Biochemical Studies
of
Cystic Fibrosis Antigen

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I declare that:-

a) this thesis has been composed by myself.

b) the work is either my own or the worker/author involved is clearly stated.

In memory of my father.

Contents

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Abbreviations

A	absorbance
ALP	alkaline phosphatase
a_2M	$a_2$macroglobulin
AML	acute myeloid leukaemia
APM	aminopeptidase-M
bp	base pair(s)
BSA	bovine serum albumin
C	cross-linking
^oC	degrees centigrade
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFAg	cystic fibrosis antigen
CFP	cystic fibrosis protein
CIE	counterimmunoelectrophoresis
cm	centimetre(s)
CML	chronic myeloid leukaemia
CNBr	cyanogen bromide
ConA	concanavalin A
cpm	counts per minute
CRP	C-reactive protein
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunoabsorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
Fig(s)	figure(s)
FPLC	fast protein liquid chromatography
g	gravitational force
GGTP	ɣ-glutamyl transpeptidase
HAT	hypoxanthine-aminopterin-thymidine
hr	hour(s)
$^{125}_{I}$	^{125}iodine
I-ALP	intestinal alkaline phosphatase
IEF	isoelectric focusing
Ig	immunoglobulin(s)
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IRMA	immunoradiometric assay
IRT	immunoreactive trypsin
kd	kilodaltons
KSCN	potassium thiocyanate
kU	kilounits

l	litre(s)
Lf	lactoferrin
M	molar
mA	milliamps
mCi	millicurie
MES	2-(N-morpholino) ethane-sulfonic acid
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MNL	mononuclear leukocytes
mRNA	messenger ribonucleic acid
MUGB	methylumbelliferylguanidinobenzoate
MW	molecular weight
NADH	nicotinamide adenosine dinucleotide
nm	nanometres
NS	not significant
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pI	isoelectric point
PMNL	polymorphonuclear leukocytes
PON	paraoxanase
PVC	polyvinyl chloride

RA	retinoic acid
RFLP	restriction-fragment length polymorphism
RIE	rocket immunoelectrophoresis
RT	room temperature
^{35}S	^{35}sulphur
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second(s)
T	total
TEMED	N,N,N'N'-tetramethyethylenediamine
TRIS	[Tris(hydroxymethyl)aminomethane]
U	unit(s)
µg	microgram(s)
µl	microlitre(s)
V	volt(s)
vol	volume(s)
v/v	volume per volume
W	watt(s)
w/v	weight per volume

Abstract

Isoelectric focusing of serum on polyacrylamide gels shows a doublet band, known as cystic fibrosis protein, at an isoelectric point of 8.46±0.05, associated with expression of the CF gene.

An antiserum (anti-IEF) produced in guinea pigs against the portion of isoelectric focusing gel containing the cystic fibrosis protein showed elevated levels of a serum protein in cystic fibrosis homozygotes, intermediate levels in heterozygotes and low levels in healthy controls. Immunoassay of serum samples, using two different batches of anti-IEF, correctly identified 94% of CF genotypes.

Biochemical studies of the serum protein, identified by anti-IEF, showed it to have several different properties to those ascribed to cystic fibrosis protein. Therefore, the protein defined immunologically by anti-IEF was called cystic fibrosis antigen.

Granulocytes from cystic fibrosis homozygotes and heterozygotes, healthy controls and chronic myeloid leukaemia patients were shown to contain high levels of cystic fibrosis antigen in their cytosolic fraction. Immunisation of guinea pigs with a chronic myeloid leukaemia granulocyte lysate produced an antiserum (anti-CML) which recognised the same protein in CF serum as anti-IEF.

Somatic cell hybrids were produced from a mouse stem-cell line and chronic myeloid leukaemia granulocytes. Segregation analysis for the expression of cystic fibrosis antigen assigned the gene to chromosome 1.

Monoclonal antibodies were produced against cystic fibrosis antigen and a two-site sandwich enzyme-linked immunoabsorbent assay developed. Comparative immunoassays of cystic fibrosis antigen using polyclonal or monoclonal antibodies showed highly significant correlations with the same elevated levels of antigen as previously observed in the presence of the cystic fibrosis gene. In order to test whether the elevated cystic fibrosis antigen present in sera from cystic fibrosis homozygotes and heterozygotes was a consequence of granulocyte proliferation, a large series of samples including patients with non-CF respiratory disorders was tested. Simultaneous measurement was made of another granulocyte derived serum protein, lactoferrin, and the acute phase reactant, C-Reactive protein. Attempts to discriminate the cystic fibrosis homozygotes from the heterozygotes and the heterozygotes from the controls were unsuccessful, even when ratios of cystic fibrosis antigen to lactoferrin and C-Reactive protein were examined. The level of cystic fibrosis antigen in the heterozygotes was significantly elevated while, lactoferrin, C-Reactive protein and granulocyte levels were similar to those of the healthy controls suggesting that there is an association between cystic fibrosis antigen and the cystic fibrosis gene.

CHAPTER ONE

INTRODUCTION

Cystic fibrosis (CF) is a common genetic disease which presents a widespread health problem throughout the world. Continuing improvements in recognition and treatment of the disease has transformed CF from a fatal disease of early childhood to a chronic disorder persisting through childhood and into adult life. Patients usually present in the first year of life, with failure to grow normally, maldigestion and repeated severe respiratory infections. In some cases symptoms are milder and diagnosis may be delayed until later childhood or even into adult life. Although the course of the disease can be variable from patient to patient, increased life expectancy may be attributed to early diagnosis and improved medical treatment.

1.1. Clinical features.

(Goodchild and Dodge, 1985)

Most of the pathology observed in CF results from abnormalities in the mucous and serous secretions. The mucus is very sticky and leads to blockage of ducts, causing obstruction in a number of organs, particularly the lungs and pancreas. The serous seretions are abnormally concentrated and high levels of sodium and chloride are present in the sweat and saliva. Although clinically the least serious problem, elevated sweat sodium and chloride concentration is the undisputed indicator of CF.

The onset of respiratory symptoms may occur at any time after birth but frequently occurs before the end of the first year. Repeated infection causes progressive damage to the lungs. In infants Staphylococcus aureus is the most common pathogen of the respiratory tract, however, in later stages of the disease Pseudomonas aeruginosa, especially the slime producing mucoid variants, often become prevalent. In the upper respiratory tract, sinus infections and nasal polyps often occur. Advanced pulmonary disease is often accompanied by hyperinflation of the chest and accompanied by severe cough, purulent sputum, shortness of breath, weight loss and general loss of energy. The cause of death is frequently as a result of pneumonia, hypoxaemia, cor pulmonale and exhaustion as a result of the prolonged period of respiratory insufficiency.

Pancreatic insufficiency occurs in approximately 90% of patients. In untreated children, severe steatorrhea, poor weight gain and abdominal distension are characteristic clinical findings. Meconium ileus (intestinal obstruction) occurs in 10-15% of children at birth. Obstruction of the large and small intestine caused by fecal mucus plugs, may occur occasionally in later life.

Evidence of liver disease is common in CF although it rarely presents clinical problems. The incidence of intrahepatic biliary obstruction increases with increased age.

Abnormalities of the salivary glands are related to the mucus content of secretions. They show marked retention of secretions, dilation of acini and some fibrosis.

In CF patients growth is slow and skeletal and sexual maturation are often delayed. Most males are sterile due to early obstruction of the vas deferens and fertility is reduced in females. Hyperglycaemia is more common in older patients with CF than in the general population. The severe malabsorption associated with the disease can result in nutritional deficiencies related to fat soluble vitamins and trace elements.

1.2. Genetics and Distribution.

The incidence of CF in Caucasian populations has been estimated at 1/2500 from new-born screening data (Wilcken et al. 1983; Heeley, 1983), with a carrier rate of 1/25. In other ethnic groups the incidence of CF is much rarer (Talamo et al., 1983).

Autosomal recessive inheritance is indicated as the mode of inheritance. Much evidence supports the existence of a single mutant locus (Romeo, 1984). This is further confirmed by recent molecular biological studies (Beaudet et al., 1986). The high incidence of heterozygotes in Caucasians has led to the proposition that there is some heterozygote advantage which maintains this high incidence of the CF gene in these populations (Danks et al., 1965; Knudson et al., 1967). However no conclusive evidence has

been presented on this matter.

The CF gene has been assigned to chromosome 7 by co-segregation of the gene with linked polymorphic markers. A genetic variation in the serum level of the enzyme paraoxanase (PON) was initially identified as being linked to CF (Eiberg et al., 1985). A CF linked restriction-fragment length polymorphism (RFLP) was identified by L-C Tsui et al. (1985). Several other laboratories tested other chromosome 7 RFLP's and also found linkage to CF (White et al.,1985; Knowlton et al.,1985; Wainwright et al., 1985). The CF locus has been more specifically assigned to the long arm of chromosome 7, probably between 7q21 and 7q31 (White et al., 1985)

1.3. Prenatal Diagnosis.

Many attempts have been made to develop an accurate and reliable test for the prenatal diagnosis of CF. Early tests included the observation of metachromasia in cultured fibroblasts (Danes and Bearn, 1968), measurement of mucociliary inhibitor in amniotic fluid and cultured amniotic fluid cells (Bowman et al., 1973) and measurement of a-mannosidase in amniotic fluid (Hosli and Vogt, 1979a). Nadler and Walsh (1980) reported that titration of amniotic fluid supernatant against the artificial substrate 4-methylumbelliferylguanidinobenzoate (MUGB) showed significantly depressed values with CF homozygotes. This work, in both retrospective and prospective studies, showed affected fetuses with values of at least two

standard deviations below the mean for normals.

Examination of isoenzyme patterns of MUGB-protease after isoelectric focusing further improved this discrimination (Walsh and Nadler, 1980). However, other laboratories could not repeat these observations (Brock and Hayward, 1982; 1983). It seems that MUGB measures an esterolytic component of albumin and other plasma proteins (Branchini et al., 1982; Schwartz, 1982).

1.3.1. Microvillar enzymes in amniotic fluid.

In 1983 Carbarns et al. reported that the activities of two microvillar enzymes, ɣ-glutamyl transpeptidase (GGTP) and aminopeptidase-M (APM) were depressed in the amniotic fluid supernatant where the fetus had CF. This finding was soon confirmed (Baker and Dann, 1983) and the measurement of microvillar disaccharidases such as sucrase, lactase, maltase and trehalase were also considerably reduced in association with a CF fetus (van Diggelen et al., 1983). The levels of GGTP and APM have been further studied in control amniotic fluid and have been shown to decrease with advancing gestation (Brock et al., 1984). The distribution of values is not normal, therefore percentiles were required to identify the level at which a value could be considered abnormal. This means that prenatal diagnosis can only be performed accurately in individuals with a 1 in 4 risk of carrying a CF fetus. The level of the intestinal isoenzyme of alkaline phosphatase (I-ALP) in amniotic fluid was also observed to

be greatly reduced where the fetus had CF (Brock, 1985).

In routine diagnosis of CF four enzymes (GGTP, APM, I-ALP and a-glucosidase) are measured, optimally, at a gestation of 18 weeks.

In general there is concordance between peptidase, I-ALP and disaccharidase activities in amniotic fluid. However, Brock and Clarke (1987) have reported an unusual enzyme pattern in 7 out of a series of 260 high risk samples. In all cases a low value of GGTP accompanied a normal APM, a high normal I-ALP and a marginal a-glucosidase. All 7 pregnancies went to term and resulted in 5 live-born CF and 2 normal infants.

The low levels of microvillar enzymes observed in the majority of amniotic fluids where the fetus has CF are most likely due to a mechanical obstruction of fetal meconium (Kleijer et al., 1985). This explanation does not account for the 7 anomalous samples. The levels of the four enzymes have been measured in fetal meconium from 19 putative CF fetuses and 32 without CF all at a gestation from 18-21 weeks (Brock and Barron, 1986). In CF fetuses the levels of enzymes were significantly elevated. By comparing the ratios of enzymes in fetal meconium with the levels in amniotic fluid GGTP, APM and a-glucosidase are very similar at approximately 100:1, whilst I-ALP has a ratio of 1000:1. This may explain why I-ALP does not always correlate with the other three enzymes. The apparently normal level of I-ALP in a small number of CF amniotic fluids may be due to an intrinsic elevation of

the enzyme level present in the fetal meconium.

Extensive data has now been published on the diagnostic potential of peptidases (Brock et al., 1984; Brock, 1985; Muller et al., 1985; Aitken et al., 1985), isoenzymes of ALP (Brock, 1985; Brock et al., 1985; Muller et al., 1985) and disaccharidases (Kleijer et al., 1985; Szabo et al., 1985). A prospective series of 200 cases at 1 in 4 risk of CF indicates an accuracy of 98% (Boué et al., 1986).

1.3.2. CF-linked DNA Probes.

The identification of CF-linked DNA probes has enabled prenatal diagnosis for CF to be undertaken using fetal material obtained by chorionic villus sampling at a gestation of 8-10 weeks (Farrall et al., 1986). These linked probes are only useful for prenatal diagnosis in families with at least one CF child. DNA must be available from an affected child, in the family being tested, to establish phase relationships between the probes and the CF gene. With the use of several linked probes; metD, metH (White et al.,1985), J3.11 (Wainwright et al.,1985), 7c22 (Scambler et al.,1986a) and B79A (Estivill et al., 1986), the majority of families investigated are fully informative and all affected offspring can be identified. Data from a collaborative study of 211 families (Beaudet et al.,1986) showed that >95% of these families showed linkage to chromosome 7q markers. In some families, where all the markers on one parent's chromosomes are

homozygous, only the the other parent's affected chromosome can be identified. In these partly informative families 50% of the offspring will have a normal phenotype. The remaining 50% will have a 50% chance of being affected with CF. In these cases the families may be advised to opt for microvillar enzyme testing.

The DNA probes have been used to confirm the diagnosis of fetuses terminated on microvillar enzyme results (Curtis et al.) In some families the index case for CF may have died and no DNA is available, but material is available from a fetus terminated on the basis of microvillar enzyme results. In these cases prenatal diagnosis may be possible using the fetus as the index case. The false positive rate for microvillar enzymes is very low (<5%). In these few cases an error in the phase relationships betwen DNA markers and the CF gene will result in half the diagnoses being wrong and a quarter will lead to the birth of an affected child (Curtis et al.). However, in the remaining 95% of cases the diagnosis will be correct. If there are other non-CF siblings in the family then it may be possible to establish the phase relationships and confirm the fetal CF diagnosis.

