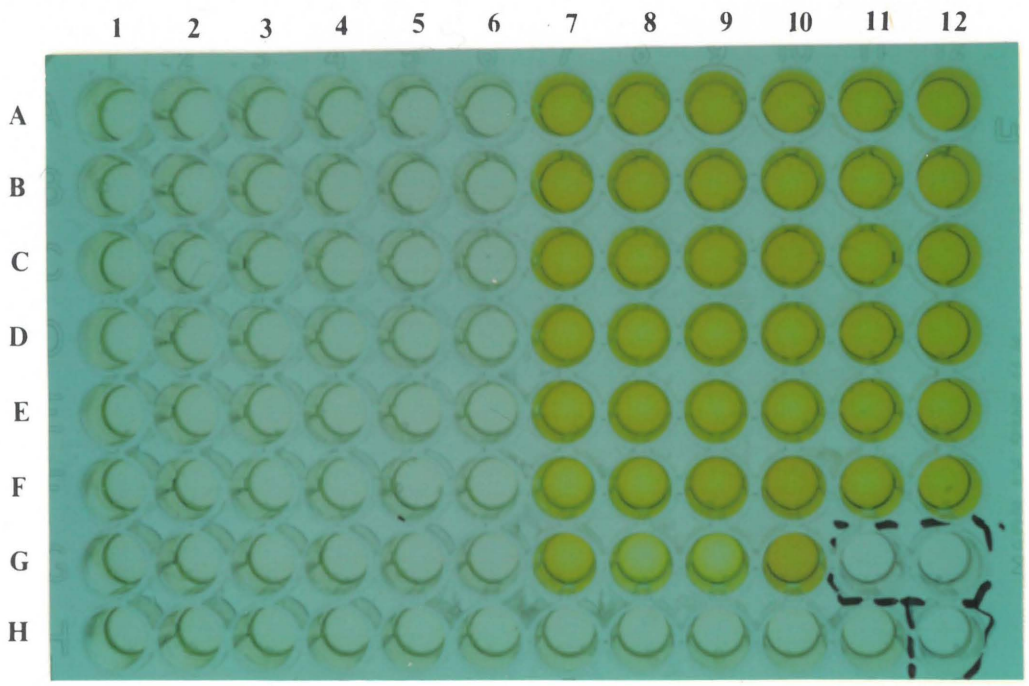

Figure 6.5. Result from ELISA screening of hybridoma culture supernatants against H. paragallinarum isolate SB86. Columns 7 - 11 represent the ELISA result from different hybridoma cultures derived from spleen cell of mice immunized against isolate SB86 which were fused with myeloma cells without the aid of PEG, while columns 1 - 6 represent the results from the appropriate control using non-immune spleen cells. Row H and wells G11-G12 were not used.

As was previously observed, the hybridoma cells subsequently relapsed and restored themselves, this time only to 25% recovery. From the restored cells, one was selected for cloning and characterization of its antibody. The cell line grew well on agar and produced an IgG2b antibody reactive against the field isolates M85 and SB86, exactly like MoAb F1 described in Table 6.1.

6.4 DISCUSSION

Different immunization protocols for the production of monoclonal antibodies against more than one immunogen or bacterial strain, may influence the specificity of the monoclonal products in several ways. Immunization with sonicates of bacteria presents the immune system with "internal" antigens as well as "internal determinants" of surface antigens. It has been reported that the internal segments of bacterial outer membrane proteins may be highly conserved within a given species (Gulig et al., 1985). This immunization strategy may therefore increase the probability of finding monoclonal antibodies of broad cross-reactivity which may not be able to distinguish between intact bacteria. Immunization with a mixture of intact whole bacteria containing all the strains to be analyzed, may yield all the different monoclonal antibody specificities from one fusion product. However, antigenic competition may bias the immune response towards shared determinants of the bacteria, resulting in cross-reactive monoclonal antibodies. An immunization strategy designed to produce monoclonal antibodies for serotyping would therefore avoid combining the different strains, while it may be the preferred method for the identification of a target antigen for the development of a universally effective vaccine. Immunization of different animals with the individual bacterial strains to be serotyped, followed by combination of the different spleens into one fusion experiment, retains the economy of doing one fusion only without sacrificing chances of finding the required type specific monoclonal antibodies. This approach was therefore best suited for the purpose of this investigation.

