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APPLICATIONS OF SPONTANEOUS HYBRIDOMA FORMATION AND CHARACTERIZATION OF A BACTERIAL INDUCER ENTITY

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Applications of spontaneous hybridoma formation and characterization of a bacterial inducer entity.

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List of Abbreviations:

(F1, V1, VF1, VF2 & VF3)	:	monoclonal antibodies
B-cell	:	Bone marrow derived lymphocyte
B16; B16-F10	:	B16 - melanoma cell line
Balb/C; C57Bl & CBA	:	Inbred mouse strains
BSA	:	Bovine serum albumin
BTA	:	Blood tryptose agar
CAPS	:	3-[Cyclohexylamino]-1-propanesulfonic acid
CD14	:	Cluster of differentiation antigen for macrophages / monocytes, receptor for LPS-LBP-complex.
CFA	:	Complete Freund's adjuvant
DMEM	:	Dulbecco's minimum essentialmedium
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme Linked Immunosorbent Assay
FACS	:	Fluorescence activated cellscanner/sorter
FCS	:	Fetal calf serum
FIA	:	Freund's Incomplete Adjuvant
FITC	:	Fluorescein isothiocyanate
HA	:	Haemagglutination
HAT	:	Hypoxanthine aminopterinthymidine
HEL	:	Hen's Egg White Lysozyme
HGPRT	:	Hypoxanthine-guanine-phospho-ribosyl-transferase
HI	:	Haemagglutination inhibition
Ig(G; M; A; E; D)	:	Immunoglobulin (G; M; A; E; D)
ip.	:	Intraperitoneally
kD	:	kilo Dalton
KDO	:	3-deoxy-D-manno-2-octuloic acid
LBP	:	LPS binding protein
LPS	:	Lipopolysaccharide
m/v	:	mass to volume
mAb	:	Monoclonal antibody
mAbs	:	Monoclonal antibodies

MM	:	Molecular mass
NAD	:	Nicotinamide
NO	:	Nitrous oxide
OD	:	Optical density
OMP	:	Outer membrane protein
p53	:	Name of a tumour suppressor gene/protein
PAF	:	Platelet activating factor
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PEG	:	Polyetheleneglycol
PVDF	:	Polyvinylidene difluoride
RBC	:	Red blood cells
SDS	:	Sodium dodecyl sulphate
SDS PAGE	:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sp2/0	:	Myeloma cell line from Balb/C linage
SPF	:	Specific pathogen free
TBS	:	Tris buffered saline
TNF-α	:	Tumour necrosis factor α
v/v	:	volume to volume

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Summary:

Spontaneous hybridoma formation was evaluated in this study. This phenomenon entails the treatment of mice with specific *Haemophilus paragallinarum* strains to induce the ability of murine splenocytes to fuse with tumour cells both *in vitro* and *in vivo*. It was descovered in the process of developing monoclonal antibodies against *H. paragallinarum.* The antigens on the relevant bacteria were further characterized to determine their chemical nature. Progress was also made in determining the optimal culture conditions for the production of bacteria that induce fusion. Two possible applications of the *Haemophilus paragallinarum* induced fusion were evaluated. The use of fusion inducing *Haemophilus paragallinarum* as an adjuvant for enhancing the local immune response and simultaneously acting as a fusing agent that simplifies the production of monoclonal antibodies against complex antigens. An animal model is described which enables the correlation of *in vivo* hybridoma formation with *in vitro* hybridoma forming ability. This provides evidence that reduced tumour growth rates observed in *Haemophilus* treated animals can be attributed to *in vivo* spontaneous hybridoma formation and that *Haemophilus* treatment therefore can have possible anti-cancer therapeutic use.

Opsomming:

Die toepassings van spontane fusie is ondersoek in hierdie tesis. Die verskynsel behels die behandeling van muise met spesifieke *Haemophilus paragallinarum* bakterieë wat 'n kondisie by miltselle induseer om met kankerselle te versmelt. Die antigene op die relevante bakterieë is verder gekarakteriseer om hulle chemiese aard te bepaal. Vordering is ook gemaak in die bepaling van optimum groeikondisies vir fusie-induserende bakterieë. Twee moontlike toepassings vir *Haemophilus paragallinarum* geïnduseerde fusie is ondersoek: Fusie-induserende *Haemophilus paragallinarum* as 'n adjuvant wat die plaaslike immuunreaksie verhoog en tegelykertyd dien as 'n fusogeen in die spontane produksie van hybridomas wat monoklonale teenliggaampies uitskei teen komplekse antigene asook die gebruik van *Haemophilus* behandeling as 'n moontlike anti-kanker middel. 'n Eksperimentele diermodel word beskryf wat korrelasie tussen *in vivo* hibriedoma vorming en *in vitro* spontane fusie aantoon. Dit verskaf getuienis dat die waargenome verlaging in tumorgroeitempo by *Haemophilus* behandelde diere aan spontane *in vivo* fusie toegeskryf kan word.

Chapter 1: Introduction

This thesis is about the phenomenon of spontaneous hybridoma formation, an *in vivo* inducible adaptation of murine B-lymphocytes enabling them to fuse with malignant cells *in vitro* and *in vivo.* In this study, specific strains of formalin inactivated *Haemophilus paragallinarum* act as the inducer agent. The study aims at furthering the understanding of this phenomenon as well as determining the possible applications it may have in hybridoma technology and cancer chemotherapy.

The introduction that follows describes how the production of monoclonal antibodies (mAb), intended to be used as characterization tools for *Haemophilus paragallinarum* isolates, lead to the discovery of spontaneous fusion. It will further review some relevant work of previous researchers on which the specific aims for this study is based.

1.1 Monoclonal antibodies against *Haemophilus paragallinarum* leading to spontaneous fusion.

Haemophilus paragallinarum is the most common bacterial infection in layer hens in South Africa. It causes coryza, an upper respiratory disease, that can be responsible for serious losses in egg production amongst infected hens. Vaccines produced to combat the problem mainly consisted of serotype representative, inactivated bacteria in an oil emulsion adjuvant. These vaccines became less protective in the early 1980's, resulting in infections that broke out amongst vaccinated birds. In order to try to address the problem Prof. L. Coetzee from Golden Lay Farms, who until recentlywas the head of the Department of Poultry Diseases, Faculty of Veterinary Science, University of Pretoria, initiated research to develop an effective disease monitoring system that could detect changes in the bacterial population, so that the vaccines could be monitored and adjusted on a regular basis (Bragg, 1996).

As one of the steps to achieve this goal, development of mAbs against *H. paragallinarum* was initiated in 1987 by J. A. Verschoor at the Department of Biochemistry of the University of Pretoria. The aim was to produce a panel of monoclonal antibodies that was able to distinguish between vaccine strains and isolates from unprotected hens (field isolates). This panel of monoclonal antibodies was to be applied to identify antigenic shift of the bacterial population, responsible for vaccine failure.

A panel of five mAbs was produced and tested for their specificity to detect field isolates and vaccine strains. One of the resultant monoclonal antibodies only reacted with field isolates and was designated F1. Another mAb reacted exclusively with vaccine strains and was named V1. Three monoclonal antibodies reacted in distinct ways with both vaccine and isolated strains and were named VF1 - VF3 (Verschoor. *et al.,* 1989).

The monoclonal antibodies were produced using standard polyetheleneglycol (PEG) fusion techniques, with hypoxanthine-guanine-phosphoribosyl-transferase deficient ($HGPRT^-$) myeloma cells (Sp0/2) and immune splenocytes as fusion partners (Galfré, and Milstein, 1981). The mice were immunized intraperitoneally (i.p.) with three consecutive doses of 10^7 cells on days 0, 14 and 59 . Selection of the *H. paragallinarum* specific monoclonal antibodies were done with an ELISA, that used whole bacteria as a solid-phase antigen. Antibodies that reacted with the bacteria were detected with a second anti-mouse Ig mAb that was coupled to peroxidase that produced a distinct colour reaction indicative of specificity (Verschoor, *et al.,* 1989).

1.2 Spontaneous fusion induction after treatment with *Haemophilus* bacteria.

While in the process of producing mAbs against *H. paragallinarum,* Verschoor observed a phenomenon where the splenocytes of *H. paragallinarum* immunized mice exhibited the ability to spontaneously fuse with myeloma cells when cocultured *in vitro* (Verschoor, *et al.,* 1990). A phenomenon of self-destructive cell fusion was described, whereby PEG fused cells would produce high yields of hybridomas that died after three weeks of culturing only to regenerate and form stable hybridoma cultures producing antibodies reactive towards *H. paragallinarum* (Verschoor, *et al.,* 1990).

The fusion experiment described above was repeated using similar immunization and fusion protocols but omitting the fusing agent (PEG). The resulting experiment produced high percentages of spontaneously formed hybridomas, illustrating a fusion inducing ability of *Haemophilus* bacteria, without the over-fusion observed previously. The phenomenon seemed to be specific for certain strains of *Haemophilus* only since mice treated with a vaccine strain seemed not to be able to generate splenocytes able to undergo spontaneous fusion with myelomas. The optimum ratio of splenocyte to myeloma cells was determined in this study and found to be 5:1.

Verschoor could also illustrate an antigen independent reaction by mixing fusion inducing *Haemophilus* bacteria with a model antigen - Hens'-Egg-Lysozyme (HEL). By

immunizing mice with this mixture, he could obtain hybridomas of which more than 10% produced HEL-specific antibodies compared to the 5% *Haemophilus* specific hybridomas (Verschoor, *et al.*, 1990). This opened up a possibility to use these bacteria as a novel aid to hybridoma technology, which is further investigated in Chapter 3.

1.3 Initial characterization of fusion inducing bacteria and hybridoma forming ability.

In his M.Sc. work, Boshoff, (1992) studied the phenomenon of spontaneous fusion by attempting to identify the molecule(s) that cause this effect. He characterized the antigens expressed by bacteria that could possibly induce *in vitro* fusion. Different chemical treatments of the bacteria were correlated to antigen recognition by the panel of monoclonal antibodies to determine their chemical composition. Experiments were concentrated on the VF3 mAb's antigen and the F1 mAb's antigen which were present on most bacteria inducing spontaneous fusion. The V1 mAb's antigen was expressed on non-fusion inducing bacteria and seemed less informative in his study as was the case for VF1 and VF2 mAbs's antigens, which were seldomly detected on isolates.

Boshoff tested the resistance of the antigens to acid and alkaline treatment as well as their sensitivity towards trypsin treatment and periodic oxidation. The latter two treatments showed the chemical nature of the antigens: trypsin treatment would affect susceptible proteins and periodic acid oxidizes di-hydroxy (diol) groups of sugar molecules abundant in the lipopolysaccharides (LPS) of the cell wall. He then also determined the fusion inducing potential of these treated bacteria.

The results showed that periodic acid treatment, acid and alkaline digestion all affected the recognition of the antigen by the VF3 mAb in ELISA. This indicated that VF3 consists of sugars, most probably LPS. The F1 mAb's antigen was affected by trypsin digestion indicating its protein nature.

The spontaneous fusion ability of the bacteria was affected by the periodate and alkaline treatment but not by trypsin digestion (Boshoff, et al., 1992). From this Boshoff could extrapolate that the fusion inducing ability could be attributed to a LPS like molecule. The only candidate antigen that fitted this description was the one interacting with the VF3 mAb.

When testing different methods of preservation of the *H. paragallinarum* bacteria it was established that freezing and thawing in PBS had a negative effect on the fusion inducing ability of *H. paragallinarum* bacteria but a lesser pronounced effect on the VF3 ELISA signal. Fusion inducing ability was not affected by freezing after dialysis against water and preservation in PBS at 4 °C.

The strongest evidence for VF3's antigen as candidate for the inducer of fusogenicity of B-cells was obtained from classical LPS extracts of *H. paragallinarum* bacteria, which were shown to induce spontaneous fusion upon its administration into mice. Extracts from fusion inducing bacteria gave high % fusion and was freeze-thaw sensitive. An immunoblot of freeze-thawed LPS extracts showed degradation of the VF3 mAb's antigen as recognized by its antibody (Boshoff, *et al.*, 1992). These results supported the hypothesis that VF3 mAb's antigen is a LPS like molecule that acts as the inducer of fusogenicity of B-cells by treating mice with certain strains of *Haemophilus* bacteria.

1.4 Immune reaction towards lipopolysaccharides.

To understand the relevance of LPS as an immuno-stimulator one needs to know more about its individual components. The LPS molecule is embedded into the outer membrane of gram-negative organisms via four sets of fatty acids chains that are either branched or unbranched depending on the bacterial species (Fig. 1.1) (Erwin, *et al.*, 1990). The fatty acids are in turn connected to two glucosamine molecules that are linked with an ester bond. Both glucosamine sugars are phosphorylated and together the fatty acids and sugars form the lipid A moiety of the LPS molecule (Fig. 1.2). The lipid A moiety is the primary immuno-stimulatory subpart of the whole molecule and is responsible for macrophage activation resulting in a cascade of immune reactions via cytokines produced by the macrophage. This also involves lymphocyte sensitisation by up-regulation of cytokine receptors and the stimulation of antibody production (Haeffner-Cavaillon, *et al.*, 1982).

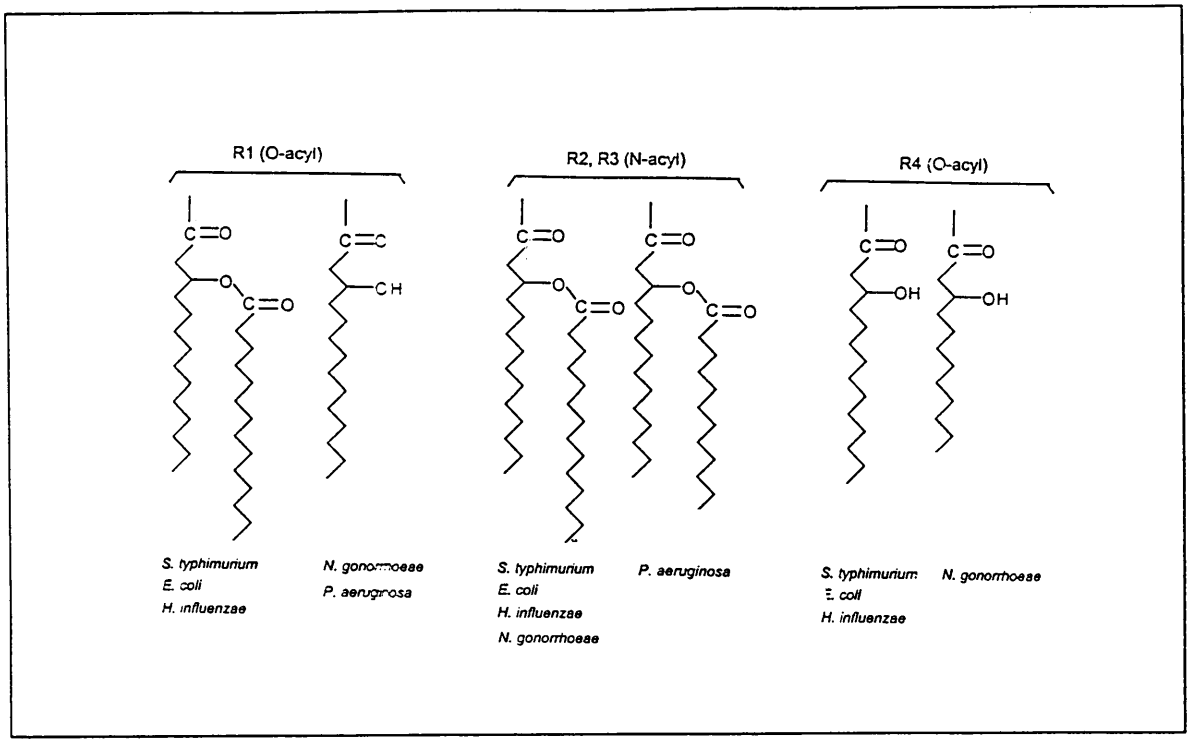


Fig. 1.1: LPS fatty acid sidechains of different gram-negative bacteria. (adapted from Erwin, *et al.*,

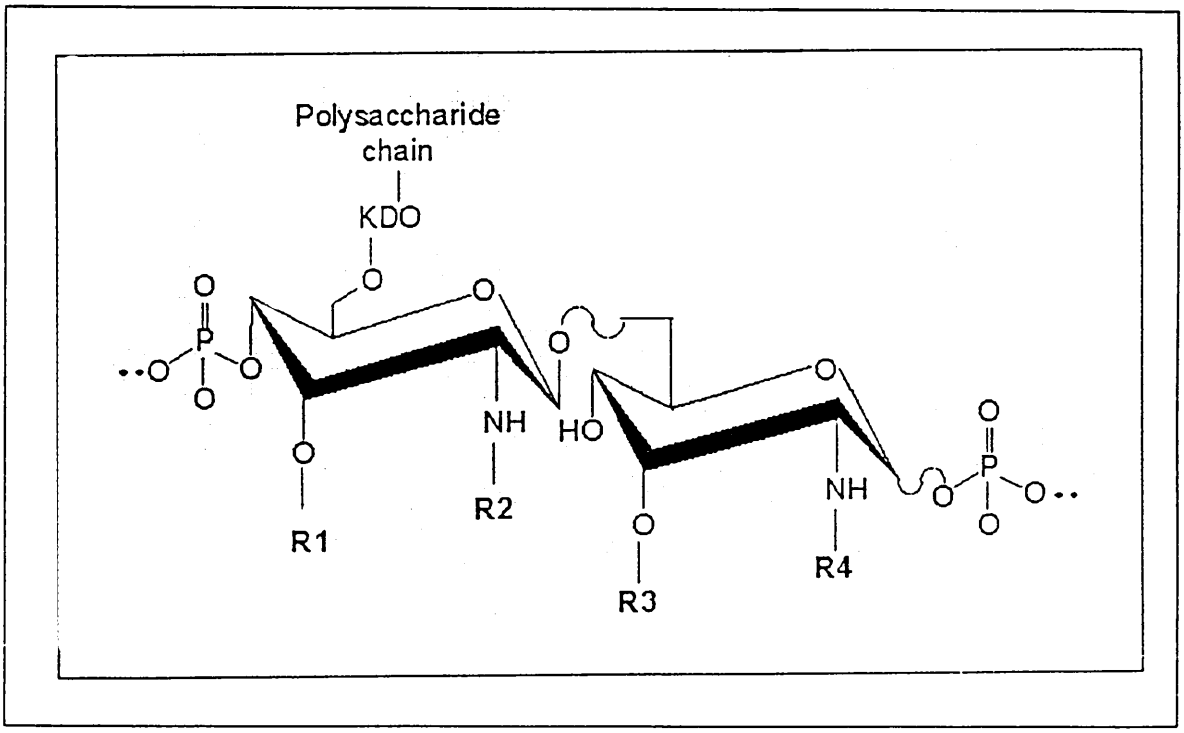


Fig. 1.2: Lipid A fine structure. (adapted from Erwin, et al., 1991)

One of the sugars is connected to the polysaccharide chain via a region called the inner core. The inner core is made up of three submolecules: the KDO (3-deoxy-D-manno-2-

octuloic acid) which connects the lipid part to the sugar chain and an ethanolamine, as well as a phosphorylated heptose molecule (Fig. 1.3). The outer core is made up of five monosaccharides and connects the so-called O-specific region to the core and lipid region. The inner core region is seldom exposed to the immune system and and is not known to elicit

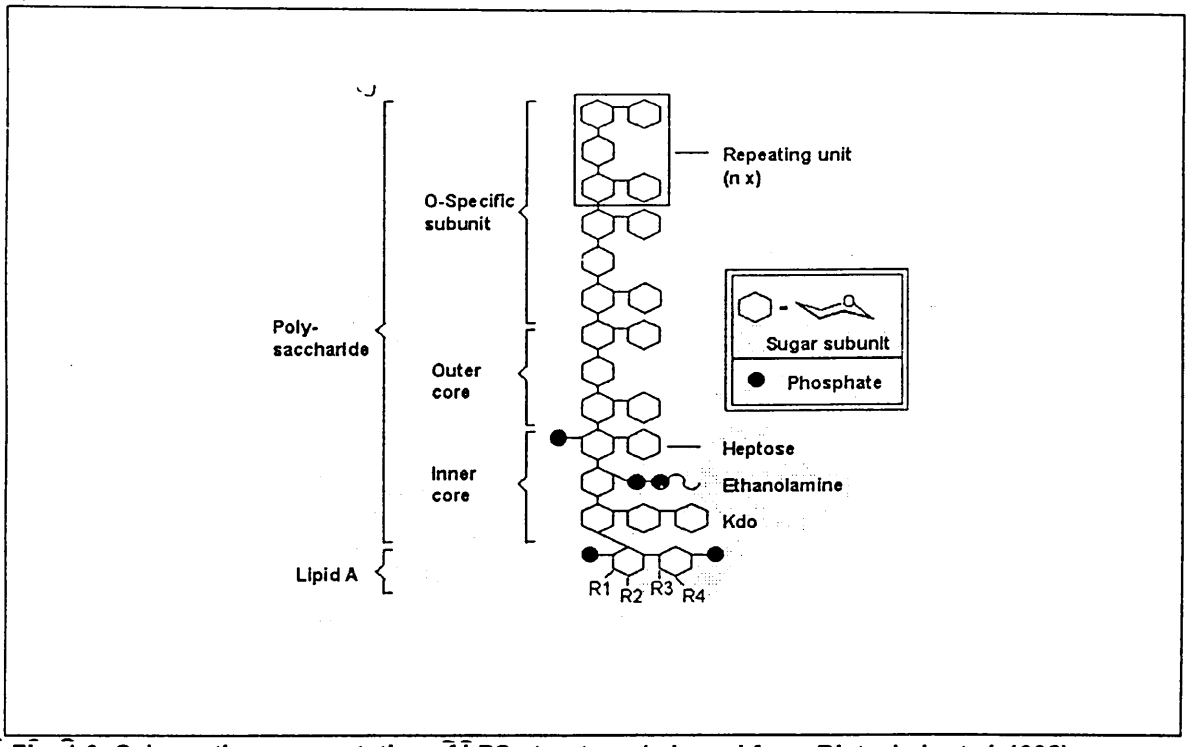


Fig. 1.3: Schematic representation of LPS structure. (adapted from Rietschel, *et al.*,1992)

any host interactions on their own. The O-specific sub-unit consists of groups of up to eight monosaccharides that repeat up to twenty times to form a surface exposed polysaccharide chain that differs from species to species. Specific antibodies to this part are therefore important tools in species characterisation (Rietschel, *et al.*, 1992).

Higher animals developed mechanisms to sense the presence of LPS molecules as such in the blood that serves as an early warning signal of a bacterial infection. A LPS binding protein (LBP) will bind any free LPS molecules in the blood stream (Schumann, *et al.*, 1990). The resulting LPS-LBP-complex is a ligand for the CD14 molecule on the surface of monocytes and macrophages. Binding to the receptor facilitates phagocytosis of the complex that activates the macrophage to produce various immunomodulating substances (Wright, *et al.*, 1990). These include cytokines like Interleukin 1, 6 and 8 (Haeffner-Cavaillon, *et al.*, 1984) as well as TNF-α (responsible for the anti-tumour properties of LPS). These cytokines are also known as inflammatory response mediators and induce lymphocyte proliferation in a non specific way (Kuby, 1993). Free radicals,

like NO, super oxide and peroxide, are also produced by the activated macrophages and can directly destroy nearby bacteria. Prostaglandins, thromboxanes and platelet activating factor (PAF) are also produced to mediate localized inflammatory reactions . The above mentioned mechanism is not the only way in which LPS can activate the immune response. It is also known that the LPS molecule can bind directly to a separate receptor on macrophages without complexing with the LPS binding protein (Rietschel, *et al.,* 1992).

With these complex immuno-stimulatory effects in mind, it is not unlikely that an LPS molecule (like VF3 mAb's antigen) on *H. paragallinarum* could be responsible for the observed effects on B-cells that lead to the induction of spontaneous fusion. This study shows that the phenomenon observed with *Haemophilus* priming of animals, is specific to LPS molecules present on only some strains of *H. paragallinarum* bacteria and is not due to a general property of LPS.

1.5 Applications of *in vitro* and *in vivo* fusion induced by *Haemophilus* treatment.

The observation by Verschoor of bacterialy induced fusion, suggested that *H. paragallinarum* bacteria might be used as additives to adjuvants with which mice can be immunized to 1.) stimulate their immune system (see par. 1.4) and 2.) induce spontaneous fusion (see par. 1.3). This could then serve as a dual purpose adjuvant, i.e. to enhance the immune response and to facilitate fusion *in vitro.* Freund's Incomplete Adjuvant (FIA) was supplemented with *H. paragallinarum* bacteria added to supply the immuno-stimulation and fusion inducing capability and mixed with an aqueous solution of model antigen, hen's egg white lysozyme (HEL). He showed that by using this method, HEL specific antibody secreting hybridomas could be produced by spontaneous fusion (see 1.4 and Verschoor, *et al., (*1990)).