Prenatal diagnosis is now widely available using DNA markers. However, it is only available to families where there is a 1/4 risk of CF and where DNA is available from an affected child.

1.4. Neonatal Screening.

Prenatal testing for CF can only be undertaken in families where there is already a CF child. Therefore, there are still many children born with CF who could not be identified by any of the available prenatal tests. For these children early diagnosis and treatment may be important, and improve their overall prognosis, although no hard evidence is available on this observation.

For many years the only method of neonatal screening available involved the measurement of albumin in meconium of newborn infants (Harries, 1978; Antonowicz and Schwachman, 1979). A raised level of albumin indicated a reduced level of pancreatic enzymes.

More recently measurement of immunoreactive trypsin (IRT) at 5 days after birth using whole blood spots has been used (Crossley et al., 1979; Heeley et al., 1982). This method, particularly if followed by a repeat test two weeks later, is a very efficient screening test. Infants born with meconium ileus tend to have normal levels of immunoreactive trypsin in their serum (Duhamel et al., 1984), however, these children are considered for possible CF diagnosis because of this disorder. Individuals with repeated high levels of IRT have their diagnosis confirmed by sweat testing, usually at 3-6 weeks after birth. IRT screening is very successful in early diagnosis of CF. However, it is not used in routine screening as intended, mainly because of the expense, but is reserved for infants with a family history of CF or showing clinical symptoms.

The CF-linked DNA probes may be of use in confirmation of diagnosis, especially in siblings of CF children where the IRT and/or sweat test results are marginal.

1.5. Heterozygote Detection.

For many years many laboratories have been searching for a heterozygote detection test for CF. The research has been characterised by a large number of experiments which have revealed opposite results in different laboratories.

Requirement for heterozygote detection can be divided into two situations either for testing of individuals in families where the CF gene has already been identified or else in the general population. In the former case the aim would be to exclude heterozygosity of individuals whose risk of heterozygote status is either greater than in the general population (siblings of affected individuals) or those who wish to have children, but whose partner is either a confirmed heterozygote (having already borne a child with CF) or homozygous for CF.

If a heterozygote test is to be used to segregate out from a large general population then the test must use an easily available tissue and be simple and rapid to perform.

1.5.1. Cell abnormalities.

Many abnormalities have been described in CF cells (Mangos and Boyd, 1984). However, the majority of reported observations have not included any data on heterozygous individuals.

Several heterozygote tests using cultured skin fibroblasts have been described. Shapiro et al. (1979) observed that the measurement of mitochondrial NADH dehydrogenase over a pH profile provided an indication of genotype. In CF patients highest activity was at pH 8.6,in heterozygotes at pH 8.3 and in normal controls at pH 8.0. Sanguinetti-Briceno and Brock (1982) have been unable to confirm these observations. Measurement of this enzyme in white blood cells and cultured lymphocytes also showed no effect of pH.

Breslow et al. (1978) demonstrated that when fibroblasts were exposed to low concentrations of dexamethasone, heterozygote cells survived for an intermediate time when compared with CF homozygotes and normals. However, at all concentrations of dexamethasone a significant overlap was observed. By simultaneously measuring sodium transport in the presence of ouabain and the dexamethasone resistance Breslow et al. (1981) could identify all three genotypes correctly. These observations could not be repeated and were withdrawn (Breslow and McPherson, 1981).

Reduced β-adrenergic responses have been demonstrated in granulocytes (Gallant et al., 1981) and in lymphocytes,

granulocytes and in the cardiovascular system of CF patients (Davis et al., 1983). In leukocytes this defect appeared at an intermediate level in CF heterozygotes, suggesting an inherited defect and not a secondary effect of disease. The basal levels of cyclic adenosine monophosphate (cAMP) were the same in both CF homozygote, heterozygote and normal lymphocytes and granulocytes, but the levels in response to isoproterenol (10^{-8}M) were significantly reduced in both cells, although there was overlap between all three groups. It was suggested that these observations indicated that the β-adrenergic receptors are normal in CF granulocytes and lymphocytes,but there is an abnormal inheritable defect in the receptor-cyclase coupling of the B-adrenergic system.

1.5.2. Bioassays of CF factors.

Many abnormalities have been described, in bioassay systems, which are caused by a substance present in the serum or secretions of CF homozygotes and heterozygotes. These substances have become collectively known as CF-Factors.

The effect of sera and other biological fluids on the sodium-dependant glucose transport system has been studied. Several laboratories (Araki et al., 1975; Gilmore et al., 1978; Tucker et al., 1979) reported a bioassay using rat jejunum which identified a diminished short circuit current with CF homozygote and heterozygote sera,

in the presence of glucose, compared with control sera. These observations showed a large overlap in all the studies. When rat brush-border vesicles were substituted for jejunum no differences were observed (Will et al., 1979), suggesting that the sodium transport inhibition detected may be tissue specific.

Rabbit tracheal assay and oyster gill assay have been the main tissues used in the assay of substances in CF homozygote and heterozygote sera which inhibit the motility of cilia. Fresh water mussel (Besley et al., 1969) and mouse sperm (Pivetta et al., 1979) have also shown similar inhibition.

Spock et al. (1967) were the first to show that CF homozygote and heterozygote sera contained substances which inhibited the motility of rabbit tracheal cilia. The material was shown to be heat labile, non-dialysable and precipitated with the euglobulin fraction of serum. Later this was identified as a substance with a molecular weight (MW) of 1000-10000 daltons which was associated with IgG (Conod et al., 1977). Others have confirmed that CF homozygote and heterozygote sera contain a ciliary dyskinesia factor bound to IgG (Wilson and Fudenerg, 1977a). This factor could be isolated from IgG by protein-A sepharose chromatography (which bound IgG and the factor) then eluting at an acidic pH (Wilson and Fudenberg, 1978). This acid-stable protein of MW 4000-6000 was also found in other biological fluids and could produce ciliary dyskinesia without IgG.

Initial work indicated that ciliary dyskinesia was specific to CF homozygote and heterozygote fluids (Spock et al., 1967). Later it was shown that sera from patients with asthma, pulmonary and autoimmune diseases could also cause ciliary dyskinesia in the rabbit tracheal assay (Conover et al., 1976). The factor in asthmatic patients' sera could be differentiated from the 4000-6000 MW factor in CF by chromatography on DEAE-cellulose followed by Sephadex G200 gel filtration (Wilson and Fudenberg, 1977b).

Phytohaemagglutinin stimulated lymphocytes, lymphoblastoid cell lines and fibroblasts from CF homozygotes and heterozygotes were shown to secrete a 1000-10000 MW substance that requires IgG to cause ciliary dyskinesia in the rabbit tracheal assay (Conover et al., 1976) Three low MW substances were also secreted from lymphocytes, lymphoblastoid cell lines and monocyte/macrophages which did not require IgG (Wilson and Fudenberg, 1978). Purification and culture of sub-populations of leukocytes showed that ciliary dyskinesia factors were secreted by T-lymphocytes and monocytes, but not by B-lymphocytes or neutrophils (Wilson and Bahm, 1980).

A glycopeptide of MW 5000, which caused ciliary dyskinesia, was purified by Blitzer and Shapira (1982a). Monoclonal antibodies were produced against this factor Blitzer and Shapira, 1982b), but none showed any immunological differences when tested against CF

homozygote, heterozygote or control sera.

A mucociliary factor was found in the sera from CF homozygotes and heterozygotes, but not in sera from controls including sera from bronchial asthmatics, by the oyster gill assay (Bowman et al., 1969).This substance was heat labile, cationic and associated with IgG (Bowman et al., 1970). A substance was purified from CF homozygote and heterozygote plasma which could be dissociated from IgG with 6M guanidinium chloride (Carson and Bowman, 1982). IgG was not required for ciliary diskinesia in the oyster gill assay. A factor was also found in fibroblast medium (Bowman et al., 1973b) and urine (Bowman et al., 1977) from CF homozygotes and heterozygotes. The factor present in urine has been purified and has a MW 4000-13000 (McNeely et al., 1982).

Several of the body fluids which have caused inhibition of ciliary motility have also induced mucus production in biological assays. Isolated acini from the rat submandibular gland have also been observed to increase secretion of glycoproteins in the presence of CF homozygote and heterozygote sera (Fleming and Sturgess, 1981; Macpherson et al., 1983).

The difficulties encountered in repetition of results in all the CF factor bioassays has led to a great deal of doubt about the observed properties of these factors and even their existence.

1.5.3. Lectins.

A lectin-like substance found in CF homozygote and heterozygote serum has been assayed by a haemagglutination assay using mouse erythrocytes (Lieberman et al., 1979). The lectin of MW 3,500-10,000 was bound to IgM but could be isolated on a fructose-sepharose column (Nordstrom et al., 1980). The assay was very variable using whole serum, but modification of the assay using the IgM fraction of sera (isolated by gel filtration) was more reproducible (Lieberman et al., 1984). In a 'blind' assay 95% of CF homozygotes, 89% of heterozygotes and 5% of controls were identified as having an elevated lectin titre. No other laboratories have reported repetition of this assay.

1.5.4. Arginine esterases.

The cascade enzymes involved in coagulation, kallikrein-kinin system and fibrinolysis have been studied in relation to CF. An excess or deficiency of enzyme activity was thought a very likely candidate for the defect in CF.

Arginine esterase activity was shown to be diminished in chloroform-ellagic acid treated plasma in CF homozygotes compared with normals (Rao and Nadler, 1974). This observation was confirmed by some investigators (Coburn et al., 1974; Chan et al., 1977), but not by others (Lieberman, 1974; Goldsmith et al., 1977). Further work demonstrated a deficiency using the artificial substrate MUGB (Walsh-Platt et al., 1979). This work has not been repeated, and in view of the substrate being

shown to measure esterolytic activity of serum proteins the results probably reflect the generally increased level of proteins in CF serum.

1.5.5. Protease inhibitors.

Many workers have studied the protease inhibitor a_2-macroglobulin (a_2M). Several abnormalities have been reported but most of these have been contradicted in other reports.

Shapira et al. (1976a; 1976b) observed normal levels of a_2M in the serum and plasma of CF patients, obligate heterozygotes and normal individuals. However, these studies showed absence of the a_2M-protease complex in CF homozygotes with an intermediate level in heterozygotes. The a_2M seemed to be antigenically similar in all three genotypes. There appeared to be a 40% reduction of sialic acid content and defective binding to Concanavalin A (Con-A) and wheat germ agglutinin, suggesting a defect in the carbohydrate residues of the protein (Ben-Yoseph et al., 1979).

In contrast other studies have shown normal trypsin binding (Parsons and Romeo, 1980), normal 2-dimensional electrophoresis patterns (Comings et al., 1980), increased trypsin binding (Schidlow and Kueppers, 1980) and increased Con-A binding (Shapira and Menendez, 1980) in CF homozygotes and heterozygotes.

A monoclonal antibody which recognised a structural variation in a_2M has been described (Eager and Kennet,

1984). The antibody showed significantly reduced binding to purified a_2M from 4/5 CF homozygotes,3/4 obligate heterozygotes compared with 13 normal controls. They hypothesised that the reason for this was either a decreased number of antigenic determinants on a_2M or an altered antigenic site.

a_1-antitrypsin, another potent protease inhibitor, has been studied extensively (Talamo et al., 1976). However, no genetic, quantitative or functional abnormalities have been identified, although increased levels are observed in CF homozygotes with increased age and progression of chronic pulmonary disease.

1.5.6. Hydrolases.

Hosli and Vogt (1979b) reported that heat inactivation of plasma a-mannosidase and acid phosphatase could be used to differentiate all three CF genotypes. The two enzymes retained nearly all their activity in normals (80-100%), 40-60% in heterozygotes and 0-10% in CF homozygotes. There was no overlap between the groups. A "blind" trial reported by Katznelson et al. (1983) showed that 45 plasma samples collected in Israel and tested by Hosli and colleagues were all correctly genotyped. Despite the apparent simplicity of this assay other laboratories have failed to reproduce these results (Harris et al., 1979; Patrick and Ellis, 1979).

1.5.7. Other Proteins.

One of the most repeatable biochemical methods reputed to track the CF gene was the identification of an abnormal doublet band after polyacrylamide gel isoelectric focusing of serum from CF homozygotes and heterozygotes (Wilson et al., 1973). This observation was the starting point for the work described in this thesis and is explained in detail in Chapter 2.

1.5.8. DNA Studies.

DNA-linkage studies on the siblings of CF homozygotes has enabled their heterozygous status to be determined. In some cases it has been possible to use linkage analysis to exclude heterozygosity in individuals who are at a higher risk of carrying the CF gene than in the normal population.

Brock et al.(1986) have described a case study where a couple enquired about prenatal diagnosis of CF. The husband's brother was married to his wife's sister and they had produced a child with CF. Therefore the couple's risk of having a CF child was 1/16, which is low for microvillar enzyme testing. By DNA typing the extended family it was possible to show that the 'at risk' wife was not a carrier of the CF gene, whilst the husband had a 50% risk. Even allowing for maximum recombination of 3% their risk of having a CF child was now reduced to 1/135. Even though this was an unusual family it illustrated that in some cases it is possible to identify heterozygote status

in individuals who are not siblings of CF homozygotes or parents of a CF child.

In a recent report Estivill et al. (1987) have described 2 new DNA probes (XV-2c and CS.7) which were derived from cosmid libraries constructed from mouse/human cell lines. These cell lines were produced using chromosome-mediated gene transfer (Scambler et al., 1986b) and were shown to contain the markers most closely linked to CF.

Probe XV-2c detects an RFLP at a frequency of 0.47 for allele 1 (2.1kb) and 0.53 for allele 2 (1.4kb) in Taq1 digests. Probe CS.7 detects an RFLP at a frequency of 0.56 for allele 1 (0.67kb) and 0.44 for allele 2 (0.47kb) in Hha1 digests. No recombination with CF was observed in 6 informative families showing crossover between CF and J3.11 or 7c22 and/or B79A. The order of loci is thus 7qter-J.311-(CF/CS.7/XV-2c)-met-7c22-B79a-7cen.