The presentation of chequered, single plate ELISA data in a three-dimensional graph as in Fig. 6.2, allows one to evaluate the individuality of all strains at a glance, even when only cross-reactive monoclonal antibodies are found. The results in Fig. 6.2 indicate large differences between vaccine strains A and B and field isolates M85 and SB86, while the individuality of all 4 strains are resolved with MoAb VF3. The relative immunodominance of the antigens with which the different monoclonal antibodies react is indicated by the frequency with which the specificities occurred in 77 hybridoma cultures.

MoAb's VF2 and VF3, distinguished by their cross-reactivity with all of the tested strains, generally reacted more strongly with the field isolates (Fig. 6.2). This difference in antigenicity is even more conspicuous in the reaction of these strains with polyclonal antisera (Fig. 6.1). The fact that a stronger response against strain A or B was found upon immunization with strain M85 or SB86, than when strain A or B itself was used for immunization, implies that the cross-reactive antigen(s) on strain M85 and SB86 are both more immunogenic and more antigenic.

These observations suggest a mechanism whereby the field isolates may have achieved their immune evasive properties: Gram-negative organisms exhibit varying O-chain lengths of outer membrane lipopolysaccharides, as for example in Salmonella typhimurium (Munford et al., 1980). The longer these side chains are, the more complement protein (C3) is found deposited on them, thereby keeping complement away from the complement sensitive proximal surface of the cells (Joiner and Frank, 1986). It is therefore possible that the observed cross-reactive antibodies recognize such longer and more antigenic structures on the field isolates M85 and SB86, allowing them to escape immunological elimination by lytic complexes.

The absolute specificity of MoAb's V1 and F1 for the vaccine and field strains respectively indicates, however, that this can not be the only difference between these 2 groups of strains.

The apparent variability in the expression of the completely cross-reactive antigen(s) recognized by MoAb's VF1 - 3, emphasizes that caution should be heeded in the use of monoclonal antibodies for serotyping purposes, as unstable antigens are poor markers for characterizing new strains of bacteria. Standardization of culturing conditions and other parameters which may influence the expression of surface antigen on bacteria will be required before monoclonal antibody panels can be implemented as standard tools for characterization of new bacterial strains.

The work is presently continued to determine the stability of the antigens recognized by the panel of monoclonal antibodies under different conditions of *in vivo* and *in vitro* culture of the bacterial isolates for vaccine purposes. New so-called untypable isolates from hens in incidental outbreaks of the disease may be characterized either by the existing panel of monoclonal antibodies, or by the development of new monoclonal antibodies against unique determinants of the problematic organism. With this it is hoped to make a further contribution to the standardization of vaccines against coryza.

The data presented under 6.3.4 support the hypothesis that "suicidal" cells are generated in the spleens of mice immunized with strain SB86 of *H. paragallinarum*. A similar phenomenon was observed during visits to Israel in 1982 and 1983. Prof. Israel Pecht and fellow scientists from the Weizmann Institute of Science in Rehovoth were working with monoclonal antibodies directed against chromolyn binding protein, a molecule in-

volved in basophil degranulation. Apparently, all positive hybridoma cultures were suicidal; the antibodies they produced recognized structures on the hybridoma cells and caused their death when allowed to build up to sufficiently high concentrations in the culture medium. Daily medium changes saved these hybridomas (Nachman Mazurek, personal communication). This detail did not appear in their published papers on the subject. Using this as a working hypothesis for the suicide observed in the Haemophilus immune hybridomas could not, however, explain the abnormally high frequency of positive antibody producers. The successful fusion of H. paragallinarum immune spleen cells with myeloma cells without using PEG as chemical fusogen, strongly suggests that the suicidal cell, or a substance produced by it, stimulates fusion between lymphoid cells and increased the frequency of viable fusion incidents such that each well on a microculture plate tested positive. This may be analogous to mammalian cell fusion effected by the fusogenic envelope proteins of viruses such as Newcastle disease virus or human immunodeficiency virus (Sodroski et al., 1986).