After it was shown that *in vitro* hybridoma formation could be achieved with *Haemophilus* priming of mice (1.3-1.4) its relevance to combat cancer *in vivo* was explored. The ability of *H. paragallinarum* treated animals to produce *in vivo* B-cell - tumour cell hybrids suggested that transfer of genetic material of normal B-lymphocytes to cancerous cells could occur and possibly suppress tumour growth. Boshoff, C.H., in his M.Sc. thesis, proposed that *in vivo* fusion of normal B-lymphocytes, with B16 melanoma cancer cells could transfer intact tumour suppressor genes to the cancer cells lacking it. The fused hybrid cells should then lose their neoplastic phenotype, by virtue of restored function of the tumour suppressor genes.

In experiments where mice were pretreated with fusion inducing *H. paragallinarum* bacteria, Boshoff (M. Sc. Thesis, 1992) could show a significant reduction in growth rate of B16 melanoma tumours. As PBS was used as placebo for *Haemophilus* in control animals, the reduced tumour growth could still be attributed to the general affect of bacterial LPS on tumour suppression (see 1.4). Further controls are needed to adequately prove the specific anti-tumour effect due to *Haemophilus* induced fusion of normal B-cells with B16 melanoma cells.

1.6 The aims of this study.

1. Characterization of the *H.paragallinarum* bacterium.

Characterization of the antigens of *H. paragallinarum* in terms of molecular mass and chemical stability was deemed important to allow the best possible quality control on the identity, growth conditions, preservation and handling of the bacteria during the study.

Determination of the optimal media conditions for production of *H. paragallinarum* was performed to enable the repoducable production of bacteria with high fusion inducing ability.

2. The application of *H. paragallinarum* as spontaneous hybridoma inducing adjuvant.

The application of *H. paragallinarum* bacteria, as immuno-stimulator and fusion inducing agent, in a dual action adjuvant to simplify monoclonal antibody production was tested.

3. The effect of *H. paragallinarum* induced *in vivo* fusion on tumour progression.

Animal models were used to determine the effect of *H. paragallinarum* treatment on tumour progression, and used to test if spontaneous *in vivo* fusion were facilitating such effects.

Chapter 2: The Characterization of antigens expressed by *H. paragallinarum.*

2.1 Introduction:

2.1.1 Production of mAb panel against *H. paragallinarum* strains.

The interest of this chapter is on the characterization of the antigens recognized by the panel of monoclonal antibodies that was produced by Verschoor by immunization of mice with whole *H. paragallinarum* bacteria in order to produce mAbs with the ability to detect the differences between vaccine strains and field isolates (Verschoor, *et al.*, 1989). The resulting mAbs reacted differently to two vaccine strains (083-serotype A and 0222-serotype B) and field isolates (M85 and SB86) used in the experiment. The mAbs reactive towards field isolates were code-named with a "F", mAbs reactive towards vaccine strains with "V" and mAbs reactive towards both groups with "VF". Five useful mAbs were selected and used in further studies on *H. paragallinarum*. These included one mAb recognizing an antigen only expressed by field isolates (F1), one mab recognizing an antigen only expressed by vaccine strains (V1) and three mAbs that recognized antigens expressed on both groups (VF1, VF2 & VF3).

2.1.2 Serotyping and characterizing isolates using mAb panel

Professor L. Coetzee of the Department of Poultry Diseases, Faculty of Veterinary Science, University of Pretoria, initiated the project of mAb production against *H. paragallinarum,* in order to be used in serotyping and characterizing the bacterial isolates from vaccinated, diseased chickens as an assessment of possible population drift of *H. paragallinarum* before and after the onset of using an oil emulsion vaccine against this bacteria in South Africa (Coetzee, *et al.,* 1983). The mAbs were subsequently evaluated by Bragg and coworkers in several studies on South African and reference strains of *H. paragallinarum.* Bragg, *et al., (*1993a) studied the distinct mAb ELISA patterns for strains representing serotypes A,B & C respectively. Strain 0083 (Serotype A) reacted strongly with the V1 mAb, strain 0222 (Serotype B) did not show any significant reaction with any of the mAbs and the Modesto strain (Serotype C) showed some reaction with the F1 mAb but no reaction with the V1 mAb. In the same study, Bragg also examined a total of 45 different South African isolates, made either

before 1974, during the 1980's or between 1990-1992 with the mAbs. It was found that none of the South African field isolates reacted with the V1 mAb, while all of the isolates made after 1974 showed some reaction with the F1 as well as the VF3 mAbs. Isolates showing the reaction with both the F1 and the VF3 mAbs were not represented in the patterns obtained from the three reference strains used to type isolates into serotypes A, B or C. Some South African field isolates also consistently showed significantly higher signals with the F1 mAb than obtained for the Modesto strain. From these results it was clear that not all of the South African isolates appeared antigenically related to either 0083 (serotype A), 0222 (serotype B) or Modesto (serotype C) according to their mAb reaction patterns. This observation urged the use of larger numbers of serovar reference strains, which was recently done. This recent study (Bragg, *et al.,*1996) showed considerable mAb ELISA pattern differences among individual isolates of a particular serovar.

Bragg also evaluated changes in antigenic profiles of bacteria, which might be related to the recent change in nutrient requirement of *H. paragallinarum* and other haemophiline bacteria. A change towards NAD independence was observed which could be transcended on NAD dependent strains by deliberate transformation (Bragg, *et al.,* 1995b).

In haemagglutination inhibition (HI) assays with serotype specific rabbit antisera, none of the monoclonal antibodies were able to inhibit serotype specific haemagglutination. This indicated that no monoclonal antibody reacted with a haemagglutinin and were therefore of little use as conventional serotyping tools (Bragg, 1996).

2.1.3 Characterization of antigens recognized by the mAb panel.

At the Department of Biochemistry, University of Pretoria, studies continued on the ability of certain *H. paragallinarum* strains to induce *in vivo* spontaneous hybridoma formation in mice immunized with the bacteria (Verschoor, *et al.,* 1990). Boshoff found evidence that the fusion inducer could be a lipopolysaccharide and possibly the antigen recognized by the VF3 monoclonal antibody (Boshoff, *et al.*,1992). In his MSc thesis, Boshoff described the first attempts to characterize the antigens recognized by the mAbs.

This study was aimed at characterizing the antigens recognized by the mAbs in terms of MM, proteolytic sensitivity, freeze-thaw sensitivity and LPS nature.

2.2 Materials and methods:

2.2.1 Bacteria:

Bacterial isolate A1383 selected for the expression of various antigens were provided by the Department of Poultry Diseases, Faculty of Veterinary Sciences, University of Pretoria, Onderstepoort, 0110, South Africa.

The bacteria were inoculated into 10 ml - 1000 ml modified Casman's medium, pH = 7,4 (unless otherwise stated), with the addition of NAD (Coetzee, *et al.* 1983), or 10% sterile chicken serum. Incubation inside a candle jar proceeded at 37 °C for 18h or until satisfactory growth could be indicated by turbidity. Bacteria were checked for purity by blood-tryptose-agar-(BTA)-plate sample evaluation, checking typical satellitism (De Blieck, *et al.,* 1932). The liquid cultures were then inactivated by adding formalin to a final concentration of 0.1% (v/v) and incubation at room temperature for 24h. Inactivated bacteria were washed 3x in PBS and used for immunization and characterization of antigens with ELISA as described below.

2.2.2 Hybridoma lines:

Hybridoma lines were used producing mAb against five different antigens of *H. paragallinarum* bacteria (Verschoor, *et al.,* 1989). These were cultured in a humidified incubator at 37°C with 5% CO_2/air ventilation with DMEM medium containing $NaHCO_3$ (3.7g/L), sodium pyruvate (1 mM), penicillin-streptomycin mixture (10 IU/ml and 10µg/ml, respectively) and 10% (v/v) fetal calf serum (FCS).

Batches consisting of several rounds of pooled mature supernatants were collected, tested for IgG concentration by ELISA using standard goat anti-mouse mAb as capture antibody and stored in 50 ml sterile tubes at -20°C. Before supernatants are used in ELISAs, they were thawed at room temperature and cleared by centrifugation at 1000 x g for 15 min.

2.2.3 Biochemical Characterization of antigens:

Antigens were tested for protein nature by treatment with trypsin. A suspension of cells (10^7 per ml in PBS) and trypsin (0.2%) was obtained by mixing a fresh stock solution of trypsin (Sigma, St. Louis, MO) in 0.001 M HCl with bacteria. After an incubation period of 16 hours at room temperature, soybean trypsin inhibitor (Boehringer Mannheim), to a final concentration of 0.32% (m/v), was added to neutralize the trypsin. As a control the same amount of inhibitor was added before the 16 hour incubation. The trypsin activity was tested prior to and after incubation of all samples.

To test antigens for carbohydrate nature, bacteria (10^7 cells per ml in PBS) were incubated in HIO_4 (Merck, Darmstadt, Germany) solution (20 mM) in PBS for one hour. The reaction was stopped by adding 100µl glycerol (10% solution v/v) to the reaction and increasing the pH to 7,2 with NaOH (1M). The control group was incubated in PBS instead of HIO_4.

The treated bacteria were then characterized by ELISA in order to estimate the effect the treatment had on mAb binding to the antigens.

The stability of the antigens towards freezing and thawing was determined by freezing bacteria (in liquid nitrogen) and thawing samples three times before electrophoresis and blotting.

2.2.4 ELISA: Enzyme-linked Immunosorbent Assay:

ELISAs were performed in order to estimate the effect of different chemical reagents on mAb binding to antigens and to determine the antigenic profiles of the strains.

Polystyrene microtitre plates (Cooke Microtitre Systems, Sterilin, South Africa) were coated with whole bacteria by pipetting: 100µl of PBS containing 10^7 *H.paragallinarum* bacteria of selected strains and in each of the wells, drying the bacteria under a fan and heating lamp before fixing with 70% methanol (200µl/well) for 10 minutes. After discarding the solvent, the plates were air dried and could be stored as such for 3 months without significant loss of ELISA signal.

Blocking was performed with a solution of 0.5% casein in Dulbeco's PBS, pH = 7,4 for 1 hour at room temperature. Wells were then flicked out and filled with 50µl of undiluted hybridoma culture supernatant and incubated at room temperature for 45 minutes. Plates were then washed with the same buffer used as blocking buffer. Excess wash

buffer was removed by vacuum suction before wells were loaded with 50µl of the secondary antibody (goat anti-mouse immunoglobulin G(H+L) peroxidase conjugate, Cappel) at 1/4000 dilution. Incubation took 30 min. and was followed by washing and vacuum suction before development took place with 50µl substrate solution containing o-phenylenediamine (10 mg) and urea-hydrogen peroxidase (8 mg) in 10 ml citrate buffer (0.1M, pH = 4.5).The plates were subsequently analyzed on a Titertek SLT-scanner.

2.2.5 SDS-PAGE and Western blotting:

Polyacrylamide gels were made according to Laemmli, U.K., (1970) on a BioRad 14 cm x 16 cm apparatus. Gels of 12% polyacrylamide with 4% stacking gels were used. Bacteria (10^8 cells) were centrifuged for 10 min. at 8500 x g, resuspended in 1 ml sample buffer and boiled for 4 minutes. The sample buffer consisted of 0.5 M Tris-HCl (pH = 6.8); glycerol (20% v/v), SDS (4% w/v) and 2-ß-mercapto-ethanol (10% v/v). Low MM Standards (Pharmacia) and bovine serum albumin were included for calibration. A volume of sample equivalent to 10^7 cells was applied per lane on the gel. Pre-electrophoresis at 60 V for one hour was followed by electrophoresis at 100 V for 3-4 hours.

After electrophoresis, gels were equilibrated in CAPS-buffer (10 mM; pH = 9.0) for 15 minutes and transferred to a PVDF membrane (Millipore, Bedford) by electroblotting for 45-50 minutes at 10 V on a semi-dry blotting apparatus (BioRad Laboratories, Richmond, CA) with a matching power supply. Lanes containing molecular mass markers were developed in Coomassie stain. Remaining lanes were cut from the membrane and blocked for one hour in a buffer containing 1% fat-free milk powder and 0.05% Tween in TBS-buffer (Tris-buffer saline; 0.02 M; pH 7.4). After overnight incubation in undiluted mAb supernatant at 4°C and washing in TBS-buffer containing 0.1% fat-free milk powder, the membrane strips were incubated for four hours in TBS-buffer containing peroxidase conjugated-goat-anti-mouse IgG (H+L) (Cappel, N.C.) diluted 1/500. The strips were finally incubated in substrate buffer prepared by adding 20 ml 4-chloronaphtol (0.3%w/v) in cold methanol to TBS (100 ml) containing 60 µl hydrogen peroxide. The reaction was terminated by soaking in distilled water after sufficient development.

2.3 Results:

2.3.1 Determination of the electrophoresis mobility and freeze lability of antigens:

Cell lysates were obtained by boiling bacteria in electrophoresis buffer and then used as sample material for electrophoresis and immunoblotting. Fig. 2.1 shows the results of a Western immunoblot probing four antigens residing on the A1383 strain. Two lanes of each antigen are shown of which one was repeatedly freeze-thawed (+) and the other not (-).

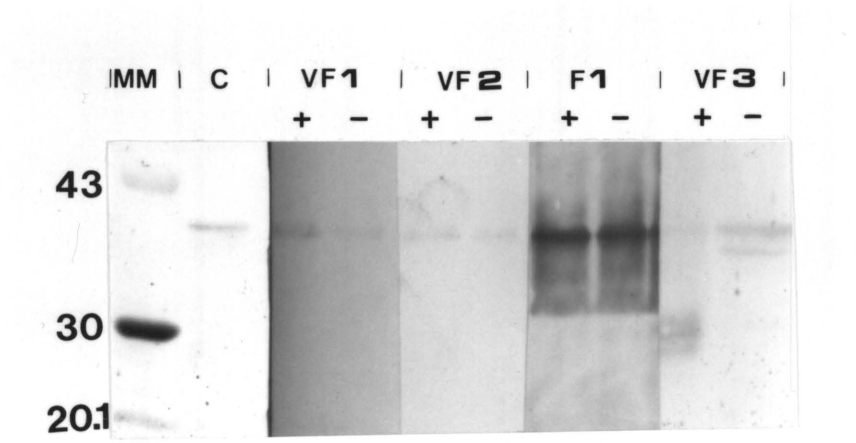


Fig. 2.1: Western immunoblot, showing the mobility of the antigens recognized by mAbs before and after freeze-thaw treatment. Lane 1: MM-markers, lane 2: A1383 bacteria stained by Coomassie, lanes marked with "+" were freeze-thawed thrice and lanes marked with "-" were not. The same batch of bacteria was used as antigen sample in all the relevant lanes.

F1 is unaffected by freezing and thawing and shows a band of 39 kDa that corresponds with a band of similar size on the Coomassie stained lane, suggesting that the F1 antigen is the primary outer membrane protein (OMP) because no other protein bands with the same intensity appeared at this concentration. A second Western blot that was exposed for a shorter time in the developing medium shows the F1 band more clearly (Fig. 2.2).

VF1 and VF2 antigens both have similar SDS-PAGE mobility, surprisingly also at 39 kDa as was observed for F1. The 39 kDa bands are not due to non-specific binding of antibody, as a non-F1 expressing *H. paragallinarum* strain (0222) does not exhibit a band at this position (Fig. 2.2, lanes 1 and 2).

The VF3 mAb's antigen is distinguishable from the other antigens by having multiple bands at 37-39 kDa which break down to multiple bands at 29-32 kDa after freezing and thawing (Fig. 2.1). None of the other antigens tested show freeze lability.

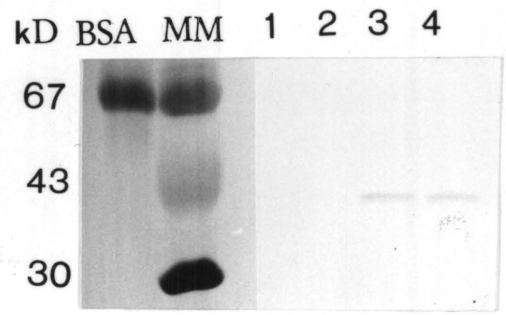


Fig. 2.2: Western immunoblot, showing the MM of the antigens recognized by F1 mAb. Lanes 1 and 2: Non-F1 carrying bacterial strain (0222). Lanes 3 and 4: F1 carrying bacterial strain (A1383) incubated with F1 mab and developed as described in methods. BSA and low molecular markers were used to determine the MM.

Fig. 2.3 shows the SDS-PAGE mobility of V1 antigen at 13,8 - 14 kDa in a Western immunoblot. The bacterium containing this antigen did not show any band in the 39 kDa region after staining with Coomassie dye, in contrast to what was found for F1 expressing bacteria and could not be successfully tested for freeze sensitivity due to its mobility to the low MM region, where visualizing of the bands became a problem.

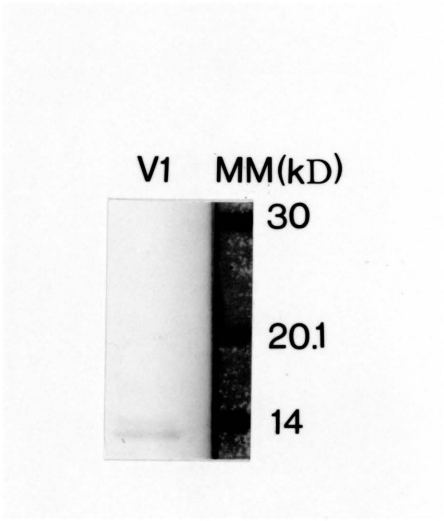


Fig. 2.3: Western immunoblot showing the SDS-PAGE mobility of the V1 mAb's antigen.

2.3.2 Protein and lipopolysaccharide character.

Treatment of the bacteria with trypsin prior to ELISA with the incubated mAbs gave the results shown in Fig. 2.4. The trypsin digestion did not significantly decrease mAb binding to the epitopes of V1 (7%) and VF3 (10%) mAbs' antigens. The ELISA signal for the F1 mAb decreased significantly by 45% after trypsin treatment providing evidence of a protein nature.

The effect of periodic acid treatment on mAb binding to V1 and VF3 antigens was also determined. Both antigens showed a decrease in ELISA signal after treatment with periodate (VF3 45% and V1 55%) while F1 showed a non-significant decrease (5-8%). These findings suggest that both V1 and VF3 antigens are polysaccharides and presumably LPS's (Fig. 2.5).

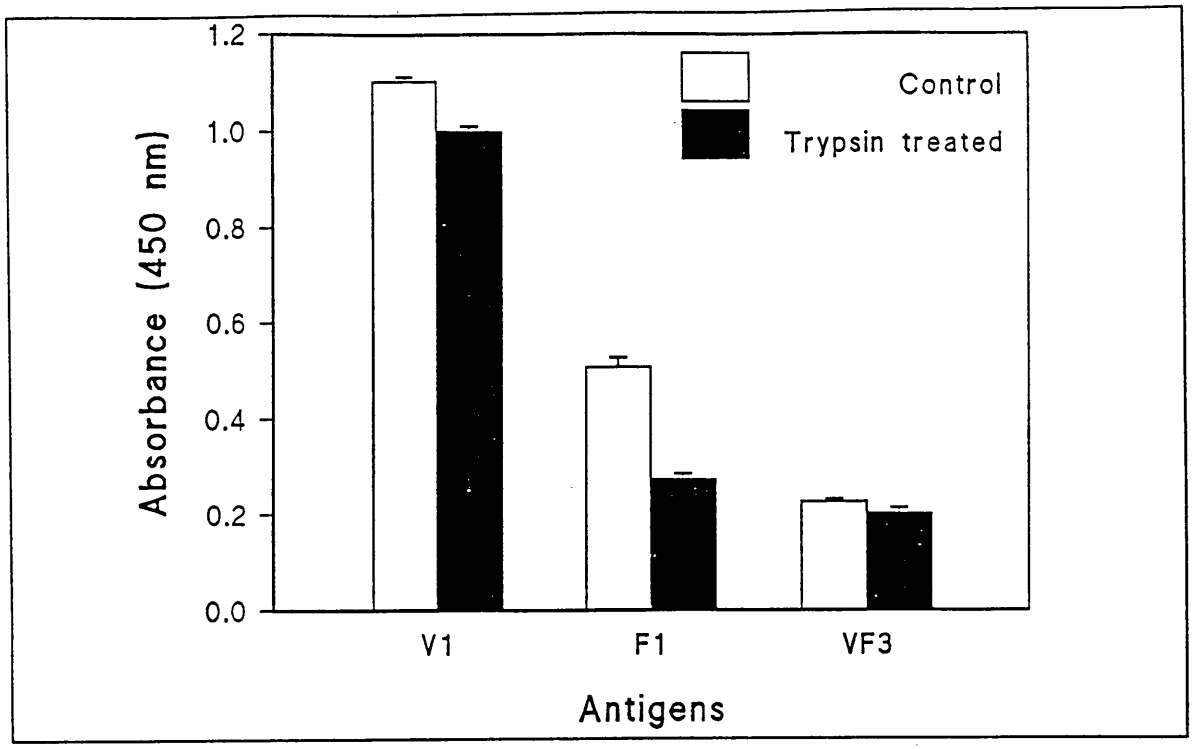


Fig. 2.4: ELISA signals of trypsin treated bacteria (Bars indicate results of quadruplicate ELISAs).

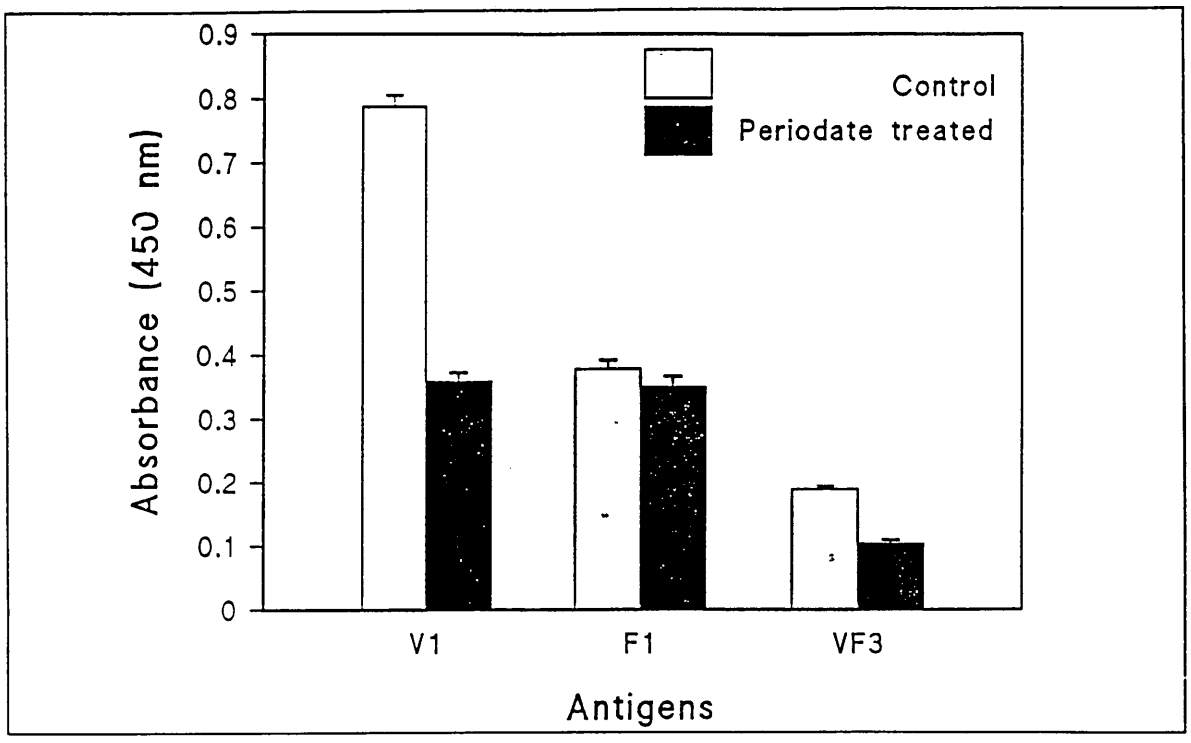


Fig. 2.5: ELISA signals of periodate treated bacteria (Bars indicate results of quadruplicate ELISAs).

2.4 Discussion:

Bragg, *et al.,* (1996) found that each of several different reference strains of *H. paragallinarum* reacted with the panel of mAbs of Verschoor, *et al.* (1989), resulting in a number of different mAb patterns not related to serovars or serogroups. For example the mAb patterns of 0083 and 221, both of serovar A were clearly different from each other. Other workers reported that these strains belonged to the same immunotype (Kume, *et al.* 1980). Because of the known cross protection between 0083 and 221 (Kume, *et al.* 1980) as opposed to clear antigenic differences between the strains, it was suggested that the antigen detected by the V1 mAb does not play an important role in protective immunity, since cross protection seems to be facilitated by antigen/s common to both strains. Moreover Bragg *et al.* (1993) evaluated 45 isolates and found that no South African isolates of *H.paragallinarum* reacted with the V1 mAb. If it is considered that the V1 expressing 0083 strain is used in vaccines in South Africa, one might argue that the antigen disappeared because of immune pressure imposed by the use of the vaccines and that V1 expression is not essential for the bacterium. V1's antigen (14 kD, Fig. 2.3) like VF3's antigen (39 kD. Fig. 2.1) appears to be polysaccharide in nature, as seen in Fig. 2.5, but they differ greatly in MM and distribution among isolates.