In 195/213 chromosomes studied the CF mutation was associated with probe XV-2c allele 1 and in 70/71 chromosomes with probe CS.7 allele 2. This suggested that the CF was closer to CS.7 than to XV-2c. One family studied, in which the CF chromosome was associated with the probe CS.7 allele 1, was the only crossover family in which XV-2c, met and 7c22 recombined with CF. The reason for this anomalous result was not clear.

A weak non-random allelic association was demonstrated in J3.11/CF and met/CF haplotypes (Beaudet et al., 1986). The markers CS.7 and XV-2c showed strong

linkage disequilibrium both for normal and CF chromosomes. In the families studied (excluding the CS.7 allele 1 associated family) 94% of CF chromosomes were XV-2c allele 1 and CS.7 allele 2, wheras only 34% of normal chromosomes showed this haplotype. Therefore, it was concluded, that an individual in a normal population who did not have this haplotype would be at a greatly reduced risk of carrying the CF gene compared with the the risk of 1/20 in the total population. Alternatively an individual who was homozygous for the haplotype would have an increased risk of 1/7 of being a carrier.

The assesment of risk for carrier status is particularly important in families where one partner either has a high prior risk or is known to be a carrier of the CF gene. In these couples, a low risk of carrier status will reduce risk of having a CF child to levels closer to that in the general population.

CHAPTER TWO

AIMS

The work described in this thesis was part of a large research programme undertaken by the Cystic Fibrosis Research Trust/Medical Research Council Research Group on Cystic Fibrosis, in Edinburgh.

The purpose of this project was to assess the potential clinical usefulness of reported biochemical methods of CF heterozygote detection.

Wilson et al. (1973) had reported an abnormal doublet band observed, after isoelectric focusing (IEF), in CF homozygote and heterozygote serum. In this laboratory, work had started on the production of a polyclonal antiserum against the portion of gel containing the doublet band (Manson and Brock, 1980). A limited quantity of antiserum was available which showed apparent quantitative differences in serum, dependent on the CF genotype. However, the titre and quality of this antiserum was extremely variable and therefore, no large scale serum testing could be undertaken.

The aims were; a) to repeat the observations made by Wilson et al. (1973), b) to produce a sufficient quantity of a consistent quality, high titre, antiserum to test a large number of serum samples and confirm the potential of using this assay in detecting CF heterozygosity and then c) to produce monoclonal antibodies against the protein identified by the polyclonal antiserum.

Once specific monoclonal antibodies were identified and a sensitive assay system devised it was hoped that measurement of the protein in serum would be simple, highly reproducible and suitable for use in CF heterozygote detection in the general population.

CHAPTER THREE

ISOELECTRIC FOCUSING

3.1. Introduction.

3.1.1. Principles.

Isoelectric focusing (IEF) is electrophoresis in a continuous stable pH gradient. The separation depends on the isoelectric point (pI) of the protein. Once a protein has reached its pI position it has no net charge and therefore can migrate no further. In this process there is a concentrating effect which produces sharp, stable protein zones.

The production of a stable linear pH gradient depends on the ampholytes. They must have a high buffering capacity to overcome any influence of the proteins themselves. They must also have an even conductance, especially at their own pI's, to maintain a flow of current through the system. These carrier ampholytes comprise many isomers and homologues of aliphatic polyamino-polycarboxylic acids with different pI's depending on the pH range required. The ampholyte pH gradient is formed when an electric field is applied. The molecules with lowest pI (highest negative net charge) will migrate towards the anode. When they reach a position where the net charge is zero, they will stop migrating, adjacent to the anode, and because of their high buffering capacity, these molecule will impart to the surrounding solution a pH equal to its own pI. All the carrier ampholyte molecules arrange themselves in series between the anode and cathode electrodes, in increasing order of

pI, and thus produce a pH gradient increasing from anode to cathode.

The pH gradient formed can be influenced by addition of substances, such as 4M Urea, which can alter the pI of proteins by several points of a pH unit. Urea is used as a solubilising and dissociating agent. High concentrations disrupt the hydrophobic bonds of proteins and cause them to unfold. Therefore the pI of a protein in the presence of urea cannot be correlated with that obtained in an aqueous environment.

The use of high power increases the speed of formation of the gradient and therefore the separation time. Adequate cooling is necessary and a temperature of $4^{o}C$ is recommended.

3.1.2. IEF of serum samples.

In 1973 Wilson et al. demonstrated, by IEF, a band in serum at pI 5.48 which was detected in control and CF heterozygotes, but not in CF homozygotes, whereas a band at pI 8.41 was demonstrated in CF homozygotes and heterozygotes but not in normal controls. Later studies observed the pI 5.48 band present in more than half of all serum samples tested. This was susequently identified as an a_2M fragment generated by proteolytic cleavage. (Wilson & Fudenberg, 1976). The detection of the pI 8.41 band in CF homozygotes and heterozygotes and occasional normals was reported by several laboratories, (Scholey et al., 1978; Nevin et al., 1981; Hallinan et al., 1981; Brock et

al., 1982; Grataroli et al., 1984; Super and Swindlehurst, 1984; Jamieson et al., 1985 and Getliffe et al., 1986) whilst other workers failed, (Smith et al., 1976; Thomas et al., 1977). Modifications of the IEF technique showed this band as a doublet at pI 8.46 ± 0.05. Wilson laid out strict methods of collection, storage and processing of sera for correct identification of this abnormal band, which he called cystic fibrosis protein (CFP). (Wilson et al., 1977; 1979).

3.2. Methods.

3.2.1. Serum samples.

Blood was collected from 23 CF homozygotes, 22 CF heterozygotes and 16 healthy controls, into glass tubes (Seward). The samples were immediately cooled to 4°C and allowed to clot for 4-6 hr before centrifugation at 4°C for 10 min at 600g. The serum was aliquotted into plastic tubes and stored frozen at -70°C until required. Each aliquot was electrofocused immediately after thawing and the excess discarded.

IgG levels in serum samples were quantitated in the Clinical chemistry laboratory, Western General Hospital, Edinburgh.

3.2.2. Preparation of gel and running conditions for IEF.

IEF was performed using the LKB Multiphor apparatus. 2mm thick gels were used with acrylamide / bisacrylamide composition T = 5%, C = 3%.

Each gel contained :

9ml acrylamide / bisacrylamide
(33g/1g in 100ml of distilled water)
50ml of urea (freshly prepared)
(12.5g made up to 50ml in distilled water)
2.9ml Ampholine pH 3.5-10 (LKB)
0.1ml Ampholine pH 2.5-4 (LKB)
0.2ml of 10% ammonium persulphate
0.3ml of 5% TEMED (N,N,N,N-tetramethylethylenediamine)

After polymerisation at room temperature (RT) for 1 hr the gel was placed at 4°C overnight. Before electrofocusing of samples, the gel was prefocused. The electrofocusing strips were soaked in electrode solutions, cathode: 5% v/v TEMED ; anode: 3% v/v phosphoric acid, and placed on the gel in contact with the electrodes. The gel was prefocused for 1 hr at 40mA on a cooling plate at 4°C. The strips used for prefocusing were replaced with fresh soaked strips before the samples were applied to 5x10mm pads of Whatman 3MM filter paper 10mm from the anode electrode strip. Undiluted serum samples, each containing 300µg of IgG , were applied together with known positive and negative controls. IEF was performed at constant power 38W, with maximum voltage 1200V for 1.5 hr at 4°C.

3.2.3. Gel staining.

The gel was fixed and stained in a solution containing 45g trichloracetic acid, 13.5g sulphosalicylic acid and 0.45g Coomassie Blue in 135ml of methanol and 280ml of distilled water. The gel was placed in this stain in a water bath at a temperature of 80°C, in a fume cupboard, and allowed to cool to RT, before being washed in distilled water to remove excess stain. Destaining was achieved by repeated washing in ethanol/distilled water/acetic acid (6/13/1 v/v). The presence or absence of the doublet band could be ascertained by observing the

gels through an orange filter and comparing with the known positive and negative controls. The protein pattern of IEF of normal, CF heterozygote and CF homozygote serum is shown in Fig 3.1. The position of the doublet band is indicated by the two bars.

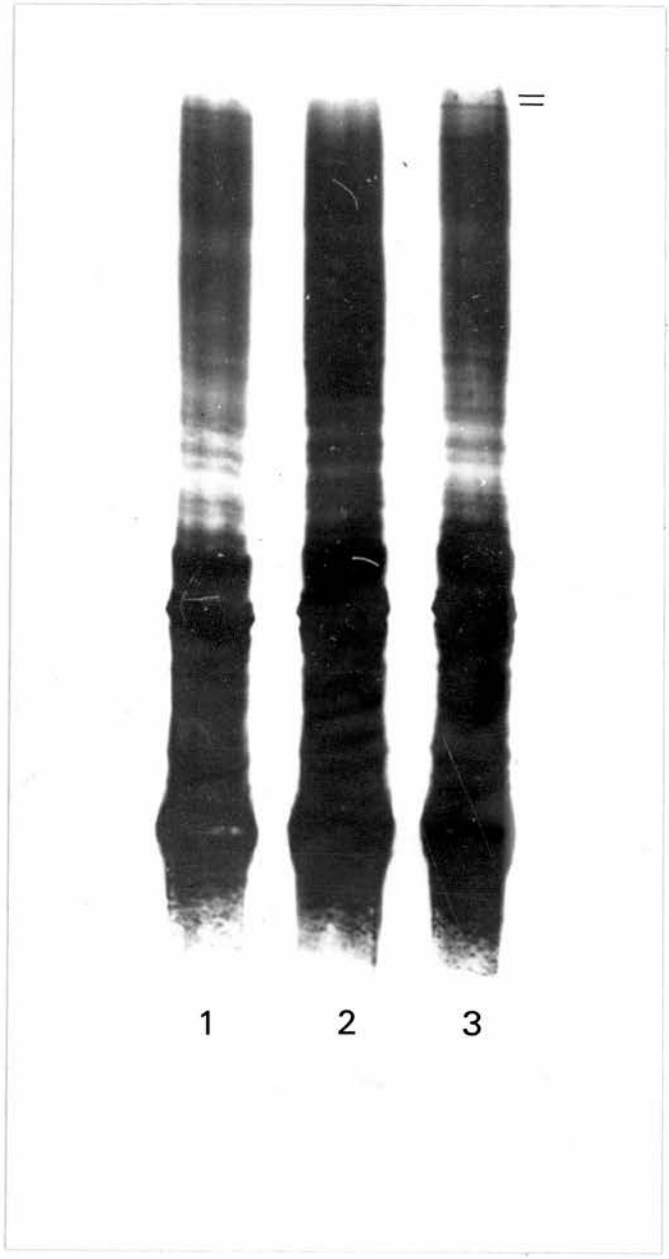


Fig.3.1. IEF of serum samples.

Track (1) CF heterozygote; (2) healthy control; (3) CF homozygote. The two bars indicate the position of the doublet band.

3.3. Results.

3.3.1. Qualitative analysis of CFP.

Three "trained" observers scored the serum samples (without being aware of donor status) by comparison with one positive and one negative control on each gel. The gel was scored with a value of 2 for a definite, 1 for a doubtful and 0 for an absent doublet band. Each sample was run on a minimum of two seperate gels allowing for six observations. Samples scored variably by the different observers were run up to 8 times

The average score for each sample was derived by summing the individual scores and dividing by the number of observations. Samples which were scored variably by different observers were run repeatedly until concordance was achieved. Since the observers were not aware of the source of the samples it is unlikely that this introduced any systematic bias towards definite, doubtful or absent scores. An average score of greater than 1 indicated a positive result. 19 out of 23 CF homozygotes, 16 out of 22 CF heterozygotes and 3 out of 16 controls showed such a score. The mean and standard deviations of the average scores are shown in Table 3.1. By using the Student t-test, the mean values of CF homozygotes and CF heterozygotes showed no significant difference, whilst the differences between CF homozygotes and controls and between CF heterozygotes and controls were highly significant ($p<0.001$).

Table 3.1. Scoring of IEF samples.

Group	Number	Mean and *SD.
Controls	16	0.45 ± 0.48
CF heterozygotes	22	1.22 ± 0.44
CF homozygotes	23	1.45 ± 0.50

*SD., standard deviation from the mean.

3.4. Discussion.

3.4.1. CFP analysis in other laboratories.

The results of this study indicate that this method is not reliable enough to distinguish the presence of the CF gene in the sample tested. The majority of samples are correctly identified but the misdiagnosis of 19% of the normal controls, 27% of CF heterozygotes and 17% of the CF homozygotes is unacceptably high for use of this test as a heterozygote detection test in a normal population. Several other laboratories have attempted to identify this protein by isoelectric focusing with varying degrees of success. These results are summarised in Table 3.2. The identification of the protein in CF homozygotes ranges from 26% to 100%, CF heterozygotes from 13% to 90% and normals from 0 to 23%. All these laboratories followed the Wilson method of blood collection in the first instance. Hallinan et al. (1981) were the least successful and despite following Wilson's IEF method meticulously only identified 26% of CF homozygotes and 13% of CF heterozygotes. They then pre-treated samples in various manners including pre-incubation at 56°C, 37°C and 4°C, in the presence and absence of protease inhibitors, EDTA and hydrochloric acid and also collected blood into plastic tubes, and anticoagulants heparin, citrate and EDTA. All these modifications had no improving effect on their detection of CFP.

Wilson et al. (1984) compared the detection of CFP in

Table 3.2. Isoelectric focusing of CFP in other laboratories.