Suicide (or murder) in hybridoma cultures was reported earlier by Kolk et al., (1984), who observed the phenomenon while raising monoclonal antibodies against Mycobacterium leprae. They attributed the phenomenon to the killing of hybridoma clones by aggressive macrophages, which were more active when Freund's adjuvant was used in the immunization program.

The phenomenon of fusogenic cells induced by immunization may have a considerable impact on the production of hybridomas in general. Cloning a fusogenic cell line from the fusion product of Haemophilus immune spleen cells and myeloma cells may facilitate the isolation and characterization of a soluble or membrane bound fusogen which could be used for generating

hybridomas. It may also unveil a natural function of the immune response which hitherto went unrecognized.

CHAPTER 7: CONCLUDING DISCUSSION

Traditional quantitative chemical analysis is under siege by enzyme-immuno-assay, a powerful combination between enzymology and immunochemistry. The body's immunoglobulin system can be seen as the analytical laboratory for foreign substances. Not only are foreign tissues such as transplants and pathogenic organisms specifically recognized and their quantity expressed by the intensity of the ensuing eliminating or clearing action by complement proteins and immune cells, but even small molecules such as antibiotics and aflatoxins, alkylated to the body's proteins, are quantified by their binding to immunoglobulins. Dihydrogriesenin provides a particularly fitting example of this last group of analytes (see Chapter 3).

In order to completely clear the body of foreign substances antibodies need to have a high functional affinity for binding to these compounds. Typical specific immunoglobulin levels in the circulation vary between 10^{-4} to 10^{-7}M after challenge with immunogen, which requires their binding affinity to be better than 10^{6}M in order to be effective (Steward, 1981). Such binding affinities provide the theoretical basis for the successful utilization of specific immunoglobulins to determine minute amounts of various analytes *in vitro*. For *in vitro* determinations, covalently conjugated enzymes proved to be well suited as indicator system for the quantification of antigen-immunoglubulin complexes, instead of the extremely labile set of complement proteins which performs this function *in vivo*, particularly on account of the increased sensitivity gained from enzymic cascadal amplification. The humoral immunoglobulin specificity

repertoire exceeds 10^9 (Schiff et al., 1988) which outnumbers by far the total number of analytes which man found useful to determine for the purpose of medicine, agriculture and industry.

In the past, the usefulness of in vitro immuno-assays was hampered by an inability to experimentally distinguish between the various antigens and antigenic determinants which served as ligands for binding to immunoglobulins. This is well illustrated in Chapter 3, where cross-reactivity of polyclonal rabbit anti-dihydrogriesenin, even with homologous rabbit serum albumin, limited the inhibition of the ELISA-signal by soluble DHG-cys to only a maximum of 50%. An explanation for this may be found in the work of Onica et al. (1978), who noticed the appearance of new antigenic determinants in serum albumin by treatment with the cross-linking agents commonly used for coupling haptens. For DHG-determination, the problem was solved by affinity purification of the antiserum, but this would cause a considerable increase in the cost of antiserum, reflecting the tediousness and effort required for this additional preparative step. Monoclonal antibodies are less affected by uninhibitable background ELISA signals as they are simply screened and selected for monospecificity towards the hapten only. This is illustrated in Chapter 4.

The advantages of monoclonal antibodies in immuno-assay were mentioned in Chapter 1. Chapters 4 and 6 narrate part of my own experience in the field and may be discussed in terms of four aspects, viz. affinity (detectability), specificity, the effects of immunomodulation on the antibody produced and applications.