The F1 mAb's antigen appears to be a protein of 39 - 40 kD (Fig. 2.2). In 1989 Blackall, and Yamamoto, could sub-divide 15 isolates of *H. paragallinarum* by means of whole cell SDS-PAGE protein profiles in two groups based on the presence of either a 42 or 40 kD antigen. In 1990 these same authors presented evidence indicating that the 42 kD and 40 kD bands actually represent a single protein (OMP C) expressed at a MM of either 39 kD or 38 kD in different isolates. The OMP-C band exhibited heat modifiable mobility in SDS-PAGE. Yamaguchi, *et al.* (1991) showed a major OMP of 39 kDa in Triton-X-100 extracts of strains they tested. Takagi, *et al.* (1991) showed that they could produce a mAb that reacted with a 39 kD protein on immunoblots of serovar-A isolates. Bacterial proteins of similar size (40 kD) are known to be associated with the peptidoglycan and function as diffusion pores for the transport of low molecular weight substances through the outer membrane (Nake, 1976). The fact that nearly all South African isolates express the F1 antigen in significant amounts (Bragg, *et al.,* 1993a) and the observation that it seems to be the most abundant protein on the bacteria expressing it (Fig. 2.1 & Fig. 2.2) underline the importance of this protein (Fig. 2.4) for the bacterium. However, recent results published by Bragg *et al.,* (1996) suggested that this antigen is not a haemagglutinin because it failed to inhibit haemagglutination of any strain and therefore would not be useful in serotyping *Haemophilus* bacteria. However, it

could still prove to be a target protein for a recombinant vaccine against (*H. paragallinarum)* coryza.

Some VF3 expressing bacteria, upon immunization into mice, have been described to induce B-cells to exhibit the ability to fuse with myeloma cells (Verschoor, *et al.,* 1990). This provided a method for spontaneous hybridoma formation which finds application in monoclonal antibody production and is extensively described in the introduction and other chapters of this study. A typical LPS extract of VF3-expressing bacteria was shown by Boshoff, *et al.* (1992) to induce this property in mice. Such an extract bound VF3 mAb in a dot blot, confirming the LPS nature of the VF3 antigen. Freeze-thaw treatment of such an extract destroyed its fusion inducing ability. The results shown in Fig. 2.1 indicates that this may be because of breaking up of the 38-39 kDa VF3 antigen into smaller fragments of 29-32 kDa. The VF3 antigen is also unique to a group of South African isolates that does not fit the mAb pattern of any of the serovar reference strains used in serotyping of the bacteria suggesting a newly developed antigenic property (Bragg, 1996).

The antigens recognized by mAbs VF1 and VF2 could not be chemically characterized because no recent isolates or reference strains were found to express these antigens in reasonable amounts in spite of their abundance in the isolates used for immunizing the mice from which the mAbs were derived (Verschoor, *et al.,* 1989). It would appear however that both have a SDS-PAGE mobility of 39 kDa as shown in Western blot (Fig. 2.1).

In conclusion, although the mAbs produced by Verschoor appear to be of very limited value for serotyping isolates of *H. paragallinarum,* the mAbs appear to be useful for monitoring antigenic change of the bacteria and predicting the spontaneous fusion inducing ability of isolates and strains. The determination of the chemical nature of the antigens aids in determining their role in the bacteria and in their interaction with the immune system of chickens and experimental mice.

The VF3 antigen seems to have a unique interaction with the immune system of mice by inducing the B-lymphocytes of the *Haemophilus* treated animal to fuse with cancer cells *in vitro.* This phenomenon forms the subject matter of the next chapter.

Chapter 3: *In vitro* hybridoma formation by VF3 expressing bacteria.

3.1 Introduction:

The subject matter of this chapter is to further characterize the ability of VF3 expressing *H. paragallinarum* bacteria to induce *in vitro* fusion between murine B-lymphocytes and Sp2/0 myeloma cells to form hybridomas. The aim is to determine and optimize physical parameters that affect VF3 expression and fusion inducing ability. This is done in order to test the applicability of *Haemophilus* induced fusion as a novel aid in hybridoma technology.

The spontaneous fusion phenomenon was first documented by Verschoor, *et al.,* (1990). The paper reported that immunization of mice with formalin treated whole *H. paragallinarum* bacteria induced spontaneous fusion of splenocytes with myeloma cells *in vitro*. This differed from other reports of enhanced fusion of murine splenocytes with myeloma cells, using mitogens as additives (Woloschak, *et al.*, 1983) because no additional fusing agent such as PEG was needed in this experiment.

Boshoff, *et al.*, (1992) linked this ability to induce spontaneous fusion to VF3 expressing bacteria (see 3.1.2). New evidence in the previous chapter substantiate the role of the VF3 antigen but external factors such as culture conditions influence its ability to reproducibly induce spontaneous fusion. This chapter deals with experiments to establish guidelines to optimize the production of VF3 expressing bacteria that will have the ability to induce spontaneous hybridoma formation and the testing of its application in monoclonal antibody production.

3.1.1 The effect of pH and supplements of the growth medium on VF3 expression and hybridoma formation.

A variable antigen expression profile in different batches of *Haemophilus* bacteria belonging to the same strain was observed by Bragg, *et al.* (1995a) in a comprehensive study into the antigen expression of the different strains and isolates using the monoclonal antibody panel produced by Verschoor, *et al.,* (1989). They reported significant changes in the antigen profiles of various isolates after alteration of the growth conditions. The biggest

influences were observed when the NaCl concentration and inoculum size was changed. The growth stages also seemed to play a role. pH variation was less prominent in effecting conversions between characteristic antigenic profiles. For VF3 expression however, pH did show a definite effect. The ELISA signal to background ratio increased proportionally to an increase of initial medium pH from 7.3 to 8.0 for the M85 strain and 8.5-8.6 for Spross and H-18 strains. For this reason initial medium pH was further tested for its effect on fusion induction via the VF3 antigen.

To test the combined effect of medium supplements and pH; two representative media pH's were tested with and without serum substitution with NAD, to establish their influence on fusion inducing potential of the M85 strain.

The following null hypotheses were tested in this regard: 1)That the pH of the growth medium has no effect on VF3 expression, 2) that the replacement of serum as source of NAD in the growth medium, with purified NAD supplement has no effect on the fusion inducing ability of the M85 bacteria, and 3) the fusion inducing potential of differently cultured batches of the M85 strain will be the same if their respective VF3 expression values are the same.

3.1.2 The effect of manipulations of M85 bacteria on hybridoma formation.

In previous work by Boshoff, (1992) it was shown that LPS extracts of fusion inducing *H. paragallinarum* bacteria, contained the ability to induce spontaneous hybridoma formation. This ability could be abolished by freeze-thaw treatment of the LPS extracts in PBS prior to treatment. An immunoblot of LPS extracts with the VF3 monoclonal antibody before and after freeze-thaw treatment showed the VF3 antigen's lability towards this treatment. In a recent publication (Bragg, *et al.,* (1997) and Chapter 2 Fig. 2.1), the 38-39 kDa VF3 antigen is shown, by SDS-PAGE, to break down to one with a mobility corresponding to a molecular mass of 28 - 33 kDa as a result of freeze-thaw treatment in the sample buffer. In order to test this effect on whole bacteria, spontaneous fusion experiments were performed with freeze-thaw treated bacteria.

H. paragallinarum has a NAD growth factor requirement that is met by the addition of chicken serum to the growth medium. Since the NAD content, acidity and other chemical parameters can be expected to differ between serum batches it might contribute to poor reproducibility among batches of *in vitro* grown bacteria. To avoid this problem purified NAD supplement was used to substitute the serum. Recently NAD independent lines were

isolated from diseased chickens (Bragg, *et al.,* 1993) which provided prospects for a strain that could be grown without the requirement for exogenous NAD. Bragg isolated DNA of some NAD independent isolates and transformed NAD dependent lines with this material. The DNA transformation rendered the treated bacteria NAD independent, suggesting that this trait was carried on a plasmid. A M85 line transfected in a similar way was provided by Bragg to be used in testing for fusion inducing potential.

The null hypothesis tested here is that DNA transformation would change the antigenic profile of the M85 bacteria in such a way that the VF3 expression and subsequent fusion inducing potential would be destroyed.

3.1.3 The use of *Haemophilus* bacteria in an adjuvant to induce hybridoma formation for monoclonal antibody production.

Verschoor, *et al.,* (1990) demonstrated that co-immunization of an unrelated antigen with *H. paragallinarum* bacteria yielded splenocytes that spontaneously fused with myelomas to form hybridomas that secreted Abs specific for this antigen. The splenocyte to myeloma cell ratio was optimized to 5:1 and this gave hybridoma colonies of which 12% were antigen specific and 3% *Haemophilus* specific. Hen's egg white lysozyme (HEL) was used as it is well-known and used as a typical antigen that was highly immunogenic and elicited high titer antibodies. To generalize the use of this technique in monoclonal antibody production it needed to be tested with a wider range of complex antigens.

A project which was aimed at the production of species specific monoclonal antibodies to a wide range of planktonic crustaceans provided an excellent opportunity to test this application on a wider scale.

The following null hypothesis were tested: 1) The use of *H. paragallinarum* bacteria in an adjuvant to simplify monoclonal antibody production is not widely applicable to other antigens. 2) Different antigens would affect spontaneous fusion differently rendering this technique useless due to unpredictable interaction with different antigens.

3.2 Materials and Methods:

3.2.1 Bacterial samples.

The M85 strain of *H. paragallinarum* bacteria obtained from the Department of Poultry Diseases, Univ. of Pretoria, Onderstepoort, was specifically grown to the requirements of the experiments as follows:

Unless stated otherwise the bacteria were cultured in liquid modified Casman's medium supplemented with either 10% sterile chicken serum (v/v) or NAD (to a final concentration of 200µg/ml) at a pH of 7.4 ± 0.2. The broth was inoculated and incubated for 18 hours at 37°C before purity testing. A sample was plated out onto a BTA plate and streaked across with an inoculum of *S. aureus* which acted as a feeder culture. After incubation for 18 hr, at 37°C in a candle jar the plates were inspected for typical satellitism, which indicated purity. The original broth culture was inactivated with formalin to a final concentration of 0.1% and left as such for 24 hr at room temperature before the cells were washed twice by centrifugation and resuspension in PBS.

The bacteria were quantified by determining the optical density at 540 nm of a 1:100 diluted sample. A OD of 0.100 was equal to a concentration of 10^8 cells per ml (Verschoor, *et al.,* 1989). For ELISAs and immunization the bacteria were diluted to a final concentration of 10^7 cells/ml.

3.2.2 ELISA.

ELISA s were performed as described in Chapter 2 (2.2.4).

3.2.3 Immunization.

A standard immunization protocol as described in 2.2. was used for immunizing mice with bacteria in PBS alone. Mice were treated on week 0, 2, 6 and then boosted three days before fusion experiments.

The protocol for the immunization of animals used to produce monoclonal antibody was similar to the method described by Verschoor, *et al.,* (1990). The antigens used were plankton species: *Calanus agulhensis* (copepod), an unspecified copepod mixture, a

mixture of Euphausiids and *Podons* (cladocerans). All samples were homogenized and suspended in a sterile saline solution. After a protein determination each antigenic sample was diluted and aliquoted to 100µg protein/mouse mixed to a stable emulsion in a 1:1 ratio with the adjuvant. The adjuvant was prepared using $3x10^8$ *Haemophilus* bacteria (M85 strain) per 1 ml Incomplete Freunds Adjuvant (IFA).

The first immunization was in both hind foot pads followed by a second immunization two weeks later at the same dose subcutaneously. This was followed by a similar booster four weeks later. Two mice of each group were then selected for fusion on grounds of polyclonal serum specificity towards antigen as determined by ELISA and Western blot analyses. Each mouse was injected i.p. with antigen in adjuvant prepared as described above and sacrificed for the fusion experiment four days later.

With the final i.p. boost, the Copepod and *Calanus agulhensis* groups received half the normal dose of *Haemophilus* bacteria but the same antigen dose. In the fusion experiments the splenocytes of each mouse were split in two equal volumes from which one half was used in a PEG fusion and the other half in a spontaneous fusion experiment. Only PEG fusions were performed with the Copepod and *Calanus agulhensis* immunized groups, to determine the affect of half the bacterial dose.

3.2.4 Hybridoma production.

3.2.4.1 Spontaneous fusion.

Splenocytes were obtained from mice immunized as stated in 3.2.3, 3 days after their final booster and co-cultured with Sp2/0 myeloma cells (Shulman, *et al.,* 1979) at a ratio of 5:1 in HAT selective medium. Splenocytes and myeloma cells were prepared according to Galfré and Milstein (1981), but PEG was omitted.

A spleen cell suspension was obtained by cutting and teasing the spleen with fine scissors and forceps over a nylon mesh of 75 µm grid. The cell suspension was then transferred to a 10 ml sterile centrifuge tube and washed three times with DMEM medium without serum. The suspension was filled up with medium to a total volume of 10 ml. A sample of the suspension was taken for cell counting with a haemocytometer. Sp2/0 cells were harvested, washed and resuspended as in 4.2.2 in DMEM medium. A sample of the suspension was taken for cell counting with a haemocytometer.

Sp2/0 cells and splenocytes were mixed and centrifuged at 100 x g for 5 minutes. The pellet was then resuspended in HAT medium (DMEM medium with supplements as in 4.2.2 with the addition of hypoxanthine (100 µM), aminopterin (0.4 µM) and thymidine (16 µM)) to a final concentration of 5 x 10^5 myeloma cells/ml. The suspension was finally distributed at 100 µL per well in 96 well microtitre plates. The growth medium was replenished every third day. After one week aminopterin was omitted from the medium and a week later hypoxanthine and thymidine were omitted as well. A weekly census was taken where each well was inspected for growing hybridoma colonies and the efficiency of fusion induction was expressed as the percentage wells containing viable hybridomas per plate.

3.2.4.2 Polyetheleneglycol fusion.

A similar procedure was followed as described in 3.2.4.1 except that a normal PEG fusion involved the resuspension of mixed splenocyte and myeloma cells in PEG and the slow dilution of the PEG with medium followed by a wash step and resuspension in HAT medium before distribution of cells in a 96 well cell culture plate (Galfré, and Milstein, 1981). A weekly census was also recorded for each of these groups of mice.

3.3 Results:

3.3.1 The influence of growth medium pH and supplements on VF3 expression and hybridoma formation.

Haemophilus bacteria were cultured under specific growth conditions. At first the pH of the starting medium was varied to see what the effect was on VF3 expression. In Fig. 3.1 ELISA profiles of M85 bacteria grown in media of increasing initial pH can be seen. It is evident that the VF3 expression is enhanced when the pH of the medium is increased. The expression of the other two antigens, recognized by F1 and V1 do not change significantly with a change in pH.

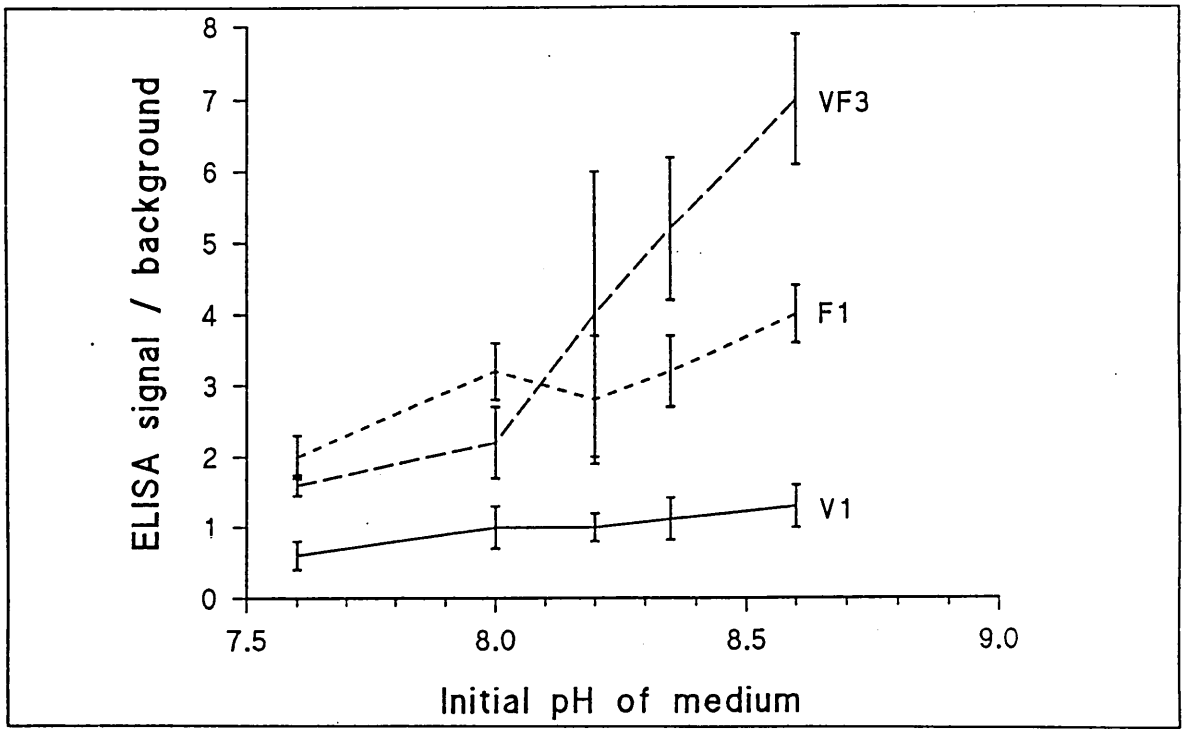


Fig. 3.1: M85 antigen expression versus initial pH of growth medium

In *in vitro H. paragallinarum* cultures chicken serum is used to provide the essential NAD (V-factor), for bacterial growth. Since this source of NAD is uncontrolled and could contain unknown components which might affect the expression of VF3, the replacement of serum with a defined, commercial NAD supplement was tested. To assess simultaneously the affect of the initial growth medium pH and serum substitution with NAD on spontaneous fusion, four batches of M85 bacteria were selected that were grown in medium at pH of 7.4

or 8.5, each with either serum or NAD supplements. The ELISA patterns of these batches are given in Fig. 3.2. Note that the VF3 expression levels of all the selected batches were significantly similar. This was intentional to normalize the results for VF3 expression. The percentage hybridomas formed per 96 well tissue culture plate were monitored for a period of seven weeks after fusion and the results are shown in Fig. 3.3. An additional PBS treated control served as negative control for induction of spontaneous fusion.

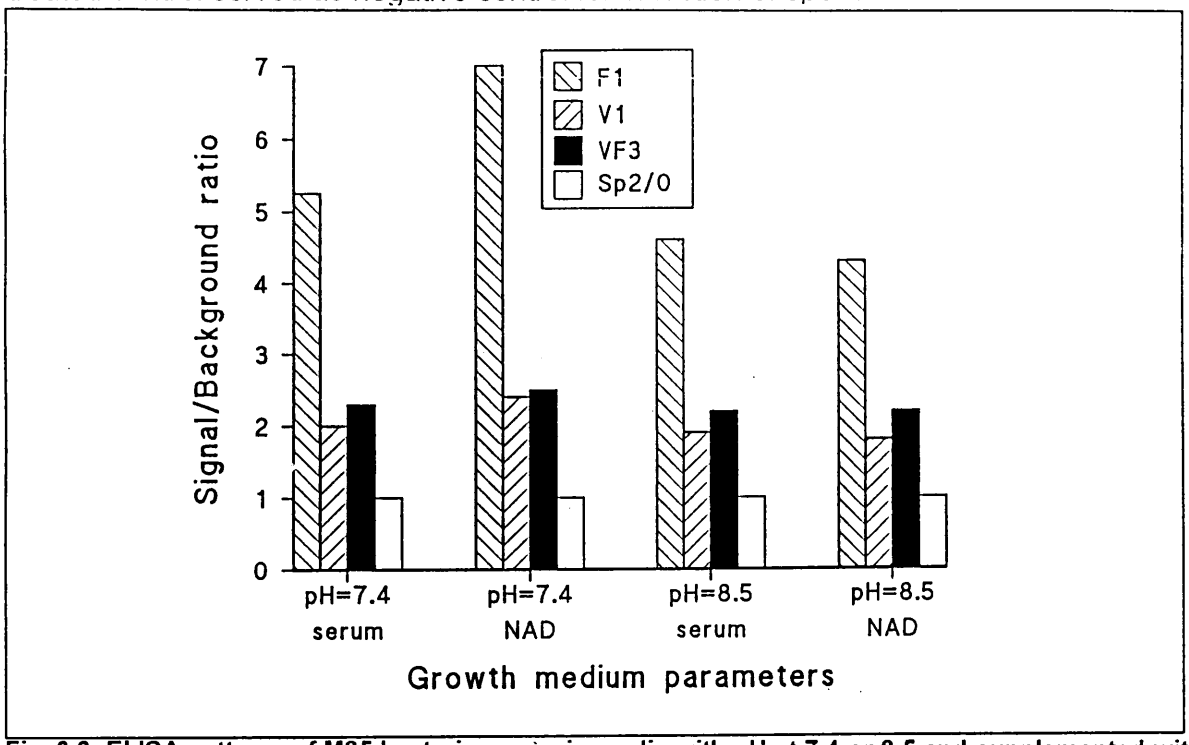


Fig. 3.2: ELISA patterns of M85 bacteria grown in media with pH at 7.4 or 8.5 and supplemented with either NAD or chicken serum.

Although the batches were selected to give more or less similar VF3 expression to simplify the comparison, big differences in spontaneous fusion inducing ability were observed. The medium with NAD supplement at an initial pH of 7.4 appeared to be the best for spontaneous fusion induction (80%), followed by the serum at the same pH (up to 50%). An increase in pH to 8.5 seemed to totally abolish the ability of the *Haemophilus* bacteria to induce spontaneous fusion, irrespective of the NAD source (5%-20% hybridoma formation). This points to an entity required for fusion induction that is very sensitive for high medium pH, a condition where the correlation between VF3 expression and spontaneous fusion induction is lost.

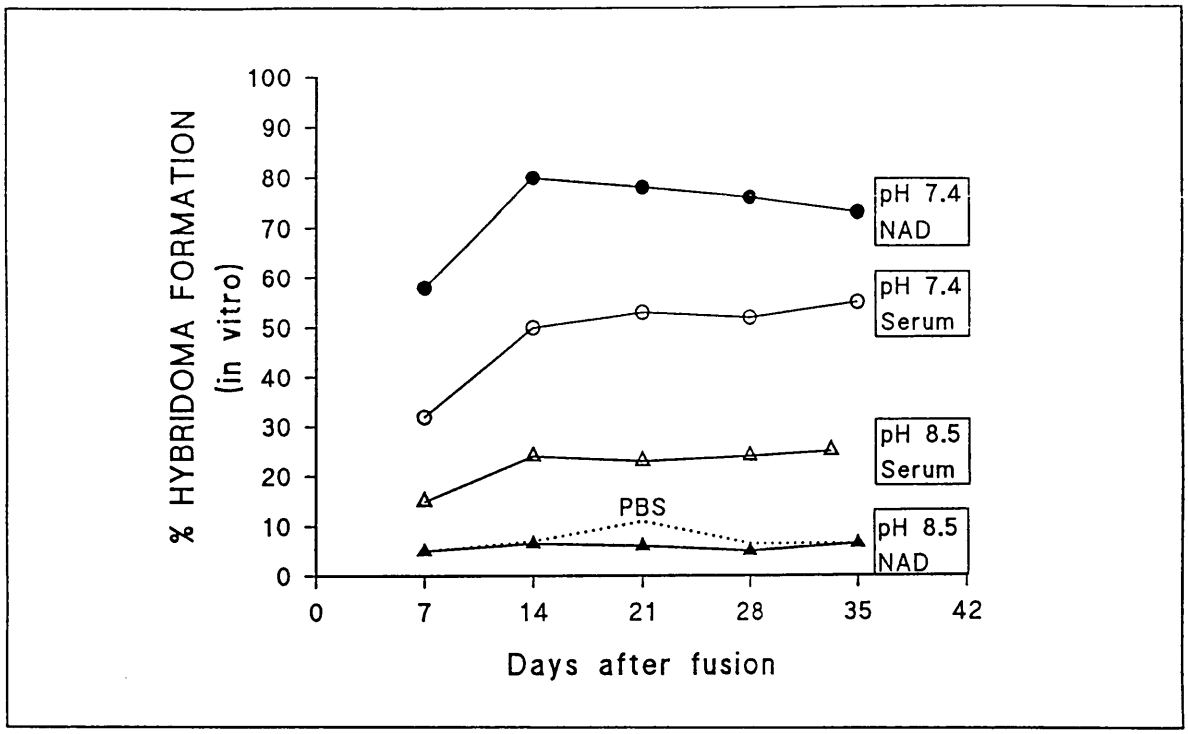


Fig. 3.3: Effects of NAD/Serum supplements and pH of growth medium on efficiency of spontaneous fusion induction, measured as % hybridoma formation. A PBS treated mouse served as negative control.