			Percentage of samples correctly classified		
			CF	HZ	N
Wilson et al.	1975	serum	97	93	9
Scholey et al.	1978	serum	70	ND	0
Nevin et al.	1981	serum	90	83	8
Hallinan et al.	1981	serum	26	13	8
Brock et al.	1982	serum	83	73	19
Wilson et al.	1984	serum	100	86	12
Wilson et al.	1984	heparinised plasma	100	90	4
Grataroli et al.	1984	serum	71	71	20
Subset of serum samples of which heparinised plasma was also collected.			75	78	40
		heparinised plasma	92	67	20
Super & Swindlehurst	1984	serum	92	88	8
Jamieson et al.	1985	serum	79	44	23
Getliffe et al.	1986	serum	91	88	8

serum and heparinised plasma. Despite initially insisting that the method only worked using carefully collected serum the results are almost identical for the two sets of samples with slightly increased identification of CF heterozygotes (4%) and decreased positivity in the normal controls from 12% in serum to 4% in heparinised plasma. Grataroli et al. (1984) published a smaller series with similar results. Jamieson et al. (1985) collected samples in the method described by Wilson but then modified the IEF technique significantly and used Pharmacia ampholytes which are a different mixture compared with those from LKB. They claim to have identified CFP by this method, but were less successful than most others in discriminating normals from carriers of the CF gene. They further changed the method by silver staining which identified even more CFP positive normals. They concluded that CFP was a "normal serum protein showing quantitative variation dependant primarily on the CF allele".

Despite the technical difficulties encountered in IEF of serum to identify CFP, the general consensus is that there is a protein present in serum which seems to be associated with the CF gene.

CHAPTER FOUR

POLYCLONAL ANTIBODIES

4.1. Introduction.

4.1.1. Polyclonal antibodies to CFP.

An antiserum to the doublet band defined as CFP (Wilson et al., 1977b; 1979) was first produced by Manson and Brock (1980). This antiserum was raised in guinea pigs by immunising with the excised, pH 8.46, portion of an IEF gel, previously homogenised in phosphate buffered saline (PBS). The antiserum produced, after absorption with CFP negative sera, was suitable for use in rocket immunoelectrophoresis (RIE) and showed a quantitative difference between CF homozygotes and CF heterozygotes as well as between CF heterozygotes and healthy controls, which appeared negative. Later that year Wilson (1980) reported an antiserum which was produced in mice. This antiserum was absorbed with CFP negative sera and then tested by counterimmunoelectrophoresis (CIE). The precipitin line for CF heterozygote serum was closer to the antigen well than for CF homozygote serum, indicating a lower level of antigen in CF heterozygotes. RIE using this antiserum showed a quantitative difference similar to that described by Manson and Brock (Wilson, 1982). The identity of the antiserum produced by Manson and Brock compared with that produced by Wilson is unknown.

4.2. Methods.

4.2.1. Preparation of IEF gel as immunogen.

6 CFP positive sera from CF homozygotes were pooled and run on IEF gels as described previously. A single sample lane from each gel was cut out and stained according to the protocol described. The doublet band was identified and the portion of unstained gel, pH 8.4-8.5, was excised and homogenised in 1ml of PBS for each gram of gel. Homogenisation, although difficult, was achieved by repeated passage through diminishing sizes of needle, using a glass syringe, until the mixture could pass through a 23 gauge needle.

4.2.2. Immunisation of guinea pigs and antisera testing.

The immunisation schedule was as described by Manson and Brock (1980), using the same inbred colony of guinea pigs. Initial immunisation was with 1ml of homogenate intramuscularly in several sites. At four week intervals 2ml of homogenate was injected intraperitoneally and from the sixth week the guinea pigs were bled weekly by cardiac puncture. The blood was clotted overnight at $4^{o}C$ and the serum removed. Before testing, small volumes of the antiserum were absorbed with increasing volumes of a pool of 6 CFP negative serum samples. The antiserum was then tested, at varying concentrations from 20%-50% by RIE, using a pool of 6 CF homozygotes as the positive control and a pool of 6 normals as the negative control. The

buffer system used was as described by Laurell and McKay (1981) containing 75mM sodium barbitone, 16mM barbitone and 1.4mM calcium lactate (pH 8.6). The 1% agarose gels were run overnight at 1 V/cm with cooling. All antisera showing a positive reaction with CF homozygote serum and a negative result with normal serum were pooled, absorbed and retested against a panel of serum samples. This antiserum was referred to as anti-IEF.

A large series of sera were tested against anti-IEF (Bullock et al., 1982). In this series the antigen level was quantitated by measuring the area under the precipitin peak. The sera were tested against two batches of antisera (AS60 and AS82/83) produced at an interval of more than a year.

These same batches of antisera were also used as the basis of an immunoradiometric assay (IRMA). Test sera of different dilutions, up to 1/50 in 0.05M phosphate buffer pH 7.5, were coated onto plastic tubes and incubated overnight at RT. The tubes were then blocked with PBS containing 5% horse serum to saturate binding sites. Guinea pig antisera were diluted up to 1/20,000 in PBS and incubated in the coated tubes overnight. In order to detect the binding of the guinea pig Ig to antigen, all tubes were then incubated with an equal quantity of ^{125}I Protein A (35,000cpm) (Amersham). The bound radioactivity was expressed as a percentage of the total counts added to each tube.

4.2.3. Immunodepletion of anti-IEF rockets.

Serum samples were run by RIE through a high concentration of several commercially available polyclonal antisera before reacting with anti-IEF, to establish that the antiserum was not against a common known protein. The principle of this technique is illustrated schematically in Fig 4.1.

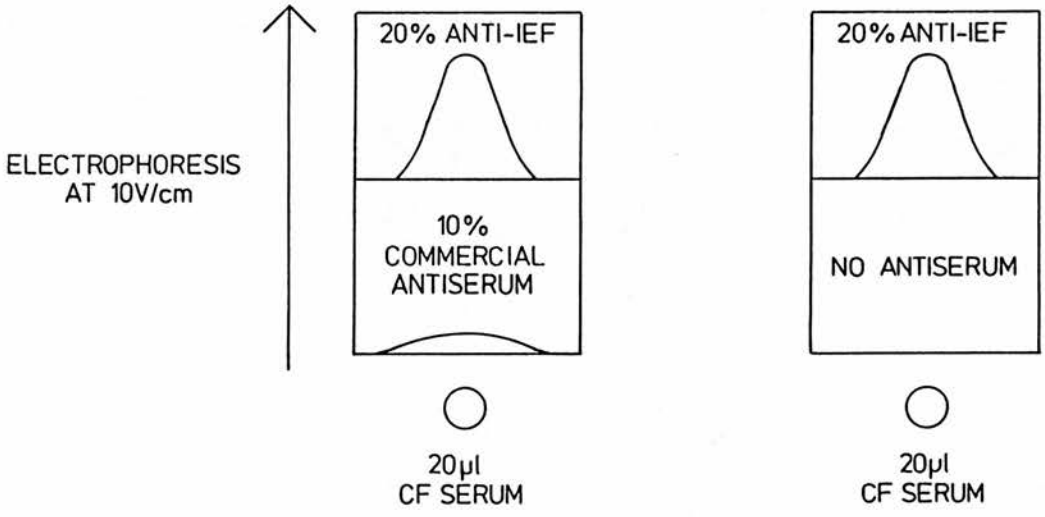
CF homozygote serum was also tested for depletion with Protein A Sepharose CL-4B. 100µl of a 10% w/v mixture of Sepharose CL-4B was incubated with 20µl of serum, for 1 hr, to remove the proteins which bind to protein A. IgG is the main serum protein to bind, but IgA and IgM will bind to a lesser extent. 100µl of Protein A sepharose will bind approximately 1mg of IgG, which is approximately double the maximum amount present in 20µl of CF serum (CF homozygote range : 13.85 - 21.81 mg IgG/ml serum, Schwartz, 1966). The serum/sepharose mixture was centrifuged and the supernatant run by RIE against anti-IEF.

4.2.4. Gel filtration of serum.

The molecular weight of the protein detected by anti-IEF in serum was estimated by Sephacryl S300 (Pharmacia) gel filtration. Sephacryl S300 is a covalently cross-linked dextran with N,N-methylene bisacrylamide giving a rigid gel with a controlled range of pore sizes and a fractionation range for proteins of MW $1x10^4$-$1.5x10^6$.

Fig.4.1. Immunodepletion of the anti-IEF rocket produced from CF serum, using commercially available polyclonal antisera.

A commercial antisera is used to deplete specific proteins from a CF serum pool. Any antiserum which cross-reacted with the protein detected by anti-IEF would show reduction or elimination of the anti-IEF rocket.


ELECTROPHORESIS
AT 10V/cm
20% ANTI-IEF
10%
COMMERCIAL
ANTISERUM
20µl
CF SERUM
20% ANTI-IEF
NO ANTISERUM
20µl
CF SERUM

A 400ml column with a bed length of 90 cm was equilibrated in PBS at a flow rate of 10ml/hr. The column was calibrated with protein standards in the same buffer and at the same flow rate as column equilibration. 5 ml of CF serum pool was fractionated on this column. 5 ml fractions were collected and tested against anti-IEF by RIE to determine those which were positive. A normal serum pool was fractionated under the same conditions.

4.2.5. Polyacrylamide gel electrophoresis.

The pooled positive fractions from a CF serum pool and the equivalent fractions from normal serum were run on 15% acrylamide-SDS gels using a discontinuous buffer system (Laemmli, 1970). This technique is characterised by the use of a stacking gel to concentrate the sample before electrophoretic separation on the resolving gel.

The electrode buffer used contained 0.025M Tris, 0.192M glycine and 0.05% SDS (w/v) with the pH adjusted to 8.3. The resolving gel contained 30ml of acrylamide solution (30g acrylamide, 0.8g N,N'methylenebisacrylamide to a final volume of 100ml in distilled water), 30ml of Tris-SDS, pH 8.8, (0.75M Tris,0.4% (w/v) SDS), 15µl of TEMED and 3ml of 1% (w/v) ammonium persulphate. The solution was mixed gently by swirling and immediately poured into an LKB 2001 Vertical Electrophoresis Unit to a height of 12cm. The solution was overlayed with butanol and left to

polymerise for 1 hr at RT. The overlay was poured off and the gel surface rinsed with distilled water to remove the butanol. Before preparing the stacking gel the distilled water was removed and the polymerised surface blotted dry with filter paper to remove any liquid which would inhibit polymerisation at the resolving gel interface. The stacking gel contained 2ml of the same acrylamide solution, 10ml of Tris-SDS pH 6.8 (0.25M Tris, 0.4% (w/v) SDS), 8ml of distilled water, 10µl of TEMED, and 1ml of 1% (w/v) ammonium persulphate solution. The solutions were mixed by swirling and poured into the electrophoresis unit over the resolving gel. A comb, which forms the sample wells, was inserted into the stacking gel and left to polymerise for 1 hr. Before sample application the comb was removed and the wells filled with electrode buffer. The samples to be electrophoresed were mixed with an equal volume of sample buffer (0.0625M Tris, 2% (w/v) SDS, 10ml of glycerol, 5ml of 2-mercaptoethanol and 0.1ml of 1% bromophenol blue made up to 100ml with distilled water) and applied to the gel along with standard molecular weight markers. The gels were run at a constant current of 100mA, for approximately 5 hr, at a temperature of 10^{o}C until the bromophenol blue reached the bottom of the gel.

The gel was removed from the electrophoresis unit and immediately placed in a protein stain. The stain consisted of 1.25% (w/v) Kenacid blue, 45% (v/v) ethanol

and 5% (v/v) acetic acid in distilled water. The gels were stained overnight at RT. Destaining was accomplished by repeated washing in a solution containing 5% ethanol and 1% acetic acid in distilled water.

4.2.6. Silver staining.

Silver-staining of proteins, first separated by electrophoresis, is a much more sensitive technique than conventional protein staining. The gels which have been previously stained with Kenacid blue can be further stained with silver. Gels which are to be silver-stained must not be touched without wearing gloves as fingerprints stain very strongly. It is also important to use deionised-distilled water for all steps to minimise background staining.

Thoroughly destained gels were rinsed in distilled water to remove destaining solution and then placed in oxidising solution (1% (w/v) potassium dichromate and 0.02% (v/v) 70% nitric acid in distilled water) for 15 min on an orbital shaker. The gels were then washed three times with distilled water before being placed in a silver nitrate solution (0.1% (w/v) in distilled water) for 30 min. After rinsing once in distilled water the stain was developed with 3% (w/v) sodium carbonate and 0.1% (v/v) formaldehyde in distilled water,changing the developer 3 times. The reaction was stopped with 5% (v/v) acetic acid when the proteins were dark against a

still clear background. After 10 min the gels were transferred to distilled water for storage.

4.2.7. Affinity purification.

The production of a polyclonal antiserum made it possible to consider purification of the protein by affinity chromatography. The use of affinity chromatography for the separation of a protein requires an antibody which can be bound covalently to a matrix and still retain its binding affinity for the protein. The desorbing substances used to remove the protein must be sufficiently gentle to retain the antigenicity of the protein. The matrix chosen for coupling of anti-IEF was CNBr-activated sepharose-4B (Pharmacia). The method used was that described in the Pharmacia booklet "Affinity Chromatography Principles and Methods".

The anti-IEF serum was first partially purified by sodium sulphate precipitation of the Ig fraction. Anti-IEF was mixed with solid sodium sulphate to a final concentration of 18% (w/v). The solution was mixed gently for 1 hr and the precipitate formed centrifuged out at 12000g. The precipitate was washed three times with 18% sodium sulphate in distilled water to remove unprecipitated proteins. The precipitate was re-dissolved in coupling buffer (0.1M sodium carbonate, pH 8.3 containing 0.5M sodium chloride). This was then dialysed aganst coupling buffer to remove the sodium sulphate. After dialysis the protein content of the Ig fraction was

measured using the method described by Lowry et al. (1951).

4.2.8. Measurement of protein.

A reagent for the measurement of protein was prepared by mixing 10 vols of 2% (w/v) sodium carbonate in 0.1M sodium hydroxide with 0.1 vol of 1% (w/v) copper sulphate in distilled water and 0.1 vol of 2% (w/v) potassium-sodium tartrate. 5µl of protein solution was made up to 20µl with distilled water, in a mirotitre plate, and 0.1ml of the above reagent was added and mixed vigorously, then allowed to stand for 10 min. 10µl of Folin-Ciocalteau reagent (diluted with an equal volume of distilled water) was added and mixed well. The mixture was incubated at RT for 40 min before reading at a wavelength of 492nm. A standard curve of bovine serum albumin (BSA) was run at the same time and the unknown protein values were calculated from this curve.