- Affinity: The competitive enzyme-linked immunoassay introduced by Yorde et al., (1976), proved particularly suitable for immuno-assay of haptens by monoclonal antibodies. Functional affinity reflects the mathematical product of all the association equilibria of Fabs in an immunoglobulin molecule involved simultaneously in binding of antigen. The immobilization of antigen on a flat polystyrene surface in CELIA provides for multivalent binding of immunoglobulin to produce a strong ELISA signal. In the presence of inhibiting concentrations of soluble hapten, the equilibrium is simply shifted towards monovalent and thus reversible binding to the solid phase (see 2.1.2). The inability of monoclonal antibodies to form circular complexes with monovalent antigens in solution, leading to lower detectability of such antigens in soluble immuno-assays when compared to polyclonal antisera (Moyle et al., 1983), is thus circumvented in CELIA. This may provide an explanation for the independence of CELIA titre observed for MoAb DCA-10 on the type of cross-reactive hapten that was immobilized on the plate (see 4.3), where the functional affinity of hapten-antibody interaction on the solid phase apparently outmatched the intrinsic affinity of the hapten-antibody interaction in solution.

The determination of the absolute intrinsic associating constant is a tedious and costly procedure, easily influenced by experimental conditions (Steward & Steengaard, 1983). As optimum detectability occurs at analyte concentrations in the range of the inverse of the association constant, its experimental determination was traditionally required as an expression of the detectability of antigen by an immunoglobulin preparation. It was recently shown (Rath et al., 1988) that hapten detectabilities approaching the inverse of the

intrinsic associating constant could be attained by some anti-hapten IgGs in CELIA. The determination of detectability by CELIA titre determination should suffice as a quantitative expression of the analytical potential of a particular monoclonal antibody in similar assays (M. Steward, personal communication). The reproducible titre of $5{,}6.10^{-9}$M found for the atrazine derivative, DCA-C_5-arm, even when using different atrazine derivatives on the solid-phase (see 4.3) lends credence to this point of view.

In my hands, CELIA does not work for IgM-type antibodies. With anti-peptide IgM (Verschoor et al., 1988) no inhibition of ELISA signal is observed at soluble antigen concentrations lower than 10^{-4}M, using monoclonal IgM as antigen detector. Two factors may contribute to this: First, IgM affinities are probably restricted to the potential available in the germline repertoire of variable region genes, which rarely exceeds $10^{5}M^{-1}$ (Karush & Tang, 1988). Second, the antigen binding valency of 10 of IgM results in its virtual irreversible binding to the solid phase. A successful CELIA determination of streptococcal antigens by monoclonal IgM was recently reported (Beatty et al., 1988). This could, however, be due to the polyvalent determinant nature of streptococcal antigen (Beachey et al., 1978), allowing the IgM to bind multivalently both with antigen in solution and on the solid phase.

The determination of monoclonal antibody isotype is therefore recommended before its use in CELIA. The method used for screening hybridoma supernatants for antibodies against atrazine derivatives (see 4.2) would automatically have excluded IgM: Only those cultures were selected which showed more than 50% inhibition in a duplicate

ELISA assay where the supernatant was pre-incubated with soluble hapten at a final concentration of 10^{-5}M. Qualitative ELISA, for example that used for fingerprinting field variants of bacteria (Chapter 6), would not be affected by the detector immunoglubulin isotype, although an increased tendency towards cross-reactivity with similar antigens may be observed with IgM (Beatty et al., 1988).

No attempt was made in this study to determine maximum detectability by manipulation of the substrate or substrate systems used in the final enzyme determination step of ELISA. Fluorogenic substrates (Curry et al., 1979) and multi-enzyme cascading (Carr et al., 1987) have been reported to significantly increase the sensitivity of detection by enzymes. If the back-ground signal can be controlled, this manipulation may increase the potency of ELISA towards attaining the limits of detection which, of course will always be dictated by the affinity of the antibody.

- Specificity: In Chapter 4, specificity of a single monoclonal antibody against various chemical derivatives of atrazine was expressed as the apparent association constants for each of the compounds determined by CELIA titres. In Chapter 6, the specificities of a panel of monoclonal antibodies were expressed as a chequered three-dimensional histogram of relative ELISA-signals against a panel of vaccine strains and field isolates of Haemophilus paragallinarum, thereby providing a distinct pattern for each strain which could not be realised with polyclonal antibodies. These experiments illustrate the adaptability of direct ELISA techniques to different requirements in defining specificity for different types of antigen. For bacteria,

dilution curves of hybridoma supernatants against different bacterial antigens have also been used as a way of expressing specificity by direct ELISA (Coates et al., 1981), but this is less accurate, as the results to be compared cannot all be produced under similar conditions on one ELISA plate.