3.3.2 The effect of freeze - thaw and DNA manipulations of M85 bacteria on hybridoma formation.

In an attempt to find a reliable negative control strain a few *in vitro* fusions were tried, with the negative control being a M85 strain that was frozen and thawed 3x in liquid nitrogen to inactivate the fusion inducing ability of the bacterial LPS. A similar treatment with LPS extracts of *H. paragallinarum* shown in previous work by Boshoff, (1992), abolished fusion inducing ability of the extract as well as the recognition of the VF3 antigen on immunoblot.

A genetically transformed strain was tested for its fusion inducing ability. The M85(T) line is a DNA transformed M85 strain. The genetic material used in the transformation was derived from a NAD independent strain (Bragg, *et al.*, 1993b) that was itself transformed with DNA from a NAD independent isolate (see Fig. 3.4). The aim with testing this NAD independent derivative of M85 was to simplify the production of fusion inducing bacteria. since the addition of exogenous NAD is expensive (commercial NAD) while chicken serum

complicates the growth medium. Should the transformed line prove to induce fusion at the same efficiency as the original strain it will simplify and economize production of spontaneous fusion inducing bacteria.

The VF3 expression of the strains used in this study as determined by ELISA are shown in Fig. 3.4. The percentage hybridoma formation (21 days after fusion) and relative VF3 expression are shown for each mouse (Fig. 3.5). The freeze thaw treatment reduced the VF3 expression from 2.5 x background (original M85 strain) to 1.75 x background, compared with 2.75 x background of the transformed strain.

Of the plasmid transformed group two mice had closely related *in vitro* hybridoma formation of >95% and the other one 40%, providing evidence that fusion inducing bacteria can be cultured without the addition of exogenous NAD. This supported the hypothesis that genetically transformed lines will also be able to induce spontaneous fusion. In addition the results show that VF3 expression correlates to induction of spontaneous fusion under the correct growth conditions in respect of pH.

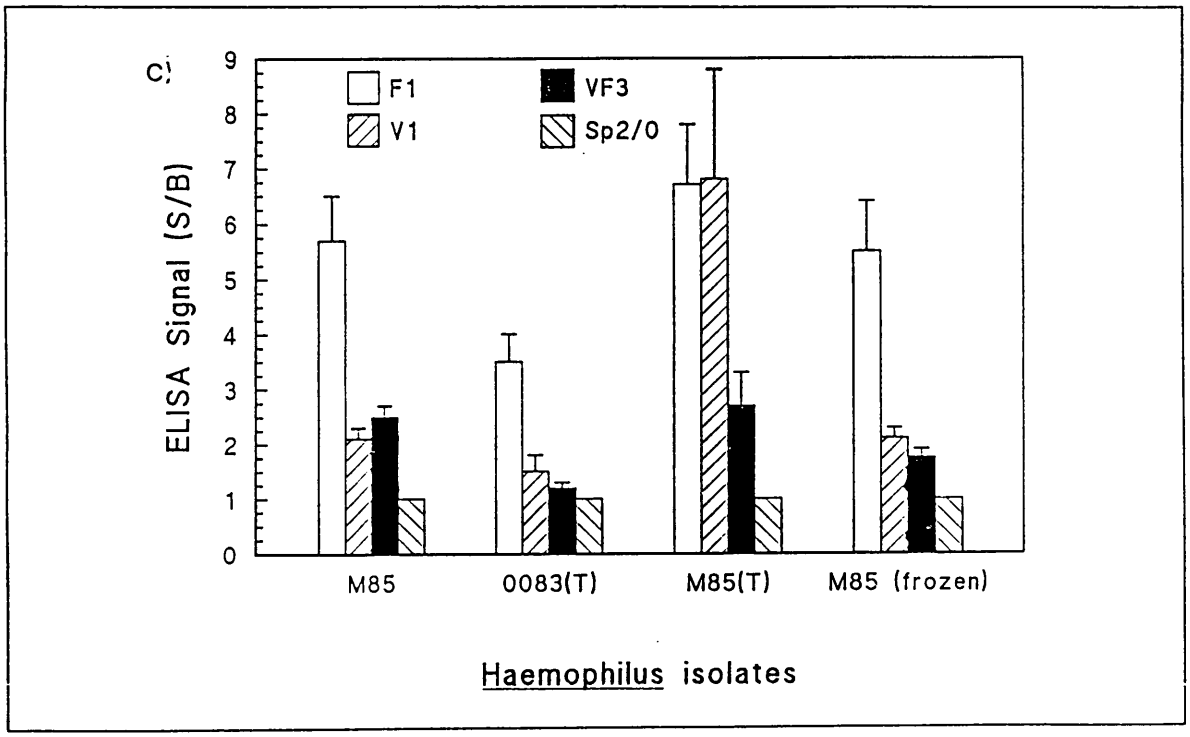


Fig. 3.4: ELISA profiles of batches of the M85 bacteria that are treated to show the effect freeze-thawing and genetic transformation have on the VF3 antigen expression. The graph shows the average and standard deviation of four assays. The M85 line is the strain generally used for treatment to induce fusion. The 0083 line was transformed to become NAD independent (0083(T)) and this line in turn was used as a source of genetic material for the

transformation of the M85 line to a NAD independent line (M85(T)). The last profile is of a sample of the original M85 line that was freeze-thawed to abolish fusion inducing ability.

The *in vitro* fusion varied considerably in the M85(T) group, but on average gave higher than 75% hybridoma formation, compared to the 54% of the original M85, 48% for the freeze-thawed group and 20% for the PBS control . After normilization (subtracting background signal), the fusion inducing ability of the transformed group was on average (55-34)/34 x 100 =62% higher than the original M85 group which in turn was (34-28)/28 x 100 = 21% higher than the frozen M85 group. The drop in spontaneous fusion inducing ability of the frozen group in comparison to the original M85 could be expected with a 30% higher VF3 expression. These results again illustrate that VF3 expression correlates to induction of spontaneous fusion

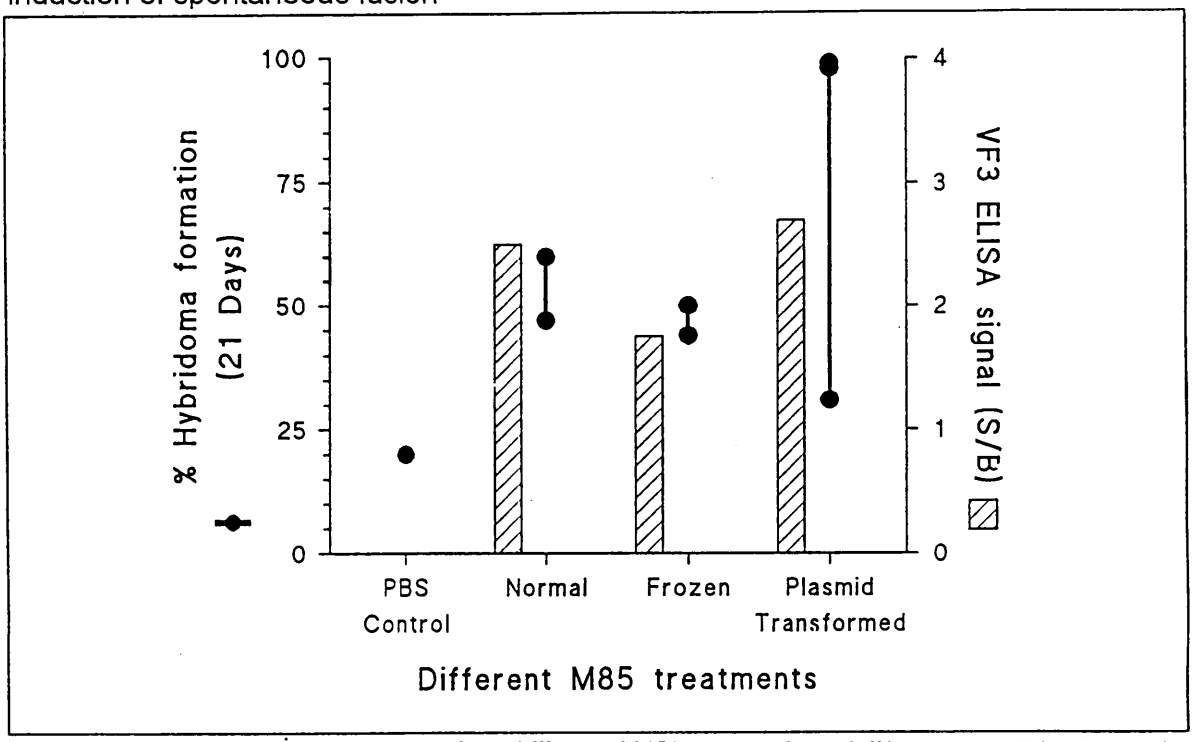


Fig. 3.5 : The *in vitro* hybridoma forming ability and VF3 expression of different modifications of the M85 isolate showing from left to right, fusion of myelomas with splenocytes from mice treated with: PBS (control), M85 bacteria, frozen and thawed M85 and transformed (NAD independent) M85 (T).

3.3.3 The use of *Haemophilus* bacteria in adjuvant to induce hybridoma formation for monoclonal antibody production.

Previously Verschoor *et al.*, (1990) used *H. paragallinarum* in Freund's incomplete adjuvant (FIA) to produce monoclonal antibodies against HEL, demonstrating that it can successfully replace *Mycobacterium* as an adjuvant, with the added advantage of inducing *in vitro*

fusion. HEL is widely used as a model antigen in various experiments, but further tests on the use of *H. paragallinarum* with different antigens could broaden the basis for evaluation of this approuch towards making monoclonal antibodies.

An unrelated project concerned with producing monoclonal antibodies to planktonic crustaceans, provided an excellent opportunity. Mice were immunized with a cocktail of different proteins, partially purified from the selected crustaceans, mixed with a given concentration of fusion inducing *Haemophilus* bacteria in an emulsion with FIA as described in 3.2.3. Four days after the final booster mice were sacrificed and splenocytes prepared for fusions.

The high variation in spontaneous fusion observed using single batches of *H. paragallinarum* required an experimental strategy to ensure antibody secreting hybridomas were obtained in the event of either low or high fusion inducing ability. PEG fusion was done using splenocytes of mice immunized with both the full and half dose of *H. paragallinarum* as adjuvant component, to provide for the event should the bacteria not be able to induce spontaneous fusion. Reducing the bacterial dose by half aimed at preventing death of hybridoma cells by over-fusion resulting from both bacterial and PEG fusion. These variations of PEG fusion were used as controls for spontaneous fusion of splenocytes derived from mice immunized with the full bacterial dose.

For these three different fusion experiments, the percentage of the wells containing growing hybridoma colonies were monitored for each antigenic group on a weekly basis. The results are depicted in Fig. 3.6.

The fusion profiles obtained from this experiment clearly shows that the addition of fusion inducing *Haemophilus* bacteria to the immunization cocktail facilitated good spontaneous fusion, but to such an extent that the addition of an additional fusing agent (PEG) caused over-fusion resulting in unstable hybrid cells in the group immunized with a full bacterial dose. Mice treated with half the *H. paragallinarum* concentration provided splenocytes with better hybridoma formation using PEG, but still compared weak to the spontaneous hybridoma forming ability unaided by PEG.

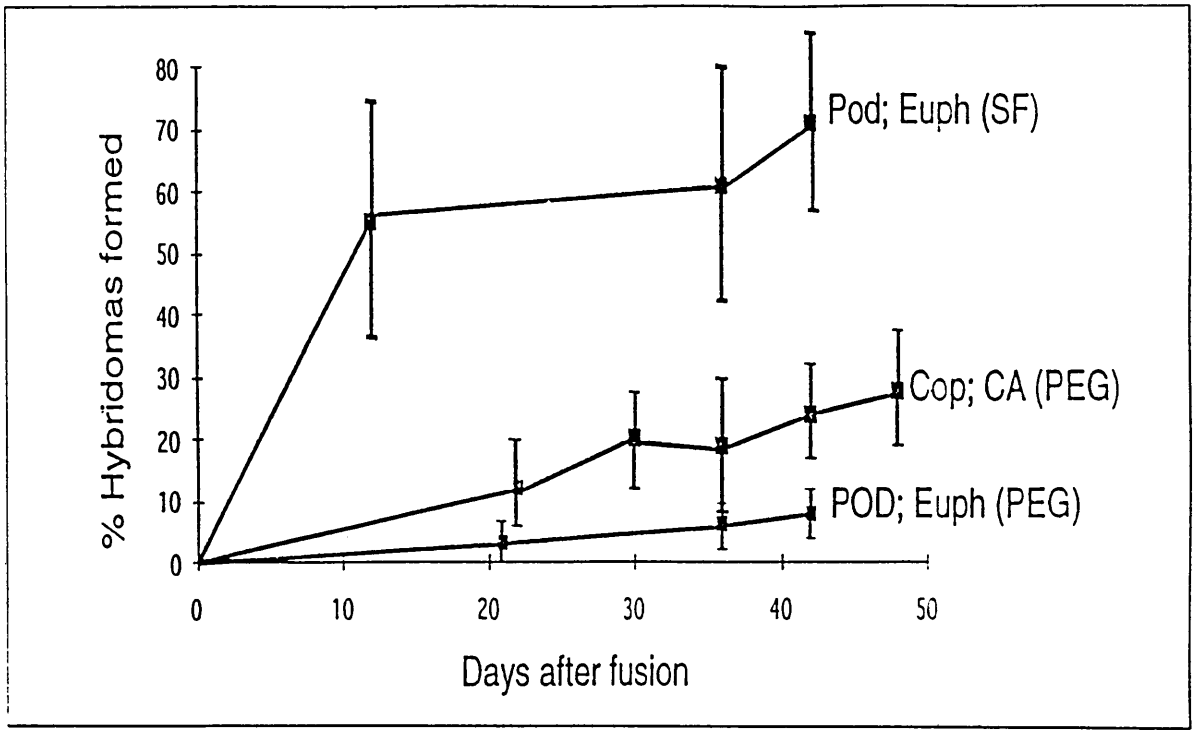


Fig. 3.6: Hybridoma formation of splenocytes derived from mice immunized with adjuvant containing fusion inducing *Haemophilus* bacteria. Hybridomas were obtained using either the spontaneous fusion procedure (one half of *Podon* and Euphausiid immunized splenocytes) or the PEG procedure (rest of *Podon* and Euphausiid splenocytes). Copepod and *Calanus Agulhensis* immunized spleens werefrom mice immunized with adjuvant and half a dose of *Haemophilus* bacteria and were only fused using the PEG procedure.

Using spontaneous fusion hybridoma technology, stable hybridomas were cloned for all antigen types. After cloning and subcloning 5 *Podon*, 4 Euphausiid, 3 copepod and 2 *Calanus agulhensis* hybridoma clones produced antibodies that showed good specificity for their respective antigens. On average 10.3% of all stable hybridomas produced antibodies specific towards their respective antigenic groups. This result compares favourably with the 12% frequency of specific antibody secretors obtained with the HEL antigen (Verschoor, *et al.*, 1990).

The experiments supported the dose dependent effect of fusion induction of VF3 expressing bacteria. It further confirmed the previous observation with lysozyme as antigen, that spontaneous fusion is not affected by the kind of antigen administered, because the different antigens showed similar hybridoma forming ability when treated with the same dose of *Haemophilus* containing adjuvant.

3.4 Discussion:

Attempts to reproducibly culture bacteria with consistent VF3 expression and fusion inducing potential was the main objective of this study. Verschoor *et al.* (1990) showed that the SB86 strain was a much better fusion inducing strain than strain 0222. SB86 became the first *H. paragallinarum* strain that was used to intentionally effect spontaneous hybridoma formation *in vitro,* i.e., without PEG as a fusion agent.

Boshoff *et al.* (1992) discovered an apparent correlation between the expression of the antigen recognized by the VF3 monoclonal antibody in ELISA assays and spontaneous fusion. They identified M85 as another fusion inducing bacterial isolate of *H. paragallinarum*. The immunization procedure was optimized in their study with the best fusion results obtained 3-4 days after a final booster treatment. The antigenicity of the VF3 antigen in respect to binding of its monoclonal antibody was shown to be affected by periodate, acid and alkaline treatment and correlated with fusion inducing potential, except for acid treated bacteria, which could still induce fusion in spite of a drop in ELISA signal. This was ascribed to the dissociation (without destruction) of the fusion agent from the bacteria by acid treatment, preventing its detection on washed ELISA plates, but still inducing the fusogenicity upon immunization of mice with the whole, treated mixture. LPS extracts from fusion inducing bacteria were shown to retain the ability to induce fusion and were still recognized by the VF3 monoclonal antibody. Both the VF3 antigen and the fusion inducing ability were freeze-thaw sensitive in the LPS extract. In Chapter 2 of this thesis this observation is corroborated by showing a chemical degradation of the VF3 antigen on SDS-PAGE Western blots after freeze-thaw treatment (see Fig. 2.1, Chapter 2).

Although evidence for the VF3 antigen being the fusion inducing entity appeared convincing, some VF3 expressing bacteria failed to induce spontaneous fusion in mice. (Boshoff, Gunter, Verschoor, and Van Wijngaardt, - unpublished). To try to explain this it was necessary to determine the effect of growth conditions and maturity of bacterial production on fusion inducing ability. The growth conditions were investigated by Bragg to establish the effect it could have on antigen expression. His concern was mainly with the differences in antigenic ELISA patterns used for serotyping the bacterial isolates.

In a comprehensive study to determine the effectiveness of a monoclonal antibody panel in typing *H. paragallinarum* and the effect of growth conditions on the antigen profiles and classification of isolates (Bragg, Ph.D.-study, 1996), Bragg commented on several changes in antigenic profiles that could be attributed to growth conditions. Some antigenic profiles

changed so dramatically that isolates could be classified in totally different monoclonal antibody pattern groups. NaCl content, initial pH of medium and growth stage of the bacteria were all found to have an effect on antigen expression. Different isolates reacted differently to the changes in growth conditions. With respect to initial pH of the growth medium, Bragg reported that strains H-18, Spross, M85 and A745/91 all showed changes in antigenic profiles as detected by monoclonal antibodies in ELISA. The effects also seemed to be highly strain or isolate specific and showed no fixed pattern that could be used to predict the effects pH had on the individual antigens. In this study the M85 line showed enhanced expression of VF3 with a rise in pH from 7.6 to 8.6, whereas V1 and VF1's antigens showed no significant change (Fig. 3.1).

Since the purpose of this study was to determine conditions for optimal bacterial growth, resulting in stable VF3 expression and spontaneous fusion induction, a set of hypotheses were tested.

The first hypothesis stated that the pH of the growth medium has no effect on VF3 expression. To test this, batches of M85 bacteria were cultured in media with five differing initial pHs. ELISAs that have been done on these bacteria (Fig. 3.1) show a distinctive rise in VF3 expression with the rise in initial pH of growth medium from 7.6 - 8.6. With this evidence the hypothesis can be rejected to favour one stating that a more basic initial pH increases VF3 expression.

To test the combined effect of medium supplements and pH, two representative media pH's were tested with or without serum substitution with NAD, to establish their influence on fusion inducing potential of the M85 strain. The batches used were selected to have similar VF3 expression (Fig. 3.2). From figure 3.3 it is clear that spontaneous fusion inducing ability is higher in batches grown at pH = 7.4 compared to batches at pH = 8.5. This proves the third hypothesis wrong and favours a hypothesis stating that the fusion inducing entity, residing on a molecule also containing the VF3 antigen, is pH sensitive. It is therefore clear that although an increase in pH will enhance VF3 expression it has no benefit for fusion inducing potential.

It can be speculated that the monoclonal antibody still recognizes the antigenic part of the molecule responsible for fusion induction after growth at high pH, although the active conformation is lost due to increased pH. The concern regarding the requirement for serum in the bacterial growth medium is also proven unfounded, since bacteria cultured in

media supplemented with commercial NAD showed higher fusion inducing potential than ones cultured in serum (at the optimal pH = 7.4).

Since 1990 various laboratories in South Africa isolated NAD independent, *H. paragallinarum* like bacteria, from chickens showing typical infectious coryza symptoms. These isolates could later be identified as NAD independent *H. paragallinarum* isolates on the basis of their carbohydrate fermentation patterns and DNA/DNA hybridization results (Mouahid, *et al.,* 1992). Bragg *et al.,* (1993) could show that these isolates also reacted with the panel of monoclonal antibodies specific for *H. paragallinarum*. Bragg could also show that the NAD independence of some *H. paragallinarum* isolates could be transferred to a NAD dependent line by transformation with a crude DNA extract from a NAD independent isolate.

To simplify the growth requirements of the M85 strain as a reproducible high fusion inducing line, the fusion inducing potential of a NAD independent isolate, transformed as mentioned in the previous paragraph, was evaluated. The M85 strain, transformed with DNA from a NAD independent line 0083(T) that was itself transformed from a NAD dependent isolate (Fig. 3.4), was obtained from the laboratory of R. Bragg. DNA transformation altered the antigenic profile in respect of V1 expression but the resulting bacteria retained expression of VF3 (Fig. 3.4). This had no effect on the fusion inducing ability of the line and high percentages of hybridomas were induced by it (Fig. 3.5). This evidence falsifies the hypothesis that DNA transformation would change the antigenic profile of the bacteria in such a way that the VF3 expression and subsequent fusion inducing potential would be destroyed or inactivated.

Freeze-thaw treatment of whole M85 bacteria does not seem to reduce the VF3 expression drastically. Mice treated with these bacteria showed a marginal reduction in ability to induce spontaneous fusion that correlated with the low reduction of VF3 expression as indicated in ELISA results (Fig. 3.5). This indicated a VF3 stabilizing effect of the intact bacterial cell wall, corroborating the results of Boshoff, *et al.*, (1992), that LPS extracts freeze-thawed in PBS showed higher lability towards fusion induction than whole bacteria treated similarly.

These results, combined with the sustained correlation observed between VF3 expression and fusion induction seen in the DNA transformed bacteria (Fig. 3.5), supports the hypothesis that the VF3 epitope is residing on a molecule that induces spontaneous fusion but that some treatments at least (increased pH) affect the fusion inducing conformation of the antigen without having an effect on the antigenicity of the VF3 epitope.

The wider applicability of inducing *in vitro* fusion by *Haemophilus* treatment of immunized animals, for monoclonal antibody production against antigens other than HEL was illustrated by combining *H. paragallinarum* bacteria with the complex antigens from planktonic crustaceans and Freund's incomplete adjuvant for immunisation of mice. In the fusion experiments the splenocytes were divided into two groups in which one group was fused using the experimental spontaneous fusion protocol while the rest were used in a normal PEG-fusion. An additional group of animals only received half of the *H. paragallinarum* dose at the final booster and were used in a spontaneous fusion protocol. The percentage hybridomas formed for each animal were monitored on a weekly basis after fusion (Venter, *et al.,* 1995).

The highest hybridoma formation was obtained from the splenocytes that were fused using the spontaneous fusion protocol. Two different antigen mixtures used in this way gave comparable hybridoma formation. The PEG fused groups had a very low survival of hybridomas after three weeks showing a similar over fused pattern as found when high fusion inducing *H. paragallinarum* bacteria were immunized alone in previous experiments (Verschoor *et al.*, 1990). The two groups of animals that were fused by PEG fusion after immunization with half a dose of bacteria showed an intermediate level of hybridoma formation illustrating a dose dependent response.

Stable, antigen specific, monoclonal antibody secreting hybridomas were cloned from all spontaneously fused groups. The percentage antigen specific hybridomas was also comparable to normal PEG fusion experiments. This data rejects the hypothesis that different antigens would affect the spontaneous fusion differently. Thus, *H. paragallinarum* appears to be able to induce spontaneous hybridoma formation resulting in specific monoclonal antibodies towards different antigens generated in a reproducible way. This illustrates the use of VF3 expressing *H. paragallinarum* bacteria as adjuvant that stimulates an immune response towards the relevant antigen as well as inducing fusion of such antigen specific B-lymphocytes.

In conclusion, it was found that VF3 expressing *H.paragallinarum* bacteria cultured in a medium with an initial pH of 7.4 and containing commercial NAD as supplement to substitute serum can induce a high percentage *in vitro* spontaneous hybridoma formation. Alternatively NAD independent lines of the M85 strain can also produce comparable fusion with the added advantage of a less complex medium composition. Both these types of *H.paragallinarum* bacteria also proved their use as versatile adjuvants capable of

stimulating an antigen specific immune response while facilitating hybridoma formation without the use of PEG. These results illustrate the applicability of *H. paragallinarum* induced fusion as a novel aid in hybridoma technology.

Chapter 4: Spontaneous *In vivo* hybridoma formation and its effect on tumour progression.

4.1 Introduction:

Spontaneous hybridoma formation as described in the previous chapter involves the fusion of *H. paragallinarum* treated murine splenocytes with myeloma cells *in vitro*. The adaptation of this system to the *in vivo* environment to test the effect that spontaneous hybridoma formation could have on *in vivo* tumour cell progression forms the essence of this chapter.