4.2.9. Preparation of CNBr-activated sepharose-4B.

The freeze-dried sepharose was swollen for 15 min in 1mM hydrochloric acid and washed on a sintered glass filter with 200ml of the same solution per gram of dried gel. 1 gram of gel gives a gel volume of 3.5 ml. Hydrochloric acid preserves the activity of the active groups which hydrolyse at a higher pH.

The swollen gel was then washed with 5ml of coupling buffer per gram of dry gel. The sodium sulphate cut of

anti-IEF was immediately added at a concentration of 5mg of protein per ml of gel. The protein solution was then coupled to the gel by end-over-end mixing for 16 hr at $4^{o}C$. The remaining active groups on the sepharose were blocked by the addition of 0.2M glycine, pH 8.0, and mixing for 2 hr at RT. The unbound protein was removed by washing the gel alternately with coupling buffer and 0.1M acetate buffer pH 4.0 containing 0.5M sodium chloride. The change in pH is critical in removing non-covalently bound protein. The gel was stored in coupling buffer, at $4^{o}C$, until use.

A 10ml column of CNBr-sepharose-Anti-IEF was equilibrated with PBS before addition of 1ml of CF serum pool diluted with 4ml of PBS. The serum sample was circulated through the column at a flow rate of 5ml per hr for 16 hr, to remove the protein binding to anti-IEF. The unbound fraction of serum was collected and the column washed with PBS.

4.2.10 Desorption of protein from CNBr-sepharose-Anti-IEF.

Desorption of protein was first attempted by using a change in pH. The 10ml column was washed with 20ml of 0.1M glycine-HCl buffer pH 2.5 at a flow rate of 2ml per hr. The pH of the eluent was immediately adjusted to pH 8.5 with solid Tris. The fractions were concentrated to the original volume of the serum in a Minicon-B concentrator (Amicon) which dialyses at the same time. The unbound protein fraction removed with PBS was similarily treated.

These fractions were tested against anti-IEF by RIE. Both the unbound fraction and the pH 2.5 eluted fraction were negative when tested against the antiserum. The same column was then re-equilibrated in PBS to remove the acidic pH buffer.

An antigen-antibody complex involves hydrophobic interactions which can be disrupted by using polarity-reducing agents such as ethylene glycol.

A 50% (v/v) solution of ethylene glycol pH 8.0 was used to elute the column at a flow rate of 2ml per hr and the fractions collected. This was followed by elution with 50% ethylene glycol, pH 11.0. The fractions were concentrated to the original serum concentration as described above, and tested against anti-IEF by RIE.

To ascertain whether the column contained any more protein, further elution using chaotropic agents was attempted. Chaotropic ions can be used to desorb proteins by disrupting the structure of water and thus reducing hydrophobic interactions. These reagents are used at a neutral pH. The column was washed with 20ml of 1M $MgCl_2$ at a flow rate of 5ml per hr and fractions collected. Then the column was left in this solution for 2 hr before further elution with 10ml of 1M $MgCl_2$. The process was repeated with 2M $MgCl_2$, 3M $MgCl_2$, 1M KSCN, 2M KSCN and 3M KSCN. The fractions collected were all concentrated to the original serum volume as above and tested against anti-IEF by RIE.

4.2.11 Identification of a tissue source.

Using IEF to produce an immunogen is an expensive, timeconsuming and technically precise method. The antiserum produced is weak, requiring large volumes of a scarce commodity for testing, and was only successfully identified in a minority of guinea pigs. Therefore the identification of a tissue source could be useful in reducing preparation time of immunogen and also enabling production of a higher titre antiserum.

The protein recognised by anti-IEF was tested for in the most easily available human materials, saliva, urine, erythrocytes, mononuclear leucocytes (MNL) and polymorphonuclear leucocytes (PMNL).

4.2.12. Saliva.

Saliva samples were collected into sterile tubes from 10 CF homozygotes, 6 CF heterozygotes and 6 healthy controls. These samples were centrifuged at 12000g to preciptate solid material and then both supernatant and precipitate, (reconstituted to 10% of the original volume in PBS and briefly sonicated) were tested against anti-IEF.

4.2.13. Urine.

Urine samples were collected from the same CF homozygotes, CF heterozygotes and healthy controls. These samples were centrifuged at 2000g to remove cells. The supernatant was either tested neat or concentrated 10-fold

in a Diaflo Ultrafilter using UM2 membranes (Amicon), with an exclusion limit of MW 1000 The cell pellet was reconstituted in 1% of the original volume and briefly sonicated before testing.

4.2.14. Blood samples.

Blood samples were collected into sodium heparin tubes from 3 CF homozygotes, 3 CF heterozygotes and 3 healthy controls. The samples were centrifuged at 1000g for 10 min and the plasma removed. The plasma was replaced with an equal volume of RPMI-1640 medium containing 20 U/ml sodium heparin. The cell suspensions were diluted with an equal volume of PBS and carefully layered onto a 1/3 volume of Lymphoprep (Nygaard). This was then centrifuged at 400g for 30 min and the cell layer at the miniscus (MNL) was removed using a pasteur pipette. The remaining supernatant, containing Lymphoprep and medium, was removed. The cell pellet, containing mainly erythrocytes and PMNL was returned to the original blood volume with RPMI-1640 containing 20 U/ml sodium heparin, mixed with 1/3 volume of 6% (w/v) Dextran 250 in 0.15M NaCl and the mixture allowed to settle for 30 min at 37^{o}C. The upper layer containing mainly PMNL was removed. The erythrocyte pellet was resuspended in PBS to the original blood volume. The cell suspensions were washed three times in PBS.

All these samples were tested against a 20% concentration of anti-IEF using a sample size of 20µl.

4.2.15. Cell counting.

MNL and PMNL were counted using a haemocytometer counting chamber. The cells were diluted 1/2 in 1% (w/v) Crystal violet in 2% (v/v) acetic acid and counted at 100-fold magnification to differentiate between MNL and PMNL.

MNL and PMNL were pelleted and resuspended at a concentration of 10^8 cells per ml, freeze-thawed twice and sonicated. The erythrocytes were checked for leucocyte contamination and finally diluted to 10% of original concentration in PBS, freeze-thawed twice and briefly sonicated.

4.2.16. Leukaemia samples.

Blood was collected into sodium heparin tubes from 14 chronic myeloid leukaemia (CML) patients. Two of whom were in blast crisis with mainly lymphoid-type cells and two with myelofibrosis as a complication of the disease.

4.2.17. Granulocyte preparation.

In many cases of leukaemia the blood contained such a large number of white cells that they could be removed by simply allowing the red cells to settle. Where this was not the case the plasma was removed by centrifugation and an equal volume of RPMI-1640 medium containing 20 U/ml of sodium heparin was added to the cells. This was mixed with 1/3 volume of 6% dextran-250 in 0.15M NaCl and allowed to

settle for 30 min at 37°C. The upper layer was removed and washed three times in PBS. The cells were counted and resuspended at a concentration of 10^8cells/ml in PBS. After freeze-thawing twice, followed by brief sonication, the cell debris was removed by centrifugation.

20µl of sample (diluted 1/10 in PBS) was tested against 20% anti-IEF by RIE.

4.2.18. Preparation of guinea pig antiserum to CML granulocyte lysate (Anti-CML).

An immunogen was prepared from a lysate of CML granulocytes from 6 patients. 10^8cells/ml were suspended in PBS and freeze-thawed twice.The debris was removed by centrifugation. The protein concentration of the lysate was 2mg/ml.

200µl of lysate was mixed with an equal volume of Freund's Complete Adjuvant (FCA) to form an emulsion. This emulsion was injected intradermally into guinea pigs in several sites. After 4 weeks the same volume was mixed with an equal volume of Freund's Incomplete Adjuvant (FIA) and emulsified before injecting intraperitoneally. At weekly intervals the guinea pigs were bled and the antiserum tested against a CF homozygote serum pool (as positive) and a normal serum pool (as negative). The guinea pigs were boosted, as the antiserum concentration decreased, with 200µl of CML lysate.

4.2.19. Comparison of anti-IEF and anti-CML by Ouchterlony double diffusion.

The identity of the 2 polyclonal antisera was established by using Ouchterlony double-diffusion.

20µl of CF homozygote serum pool or 20µl of CML lysate (1/10 dilution) was allowed to diffuse against 20µl of each of anti-IEF and anti-CML. The gels were covered and allowed to diffuse overnight at RT. The gel was washed repeatedly with shaking, in 0.05% Tween 20 in PBS. The agarose was then pressed with blotting paper and dried in an oven. The precipitates were stained in 0.5% Kenacid Blue in 5/5/1 (v/v) methanol/distilled water/acetic acid for 10 min and destained in repeated changes of methanol/distilled water/acetic acid as above. When the background was clear, the gel was dried at RT.

4.2.20. Chromosomal assignment.

To observe the chromosomal segregation of the protein identified by anti-IEF with a human chromosome a series of somatic cell hybrids were produced from the mouse myeloid stem-cell line WEHI-TG and cells from four different myeloid leukaemia patients (van Heyningen et al., 1985). Fusion of the leukaemic cells and WEHI-TG was effected at a ratio of 10 to 1 in the presence of 0.5ml of 25% (v/v) polyethylene glycol 1500 containing 1,250 haemagglutinating units of B-propriolactone-inactivated Sendai virus. The fused cells were grown in hypoxanthine-aminopterin-thymidine (HAT) supplemented

medium. The mutant myeloma cells cannot grow in HAT supplemented medium, therefore the hybrid cells can be selected out. Sub-cloning was by limiting dilution until reasonable chromosomal stability was achieved. Subclones were then grown up so that cells from one to three sequential generations were pelleted for testing against anti-CML and karyotyped by sequential trypsin banding and Giemsa-11 staining.

4.3. Results.

4.3.1. Antiserum to the IEF doublet band.

In total 128 guinea pigs were immunised with the homogenate from the IEF doublet band, of which 50 showed a positive reaction in initial testing. The volume of antiserum available from each guinea pig was limited to 5ml per bleed and the total was dependent on the persistence of the antibody reaction, which varied from animal to animal. Any antibody, produced in this manner, which showed apparent specificiy for a component in CF serum was referred to as anti-IEF.

The guinea pig serum in its unabsorbed state showed several immune reactions against both normal and CF serum (Fig.4.2a) and on optimum absorption (normally 1 vol of normal serum to 5 vol of antiserum) showed a monospecific reaction with CF homozygote serum by RIE (Fig.4.2b). The optimum antiserum concentration for visualisation of immunoprecipitin rockets, using 20µl of serum, varied from batch to batch, but ranged from 20% to 50%.

4.3.2. Quantitive immunoassay of serum samples.

The antiserum produced by Manson and Brock (1980) showed a positive reaction by RIE with sera from 16 out of 17 CF homozygotes, 8 out of 9 obligate heterozygotes and 1 out of 15 normal subjects. The precipitin peaks were larger in CF homozygotes than in heterozygotes suggesting the possibilty of a quantitative biochemical test for CF

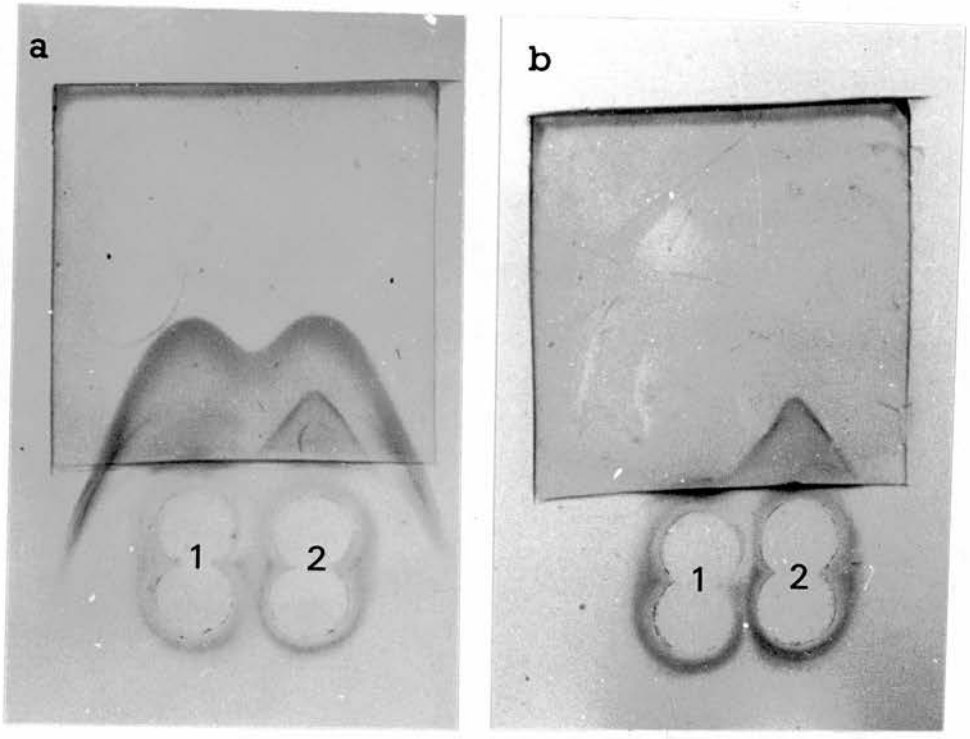


Fig.4.2. RIE of a normal and a CF serum pool against anti-IEF.

20µl of a normal (1) and a CF (2) serum pool were tested against unabsorbed anti-IEF (a) and the same anti-IEF absorbed 5:1 with a normal serum pool (b). Both the unabsorbed and the absorbed antisera were at a concentration of 20%.

heterozygosity. Only the samples which scored positive for the CFP doublet showed a positive reaction with antiserum.

Sera from 14 CF homozygotes, 28 CF heterzygotes and 23 normal controls were tested against two batches of anti-IEF (AS60 and AS82/83), by RIE and IRMA. By selecting an arbitrary cut-off, between the three different groups, 94% of the samples were correctly assigned as CF homozygotes, CF heterozygotes or normals.