The extreme sensitivity of an anti-atrazine monoclonal antibody for the kind of substituents on the triazine ring would have been difficult to detect with polyclonal antisera by CELIA, due to the high background and presence of other antibodies with possible less strict recognition properties, as was illustrated with polyclonal antiserum against DHG in Chapter 3. The antigenicities of different atrazines in binding to a particular monoclonal antibody, as compared in Chapter 4, underline the importance of the design of a hapten-carrier conjugate to be used as immunogen in raising anti-hapten monoclonal antibodies (or antiserum) for use in quantitative analysis. This represents the most intricate step in the development of such assays. In the case for the determination of atrazine in soil, it became clear that an immunogen had to be designed which retained the exocyclic chlorine in the same position as in atrazine. On the other hand, the relative insensitivity towards binding specificity of the hapten conjugate to be used for immobilization on the ELISA solid phase is encouraging, as this eases the need for strict control over its design and synthesis.

- Effects of immunomodulation on antibody-production: The nature of the immunogen and the way it is presented to the animal may have a notable effect on the isotypic and antigenic repertoire of the antibodies

produced, as well as their clonotypic frequency and amplitude. Some examples from the literature include the following:

Affinity of antibodies in antiserum is directly proportional to duration and frequency of immunizations and indirectly proportional to the dose of immunogen (Ruoslahti, 1976). The amplitude of the antibody response need not, however, steadily increase or remain at a high plateau for the duration of the immune response.

Freund's complete adjuvant causes a bias towards an IgG response, while alum precipitates of immunogen evoke IgE antibodies preferentially (Ishizaka, 1986).

A high hapten density may restrict the resulting antibody response towards IgM only (Klaus & Cross, 1974).

Certain bacterial antigens may suppress an ensuing specific antibody response, e.g. Mycobacterium leprae (Rook, 1987).

Certain bacterial infections may give rise to an abrogation of self-tolerance, leading to the production of autoreactive antibodies (Grooten et al., 1980).

From the research reported in this thesis, two cases of immunogen driven immunomodulations deserve further discussion, viz.. the enhancement of the antibody response against immunorecessive determinants (Chapter 3) and the appearance of suicidal cells with the ability to effect fusion between lymphoid cells in vitro (Chapter 6).

The production of antibodies to immunorecessive determinants is important for broadening the application spectrum of antibody based immuno-assays. Several approaches towards achieving this goal have been reported. Suppression of immunodominant epitopes by simultaneous injection of the immunogen and homologous antibodies directed at the immunodominant determinants (so-called passive immunization) resulted in increased titres of antibodies directed at immunorecessive determinants (Thalhamer & Freund, 1985). A similar result was obtained by eliminating the B-cell clones reactive against immunodominant determinants by treatment of immunized animals with chemical killers of rapidly dividing cells e.g. cyclophosphamide, followed by *in vitro* immunization of spleen cells with a similar immunogen, but containing an added immunorecessive determinant (Matthew & Patterson, 1983). In Chapter 3, dihydrogriesenin was observed to be an immunorecessive determinant on its serum albumin carrier. A significant antibody titre against DHG was brought about by carrier modulation, the principle of which was demonstrated by Paul *et al.*, (1967). This was brought about by alternating the serum albumin carrier used for conjugation with DHG with other heterologous serum albumins in subsequent booster injections. The approach is probably superior to that of passive immunization, which was applied, with only limited success, in an attempt to increase the antibody response against the immunorecessive loop peptide determinant of lysozyme (Verschoor *et al.*, 1988). However, an observed immunomodulatory effect of one antigen does not necessarily apply to another antigen system.

The generation of *in vitro* fusogenic spleen cells from mice that were immunized with field isolates of *H. paragallinarum* (see 6.3) raises

several questions regarding the type of cell involved and the mechanism whereby it induces fusion between lymphoid cells.