To achieve this, a strategy was designed which can be divided into the following three parts: a) The establishment of an animal model that could indicate the effects of *H. paragallinarum* treatment on tumour progression by evaluation of parameters like tumour growth rate, tumour volumes and metastatic activity. b) The characterization of the *in vivo* effects, so that predictions can be made on the possible application of a *H. paragallinarum* based cancer treatment. c) The design of an experiment that could provide direct evidence that the effects observed are in fact due to elicitation of *in vivo* fusion in the experimentally treated animals as compared to adequate controls.

Previous work by Boshoff, (1992) demonstrated reduced tumour growth in mice pretreated with *H. paragallinarum* bacteria before inoculation with B16-F10 melanoma cells. In these experiments he also evaluated metastatic colonization of the B16 cells to the lungs of infected mice, but could show no significant trends in the number or size of metastatic tumours, due to high variation of the results. PBS treated mice served as controls for all experiments reported in his M.Sc. thesis. The presence of bacterial LPS in the samples used in the experimental treatments, but not in the control samples, complicated the interpretation of the results. This was because tumour growth suppression, through the activation of lymphocytes and macrophages, is a well-documented biological activity of bacterial LPS (Goodman, 1979 & Haeffner-Cavaillon, 1982).

The experiments of Boshoff and coworkers were initially hampered by unreliable expression of the VF3 antigen on the bacteria used, a property that appeared to correlate with the induction of spontaneous fusion (Boshoff, *et al.* 1992). The results of the cancer experiments reported in his M.Sc. thesis were all obtained from a single well-characterized batch of *H. paragallinarum* expressing VF3, but problems with variable tumour take and

high standard deviations of tumour size were still encountered. This rendered his findings preliminary evidence and not yet publishable (Boshoff, M.Sc. thesis, 1992).

The B16-F10 melanoma cell line, used for all tumour progression studies up to date, was derived from a less metastatic B16-F1 line but was selected for its metastatic colonization to the lungs by repeated passaging in mice (Fidler, *et al.*, 1973). The resulting cell line had a similar low MHC class I antigen expression as well as low immunogenicity like the original F1 line (Fidler, *et al.*, 1976; Hanna, & Fidler, 1981). Differences between the lines that seemed to correlate to their different metastatic potential were: the F10 line's higher collagenase type IV activity (Garbisa, *et al.*, 1980), higher fibrinolytic activity (Wang, *et al.*, 1980), higher capacity to penetrate the chorioallantoic membrane (Fidler, 1978) and higher adhesiveness to lung cells (Nicolson, & Winkelhake, 1975). This line was selected for its lung metastatic ability and the ease of distinction between its tumours and other tissue material.

Spontaneous fusion between normal B-cells and tumour cells *in vivo* could effect the transfer of normal genes to the latter, similar to gene transfer technologies such as, somatic cell hybridization (Ringertz *et al.,* 1976; Larizza, & Schirrmacher, 1984), micro cell mediated chromosome transfer (Fournier, 1982; McNeil, *et al.,* 1980) and chromosome and DNA transfection (Klobutcher & Ruddle, *et al.,* 1979).

Several mechanisms may be considered for suppression of tumour growth by fusion of neoplastic cells with normal cells:

Porgador, *et al.* (1989) reported that transfection of a high-metastatic tumour cell line with H-2K^b genes resulted in the conversion of this line to a non-metastatic tumour line with increased immunogenic competence, indicating that genetic transfer can switch a cell from one cancerous stage to a less cancerous one. A similar conversion to a less metastatic and more immunogenic B16 line could be accomplished by fusion with B-lymphocytes with normal H-2K^b expression.

Alternatively the fusion with B-cells could reintroduce or restore mutated or non-functional suppressor genes, like p53, in the hybrid cell that could change the genotype of the hybrid resulting in a less neoplastic cell.

Weinberg, *et al.* , (1994) studied the effect of an increase in the gene copy number of the p53 tumour suppressor gene in keratinocytes expressing a specific oncogene. After

transforming cells with the p53 gene they inoculated mice with cells of different p53 copy number and documented mean tumour volumes in mice, at four weeks after grafting, of 2400 mm^3 (p53 -/-), 1800 mm^3 (p53 +/-) and 200 mm^3 (p53 +/+), demonstrating an inversely proportional relation between wild-type p53 gene copy number and tumour growth tempo.

The hypothesis that *H. paragallinarum* induced *in vivo* spontaneous fusion between B-lymphocytes and cancer cells, and that this causes a reduced tumour growth rate and/or metastatic colonization, could be explained by a similar suppressor gene reconstitution model. The increase of wild-type p53 (or similar tumour suppressor) gene copy number by *in vivo* fusion of a normal (p53 +\+) B-lymphocyte with a malignant B16 melanoma or Sp2/0 lymphoma cell (possibly p53 -/- or p53 +/-), might provide a likely mechanism for the reduced tumour growth in mice pretreated with spontaneous fusion inducing (*in vitro) H. paragallinarum* (Boshoff, M.Sc.- thesis, 1992).

This study builds on the previous work but tries to give more precise answers by using a non-fusion inducing (low VF3 expression) 0222 *H. paragallinarum* line as a control. This could show that lipopolysaccharides, present in both mixtures, are not the cause of the reduced growth rate.

Boshoff used chromosome counts to confirm the formation of *in vitro* hybridomas (M.Sc.-thesis, 1992). Here a flow cytometric analysis of DNA content is used, because it provides rapid and statistically more significant data (Rampoldi, *et al.,* 1989).

To confirm that *in vivo* hybridomas originated from fusion with normal B-cells, the IgG expression on the membrane structure is measured by flow cytometry, making use of a FITC conjugated goat-anti-mouse-IgG monoclonal antibody. When cells fuse, their phenotype is typically a mixture of the ancestral phenotypes and would result in the expression of IgG on the surface of the hybridoma cells, if one of the parent cells was a B-cell. If both ancestors of the hybridoma were melanoma cells no IgG expression would be detectable.

A final experiment attempts to show differences in the hybridoma to tumor cell ratio in mice treated with bacteria of different *in vitro* fusion inducing ability. In this experiment, a higher hybridoma to tumour cell ratio would be indicative of better *in vivo* fusion. This enabled direct comparison between *in vitro* and *in vivo* fusion inducing potential in the same animal, in an attempt to show a correlation.

In order to answer these questions logically, the following null hypotheses are tested:

1. The C57Bl/6 mouse /B16 melanoma and Balb/c mouse /SP2/0 myeloma models are not suitable to demonstrate the effect of *in vivo* spontaneous fusion induced by *H. paragallinarum.*

2. Treatment of mice with fusion inducing *H. paragallinarum* bacteria have no specific suppressive effect on tumour progression over and above the non-specific effect induced by LPS in general

3. The ability of *H. paragallinarum* bacteria to induce *in vitro* spontaneous hybridoma formation has no correlation with *in vivo* spontaneous hybridoma forming ability, and thus provides no explaination for reduced tumour growth rate in mice treated with *in vitro* fusion inducing bacteria.

Proving these hypotheses wrong could provide evidence to assume the opposite.

4.2 Materials and methods:

4.2.1 Bacterial strains:

H.paragallinarum was cultured from stocks maintained at the Department Poultry Diseases, Veterinary Faculty, University of Pretoria. The M85 line is a good expressor of both the F1 and VF3 antigens, of which the latter is apparently associated with induction of spontaneous fusion, while the 0222 line expresses hardly any antigens recognized by the mAbs and does not induce spontaneous fusion to any significant extent. A NAD independent M85 strain (M85T) was obtained from Dr. R.R. Bragg, Department Poultry Diseases, Veterinary Faculty, University of Pretoria, who developed this strain by transformation of a NAD dependent M85 strain with DNA extracted from NAD independent strains (see Chapter 3 and Bragg *et al.*, 1993)

Bacteria were cultured in modified Casman's medium in glass containers. The pH of all media was adjusted to 7.5 and nicotinamide was used as additive, except for the M85 T line where it was omitted. Growth took place at 37°C with shaking for 18 hours (Coetzee, *et al.,* 1983).

Samples of the cultures were tested for purity by microscopic evaluation of gram-negative stained smears and checking colony morphology (Bragg Ph.D., 1996). Harvested cultures were inactivated with 0,1% formalin and incubated at 10°C for 24 hours before centrifugation. Afterwards cells were washed 3 times in PBS, (pH = 7.2) by centrifugation. Harvested cells could be stored at 4°C for up to 1 year (Boshoff, *et al.,* 1992).

4.2.2 B-16- and Sp2/0 tumour cell cultures

B16 melanoma cells (Fidler, *et al.,* 1973) and Sp2/0 cells (developed by Shulman, *et al.,* 1978 and obtained from Onderstepoort Veterinary Research Institute, SA) were cultured at 37 °C in a humidified incubator at 5% CO_2/air ventilation. The culture medium consisted of DMEM supplemented with $NaHCO_3$ (3.7 g/L), sodium pyruvate (1 mM), penicillin streptomycin mixture (10 IU/ml and 10 μg/ml, respectively) and 10% v/v fetal calf serum. All growth media, cell lines, supplements and serum were purchased from Highveld Biological (Pty.) Ltd., Kelvin, South Africa, while all sterile plastic ware was obtained from Cell-Cult, Sterilin, Middlesex, U.K..

B16 cells were harvested by overlaying with a thin layer of 0.25% trypsin for 2 min. at 37°C and reconstituted regularly into new containers to ensure growth in a single cell layer and avoid clump-formation by overcrowding. Sp2/0 cells were also harvested regularly to promote rapid growth and minimize the non-viable floating cell concentration.

Cells were harvested by means of trituration with a Pasteur pipette followed by washing in cold DMEM, centrifugation at 100 x g for 5 minutes and resuspension in cold DMEM. The viable cells were counted with a haemocytometer (Neubauer, improved-counting chamber) after trypan blue treatment.

Harvested cells were used in inoculations of *H. paragallinarum* treated mice in tumour progression experiments. The Sp2/0 line was further used in *in vitro* spontaneous hybridoma formation experiments.

4.2.3 Induction of spontaneous fusion in mice.

Two mouse strains (Balb/c and C57Bl x CBA), obtained from H. A. Grové Research Centre, Pretoria, were used in different experiments. Analyses of the progression of B16 tumours were performed using a F1 cross between C57Bl and CBA mice between the ages of 6 and 12 weeks. Balb/c mice of similar age groups were grafted with Sp2/0 tumour cells. In both cases the *Haemophilus* treatment regime was similar.

Mice were treated with different strains of formalin inactivated *H. paragallinarum* bacteria as described in the results section. The strains included: NAD dependent M85, 0222 as control line, and the NAD independent (genetically transformed) M85 T. An additional control in some experiments was the M85 freeze treated bacteria, that was frozen in liquid nitrogen and thawed tree times prior to use in treatments.

All mice received $5x10^6$ formalin inactivated bacteria in 500µl PBS i.p. as pretreatment. The first treatment (day 0) was followed by a second (day 14) and third (day 42). Tumour grafting or spontaneous fusion experiments took place three days after the final treatment. In cases where tumour grafting was followed by examination of spontaneous fusion ability of splenocytes an additional treatment was given three days prior to the fusion experiment.

4.2.4 Tumour inoculation, monitoring and dissection.

B16 or Sp2/0 cells cultured as described in 4.2.2 were prepared by harvesting and washing (2x) in cold DMEM without any supplements. Cell suspensions were diluted to 10^7 cells/ml and kept on ice. Each mouse was inoculated with 20 µl (standard volume) cell suspension (thoroughly mixed prior to every grafting) three days after the final *Haemophilus* treatment as described (4.2.3). In the B-16 tumour progression evaluation experiments the mice were treated with one standard volume of the tumour cell suspension in the right hind footpad. After inoculation mice were kept under close observation till the first sign of actively growing tumours appeared. Hereafter tumours were measured with a veneer caliper, every second day to monitor growth rate.

The right hind feet containing the B16 tumours were amputated 20 days after tumour grafting. The feet were carefully dissected to obtain all dark tumour tissue suspended in saline. Tumour cells were centrifuged for 10 minutes at 1000 x g to obtain a pellet in the bottom of a conical tube that was marked to estimate the actual volume of the tumour in cm^3. A sample of cells was taken and cultured in medium as described above, but with the substitution of fetal calf serum with the same amount of horse serum (Highveld Biological (Pty.) Ltd., Kelvin, SA). These cells were later used for FACS analysis.

The mice used in the Sp2/0 experiment were treated with one volume of cultured myelomas in the hind foot pad and another subcutaneously on the left lateral side, above the spleen. This enabled aseptic removal of the tumour and spleen concurrently. The sizes of the subcutaneous tumours of the mice were monitored by a veneer caliper until they were 10 mm in diameter. Each mouse was then treated with the same *Haemophilus* sample as for pretreatment, to prime it for an *in vitro* fusion experiment (see later methods). Three days after the final treatment the mouse was sacrificed and the spherical tumour was carefully removed under sterile conditions. After the tumour's mass was determined it was assessed for its hybridoma content using the HAT selection test (see 4.2.7).

4.2.5 Flow Cytometry.

4.2.5.1 DNA content measurement.

COULTER DNA-prep kit protocol and reagents were used in determining the DNA content of freshly cultured tumour cells (COULTER DNA-prep kit manual, COULTER Diagnostics). The method involves dilution of cell samples, to a final concentration of 3-10 x 10^4cells/ml, in PBS or COULTER sheath fluid. Samples of 100 µl were automatically mixed to the correct amount of COULTER DNA-prep Lysing and permeabilizing reagent (LPR) (containing 0.1% KCN, 0.1% NaN_3, nonionic detergents, saline and stabilizers). This

reagent facilitates red blood cell lysis and membrane permeabilization. Following this step the COULTER DNA-prep stain (containing 0.5% propidium iodide, bovine pancreas RNAse (4 000 U/ml), 0.1% NaN_3, saline and stabilizers) was added to the sample, thoroughly mixed and left to incubate for 30 min. The samples were calibrated with COULTER DNA-prep reference, which contains chicken erythrocytes. The anti-F1 and anti-Coxsackie monoclonal antibody secreting hybridomas and B16 melanoma cells, which were not passaged in mice, were used as positive controls for fused and non-fused cells respectively.

4.2.5.2 Determination of surface IgG content

Tumour cells were obtained by cutting the tumour tissue with a pair of scissors and forceps while covered with cold PBS. The resulting suspension was then forced trough a nylon sieve (75 μm grid) to obtain single cells. A sample of the suspension was taken for counting under the haemocytometer using trypan blue exclusion as an indication of viability. Cells were then centrifuged for 5 minutes at 600 x g followed by resuspension in 200μl of fresh PBS containing 0.5% BSA and 0.02M sodium azide. Three wash steps followed, comprising centrifugation for 10 sec. (11 000 x g) at 4°C and resuspension in 200μl BSA/PBS buffer.

After the third centrifugation 25μl of a 1% FITC-labeled Goat-anti-Mouse-IgG (FITC-GαM, Cappel) in PBS (containing 0.5% BSA and 0.02M azide) were added to each Eppendorf tube (Merck SA). The FITC-GαM mixture was preincubated in horse serum (2%) for 1 hour, on ice, to eliminate nonspecific binders. The sample-antibody mixture was left to incubate for 30 min. on ice (<4°C) in a dark cabinet, followed by dilution to 200μl with the PBS buffer, two wash cycles as described above and finally resuspended in 50μl PBS. The samples were kept on ice prior to FACS analysis.

Similarly treated positive controls were used to adjust the flowcytometer, including a hybridoma line secreting mAbs against Coxsackie virus, *in vitro* cultured B16 cells as well as tumour cells of PBS treated mice. An Epics Profile-II flowcytometer was used with Epics software. Ten thousand cells were counted per sample and graphs of count versus log fluorescence were generated as well as data on peak position of fluorescence and average fluorescence for each sample.

4.2.6 *In vitro* spontaneous fusion procedure.

Splenocytes were obtained from *Haemophilus* treated mice 3 days after their final booster and cocultured with Sp2/0 myeloma cells (Schulman, *et al.,* 1978) at a ratio of 5:1. Splenocytes and myeloma cells were prepared according to procedures of Galfrè and Milstein (1981), but PEG was omitted.

The spleen cell suspension was transferred to a 10 ml sterile centrifuge tube and washed three times with DMEM without serum. The suspension was filled up with medium to a total volume of 10 ml. A sample of the suspension was taken for cell counting with a haemacytometer.

Sp2/0 cells were harvested, washed and resuspended in DMEM medium (section 4.2.2). A sample of the suspension was taken for cell counting with a haemocytometer.

Sp2/0 cells and splenocytes were mixed and centrifuged at 100 x g for 5 minutes. The pellet was then resuspended in HAT medium (DMEM medium supplemented with hypoxanthine (100 µM), aminopterin (0.4 µM), thymidine (16 µM) and horse serum (10% v/v)) to a final concentration of 5 x 10^5 myeloma cells/ml. The suspension was finally distributed at 100 µL per well in 96 well microtitre plates.

The growth medium was replenished every third day. After one week aminopterin was excluded from the medium and a week later hypoxanthine and thymidine were omitted as well. A weekly census was taken of wells containing growing hybridoma cells. The percentage of wells containing hybridomas indicated efficiency of fusion induction.

4.2.7 *In vitro* HAT selection to determine *in vivo* fusion from dissected tumours.

Dissected tumours from Balb/C mice were fragmented with scissors and forceps and forced through a 75 µm nylon mesh, diluted in DMEM medium, washed and centrifuged as described for splenocytes (4.2.6). The tumour cells were then diluted to a concentration of 10^7 cells per ml in HAT medium with 10% horse serum (see 4.2.6). The suspension was then distributed at 100 µl per well in only the first column of the microtitre plate. The other wells were each filled with 50 µl of HAT medium. By taking 50 µl from the cell suspensions in the first column and transferring them to the next well, followed by mixing, a two fold dilution range covering tumour cell concentrations from 10^7 to 10^4 per well was constructed .

A titer of cell growth was determined for each tumour, defined as the highest dilution where >3/8 (i.e. 50% or more) wells contained hybridomas after three weeks of culturing. Because each column on the plate had a known tumour cell concentration at the time of preparation and because one surviving hybridoma cell per well is enough to give rise to a hybridoma colony, the titer could be used to calculate the number of hybridomas per tumour cells, thus indicating the frequency of *in vivo* fusion.

4.3 Results:

4.3.1 Size distribution of B16 melanoma tumours after *Haemophilus* pretreatment.

In the work of Boshoff and the previous chapters of this thesis experimental evidence points to a correlation between VF3 expression and spontaneous fusion inducing ability of *H. paragallinarum* bacteria. This correlation is assumed to apply in the experimental design that follows.

To provide evidence that *Haemophilus* lines with differing *in vitro* fusion inducing ability, would affect B16 melanoma growth rate differently, two bacterial samples, with VF3 expression as shown in Fig. 4.1, were used to pretreat mice. The M85 line is similar to other batches used to induce high *in vitro* hybridoma formation (see Chapter 3). The 0222 line is used as a LPS containing control that should be unable to induce *in vitro* hybridoma fusion above background level. The mice used in this experiment were a F1 cross between CBA and C57Bl to minimize the heterogenisety of haplotypes, as a measure of lowering the experimental result variation in the groups.

After a full treatment period one mouse of each group was sacrificed in an *in vitro* fusion experiment (4.2.6) to determine the fusion inducing ability of the bacteria. The percentage hybridomas found in the M85 and 0222 treated groups after two weeks of culturing, was 31% and 7% respectively and remained constant for the following weeks.

The remaining mice were then inoculated with B16-F10 melanoma cells in the hind foot pad three days after the final treatment as described in 4.2.3. The tumour growth was monitored and feet amputated 20 days after inoculation. Volumes of the individual tumours and average tumour volume for the two groups are shown in Fig. 4.2.

The experimental mice exhibited a significant ($p < 0.1$, with students T-test) 49% lower average tumour volume indicating an average suppression in tumour growth rate of 4.65 mm^3 per day in comparison to control mice. This result is further significant because both groups were treated with LPS containing *H. paragallinarum* bacteria indicating that the observed difference in tumour growth suppression is not an LPS effect but rather due to differences in the fusion inducing entity of the $VF3^+$ *H. paragallinarum* bacteria with which the mice were pretreated.

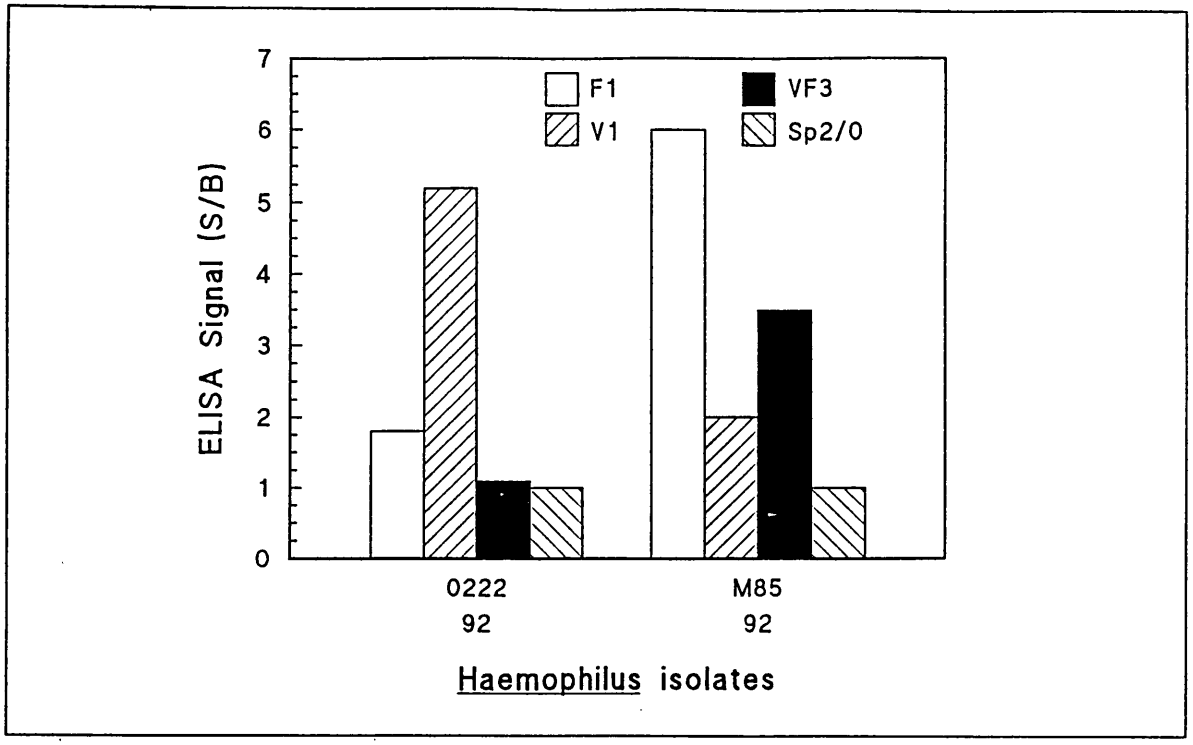


Fig. 4.1: ELISA profiles of the bacterial lines used to pretreat mice before B16 inoculation.