The RIE peak values depended on the batch of antiserum used. The results are shown in Fig 4.3.. Each group of samples has a different median values for the two different antisera. These are summarised in Table 4.1.. The data was not normally distributed and non-parametric tests of significance were used in analysis, the Median test for differences between the medians and the Spearman rank test for correlation coefficients (Siegel, 1956) The median values between normal and CF heterozygotes and between CF heterozygotes and CF homozygotes showed highly significant differences ($p<0.001$ in both cases for both antisera). The correlation coefficient between the values for the two different antisera was 0.8627, $p<0.001$.

In the IRMA the percentage of ^{125}I Protein A bound to the different sample groups is shown in Fig 4.4.. The median values are shown in Table 4.2.. The median values, between different sample groups, again showed highly significant differences ($p<0.001$). The correlation coefficient between the values for the two different antisera was 0.8432, $p<0.001$.

Fig.4.3. Relative peak areas of RIE of serum samples using two different batches of anti-IEF.

CF, CF homozygotes, HZ, obligate CF heterozygotes, N, normal controls. The median value of peak areas (mm^2) for each group of sera is indicted by the solid line, the arbitrary cut-off points for each group is indicated by the dashed line.

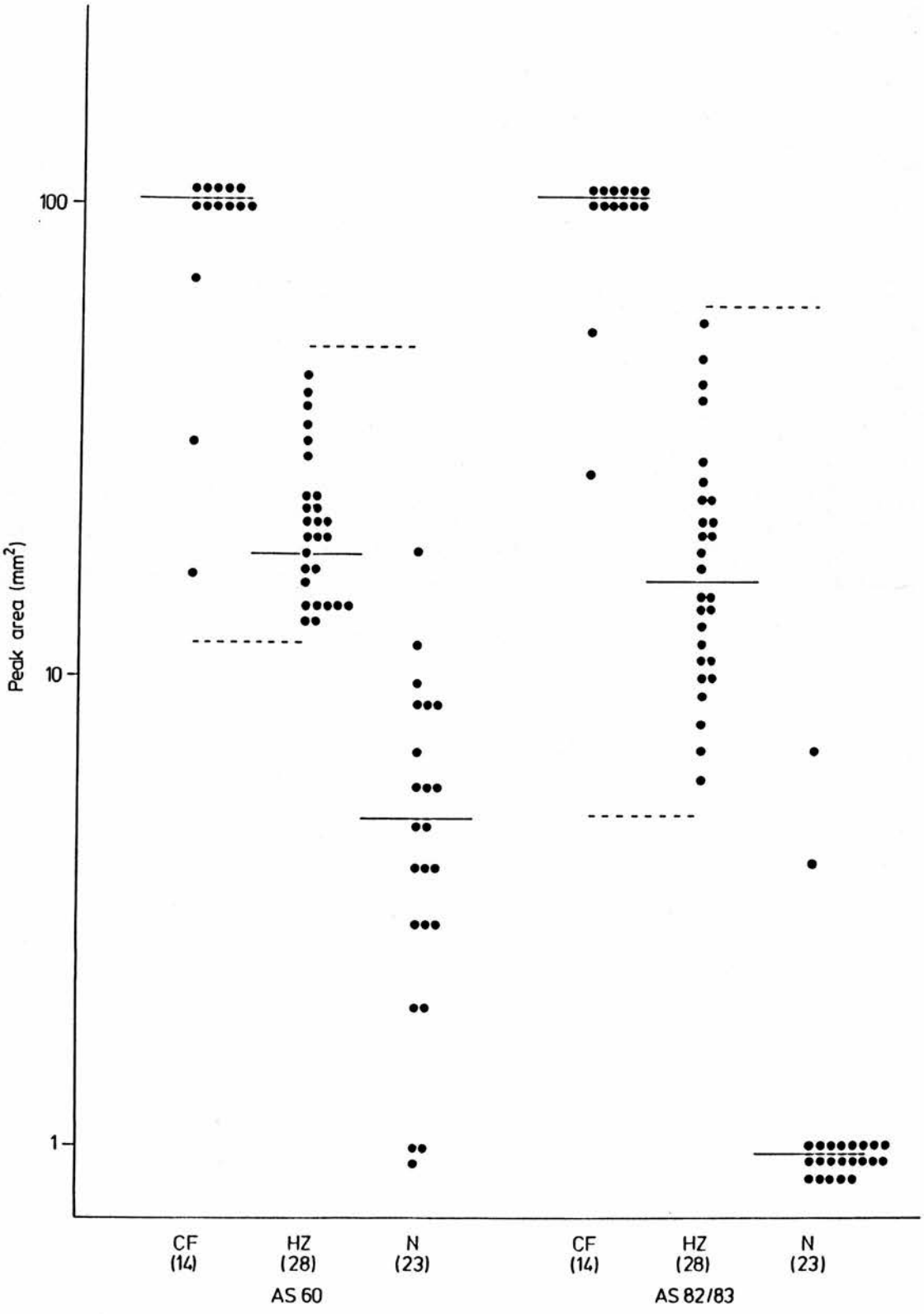

100
10
1
Peak area (mm²)
CF
(14)
HZ
(28)
N
(23)
AS 60
CF
(14)
HZ
(28)
N
(23)
AS 82/83

TABLE 4.1. Median values of peak areas* for RIE assay.

Sample	Number	Median AS60	Median AS82/83
CF homozygote	14	>100	>100
CF heterozygote	28	18	15
Normal	23	5	0

* Peak area measured in mm^2

Fig.4.4. Relative percentages of bound radioactivity of an IRMA of serum samples.

CF, CF homozygotes, HZ, obligate CF heterozygotes, N, normal controls.
The median value of the bound radioactivity for each group of sera is indicted by the solid line, the arbitrary cut-off points for each group is indicated by the dashed line.
Radioactivity bound is expressed as a percentage of the total radioactivity added.

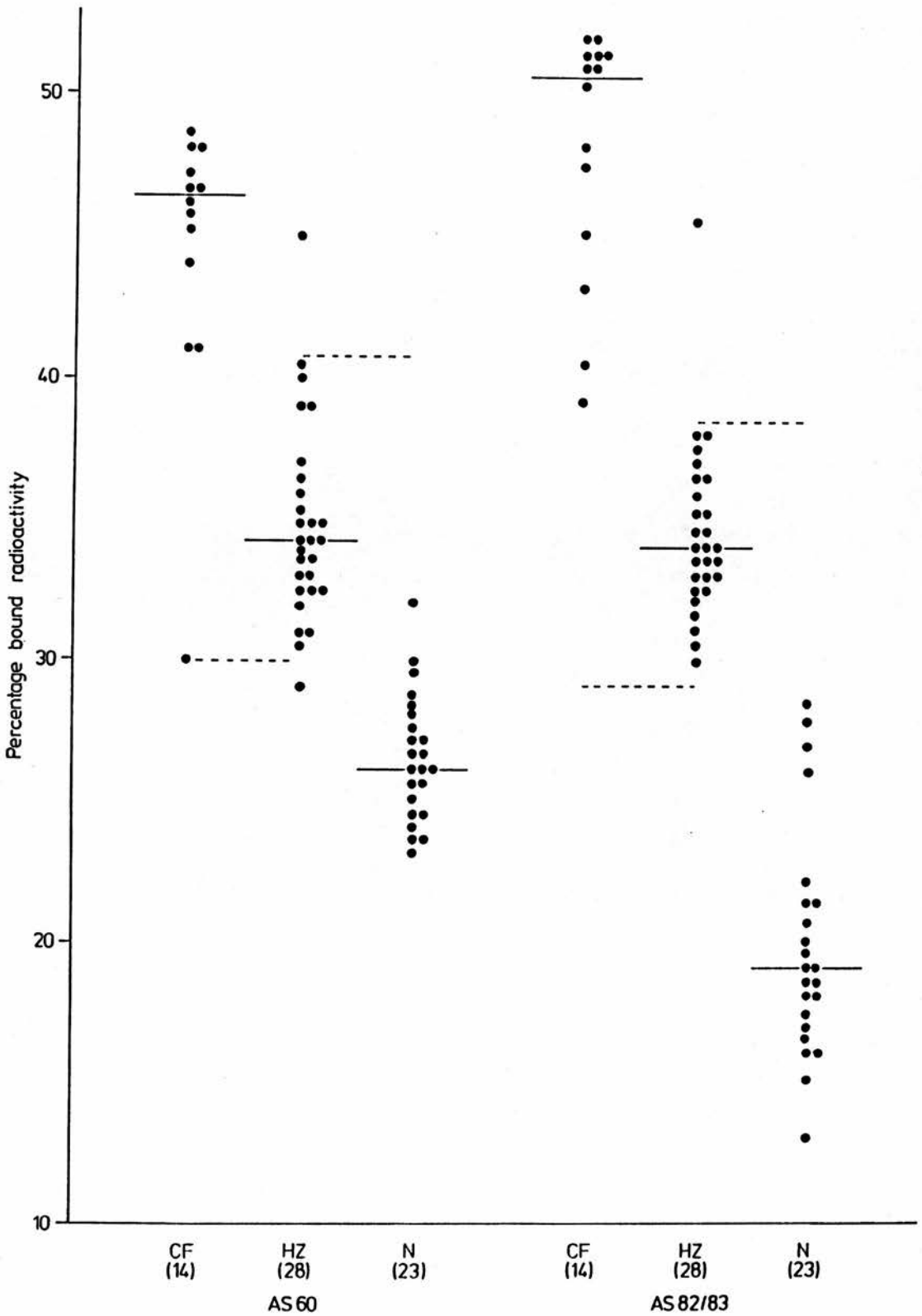

Percentage bound radioactivity
50
40
30
20
10
CF
(14)
HZ
(28)
N
(23)
AS 60
CF
(14)
HZ
(28)
N
(23)
AS 82/83

TABLE 4.2. Median values of radioactivity bound*, by IRMA.

Sample	Number	Median AS60	Median AS82/83
CF homozygote	14	56	51
CF heterozygote	29	34	34
Normal	23	26	19

* Radioactivity bound expressed as a percentage of the total radioactivity added.

These immunoassays showed the potential of using anti-IEF antisera for detecting CF heterozygotes in the general population. The blood samples in these series were all collected in a standard manner, as described for IEF, and this may be critical in the correct assignment of CF status.

4.3.3. Immunodepletion of anti-IEF rockets.

To establish that the antiserum, produced in guinea pigs, was not against a common known protein, serum samples were run by RIE through a high concentration of several commercially available polyclonal antisera before reacting with anti-IEF.

The antisera which were tested for immunodepletion of the anti-IEF peak are shown in Table 4.3..

All of these antibodies failed to immunodeplete the anti-IEF precipitin peak and the protein was therefore not one of these common well characterised serum proteins.

CF homozygote serum was also tested for depletion of the anti-IEF peak with Protein A-Sepharose CL-4B. This depletion experiment showed no reduction in the level of antigen in serum.

4.3.4. Gel filtration of serum.

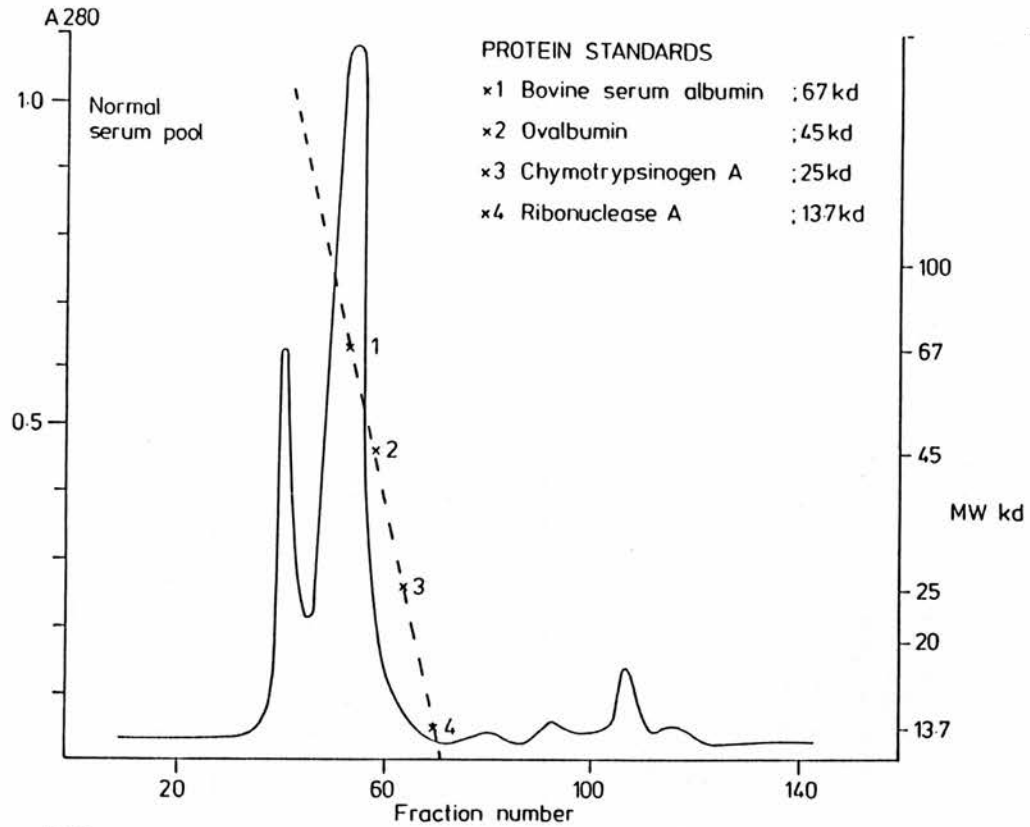
The profiles of sephacryl S300 gel filtration of a normal serum pool and a CF serum pool are shown in Fig.4.5.. By plotting the MW of the protein standards versus their elution volume the MW of the anti-IEF

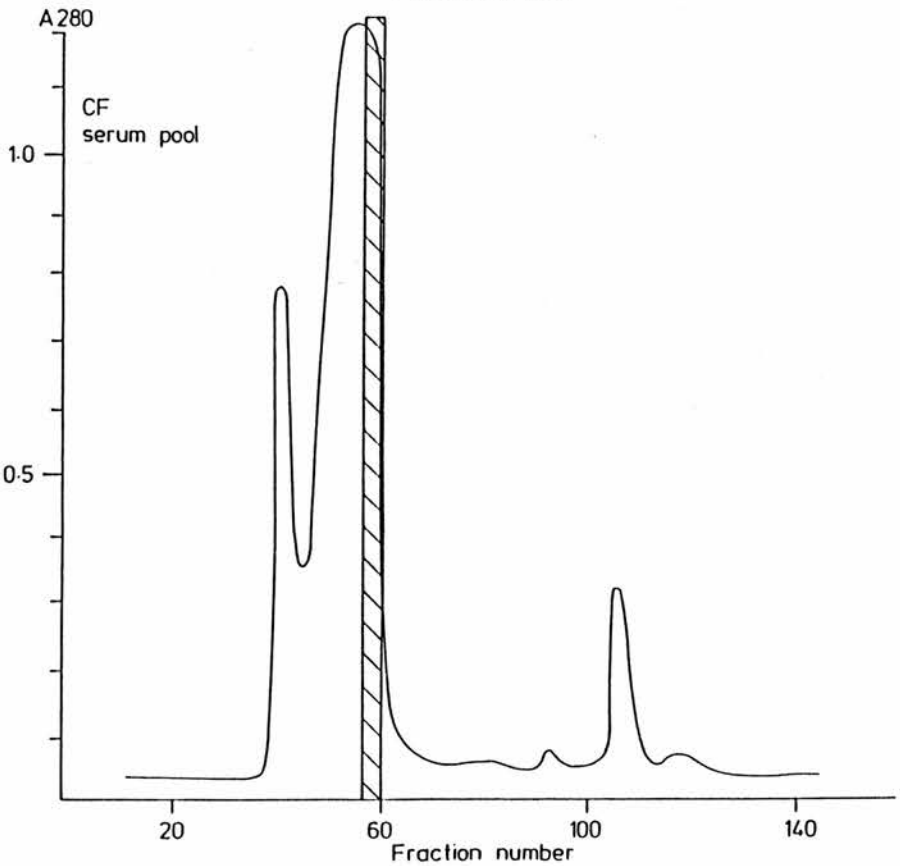
Table 4.3. Polyclonal antisera used to immunodeplete the protein detected by anti-IEF.