Regarding the cell type, it could be either a primary cell or a product of fusion with myeloma cells. The available data do not allow a choice to be made between the two possibilities. The fact that self destruction of developing hybridoma colonies down to a few individual cells occurs, followed by rapid expansion of a second generation of hybridoma cells which remains unaffected during further in vitro culture, argues for a primary cell. This cell probably generates triomas and tetromas which are unstable and eventually die off. The primary cell type from which the fusogenic cell derives could be either a macrophage (Kolk et al., 1984), an autoreactive T-cell such as a veto cell (Miller, 1986) or a B-cell producing autoreactive antibodies (Grooten, J. et al., 1980).

Two possible mechanisms can be considered whereby fusogenic cells effect fusion of lymphoid cells, viz. fusion by a secreted fusogenic factor, or fusion effected by cellular contact. These possibilities are presently investigated by determining the fusogenicity of mature supernatant from a cloned, apparently fusogenic cell line.

- Applications: The application of ELISA to a particular antigen depends much on the creation of a suitable antigen coated solid phase. In this study two such problems were encountered, the solution of which deserves some general discussion:

The non-polar nature of atrazine is mainly responsible for its extremely low solubility in water which, in turn, made its conjugation

to water-soluble protein very difficult (see 5.1). Normally, this problem is overcome by the chemical addition of a polar moiety to the hapten, before it is conjugated to the carrier protein (Chase et al, 1976). The conjugation of atrazine to dissolved nylon (Chapter 5) provides a fresh approach which may contribute to broaden the application of ELISA and the production of hapten specific antibodies. The adsorption isotherm (Fig. 5.5) that was demonstrated for haptenated nylon binding to polystyrene may be extended to include materials such as polycarbonate, polyethylene and polyvinylchloride, which are often used in solid phase immunoassays. Adsorption of haptenated nylon to powdered clays and silicas may render it immunogenic, with the possibility that only hapten specific antibodies will be obtained, albeit after purification by simply passing the antiserum through a nylon column. Haptenated nylon may also be applied in the affinity purification of hapten specific antibodies, but the synthesis described in Chapter 5 would probably not prove advantageous to the method of Hendry and Herrman (1980), of covalently coupling haptens to nylon balls in aqueous buffer.

A limitation of the haptenated nylon system described in Chapter 5 is that it requires approximately 100 times more conjugate for coating of ELISA plates than with protein conjugates (Cantarero et al, 1980), although this was compensated for, in the case of atrazine, by the improved solubility and stability of the haptenated nylon compared to protein-conjugates. Another limitation, caused by the nature of the solvents that solubilize nylon (cresols and strong acids), is the inconvenience of hapten density determination. The demonstration of a point of saturation of covalent attachment of hapten and the limited availability of free amino groups on nylon due to polymer size may,

however, obviate the need for hapten density determination for most applications.

In conclusion, little doubt remains that monoclonal antibodies in combination with ELISA can be used to quantify or characterize almost any analyte, if one takes cognizance of the avoidable pit-falls, and may soon replace mass screening of samples for most analytes in medicine, agriculture and industry. However, it will never replace the existing chemical techniques completely, simply for the reason that analyses of samples for a particular analyte are best confirmed by several analytical techniques based on different principles. The opinion expressed by Sir Gustav J.V. Nossal at a public lecture in 1981 at the Weizmann Institute of Science in Rehovoth, Israel, that "one can in principle raise antibodies against any substance if only one needs it badly enough" may be rather optimistic in the light of my own experiences of immunogen directed isotype restriction, hybridoma suicide and immunorecessiveness. On the other hand, the development of methods with which to modulate the immune response of experimental animals may soon put my apprehensions to rest.

OPSOMMING

Monoklonale teenliggame verteenwoordig die maksimum bereikbare spesifisiteit in serologiese analise en diagnose. In kombinasie met die sensitiwiteit en aanpasbaarheid van ensiem gekoppelde immuno-adsorbente analise (ELISA), verskaf dit 'n kragtige hulpmiddel om te onderskei tussen nouverwante analiete, in gevalle waar dit moeilik bereikbaar is met konvensionele tegnieke. Die doel van hierdie studie was om die toepassingsmoontlikhede van ELISA en monoklonale teenliggame te verbreed of om verklarings te vind vir tekortkominge in geselekteerde gevalle van belang in die landbou.