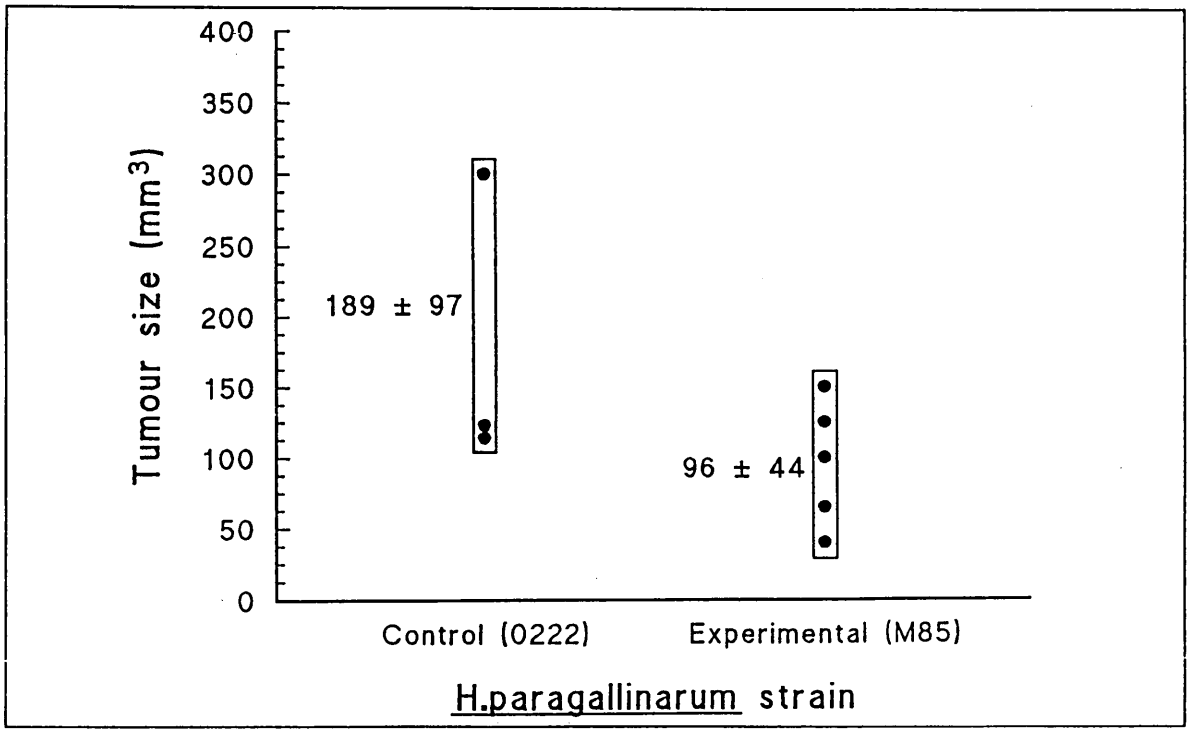
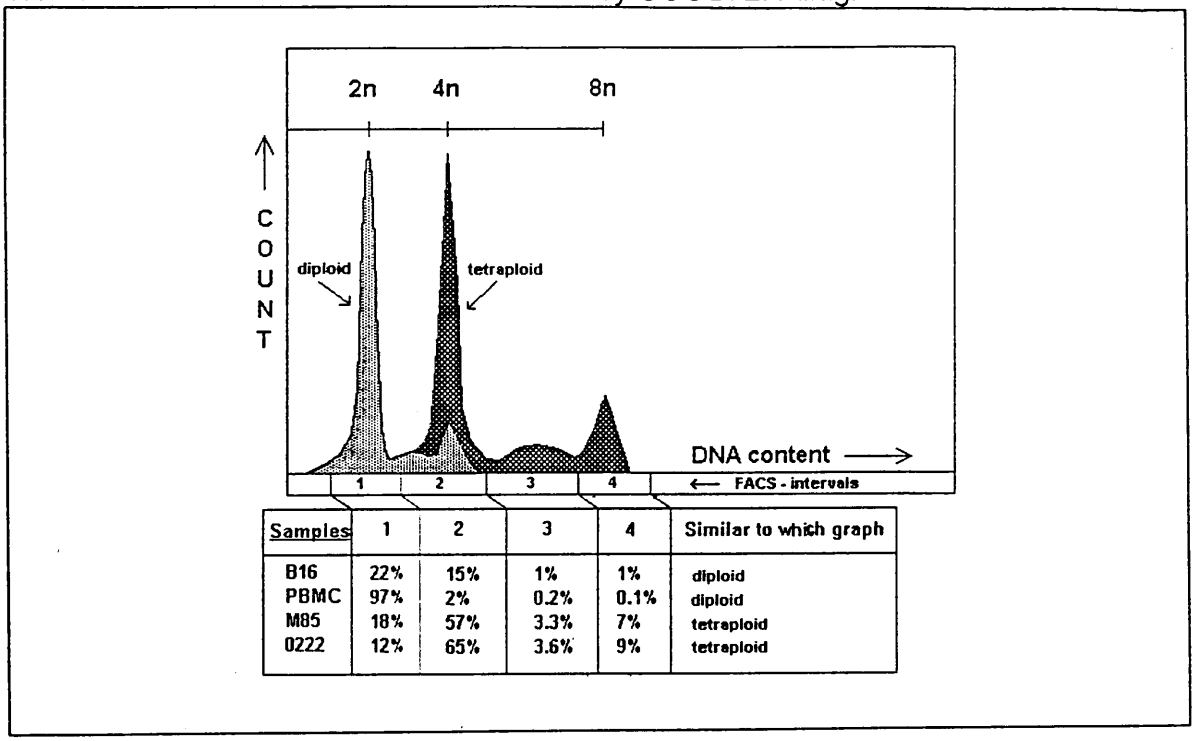


Fig. 4.2: Average B16 tumour volumes (20 days after grafting) of CBA x C57Bl mice, treated with two different *Haemophilus* strains.

To determine if *in vivo* fusion might be the reason for reduced tumour growth rate samples of the M85 treated and 0222 treated tumours were analyzed for their hybridoma content.

4.3.2 FACS analysis of hybridoma content of tumour cell samples.

Flow cytometric analyses of DNA content and IgG expression, were performed on the tumour cell samples. The assumption was that if two cells fused, the resulting hybridoma would have approximately double the G0/G1 DNA content (4n) of its ancestors. This information could be ascertained by DNA FACS analysis of dissected tumours by making use of the DNA intercalating agent propidium iodite (PI) provided in a ready to use kit for determination of DNA content of intact cells by COULTER diagnostics.



Samples	1	2	3	4	Similar to which graph
B16	22%	15%	1%	1%	diploid
PBMC	97%	2%	0.2%	0.1%	diploid
M85	18%	57%	3.3%	7%	tetraploid
0222	12%	65%	3.6%	9%	tetraploid

Fig. 4.3: Diagram of a representative FACS-DNA profile of diploid and tetraploid cells. The diagram shows the intervals set on the flow cytometer using the *in vitro* cultured B16 cells and peripheral blood monocytes (PBMC) of a mouse as controls. Tumour cell samples of animals treated with M85 and 0222 are compared to these profiles to determine their ploidy. Data of the percentage cells falling in the designated interval for each of the samples are shown.

In Fig. 4.3 an example is given of a DNA content profile of typical diploid and tetraploid cells. For diploid cells growing normally most cells are in the 2n (G0/G1) peak and all the rest either in the synthesis (S - phase) or 4n (G2 - phase) peak. Replicating tetraploid cells however, would have their G0/G1 peak at 4n and a G2 peak at 8n.

DNA analyses on tumour cell samples indicated replicating tetraploid cells for both M85 treated (exp.) and 0222 treated (contr.) groups (Fig. 4.3) as compared to the normal B16 and peripheral blood mononuclear cells (PBMC). No significant differences in the amount of cells showing a tetraploid DNA content were noticed between the groups. The observed

tetraploid DNA profiles on their own privide no proof of fusion between melanoma and B-cells, since the phenotypic origins of the parental cells forming the hybrid are not determined by this method.

Similar tumour cell samples from the same group of mice treated with the same batches of bacteria and showing similar DNA profiles were analyzed for the expression of surface IgGs (B-cell marker) on the tumour cells as an indication of hybridoma formation with B-cell fusion partners. As negative control a non-B-cell murine line (BW cells) and *in vitro* cultured B16 melanoma cells were analyzed, while an antibody secreting F1 hybridoma served as positive control (Fig. 4.4).

Fig. 4.4 shows enhanced IgG expression in both experimental and control tumour samples shortly after dissection. A significant rise in IgG expression could indicate a high rate of successful B-cell-melanoma fusion. It is, however, apparent that no significant differences in fluorescence are present between the tumour samples indicating a non-specific effect, unrelated to the fusogenicity of the bacteria.

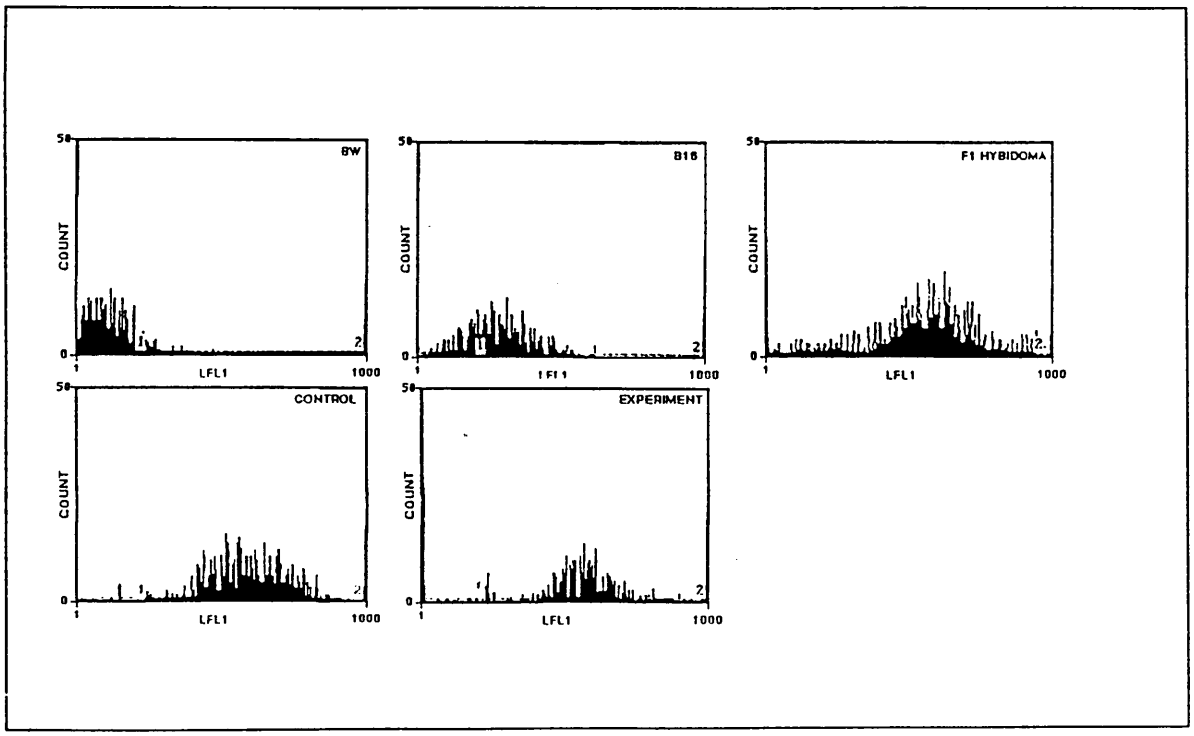


Fig. 4.4: FACS-analyses of M85 (experimental) and 0222 (control) pretreated tumour cell samples, determining B-cell marker expression using a FITC conjugated Goat α mouse-IgG antibody.

An experiment to determine the effect that the mouse serum has on the analysis of freshly isolated tumour cells, was performed to determine whether the signal could be due to non-specific adherent mouse IgGs on the tumour cells. B-cell marker analyses were performed

on control cells cultured in medium containing mouse serum versus Fetal calf serum (FCS) and the results are shown in Fig. 4.5.

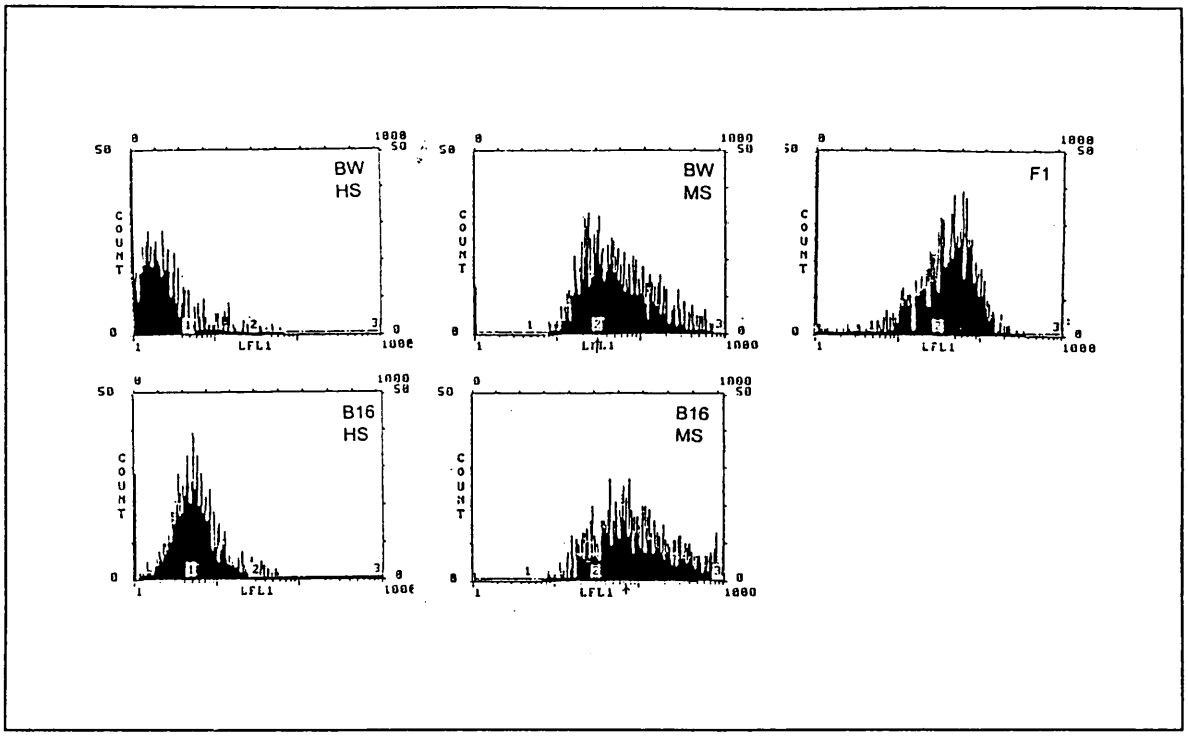


Fig. 4.5: The effect of nonspecific adhesion of mouse immunoglobulins, to the surface of tumour cells, on B-cell marker determination. The effect of recent dissection was simulated by culturing cells in mouse serum.

The average fluorescence (apparent IgG expression) was 15 x higher for both BW cells and B16 cells if cultured in media containing mouse serum versus FCS indicating a definite nonspecific IgG adherence. Culturing the two control cell lines in mouse serum for five days clearly indicates that the apparently enhanced expression of surface IgG in the tumour cell samples could be an artifact of the mouse serum. The experiment was subsequently repeated using M85 treated (Experimental) and PBS treated (control) mice, so that the difference in fusion inducing potential between groups would be as high as possible to give the best possible chance of detecting differences in hybridoma content of the tumours. Cells were now cultured in horse serum for five days before FACS analysis, the F1 hybridoma control was replaced with an anti-Coxsacki virus antibody secreting hybridoma and the BW cell control was omitted.

The results show no significant difference in B-lymphocyte derived hybridoma content as is apparent from the difference between the fluorescence peak of the tumour cells and the *in vitro* cultured B16 cells (Fig. 4.6).

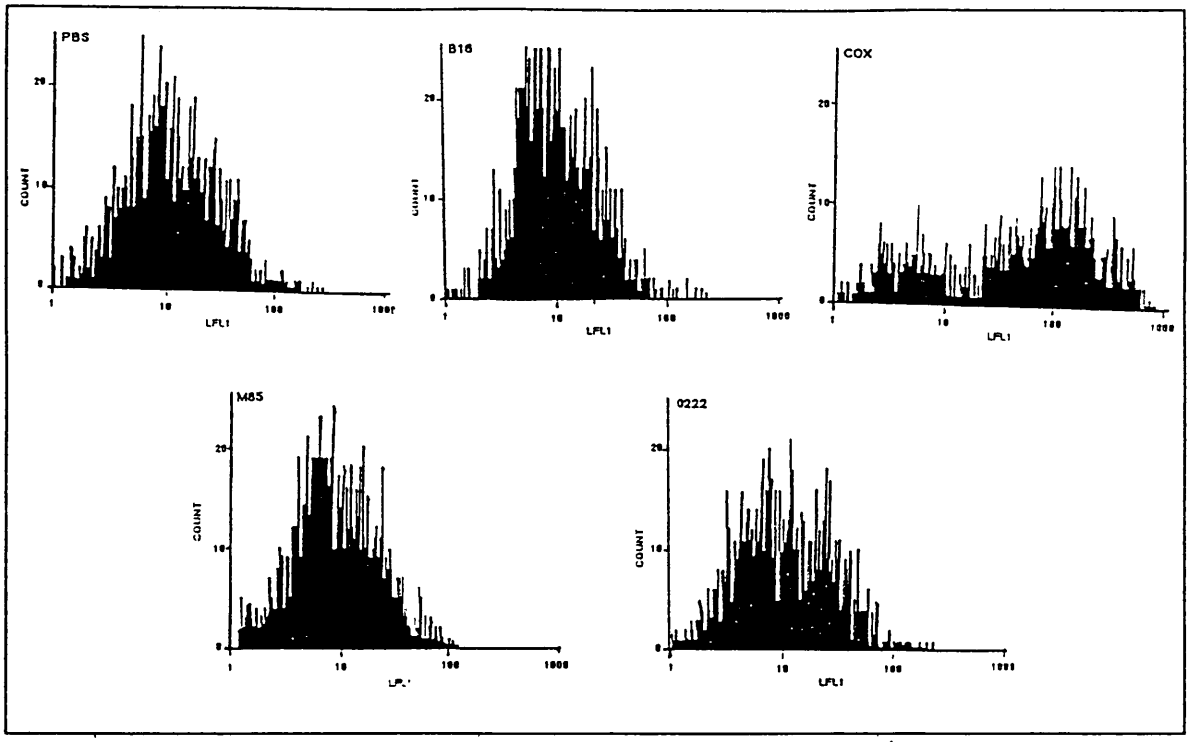


Fig. 4.6: B-cell marker expression analyses of tumour cell samples. The mean log fluorescence values are: 6.2 (B16); 6.0 (PBS); 5.9 (0222); 6.0 (M85); 122,6 (α-Coxsacki). Note that all samples show similar mean peak positions as B16 cultured cells but differ considerably (20x lower) from the α-Cox hybridoma. All analyzed samples were cultured in horse serum prior to analysis.

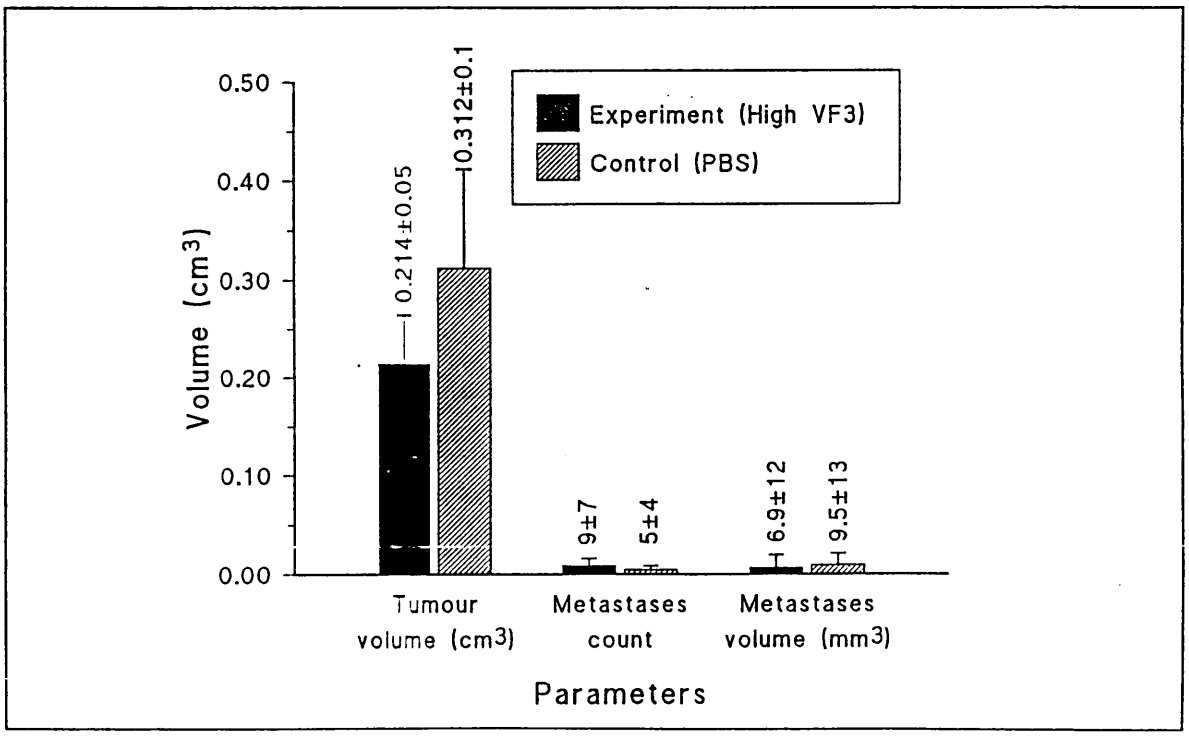


Fig. 4.7: The influence of *Haemophilus* treatment on tumour growth rate and metastatic progression in mice grafted with B16 tumours. The experimental and control groups contained 5 and 4 mice respectively. A significant difference in results were only obtained for the tumour volume data set.

Tumour volumes and metastases were also evaluated (Fig.4.7), but were not significantly different between PBS and *Haemophilus* treated tumours. However, tumour growth rate, as determined by tumour volume at 21 days were reduced in the M85 treated animals. A difference in mean tumour volume of 31% was observed between the experimental and control group, illustrating a 4.7 mm^3/day difference in growth rate of tumours in experimentally treated animals. This correlates well with the reduced tumour growth rate of 4.65 mm^3/day of the experiment with the 0222 line as control, indicating that previous results obtained by Boshoff (using PBS as control) are comparable to results obtained with a LPS containing *Haemophilus* control.

FACS analysis, although able to indicate the ploidy of the tumour cells, appeared to be not sensitive enough to detect any difference of hybridoma formation between the experimental and the control groups. An experiment that gives the hybrid cells a selective advantage would seem more sensitive and able to distinguish better between the groups.

4.3.3 Direct comparison between *in vitro* and *in vivo* hybridoma formation using HAT selection.

Quantification of the hybridoma frequency in tumours was performed by making use of a modification of the HAT selection test (Galfré & Milstein, *et al.,* 1983). This test is normally used as a method of selecting hybridomas in monoclonal antibody production. Tumours of *Haemophilus* treated mice were dissected and grown in HAT media to select for *in vivo* formed hybridomas that would be able to grow in this selective medium. By analyzing the tumour cell:hybridoma ratio and other tumour data and comparing it to tumour data of mice treated with bacteria of different fusion inducing capabilities the experiment attempted to shed light on the effects of *in vivo* hybridoma formation on tumour growth.

Even though the correlation between fusion inducing ability and reduced tumour growth could be shown in two separate experiments of which one showed that the effect was probably not due to a non-specific LPS effect, neither of the experiments could produce conclusive evidence that the effect was due to an increased ability to form *in vivo* hybridomas. The problem could be due to the low sensitivity of the techniques used, based on the following argument:
If the tumour cells lose their malignant phenotype after fusion with a non-malignant B-cell, it is not surprising that the ratio of tumour cells to hybridomas in such a tumour would be big since the malignant cells would overgrow the slower growing hybrid cells. This could be the

explanation for the inability of flowcytometric techniques to show differences in hybridoma content between groups.

To overcome this problem, an experiment similar to that described above, with Sp2/0 cells as the malignant cell line that is inoculated in syngeneic mice (Balb/c), were used to determine the amount of *in vivo* formed hybridomas. Since these cells are HGPRT$^-$ they can be selectively grown *in vitro* in HAT medium after dissection from the tumour. By using such dissected tumour samples to construct an eight-fold replicate dilution series with known concentrations of tumour cells in a microtiter plate, a good estimation of the tumour cell/hybridoma ratio could be obtained for each tumour. A titre value was awarded to each tumour sample to indicate the lowest concentration of tumour cells that gave hybridoma colonies in more than 50% replicates of the same dilution.

Hypothetically if a sample of tumour cells from a mouse treated with fusion inducing bacteria would on average contain more hybridomas than a control sample, this would be observed as a higher titre, enabling direct comparison between the *in vivo* fusion inducing ability of different animals. This could be correlated to the observed *in vitro* hybridoma forming ability of the same animal by performing a spontaneous fusion experiment with its splenocytes. If a correlation exists this would then serve as corroborating evidence that previously observed reductions in tumour growth rate in mice treated with fusion inducing *H. paragallinarum* bacteria could be due to enhanced *in vivo* hybridoma formation.

By combining this data of each mouse with data on the growth rate of the tumour and weight at time of dissection more evidence could be gathered to understand the effect that bacterial induced *in vivo* fusion has on tumour progression.

Using this experimental protocol, four groups of mice were designated according to the bacterial strain they were treated with: a M85 group (7 mice); a plasmid transformed M85 group (2 mice); a frozen and thawed M85 group (7 mice) as well as a group treated with PBS (4 mice). The tumour growth was monitored every second day until a tumour reached 10 mm diameter at which time the date was noted. The mouse was treated again to prime for *in vitro* fusion and placed in a separate cage. After sacrificing each mouse, three days after priming, both tumour and spleen were dissected and used in the HAT selection and *in vitro* spontaneous fusion experiments as described in methods.

After inspecting mice for 40 days post-inoculation it was noted that only 10 mice of a possible 20 had actively growing subcutaneous tumours while none had any tumours

growing in the foot pads. Four M85 treated, five freeze treated M85 mice and one PBS mouse had tumour progression to the desired 10 mm diameter size. No mouse treated with plasmid transformed bacteria had any tumour growth.

All spleens dissected from tumour growing animals were considerably larger than spleens of primed mice dissected for mAb production, indicating an immune response towards the Sp2/0 cell line. This was unexpected considering that Sp2/0 cells should be syngeneic to Balb/c mice. Reasons for the effect could be genetic drift of the Sp2/0 cell line due to numerous *in vitro* multiplications since the line was acquired, resulting in either mutations or chromosome loss and thus divergence from the original genotype. The observation might explain the absence of tumours in the feet of mice but seems highly unlikely since these cells were cloned before inoculation and should be similarly rejected in all mice. The errant tumour-take also indicated that the mice were not genetically homogeneous,a fact that was confirmed by establishing post-factum from the suppliers of the mice, that the animals were not bred by sequential brother-sister pairing of siblings..