Antiserum		Commercial Source
Anti-human	IgG	Dako
"	$a_2$macroglobulin	"
"	albumin	"
"	IgG,IgM.IgA mixture	"
"	IgG Fc fragment	Behringwerke
"	IgG Fab fragment	"
"	ceruloplasmin	"
"	IgM	"
"	$a_1$antichymotrypsin	"
"	$a_1$antitrypsin	"
"	transferrin	"
"	prealbumin	"
"	C3a	"
"	C1q	"
"	C4	"
"	haemopexin	"
"	haptoglobin	"
"	kininogen	Nordic
"	prekallikrein	"

Fig.4.5. Sephacryl S300 gel filtration profiles of normal and CF serum pools.

The anti-IEF positive fractions on the CF serum profile are indicated by the hatched area. The MW of these fractions was estimated by comparing their elution volume with the elution volume of a series of standard proteins as shown on the normal serum profile.


A 280
1·0
0·5
Normal
serum pool
PROTEIN STANDARDS
×1 Bovine serum albumin ; 67 kd
×2 Ovalbumin ; 45 kd
×3 Chymotrypsinogen A ; 25 kd
×4 Ribonuclease A ; 13·7 kd
1
2
3
4
100
67
45
MW kd
25
20
13·7
20
60
100
140
Fraction number


A 280
1·0
0·5
CF
serum pool
20
60
100
140
Fraction number

positive fractions can be estimated. the anti-IEF positive fractions elute at a MW range of 51-60 kilodaltons (kd).

4.3.5. Polyacrylamide gel electrophoresis.

The pooled anti-IEF positive fractions from sephacryl S300 gel filtration of a CF serum pool and the equivalent fractions from a normal serum pool were run on 15% acrylamide-SDS gels. The gels were stained with Kenacid Blue (Fig.4.6.a) followed by the more sensitive silver staining (Fig4.6.b).

There were no observed differences in the electrophoretic patterns of CF and normal fractions with Kenacid Blue or silver staining. The protein reacting with anti-IEF may be masked by one of the other serum proteins or the level may be too low to observe by these methods.

4.3.6. Affinity purification.

The production of a polyclonal antiserum made it possible to consider purification of the protein by affinity chromatography.

The immunologically reactive component of a CF serum pool was bound to a CNBr-sepharose anti-IEF column. Attempts to elute the bound fraction by change in pH were unsuccessful. Use of the polarity reducing reagent ethylene glycol at pH 8.0 and pH 11.0 resulted in a fraction which reacted with anti-IEF. The results are

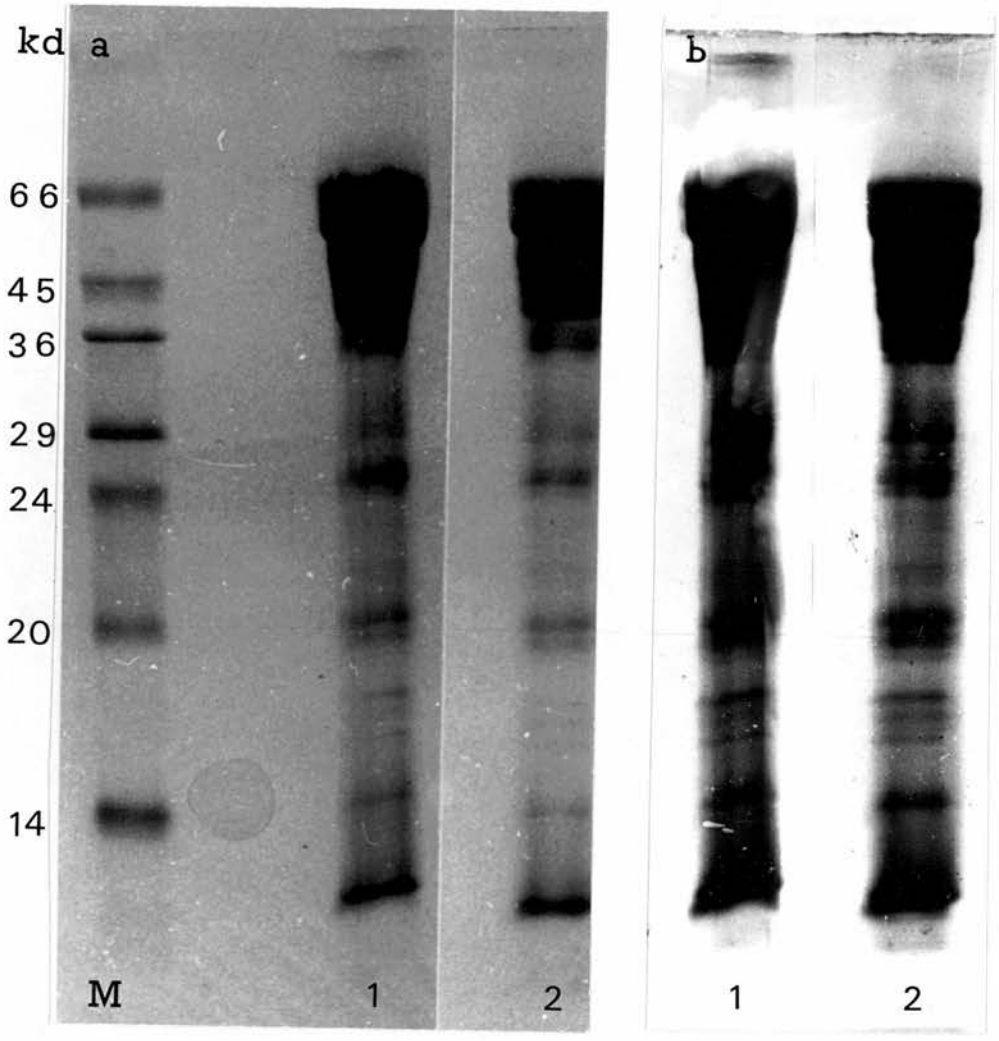


Fig.4.6. SDS-PAGE of gel filtration fractions.

SDS-PAGE of serum fractions separated on a sephacryl S300 gel filtration column : 1) the pooled anti-IEF positive fractions (MW 51-60kd) from a CF serum pool, 2) the pooled MW 51-60kd fractions from a normal serum pool, stained with Kenacid Blue (a) and silver (b). Lane M shows the MW markers.

illustrated in Fig 4.7..

The purification of the protein from CF serum by affinity chromatography was seen to be possible. However, the purified protein appeared extremely unstable. The protein isolated by eluting with 50% ethylene glycol, pH 11.0, when retested 24 hr after purification, no longer showed a positive reaction. The reason for this is unknown. The amount of material purified by this method was judged to be less than 20% of the original concentration in serum by measuring the area under the precipitin rocket. This indicated that 80% of the protein was either still bound to the CNBr-Sepharose anti-IEF, or was lost in the concentration step, or that the majority of the protein had lost its immunological activity. To acertain whether the column contained any more protein, further elution using chaotropic agents was attempted. The column was washed sequentially with 1M $MgCl_2$, 2M $MgCl_2$, 3M $MgCl_2$, 1M KSCN, 2M KSCN and 3M KSCN. The fractions were collected, concentrated to the original serum volume and tested against anti-IEF by RIE. These were all negative and no further detectable antigen could be eluted.

The persistent problems of purification may be due to the antigen being unstable. If the material could be purified at higher concentrations then this problem might be overcome. However, limited quantities of anti-IEF and CF serum made this method for producing large quantities of purified material impractical.

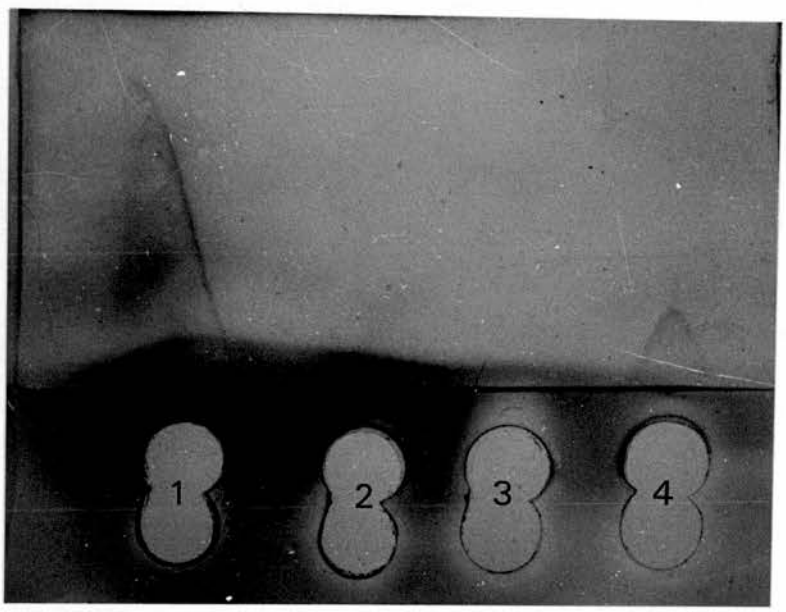


Fig. 4.7. RIE of CF serum fractions eluted from an anti-IEF immunoaffinity column.

A CF serum pool (1) was loaded onto a CNBr-sepharose anti-IEF immunoaffinity column. The unbound fraction was eluted with PBS (2) and the bound fractions were eluted with 50% (v/v) ethylene glycol either pH 8.0 (3) or pH 11.0 (4). 20µl of each of the concentrated fractions (2-4) and the CF serum pool were run against 20% anti-IEF.

4.3.7. Comparison of the properties of the protein identified by anti-IEF with CFP.

The properties of CFP described by Wilson (1982) and the properties of the protein defined by anti-IEF are summarised in Table 4.4. The pI of CFP is 8.46 and it can only be assumed that the anti-IEF protein has the same pI, as the antiserum was produced from the portion of gel at that pH. The discrepancy in molecular weight, the apparent difference in complexing to IgG and binding to Protein A indicate that the 2 proteins are different. Therefore the protein defined by anti-IEF was named CF Antigen (CFAg).

4.3.8. Identification of a tissue source for CFAg.

CFAg was tested for in the most easily available human materials, saliva, urine, erythrocytes, MNL and PMNL.

All saliva and urine samples were negative when tested against anti-IEF.

Blood samples were collected into sodium heparin tubes from 3 CF homozygotes, 3 CF heterozygotes and 3 healthy controls. The samples were fractionated into three cell types, erythrocytes, MNL and PMNL. Each cell type was divided into a cytoplasmic and a membrane fraction. The erythrocytes and MNL were negative when tested against anti-IEF. However, the cytoplasmic fraction of PMNL showed a very strong reaction with anti-IEF in all three genotypes

Identification of PMNL as a source of CFAg indicated

Table 4.4. Comparison of the properties of CFP and the protein defined by anti-IEF.

CFP	Protein defined by anti-IEF
pI 8.46±0.05.	pI between 8.4 and 8.5
Binds to Protein-A.	Does not bind to Protein-A
Appears to complex with IgG.	Does not appear to complex with IgG
MW 90-180 kd in serum.	MW 51-60 kd in serum.
Found in the sera from CF homozygotes and heterozygotes.	Found in the sera from CF homozygotes and heterozygotes.

the possibilty of using leukaemia cells, which are available in large numbers in a small blood sample.

Cells were separated from blood collected from 14 CML patients. The cytoplasmic fraction of 12 of these samples was strongly positive against anti-IEF. The 2 samples from patients in blast crisis were negative.

4.3.9. Guinea pig antiserum to CML granulocyte lysate (anti-CML).

8 guinea pigs were used to produce antiserum to CML lysate. The antiserum in its unabsorbed state produced a monospecific antiserum, by RIE, against a component in CF homozygote serum which was absent in normal serum. (Fig.4.8.)

The quantity of antiserum required for visualisation of rockets was much lower than with anti-IEF and was optimised at 3% with a 20µl serum sample.