1. Met die ontwikkeling van 'n ELISA vir die bepaling van sisteïenieldihidrogriesenien, 'n aminosuuradduk van 'n seskwiterpeenlaktoontoksien geïsoleer uit Geigeria spp, het die hapteen immunoresessiwiteit geopenbaar op 'n serumalbumiendraer. Die probleem is opgelos deur verskillende serumalbumiene afwisselend te gebruik as draers in aanjaerimmuniserings en deur affiniteitsuiwering van die immuunsera.

2. In 'n poging om met monoklonale teenliggame die onkruiddoder, atrasien, kwantitatief te bepaal in ELISA met serum-albumien-atrasien konjugate as soliede fase, het dit geblyk dat substitusie van die 2-chlorogroep op die triasienring van atrasien met 'n aminokaproësuurspasieerder vir die immunogeen, teenliggame van onvoldoende affiniteit verwek het. Bowendien was die atrazien-serumalbumienkonjugate onstabiel en is die sintese daarvan bemoeilik

deur die beperkte wateroplosbaarheid van die haptene. Die monoklonale teenliggame wat wel so verwek is, is aangewend om 'n metode te ontwikkel vir die sintese van nylon-atrasienkonjugate as stabiele soliede fase vir gebruik tydens sifting van hibriedomakultuurbostande. Die gebruik van nylon as 'n algemene hapteendraer vir soliede fase antigeen verteenwoordig 'n nuwe benadering in ELISA.

3. Die identifisering van enstof- en veldstamme van Haemophilus paragallinarum, die siekteveroorsakende agent van besmetlike korisa in pluimvee, is suksesvol uitgevoer deur die vestiging van 'n paneel van monoklonale teenliggame wat aangewend is in 'n direkte ELISA. 'n Fusogeniese aktiwiteit is waargeneem in vitro in Haemophilus-immuun miltselle wat poli-etileenglikol oorbodig gemaak het in die versmeltingsproses met miëlomaselle om monoklonale teenliggaamproduserende hibriedomas te lewer. Die moontlikhede om deur antigeengerigte immunomodulering funksioneel nuttige hibriedomas te skep, is bespreek.

SUMMARY

Monoclonal antibodies represent the ultimate in specificity of serological analysis and diagnosis. Combined with the sensitivity and versatility of enzyme linked immunosorbent assay (ELISA), it provides a powerful tool to distinguish between closely related analytes where this is not easily accomplished by conventional means. The purpose of this study was to extend the application of ELISA and monoclonal antibodies, or account for imperfections in a selected number of situations of interest to the field of agriculture.

1. In the development of an ELISA for the detection by polyclonal antibodies of cysteinyldihydrogriesenin, an amino acid adduct of a sesquiterpene lactone toxin from Geigeria spp, the hapten exhibited immunorecessiveness on its serum albumin carrier. This problem was solved by alternating different serum albumins as carriers in booster injections and by affinity purification of the immune sera.

2. An attempt towards the quantitative determination by monoclonal antibodies of derivatives of the atrazine herbicide with ELISA, using covalent serum albumin-atrazine conjugates as ELISA solid phase, revealed that substitution of the 2-chloro-group on the triazine ring of atrazine with an amino-caproic-acid spacer in the immunogen, elicited antibodies of insufficient affinity. In addition, the atrazine-serum albumin conjugates were unstable and their syntheses difficult to execute due to the weak solubility of the haptens in water. The monoclonal antibodies that were obtained this way, were

applied to develop a method for the synthesis of nylon-atrazine conjugates which provided a stable solid phase antigen for screening of hybridoma supernatants. The application of nylon as a general hapten carrier for solid phase antigen represents a new approach in ELISA.

3. The identification of vaccine- and field-strains of Haemophilus paragallinarum, the causative agent of infectious coryza in fowl, was successfully carried out by establishing a panel of monoclonal antibodies which were applied in direct ELISA. A fusogenic activity was demonstrated in vitro in Haemophilus immune spleen cells, which obviated the need for polyethylene glycol in their fusion with myeloma cells to produce monoclonal antibody secreting hybridomas. The scope of antigen directed immunomodulation to create functionally useful hybridomas is discussed.

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