Data collected in this experiment failed to illustrate the effect of freezing and thawing on the ability of the whole bacteria to reduce spontaneous hybridoma formation. In addition, only one of the remaining negative controls (PBS treated) produced a tumour and also showed an unusually high percentage of *in vitro* fusion, but failed to show any surviving hybridoma colonies in the HAT selective medium even at the highest tumour cell concentration. These shortcomings hampered the interpretation of the results and the statistical evaluation.

Evaluation of all parameters to find interpretable trends provided little significant insights as can be seen from the following averages:

		(M85)	(M85F)	(PBS)
Number of animals	:	4	5	1
In vitro hybridoma formation	:	22.4 ± 27	27.6±22	25
Tumour cell : Hybridoma ratio (x = 10^6)	:	1.8 x : 1	1.5 x : 1	>10 x : 1
Tumour size (grams at dissection)	:	1.6 ± 11.9 ± 1.7	1.1	
Growth rate (mm^3/day)	:	20.9 ± 8	22.8 ± 2.5	22.8

Further evaluation accommodated the fact that the mice actually represented a heterogeneous group. The results were ordered according to *in vitro* hybridoma forming ability and subsequently tested for any correlation between *in vitro* fusion ability and *in vivo* hybridoma forming ability as determined by fusion percentage and HAT survival respectively. This was done using regression analysis of the plot showing percentage *in vitro* hybridoma formation vs. titre (hybridoma to tumour cell ratio) (Fig. 4.8). The regression line showed a significant correlation between *in vitro* and *in vivo* hybridoma forming ability of mice pretreated with *H. paragallinarum* ($p < 0.12$).

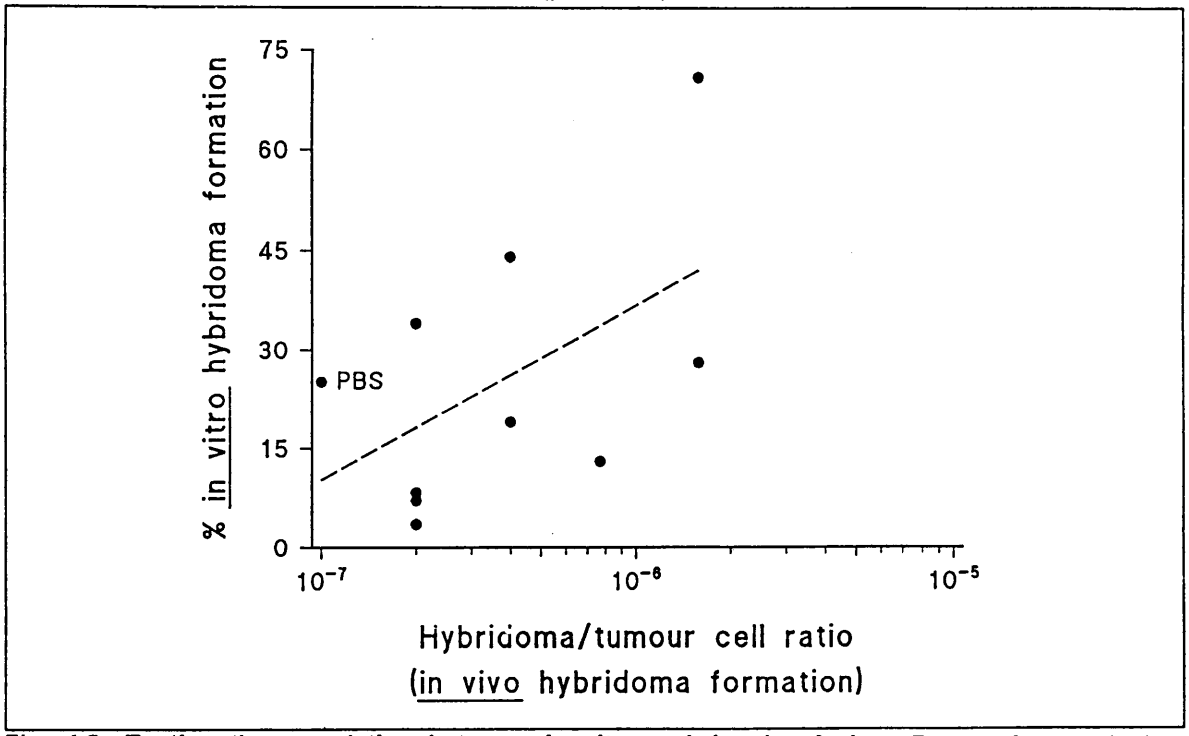


Fig. 4.8: Testing the correlation between *in vitro* and *in vivo* fusion. Regression analysis of percentage *in vitro* hybridoma formation vs. titre (Hybridoma : Tumour cell ratio).

This experimental technique provided significant evidence that a correlation between *in vitro* hybridoma forming ability and *in vivo* hybridoma formation exists. This is the first evidence, albeit subtle, that shows the possible application for *H. paragallinarum* treatment as a novel anti-tumour therapy.

4.4 Discussion:

The possible application of *H. paragallinarum* induced *in vivo* spontaneous hybridoma formation as a novel anti-tumour treatment was first postulated by Boshoff, in 1992. He tested the effect of *H. paragallinarum* treatment of mice, before inoculation with B16-F10 melanoma cell line, on metastatic colonization of the lungs and tumour growth of the inoculated animals. Boshoff could show a reasonable suppression of tumour growth rate in two separate experiments and postulated that the reason for this reduction was attributed to *in vivo* fusion of B-lymphocytes with the tumour cells, induced by the fusogenic properties of the VF3 antigen, restoring a defective tumour suppressor function (Boshoff, 1992). The results were compared to PBS treated controls, which failed to rule out the possibility of LPS induced tumour suppression that is well established (Goodman, 1979; Haeffner-Cavaillon, 1982). Further problems were experienced with high standard deviations and unreliable expression of the VF3 on the bacteria.

The B16-F10 / C57Bl mouse cancer model was used to confirm and expand Boshoff's findings. In order to reduce possible heterogeneity due to haplotype differences in experimental animals, mice derived from a F1 cross of C57Bl and CBA mice were used, and proved effective.

To eliminate the contribution of LPS in reduction of tumour growth rate, pretreatment of the mice with the fusion inducing M85 line before inoculation with B16-F10 melanoma cells were performed, which once again showed a significant reduction in tumour growth rate of 4.6 mm^3/day or 49% ($p < 0.1$, taken on day 20 post inoculation) compared to mice pretreated with non-fusion inducing, 0222 line (LPS containing) (Fig. 4.2). A repeat of the experiment using a PBS control produced similar tumour growth suppression verifying that the results were purely due do to the fusion inducing potential and not a general LPS-induced effect. This evidence rejects the null hypothesis and corroborate the results of Boshoff with the additional justification of a LPS containing control. No significant change in metastatic potential could be indicated in either of the experiments (Fig. 4.7)

To determine the degree of spontaneous *in vivo* melanoma/B-cell fusion, dissected tumour samples of the mice were tested for DNA content, making use of a flow cytometric analysis. All samples showed a high tetraploid DNA content as compared to *in vitro* cultured B16 cells and mouse PBMCs (Fig. 4.3). This unexpected high number of tetraploid cells could be attributed to the intrinsic character of B16 cells to become tetraploid if the growth area becomes restricted.

To determine the % of fused cells resulting from B- and tumour cell fusion the same samples were tested for IgG (B-cell marker) expression using a goat-anti-mouse -IgG mAb conjugated with FITC in a flow cytometric analysis. The use of a B-cell marker that is not expressed by all B-cells (only mature B-cells) might not seem efficient but in an article of Clark, and Milstein, (1981) it is reported that hybrids of mouse lymphocytes and tumour cells have a strong selection for the IgG secreting phenotype, justifying this technique. Results obtained in this experiment showed no significant difference in surface IgG expression for either PBS, 0222 or M85 treated tumours (Fig. 4.6).

This led to the reevaluation of the experiment in order to consider alternative explanations for the low abundance of hybridomas in the tumours that showed retarded growth. One consideration was that the spontaneous hybrid would be overgrown by unfused tumour cells, exactly by its reduced growth rate. This would imply that hybridomas in tumours would seldomly be expected to multiply to any significant abundance relative to the normal tumour cells.

The obvious way to resolve the problem was to use a model were the hybridomas formed in the tumours had a selective advantage above the normal tumour cells. An improved experiment used a sensitive selection system able to indicate hybrid cells between B-lymphocytes and Sp2/0 cells. The method is similar to the HAT selection procedure used in monoclonal antibody production (Galfré and Milstein, 1981). It makes use of the inability of $HGPRT^-$ myealoma cells to grow in HAT medium because they cannot synthesize nucleic acids from the hypoxantine and thymidine precursors. Normal cells have an intact HGPRT enzyme but are unable to sustain growth and replication in tissue culture without exogenous growth factors. Hybrid cells however have both the HGPRT gene and a neoplastic phenotype (if the suppressor genes of these cells are lost) enabling the cells to grow in HAT medium which inhibits *de novo* pyrimidine and purine synthesis.

This experimental model was tested using dissected Sp2/0 tumours of mice pretreated with *H. paragallinarum* bacteria with different fusion inducing potential. By diluting a suspension of dissected tumour cells to $1x10^7$ cells/well a single surviving hybridoma in this well would indicate a $1x10^7$:1 tumour cell to hybridoma ratio. By determining a titre, it was possible to quantify the hybridoma to tumour cell ratio up to 10^4 :1 for all tumour samples. Analysis of this data enabled direct comparison of *in vivo* and *in vitro* hybridoma forming ability of the same animal for all animals in the different groups.

Although the experiment failed to provide any new correlations besides the reduced tumour growth rate of *Haemophilus* vs control treated animals due to the genetic non-homogeneity of the mouse-strain used and the variation possibly caused by inadequate animal facilities, regression analysis of the *in vitro* hybridoma formation and tumour cell/hybridoma titers (indicative of *in vivo* hybridoma forming ability) provided evidence of correlation between these parameters ($p<0.12$, Fig. 4.8).

This evidence convincingly rejects the null hypothesis and provides reason to believe that previously observed reduced tumour growth might be atributed to a *H. paragallinarum* induced *in vivo* hybridoma forming ability. This evidence would be the first indication that *H. paragallinarum* treatment could provide a novel anti-tumour therapy. The experiment should be repeated with a higher number of properly inbred animals fully compatible with the cancer cell line used to statistically verify the results in a more controlled manner. It would also be advisable to perform such an experiment under SPF conditions to reduce the possible occurrence of false positives due to for example incidental upper respiratory infection.

Experimental data of other researchers provide a rationale for the concept of tumour suppression by spontaneous fusion: A spontaneous tumour could result from a mutation in a tumour suppressor gene, that results in loss of cell growth control (Vogelstein, *et al.,* (1992, 1993)). One well documented tumour suppressor that is implicated in mouse and human cancer is the p53 protein. The p53 gene encodes a protein that can bind DNA in a sequence specific manner (Foord, *et al.,* 1993), interact with transcription factors (Borellini, *et al.* 1993; Truant, *et al.,* 1993; Martin, *et al.,* 1993; Seto, *et al.,* 1992; Maheswaran, *et al.,* 1993) and in so doing suppresses transcription of several genes (Chin, *et al.,* 1992). The wild-type p53 acts as a cell cycle checkpoint, halting the cell cycle in G1-phase when required (Livingstone, *et al.,* 1992; Zhan, *et al.,* 1993; Rotter, *et al.,* 1993) and also controls differentiation and apoptosis (Kastan, *et al.,* 1991; Fujiwara, *et al.,* 1993). The loss or mutation of this gene will therefore result in a cell with neoplastic phenotype (Weinberg, *et al.,* 1994). The p53 gene loss is a frequent occurrence in human cancers (Harris, *et al.,*1993) and transgenic mice deficient in the p53 gene are developmentally normal but acquire a wide range of spontaneous tumours at about 20 weeks of age (Donehower, *et al.,* 1992). Weinberg, *et al.,* (1994) reported that p53 gene frequency alone is enough to delay growth and malignant progression of certain skin cancers. They showed 25% and 82% reduction in tumour volume of malignant tumours transfected to be heterozygotic and homozygotic towards the wild-type p53 respectively.

In another article Van Kessel, and Den Boer (1981) reported partial suppression of tumourigenicity of mouse and hamster tumour cell lines after fusion with either normal or leukemic human leucocytes.

These articles illustrate that fusion of normal non-tumourous cells with tumourigenic cells could suppress the tumourigenicity of the latter. Since reduced tumour growth could be indicated in two separate instances in this study and twice previously by Boshoff. after treatment with fusion inducing *H. paragallinarum* bacteria, it strongly suggests that a similar model of reduced tumourigenicity after fusion could be responsible for this reduction. Evidence that the anti-tumour effects of LPS do not play a significant role in these experiments have been produced, by making use of a LPS containing negative control (0222) which showed a higher tumour growth rate. The correlation between *in vitro* and *in vivo* hybridoma forming ability of *H. paragallinarum* treated murine B-cells further persuades that this treatment has the ability to produce a novel anti-tumour chemotherapy, that could be purified from bacteria expressing the fusogen.

The study provides future research with the techniques and animal models to successfully test the influence of bacterial induced *in vivo* fusion on the tumour progression of different cancer cell lines to determine the wider application of this kind of approach in cancer therapy.

Tests could also be done with tumour cell lines with known suppressor deficiencies to estimate which kind of cancer reacts best on spontaneous fusion therapy as well as to gain more insight into the dominance of certain suppressor genes.

Chapter 5: Concluding Discussion

This study aimed at furthering the understanding of the spontaneous hybridoma formation phenomenon as well as determining the possible applications it has in hybridoma and cancer chemotherapy.

The phenomenon of bacterially induced spontaneous hybridoma formation (spontaneous fusion for short) was first described by J.A. Verschoor while producing mAbs to be used as tools in the study of *H. paragallinarum* bacteria. In order to produce immune splenocytes, mice were immunized intraperitoneally with 10^7 *H. paragallinarum* bacteria suspended in saline followed by two booster immunizations of similar dose. Four *H. paragallinarum* strains were used, two each of the vaccine and field isolate groups (Verschoor, *et al.*, 1989). After PEG fusion of immune splenocytes with Sp2/0 myeloma cells a phenomenon of over-fusion was observed. This manifested as a high number of hybridoma colonies (approaching 100%) on day 20 post-fusion that rapidly died off, a day or two later. The surviving colonies formed some stable antibody secreting hybridomas. A subsequent repeat of the experiment omitting the fusion agent (PEG) resulted in hybridoma formation in approximately 70 % of the wells. These stable spontaneously fused cells secreted antibodies at a frequency comparable to that obtained with conventional fusion techniques (Verschoor, *et al.*, 1990).

As described in the Introduction and in Chapter 2 the mAbs recognized field isolates and vaccine strains differently. Characterization of the specificity of the panel of monoclonal antibodies produced was performed (Verschoor, *et al.*, 1989). These antibodies were extensively used by Bragg to study the population dynamics of *H. paragallinarum* bacteria isolated from South African chickens (Bragg, 1996). The mAbs were found to be specific for *H. paragallinarum* bacteria and were therefore investigated for their use as serotyping tools, but were found insufficient due to variance of the ELISA patterns with changes in bacterial growth medium composition (Bragg, 1996). The conventional method of typing serogroups using a haemagglutination assay with serogroup specific sera to assign isolates to one of four groups (Kume, *et al.*, 1983), remained the better serotyping method.

The mAbs were however useful in following the change in antigen patterns in wild type populations after the introduction of an oil emulsion vaccine in 1983 using the 083 and 0222 vaccine strains. Since 1983 there was a definite shift in antigenic make-up and growth requirements of *H. paragallinarum* isolates, that could be determined with the mAb panel combined with standard biochemical classification assays (Bragg, *et al.*, 1993a).

One property of the bacteria that changed was its dependence on NAD in *in vitro* growth medium (Blackall & Reid, 1982). NAD independent strains were isolated in Natal, South Africa, since 1990 (Mouahid, *et al.,* 1992). These isolates proved to be identical to *H. paragallinarum* in every respect, but their independence on NAD. Similar changes in growth requirements were also reported for other *Haemophilus* species. NAD dependence proved to be a plasmid carried trait (Windsor, *et al.,* 1991). Bragg used the mAb panel to monitor the change in antigenic profile after experimental transfer of DNA from NAD independent strains to NAD dependent strains. The transformation rendered the bacteria independent of NAD without loosing their other species specific traits. Because of their specificity the mAb panel could convincingly demonstrate that the NAD independent bacteria isolated since 1990 were indeed *H. paragallinarum* that acquired additional genetic material (probably plasmid carried) responsible for the change to NAD independence (Bragg, *et al.,* 1993b, 1995b).

The ability of *H. paragallinarum* bacteria to induce spontaneous fusion also appeared to be a variable in the population dynamics of *H. paragallinarum.* This could be illustrated by the ability of M85 and SB86 to induce fusion, but which was not achievable with vaccine strains or more recent field isolates. The biological activity of the fusion inducing strains also appeared to decrease with continued subculturing.

C. H. Boshoff used the panel of monoclonal antibodies to determine the fusion inducing potential of *H. paragallinarum* bacteria after observing that it seemed to correlate with the expression of the VF3 antigen (Boshoff, *et al.*, 1992). He showed that the VF3 antibody reacted to the antigen in an immuno-dot-blot of a LPS extract that could induce spontaneous fusion. After freezing and thawing the LPS extract, the VF3 antibody no longer recognized it and it was no longer able to induce fusion. Boshoff also attempted to characterize the VF3 and F1 antigens to determine their protein or polysaccharide nature of the antigens. He showed that VF3 was sensitive to periodic oxidation, typical of polysaccharide nature and that F1 showed trypsin sensitivity typical of protein nature. Other antigens were not tested (Boshoff, 1992).

In corroboration with results of C. H. Boshoff in his M.Sc. thesis, the antigen recognized by the F1 monoclonal antibody was here illustrated to be a protein of molecular mass of 39 kDa, determined by Western blotting of a SDS-PAGE gel. A band of similar electrophoretic mobility visualized by Coomassi staining appeared to be dominant in *Haemophilus* lysates that are F1 positive. The F1 protein corresponds in MM and abundance to the OMP-C

protein recognized by polyclonal sera in immunotyping experiments of *Haemophilus* bacteria (Blackall, *et al.,* 1990). No haemagglutination inhibition could be illustrated using purified F1 mAb to any of the serogroups which led to the conclusion that the F1 antigen was not the haemagglutinin (Bragg, *et al.,* 1997).

The antigens recognized by the VF1 and VF2 mAbs could be shown to have a MM of 39 kDa. Since these antigens were not found on any recent isolates, further characterization was deemed unnecessary.

The V1 mAb's antigen was not previously characterized and is shown here to have a polysaccharide nature and electrophoretic mobility of 14 kDa as determined by periodic oxidation and SDS-PAGE immunoblot. The antigen is trypsin resistant and unable to inhibit haemagglutination of *H. paragallinarum* reference strains. This data illustrates that the antigen is not a protein.

By Western blotting of a SDS-PAGE gel of fresh and freeze-thawed bacteria it could now be shown that the VF3 antigen is chemically cleaved, resulting in an increased mobility in SDS-PAGE. Multiple bands at 38-39 kDa were converted into similar bands at 29-32 kDa after freeze-thaw treatment. This evidence strongly supported the notion that the antigen recognized by the VF3 mAb could be the fusion inducing entity that is extractable by LPS extraction (Boshoff, 1992), since this trait is also sensitive to freeze-thawing in PBS. The F1,V1,VF1 and VF2's antigens remained stable upon freezing and thawing in terms of electrophoretic mobility and antigenicity, making them unlikely candidates for the fusion inducer agent.

Problems to culture the *H. paragallinarum* bacteria with reproducible VF3 expression and fusion inducing potential prompted the further optimization of the culture conditions of the bacteria (Bragg, 1996). The VF3 expression of the M85 strain was shown to increase with a higher initial pH (≥ 8,0) of culture medium. This could not be correlated with an increase in spontaneous fusion induction. At an optimum pH of 7.4 however, good correlation between VF3 expression and fusion inducing ability was found. This lead to the conclusion that the VF3 antigen and fusion inducing entity are residing on the same molecule but occupy different parts of the structure . Further evidence for this conclusion was found by observing that freezing and thawing chemically degrades the VF3 molecule to a smaller entity and reduces its fusion inducing ability without significantly affecting the ability of the mAb to recognize the VF3 epitope on an immunoblot of a SDS-PAGE gel (Gunter, *et al.,* 1994). After similar treatment of LPS extracts and its application on an immuno-dotblot

membrane, the enzyme-immuno-assay signals were reduced because the VF3 carrying part was probably lost in the wash steps.

Chicken serum was commonly used in culture conditions to satisfy NAD requirements. This introduced a variable in the culture which was difficult to control from batch to batch and could also affect VF3 expression. To rectify this, commercial NAD was tested as a supplement and found not to hamper VF3 expression or fusion inducing potential.

A NAD independent strain was tested for VF3 expression and fusion inducing ability. It proved just as good in VF3 expression and fusion inducing potential as the normal M85 strain. This new strain will reduce the cost to culture fusion inducing *H. paragallinarum* bacteria, by its less rigorous demand for medium supplements.

The use of *H. paragallinarum* as a dual purpose adjuvant to aid in hybridoma technology was demonstrated previously to generate monoclonal antibodies to hen's egg white lysozyme with reasonable success (Verschoor, *et al.,* 1990). The mice were immunized with the antigen mixed with *H. paragallinarum* bacteria in FIA. Following this, the immune splenocytes were co-incubated with Sp2/0 myeloma cells resulting in spontaneous hybridoma formation, without the use of PEG. The hybridomas were then selected for secretion of the desired mAb. It was shown that 12% of hybridomas formed, secreted HEL specific antibodies that were comparable to what was obtained by PEG fusion (Verschoor, *et al.*, 1988).

In an attempt to show that this technology is wider applicable to a broader spectrum of antigens, mice were immunized with various crudely purified, planktonic sonicates mixed with IFA and *H. paragallinarum* in a similar manner as stated above. The M85 strain cultured in serum free NAD supplemented medium was used in these experiments. A high percentage of spontaneous hybridoma formation was observed from which several hybridomas were cloned, secreting antibodies with high specificity for the individual planktonic species. This proved that the *Haemophilus* aided hybridoma formation technology is wider applicable, even with very crude preparations of antigen. The advantage of this procedure is that the *Haemophilus* bacteria stimulate the local immune response in a similar way to mycobacterial antigens in FCA, while acting as a fusion inducing agent that obviates the need for PEG in the hybridoma production.

The same technique was used by S. Krishnan at the Department of Biochemistry, Cancer Institute, University of Madras, India, who used the same M85 *H. paragallinarum* strain to

produce a highly specific IgG1 monoclonal antibody (28K5) to Dalton's lymphoma associated antigen (DLAA). A radio-labeled 28K5 mAb was used in tumour bearing mice to generate clear *in vivo* tumour images using gamma camera technology (Krishnan, *et al.,*1997). This provided another illustration of the principle that monoclonal antibodies produced by spontaneous fusion improves on the conventional method for hybridoma production.

The application of the spontaneous fusion inducing ability of *H. paragallinarum, in vivo* as a therapy to inhibit tumour progression, was also evaluated. Boshoff initiated this investigation and came up with some encouraging results (Boshoff, 1992). Mice treated with *H. paragallinarum* (VF3 expressing M85 strain) before inoculation with B16-F10 melanoma cells, exhibited a reduced tumour growth rate. This observation was made using PBS treated mice as controls. From the literature however it is known that LPS (a constituent of gram-negative bacterial cell walls) have tumour reducing properties due to activation of macrophages (see Chapter 1 and 4). Since the *Haemophilus* treated animals were treated with LPS containing preparations, the reduced tumour growth could have been as a result of this.

Here, various experiments were conducted to find evidence that *H. paragallinarum* induced *in vivo* fusion is responsible for reduced tumour growth rate. In an experiment using the B16-F10 melanoma model, *Haemophilus* treatment with a LPS containing, but non-fusion inducing control, a 49% drop in tumour growth rate could be obtained. This illustrated that the non-specific effect of LPS can be ignored in these experiments and that the growth rate differences were mostly due to the *Haemophilus* strain specific trait.

Most spontaneous tumours result from a mutation in a tumour suppressor gene, which results in loss of cell growth control (Harris, *et al.,* 1993). This unregulated replication of the resulting neoplastic mutants is responsible for the formation of tumours with subsequent further progression to metastatic tumours with the ability to cross tissue barriers and infiltrate the bloodstream from where it can spread to other tissue to start new tumour colonies (Vogelstein, *et al.,* 1993).

As early as 1969, Sinkovics observed the fusion of normal B-cells with neoplastic cells when the latter were passaged through mice (Sinkovics, 1981, 1985, 1990). In the same year, Harris *et al.* reported that fusion of malignant cells with normal cells could generate hybrids in which malignancy is suppressed (Harris, *et al.,* 1969). Since 1996 several authors reported the *in vivo* fusion between leukocytes and neoplastic cells (Wiener, *et al.,*

1972; Larizza, *et al.,* 1984; De Baetselier, *et al.,* 1984). The theory of suppressor genes became more acceptable after the characterization of a few of these genes (Klein, *et al.,* 1987; Marshall, *et al.,*1991; Vogelstein, *et al.,* 1993). One example is the recently well documented tumour suppressor that is implicated in mouse and human spontaneous cancer development, the p53 protein (see 4.1 and 4.4).

The increase of wild-type p53 (or similar tumour suppressor) gene copy number by *in vivo* fusion of a normal (p53 +\+) B-lymphocyte with a malignant B16 melanoma or Sp2/0 lymphoma cell (possibly p53 -/- or p53 +/-), might provide a likely explanation for the observed tumour growth reduction after *H. paragallinarum* treatment.

In this study flow cytometric techniques were found to be inadequate for determining the hybridoma cell : tumour cell ratios but clearly indicated that B-cell/tumour cell hybridomas, if existing, were present at very low frequency probably due to there growth disadvantage.

An experiment applying a modification of the HAT selection test selectively favoured hybrid cells of the B-cell/tumour-cell phenotype and could thus give better indications of the hybridoma : tumour cell ratios *in vivo*. With this approach, a hybridoma : tumour-cell titre for each animal could be correlated with *in vitro* fusion ability. Despite the problems that were encountered with the in-bred quality of the animals used, it was possible to show a correlation between the *in vitro* fusion inducing ability and *in vivo* hybridoma frequency in different animals.

This represents the first evidence that *H. paragallinarum* treatment of animals before inoculation with tumour cells can retard the growth of tumours much more than can be effected by the LPS molecules of the bacteria. This is also the fourth experiment, and with two different mouse tumour models, showing that *H. paragallinarum* treatment reduces tumour growth rate in mice.

Since a correlation between *in vivo* fusion and *in vitro* fusion inducing ability was demonstrated, it is more than likely that the observations of Boshoff, on reduced tumour growth upon pretreatment with fusion inducing *H. paragallinarum* bacteria, can also be explained by the stimulation of *in vivo* fusion.

The actual mechanism whereby fusion of B-cells and neoplastic cells accomplish a reduction in tumour growth rate is unclear at the moment but two different theories exist in the litarature: The theory described above has to do with the reconstitution of defect tumour

suppressor genes. A second, more recent theory relies on the ability of the hybrid cell to act as an antigen presenting cell to present cancer cell specific antigens to T-cells, thus enabling a cytotoxic immune response on the hybrid as well as unfused tumour cells.

Recent papers in *Nature Medicine* and *Science* demonstrate that B cell and dendritic cell hybrids with tumour cells have the ability to express MHC class I and class II molecules as well as other essential T-cell activating molecules such as LFA-1, B7 and ICAM-1 and can act as an immunotherapy to protect against parental tumour cells (Gong & Kufe, *et al.,* 1997; Guo, *et al.,* 1994) The paper in *Science* by Yajun Guo *et al.,* reports on fusing hepatoma and activated B-cells using PEG to produce hybrid cells with reduced ability to form tumours. These cells could however colonize murine liver and could protect mice from simultaneous and subsequent challenge with unfused hepatomas. The researchers could show enhanced T-cell infiltration to the area surrounding the hybrids ($CD8^+$ (70%) and $CD4^+$(30%)) but no similar effect in animals injected with normal tumour cells alone. Depletion studies indicate that both $CD4^+$ and $CD8^+$ cells are involved in the reaction but that $CD8^+$ cells provide sufficient protection after treatment with hybrids. Injection of either B-cells, tumour cells and/or PEG alone did not confer protection (Guo, *et al.,* 1994).

A combination of enhanced immunogenicity and acquired replenishment of tumour suppressor genes could account for the observations made in this study. Initially the growth of fused cells alone are stunted by tumour suppressor gene supplementation but they can then act as activators of a specific anti-tumour immune reaction and subsequently affect the unfused cells. The optimization of the treatment to achieve higher *in vivo* hybridoma formation could produce improved results, since even the low percentage detectable hybrids obtained in this study already affected growth significantly.

The establishment of a suitable animal model to test the effect of *in vivo* spontaneous fusion on cancer growth is a major advance contributed by this research. This model can now be applied on different cancers by manipulating the specific tumour cells to become $HGPRT^-$ (Kaeffer, *et al.,* 1991) and using them in similar experiments to determine the degree of correlation of reduced tumour growth with *in vitro* fusion inducing potential. This could indicate the relative susceptibility of different tumours to this treatment, to assess its broader application as a cancer therapy.

Further investigation should substantiate the findings with a more reliable inbred mouse strain in an adequate mouse facility. The use of pathogen free mice would eliminate the possibility of accidental infection with gram negative bacteria causing false positive results.

The possible application of this work in human cancer therapy involves numerous ethical considerations. The normal route of first testing in non-human primates involves the treatment of animals with carcinogens to produce syngeneic tumours. However, it is now possible to test the protocol in a simulated human immune system. This was made possible by the development of SCID-human mice (Mosier, *et al.,* 1988).

A CB-17 mice strain fails to develop mature T- and B-cells due to an autosomal recessive mutation in a gene facilitating V-(D)-J recombination (Alt, *et al.,* 1992). The mice are susceptible to any bacterial or parasitic disease and should be kept in pathogen free facilities. These animals can except grafts from any mouse or non-mouse donor because they can not launch a rejection reaction. Inoculation with human B- and T-cell precursors can reconstitute mice with human immune cells. Such mice are called SCID-human mice. Although very expensive and difficult to come by, it might be extremely informative to test *H. paragallinarum* treatment in this system.

Two approaches exist to test human immune function in SCID mice. The first is a thymus-SCID-human model which allows full haemapoetic development of B- and T-cells. Human thymus and lymphoid tissue obtainable from tissue bank facilities are implanted under the kidney capsule of a SCID mouse to function as both primary and secondary lymphoid tissue for the human lymphoid cells to mature and undergo selection. Human lymphoid stem cells are then injected into the animal to reconstitute a "SCID-human" immune system (Dorshkind, *et al.,* 1986). The animal will develop a full complement of immune cells that can be transferred to other identical SCID mice (also grafted with human secondary lymphoid organs) to facilitate identical controls.

A second, less complex approach entails so-called (PBL)-SCID mice. Human peripheral blood lymphocytes serve as source of lymphocytes for populating a human implanted lymph node. The lymph node will act as a human secondary lymphoid organ with similar size and function in the SCID mouse as a spleen normal mice. This model provides fully functional B-cells and mature T-cells. The disadvantage of this approach is that T-cells that matured in the human donor, might react with non-self antigens of the mouse with undesirable side effects. The function of B- and T-cells could however be tested separately by selective grafting of either.

H. paragallinarum induced spontaneous *in vivo* fusion might turn out to be just the way to eliminate individual tumour cells or residing tumour tissue after standard chemotherapy or

dissection of malignant tissue. It could reduce the rate of reemerging tumour growth which can make the chemotherapy more effective. The treatment does not involve the administration of any toxic chemicals and uses the immune system to accomplish its effect with no undesired side-effects on the patient.

References:

1. Alt, F.W. Oltz, E.M., Young, F., 1992. VDJ recombination. *Immunol. Today*, 13:306.

2. Blackall, P.J., Reid, G.G., 1982. Further characterization of *H. paragallinarum* and *Haemophilus avium. Vet. Microbiol.*, 7:359-367.

3. Blackall, P.J., Yamamoto, R., 1989. Whole cell protein profiles of *H. paragallinarum* as detected by polyacrylamide gel electrophoresis. *Avian Dis.*, 33:168-173.

4 Blackall, P.J., Rogers, D.G., Yamamoto, R., 1990. Outer membrane proteins of *H. paragallinarum. Avian Dis.*, 34:871-877.

5. Borellini, F., Glazer, A., 1993. Induction of Sp1-p53 DNA-binding heterocomplexes during granulocyte/macrophage colony-stimulating factor-dependent proliferation in human erythroleukemia cell line TF-1. *J. Biol. Chem.*, 268:7923-8.

6. Boshoff, C.H., 1992. Spontaneous cell fusion induced by bacterial immunization. *M.Sc. thesis, University of Pretoria, Faculty of Natural Science.*

7. Boshoff, C.H., Coetzee, L., Visser, L., Verschoor, J.A., 1992. Spontaneous hybridoma formation induced by immunization with *H. paragallinarum*: evidence for a lipopolysaccharide fusion inducer. *Hybridoma,* 11:257-266.

8. Bragg R.R., Gunter N.J., Coetzee, L., Verschoor, J.A., 1997. Monoclonal antibody characterization of reference isolates of different serogroups of *H. paragallinarum. Avian Pathol.* 26:749-764.

9. Bragg, R.R., 1996. Monoclonal antibody detection of both growth stage and isolate dependent antigen expression of *H. paragallinarum*, *Ph.D. Thesis, University of Pretoria, Faculty of Veterinary Science.*

10. Bragg, R.R., Coetzee, L., Verschoor, J.A., 1993a. Monoclonal antibody characterization of South African field isolates of *H. paragallinarum.* , 60:181-187.

11. Bragg, R.R., Coetzee, L., Verschoor, J.A., 1993b. Plasmid encoded NAD independence in some South African isolates of *H. paragallinarum*. , 60:147-152.

12. Bragg, R.R., Coetzee, L., Verschoor, J.A., 1997. Effects of growth conditions and incubation times on the expression of antigens of *H. paragallinarum* as detected by monoclonal antibodies. *Onderstepoort J. vet. Res.* 64:57-63.

13. Bragg, R.R., Purdan, G., Coetzee, L., Verschoor, J.A., 1995b. Effects of transformation on the hemagglutinins of *H. paragallinarum*. *Onderstepoort J. vet. Res.*, 62:261-270.

14. Chin, K.V., Ueda, K., Pastan, I., Gottesman, M.M., 1992. Modulation of the activity of the promoter of the human MDR1 gene by ras and p53. *Science*, 255:459-462.

15. Coetzee, L., Rogers, E.J., Velthuysen, L., 1983. The production and evaluation of a *H. paragallinarum* (infectious coryza) oil emulsion vaccine in laying birds. *Proc. Int. Union Immunol. Soc.*, 66:277-283.

16. De Baetselier, P., Roos, E., Brys, L., Remels, L., Feldman, M., 1984. Generation of invasive and metastatic variants of a non-metastatic T-cell lymphoma by *in vivo* fusion with normal host cells. *Int. J. Cancer*, 34:731-738.

17. De Blieck, L., 1932. A haemoglobinophilic bacterium as the cause of contagious catarrh of fowl. *Vet. J.*, 88:9-13.

18. Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., Bradley, A., 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, 356:215-221.

19. Dorshkind, K., Denis, K.A., Witte, O.N., 1986. Lymphoid bone marrow cultures can reconstitute heterogeneous B and T cell-dependent responses in severe combined immunodeficient mice. *J. Immunol.*, 137:3457-63.

20. Erwin, A.L., Munford, R.S., 1990. Deacylation of Structurally Diverse Lipopolysaccharides by human Acyloxyacyl Hydrolase. *J. Biol. Chem.*, 265, 27:1644-49.

21. Erwin, A.L., Mandrell, R.E., Munford, R.S., 1991. Enzymatically deacylated *Neisseria* lipopolysaccharide (LPS) inhibits murine splenocyte mitogenesis induced by LPS. *Infect. Immun.*, 59, 6:1881-1887.

22. Fidler, I.J., 1973. Selection of successive tumour lines for metastasis. *Nature New Biol.*, 242:148-149.

23. Fidler, I.J., 1978. Tumour heterogeneity and the biology of cancer invasion and metastasis. *Cancer Res.*, 38:2651.

24. Fidler, I.J., Nicolson, G.L., 1976. Organ selectivity for implantation survival and growth of B-16 melanoma variant tumour lines. *J. Nat. Cancer Inst.*, 57:1199-02.

25. Foord, O., Navot, N., Rotter, V., 1993. Isolation and characterization of DNA sequences that are specifically bound by wild-type p53. *Mol. Cell. Biol.*, 13:1378-84.

26. Fournier, R.E.K., 1982. Microcell-mediated chromosome transfer. *Techniques in Somatic Cell Genetics, New York, Plenum Press*, 309-327.

27. Fujiwara, T., Grimm, E.A., Mukhopadhyay, T., Cai, D.W., Owen-Schaub, L.B., Roth, J.A., 1993. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res.*, 53:4129-33.

28. Galfré, G., Milstein, C., 1981. Preparation of monoclonal antibodies: strategies and procedures. *Meth. Enzymol.*, 73:3-46.

29. Garbisa, S., Kniska, H., Tryggvason, K., Flots, C., Liotta, L.A., 1980. Quantitation of basement membrane collagen degradation by living tumour cells *in vitro*. *Cancer Letters*, 9:359.

30. Gong, J., Chen, D., Kashiwaba, M., Kufe, D., 1997. Introduction of antitumour activity by immunization with fusions of dendritic and carcinoma cells. *Nature Med.*, 5:558-561.

31. Goodman, G.W., Sultzer, B.M., 1979. Further studies on the activation of lymphocytes by endotoxin protein. *J. Immunol.*, 122(4):1329-1334.

32. Gunter, N.J., Bragg, R.R., Boshoff, C.H., Verschoor, J.A., 1994. Properties of bacteria that induce spontaneous hybridoma formation in mice. *Abstr. 12th Biochemical Society Congress, University of Stellenbosch., University of Stellenbosch,* January 1994, 52.

33. Guo, Y., Wu, M., Chen, H., Wang, X., Liu, G., Li, G., Ma, J., Sy, M-S., 1994. Effective tumour vaccine generated by fusion of hepatoma cells with activated B cells. *Science*, 263:518-520.

34. Haeffner-Cavaillon, N., Cavaillon, J.M., Moreau, M., Szabo, L., 1984. Interleukin 1 secretion by human monocytes stimulated by the isolated polysaccharide region of the *Bordetella pertussis* endotoxin. *Mol. Immunol.*, 21(5):389-395.

35. Haeffner-Cavaillon, N., Cavaillon, J-M., Szabo, L., 1982. Macrophage dependent polyclonal activation of splenocytes by *Bordetella pertussis* endotoxin and its isolated polysaccharide and Lipid A regions. *Cell. Immunol.*, 74:1.

36. Hanna, N., Fidler, I.J., 1981. Relationship between metastatic potential and resistance to killer cell-mediated cytotoxicity in three murine tumour systems. *J. Nat. Cancer Inst.*, 66:1183.

37. Harris, C.C., Hollstein, M., 1993. Clinical implications of the p53 tumour-suppressor gene. *N. Engl. J. Med.*, 329:1318-27.

38. Harris, H., Miller, O.J., Klein, G., Worst, P., Tachibana, T., 1969. Suppression of malignancy by cell fusion. *Nature*, 223:363-368.

39. Kaeffer, B., Bottreau, E., Marcon, D., Olivier, M., Lantier, I., Salmon, H., 1991. Histocompatible miniature pig (d/d haplotype): generation of hybridomas secreting A or M monoclonal antibody. *Hybridoma*, 10:739-744.

40. Kastan, M.B., Radin, A.I., Kuerbitz, S.J., Onyekwere, O., Wolkow, C.A., Civin, C.I., Stone, K.D., , Woo, T., Ravindranath, Y., Craig, R.W., 1991. Levels of p53 protein increase with maturation in human hemapoietic cells. , *Cancer Res.*, 51:4279-86.

41. Klein, G., 1987. The approaching era of the tumour suppressor genes. *Science,* 238:1539-1545.

42. Klobutcher, L.A., Ruddle, F.H., 1979. Phenotype stabilization integration of transferred material in chromosome-mediated gene transfer. *Nature*, 280:657-660.

43. Krishnan, S., 1997. Studies on Radioimmunolocalization of technetium-99m in murine lymphoma. *PhD thesis: University of Madras, Department of Biochemistry.*

44. Kuby, J., 1993. *Immunology, Second Ed.(Relevant Sections), W. H. Freeman and Company. New York, USA.*

45. Kume, K., Sawata, A., Nakase, Y., 1980a. Immunologic relationship between Page's and Sawata's serotype strains of *H. paragallinarum. Am. J. Vet. Res. ,* 41:757-760.

46. Kume, K., Sawata, A., Nakase, Y., Matsumoto, M., 1983. Serological classification of *H. paragallinarum* with a haemagglutinin system. *J. Clin. Microbiol.*, 17:958-964.

47. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685.

48. Larizza, L., Schirrmacher, V., 1984. Somatic Cell fusion as a source of genetic rearrangement leading to metastatic variance. *Cancer Metas. Rev.*, 3:193-222.

49. Larizza, L., Schirrmacher, V., Graf, L., Pfluger, E., Peres-Martinez, M., Stohr, M., 1984. Suggestive evidence that the highly metastatic variant ESb of the T-cell lymphoma Eb is derived from spontaneous fusion with a host macrophage. *Int. J. Cancer,* 34:699-707.

50. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., Tlsty, T.D., 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53.1 *Cell*, 70:923-935.

51. Maheswaran, S., Park, S., Bernard, A., Morris, J.F., Rauscher, F.J. III, Hill, D.E., Haber, D.A., 1993. Physical and functional interaction between WT1 and p53 proteins., *Proc. Nat. Acad. Sci. USA.*, 90:5100-104.

52. Marshall, C. J., 1991. Tumour Suppressor genes. *Cell*, 64:313-326.

53. Martin, D.W., Munoz, R.M., Subler, M.A., Deb, S., 1993. p53 binds to the TATA-binding protein-TATA complex. *J. Biol. Chem.*, 268:13062-7.

54. McNiel, C.A., Brown, R.L., 1980. Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc. *Nat. Acad. Sci. USA,* 77:5394-5398.

55. Moberg, K.H., Tyndall, W.A., Hall, D.J., 1992. Wild-type murine p53 represses transcription from the murine c-myc promoter in human glial cell line. *J. Cell. Biochem.*, 49:208-215.

56. Mosier, D.E., Gulizia, R.J., Baird, S.M., Wilson, D.B., 1988. Transfer of functional human immune system to mice with severe combined immunodeficiency. *Nature*, 335:256-259.

57. Mouahid, M., Bisgaard, M., Morley, A.J., Mutters, R., Mannheim, H., 1992. Occurrence of V-factor (NAD) independent strains of *H. paragallinarum*. *Vet. Microbiol.,* 31:363-368.

58. Nake, T., 1976. Outer membrane of *Salmonella typhimurium*: isolation of protein complexes that produce transmembrane channels. *J. Biol. Chem.*, 254:1457-1461.

59. Nicolson, G.L., Winkelhake, J.L., 1975. Organ specificity of blood-borne tumour metastasis determined by cell adhesion ? *Nature*, 255:230.

60. Porgador, A., Feldman, M., Eisenbach, L., 1989. H-2Kb Transfection of B16 Melanoma cells resulted in reduced tumourigenicity and metastatic competence. *J. Immunogen.*, 16:291-303.

61. Rampoldi, E., Larizza, L., Heltai, S., Erba, E., Ubezio, P., Shirrmacher, V., 1989. Cytologic and flow cytometric DNA analysis of multinucleated tumour cells and derived microcells. *Analytical and Quantitative Cytology and Histology.*, 11(1):59-66.

62. Rietschel, E.T., Brade, H., 1992. Bacterial Endotoxins: Molecular relationships between structure and activity. *Infectious Disease Clinics of North America*, 5(4):753-779.

63. Ringertz, M.R., Savage, R.E., 1976. Virus induced cell fusion: Mechanism of cell fusion. *In: Cell Hybrids, Academic Press, New York,* 29-60.

64. Rotter, V., Schwartz, D., Almon, E., Goldfinger, N., Kapon, A., Meshorer, A., Donhower, L.A., Levine, A.J., 1993. Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. *Proc. Nat. Acad. Sci. USA*, 90:9075-79.

65. Schulman, M., Wilde, C.D., Köhler, G., 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature*, 276:269-270.

66. Schumann, R.R., Leong, G.W., Flaggs, P.W., Gray, S.D., Wright, J.C., 1990. Structure and function of lipopolysaccharide-binding protein. *Science*, 249:1429.

67. Seto, E., Usheva, A., Zambetti, G.P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A.J., Shenk, T., 1992. Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proc. Nat. Acad. Sci. USA.*, 89:12028-32.

68. Sinkovics, J.G., 1981. Early history of specific antibody-producing lymphocyte hybridomas. *Cancer Res.*, 41:1246-47.

69. Sinkovics, J.G., 1985. Discovery of the Hybridoma principle in 1968-69:Immortalization of the specific antibody-producing cell by fusion with a lymphoma cell. *J.Med.*, 16:509-524.

70. Sinkovics, J.G., 1990. The earliest concept of the 'Hybridoma Principal' recognized in 1967-1968. *Front. Radiat. Ther. Oncol.,* Ed.:Vaeth, J.M., Meyer, J.L., *Karger, Basel,* 24:18-31.

71. Subler, M.A., Martin, D.W., Deb, S., 1992. Inhibition of viral and cellular promoters by human wild-type p53. *J. Virol.*, 66;4757-62.

72. Takagi, M., Hirayama, N., Makie, H., Ohta, S., 1991. Production, characterization and protective effect of monoclonal antibodies to *H. paragallinarum* serotype A. *Vet. Microbiol.,* 27:327-338.

73. Truant, R., Xiao H., Ingles C.J., Greenblatt, J., 1993. Direct interaction between the transcriptional activation domain of human p53 and the TATA box-binding protein. *J. Biol. Chem., 268*:2284-7.

74. Van Kessel, G.A.H., Den Boer, W.C., Van Agthoven, A.J., Hagemeijer, A., 1981. Decreased tumourigenicity of rodent cells after fusion with leucocytes from normal and leukemic donors. *Somatic Cell Genet.,* 7(6):645-656.

75. Venter, J.D., Gunter, N.J., Verheye, H.M., Lipinski, M.R., Verschoor, J.A., 1995. Monoclonal antibodies for immunoassay of the diet of chokka squid paralarvae., *Abstr. 13th Biochemical Society Congress, University of the Orange Free State.* April, 95.

76. Verschoor, J.A., Boshoff, C.H., Coetzee, L., Van Wyngaardt, S., Visser, L., 1990. Spontaneous fusion between splenocytes and myeloma cells induced by bacterial immunization. *Hybridoma*, 9:511-517.

77. Verschoor, J.A., Coetzee, L., Visser, L., 1989. Monoclonal antibody characterization of two field strains of *H. paragallinarum* isolated from vaccinated layer hens. *Avian Dis.*, 33:219-225.

78. Verschoor, J.A., Gunter, N.J., Boshoff, C.H., 1993. The induction of spontaneous hybridoma formation as a potential cancer chemotherapy., *Abstr. 12th Biochemical Society Congress, University of Stellenbosch., University of Stellenbosch.* January 94, 51.

79. Vogelstein, B., Kinzler, K., 1992. p53 function and dysfunction. *Cell*, 70:523-526.

80. Vogelstein, B., Kinzler, K., 1993. The multistep nature of cancer. *Trends Genet.*, 9:138-141.

81. Wang, B.S., McLoughlin, G.,A., Richie, J.P., Mannick, J.A., 1980. Correlation of the production of plasminogen activator with tumour metastases in B16 mouse melanoma cell lines. *Cancer Res.*, 40:288.

82. Weinberg, W.C., Azzoli, C.G., Kadiwar, N., Yuspa, S.H., 1994. p53 gene dosage modifies growth and malignant progression of keratinocytes expressing the v-rasHa oncogene. *Cancer Res.*, 54:5584-92.

83. Wiener, F., Fenyo, E.M., Klein, G., 1972. Fusion of tumour cells with host cells. *Nature New Biol.*, 238:155-159.

84. Windsor, H.M., Gromkova, R.C., Koornhof, H.J., 1991. Plasmid mediated NAD independence in Haemopilus parainfuenza. *J. Gen. Microbiol.*, 137:2415-21.

85. Woloschak, G.E., Senitzer, D., 1983. Splenocytes from NZB mice exhibit spontaneously high rates of fusion compared to control strains. *Cell. Immunol.,* 79:164-172.

86. Wright, S.D., Ramos, P.S., Tobias, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of LPS and LPS binding protein. *Science*, 249:1431.

87. Yamaguchi, T., Kobayashi, M., Masaki, S., Iritani, Y., 1993. Isolation and characterization of a *H. paragallinarum* mutant that lacks a hemagglutinating antigen. *Avian Dis.*, 37:970-976.

88. Yamaguchi, T., Blackall, P.J., Takigami, S. , Iritani, Y., Hayashi, Y., 1991. Immunogenesity of *H. paragallinarum* serovar B strains., *Avian Dis.*, 35:965-968.

89. Zhan, Q., Carrier, F., Fornace, A.J., 1993. Induction of cellular p53 activity by DNA-damaging agents and growth arrest., *Mol. Cell. Biol.*, 13:4242-50.