4.3.10 Comparison of anti-IEF and anti-CML.

The identity of the 2 polyclonal antisera was established by using Ouchterlony double-diffusion. The antigen and antibody were placed in wells punched in agarose gels and allowed to diffuse. A straight line of precipitate is formed where concentrations of diffusing antibody and antigen reach equivalence. The ideal concentrations of antibody and antigen will produce a precipitin line midway between the two wells. In an unbalanced situation, in which there is a great excess of

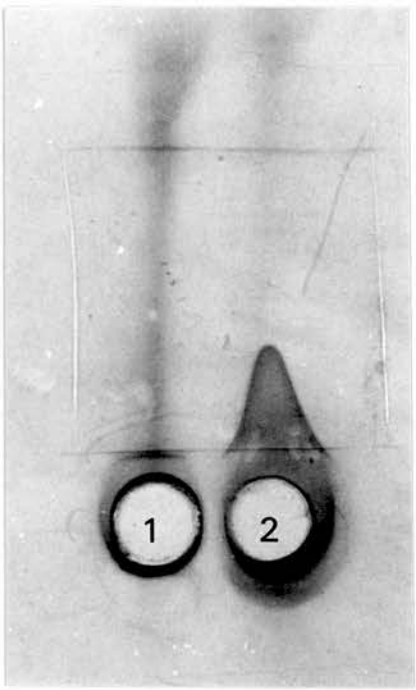


Fig 4.8. RIE of a normal and a CF serum pool against unabsorbed anti-CML.

20µl of a normal (1) and a CF (2) serum pool were tested against unabsorbed anti-CML at an antiserum concentration of 20%.

antigen or antibody, multiple precipitates may form due to alternate solubilisation and reprecipitation. To establish optimum concentrations, antigen or antibody is placed in a central well with serial dilutions of the other round it. In a reaction of complete identity there is a continuous precipitate formed.

Results are shown in Fig 4.9.. The continuous precipitate formed shows complete identity between anti-IEF and anti-CML using CF homozygote serum or CML lysate as an antigen source. This technique also shows a line of complete identity between the 2 antigen sources.

4.3.11. Chromosomal assignment of CFAg.

Four subclone series of somatic cell hybrids (WEMAD 1., WEMAD 2, WEH and WEP) were produced, each derived from an independent fusion.

Cell lysates were produced from 15 subclones derived from the 4 hybrid lines and tested against anti-CML by Ouchterlony double diffusion. The results are summarised in Fig 4.10.. Lysates of the leukaemic cells and WEHI-TG from which the hybrids were derived were also tested by the same method.

All four leukaemic cells were positive for CFAg, whilst the mouse myeloid stem-cell line was negative. Analysis of the Ouchterlony results and the karyotypes showed that CFAg segregated with human chromosome 1 with complete concordance.

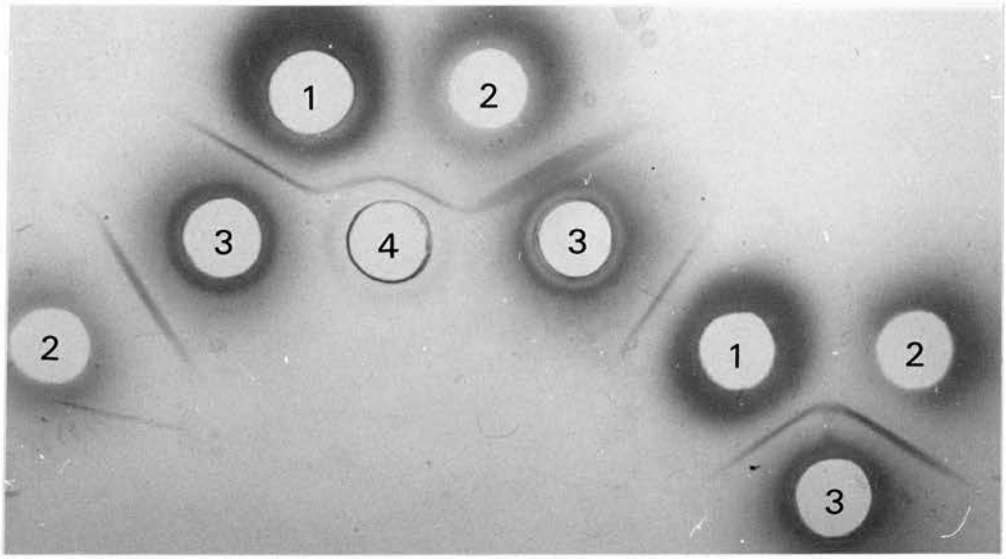
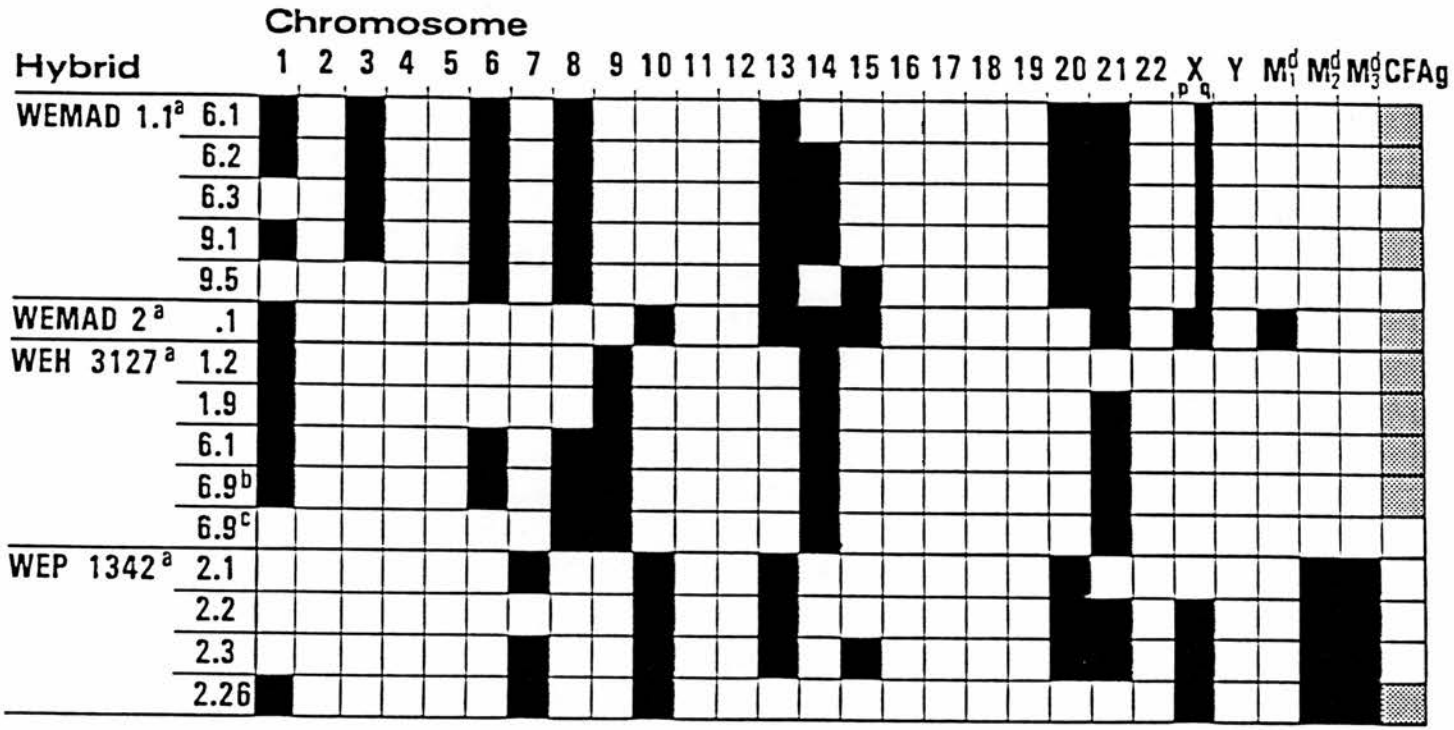


Fig 4.9. <u>Ouchterlony double diffusion of anti-IEF and anti-CML against CF serum and CML granulocyte lysate.</u>

1, 20µl of anti-IEF. 2, 20µl of anti-CML.
3, 20µl of CF serum pool. 4, 20µl of $a_8$1/10 dilution of CML granulocyte lysate (10^8 cells/ml of PBS).
A line of complete identity is formed between anti-IEF and anti-CML against both CF serum and CML granulocyte lysate. There is also a line of complete identity between CF serum and CML granulocyte lysate against both antisera.

Fig.4.10. Segregation of human chromosomes and CFAg in hybrid cell lines.

The filled squares show the presence of a human chromosome in more than 30% of cells karyotyped.
Open squares show the absence of the chromosome in more than 95% of cells karyotyped.
The shaded squares show the presence of CFAg when tested by Ouchterlony double diffusion against anti-CML.


Chromosome
Hybrid
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y M_1^d M_2^d M_3^d CFAg
p q
WEMAD 1.1[a] 6.1
6.2
6.3
9.1
9.5
WEMAD 2[a] .1
WEH 3127[a] 1.2
1.9
6.1
6.9[b]
6.9[c]
WEP 1342[a] 2.1
2.2
2.3
2.26

4.4. Discussion.

A polyclonal antiserum has been produced against the portion of IEF gel pH 8.4 to 8.5 as described by Manson and Brock (1980). A large series of serum samples, collected in a standardised way, were tested against two batches of anti-IEF by RIE. The results confirmed the earlier observation that there was an elevated level of a specific protein in CF homozygotes, an intermediate level in obligate heterozygotes and a low level in healthy controls. The same samples were also assayed by an IRMA based on the same two batches of anti-IEF. Both these assay systems detected 94% of the genotypes correctly and showed the potential of using these assays to identify CF heterozygosity in a general population.

The availability of anti-IEF enabled some biochemical characterisation of the protein which it identified. Immunodepletion of CF serum using several commercially available polyclonal antisera or depletion with Protein-A sepharose failed to remove or reduce the immunological activity of the protein. Gel filtration of a CF homozygote pool of serum indicated a fraction of MW of 51-60kd which reacted with anti-IEF. SDS-PAGE of the positive fraction showed no difference in the banding pattern when compared with the equivalent fraction from a normal serum pool.

The protein defined by anti-IEF showed several different properties to CFP as described by Wilson (1982). Immunodepletion experiments show that, unlike CFP, it does

not bind to protein-A or IgG. The apparent MW in serum is 51-60kd compared with 90-180kd for CFP. Therefore, this protein was named Cystic fibrosis antigen (CFAg).

Immunopurification of CFAg, from CF serum, was attempted using solid phase anti-IEF. It was possible to elute immunologically active CFAg with 50% ethylene glycol, pH 11.0. However, this material appeared extremely unstable and if retested after a short period of storage at $4^{o}C$ was no longer detectable immunologically.

The tissue source of CFAg in serum was identified as granulocytes. Antiserum to CFAg was more easily produced in guinea pigs using a CML granulocyte lysate as the immunogen. This antiserum (anti-CML) appeared monospecific against a component in CF serum when tested by RIE.

Anti-IEF and anti-CML reacted with the same protein in serum and CML granulocyte lysate as shown by lines of complete identity when tested by Ouchterlony double diffusion. This method also showed that the antigen in CF serum was identical to the antigen in CML granulocyte lysate.

Localisation of CFAg in CML granulocytes made it possible to produce somatic cell hybrids using a mouse stem cell line and CML granulocytes. The cell lines were checked for expression of CFAg by Ouchterlony double diffusion. All cell lines produced were karyotyped and expression of CFAg was restricted to those cell lines which retained human chromosome 1. The localisation of CFAg on chromosome 1 excludes the possibility of it being

the basic defect in CF, as linkage studies have shown that the mutant gene for CF is on chromosome 7 (Beaudet et al., 1986). Nevertheless, the observed elevation of CFAg in the serum of CF homozygotes and heterozygotes may be due to the involvment of CFAg in some biochemical or physiological pathway which is altered in CF.

There were many problems encountered both in the production of antiserum and in using this antiserum to characterise and purify CFAg. It was possible that if monoclonal antibodies could be produced against CFAg, they would not only improve the assay system, but also be useful in purification of the protein.

CHAPTER FIVE

MONOCLONAL ANTIBODIES

5.1. Introduction.

5.1.1. Principles.

In 1975 Kohler and Milstein described a technique to produce large quantities of antibodies by fusing myeloma cells with B-lymphocytes.

By immunising an experimental animal (usually a mouse) with an antigen of interest it is possible to stimulate lymphocytes to produce antibodies which recognise this antigen. The mouse is then killed and the lymphocytes collected from the spleen. These are then fused with a myeloma cell line to produce hybrids which grow as rapidly as myeloma cells but still retain their antibody production derived from the β-lymphocytes. This method of priming mouse lymphocytes to produce specific antibodies still leaves many fused lymphocytes producing irrelevant antibodies. Even the best immunisation schedules with highly immunogenic, partially purified, antigen may only produce a few percent of fusions secreting the specific antibody desired.

In all fusions, before further cloning, these antibody secreting hybrids must be selected by screening. Essentially this primary screening must be simple, cheap and fast to deal with large numbers of hybrids and identify those of interest. This is particularly important in highly successful fusions where several hundred hybrids may result, since it is impractical to maintain them all in culture for any length of time.

The choice of immunogen and screening method is dependent on the antibody required. With carefully designed screening it is possible to isolate a specific antibody from a wide variety of antibodies produced by immunisation with crude material.

5.1.2. Monoclonal antibodies to CFAg.

The production of polyclonal antibodies to CFAg indicated the potential of using this protein to detect CF heterozygosity in the general population. In the expectation of improving this detection test it was decided to raise monoclonal antibodies to this protein even though it had not been purified or well characterised.

5.1.3. Strategy.

Monoclonal antibodies can be made against any substance that can be recognised as an antigen by cells in an animal immune system. When a crude mixture of substances is injected into an animal a series of antibodies are made which recognise different components of the antigen mixture. In the process of fusion the whole spleen is used as the source of lymphocytes. Many fused lymphocytes will be against antigens of no interest. Therefore, each hybridoma supernatant must be screened to select the specific monoclonal antibodies required.

a) Immunogens.

The first step in producing a monoclonal antibody to a defined antigen is to select a suitable preparation of the antigen for immunisation.

When it was first decided to produce monoclonal antibodies against CFAg the only known source of CFAg was CF homozygote or heterozygote serum. Some form of partially purified CFAg, derived from CF serum, could be used to immunise mice and the specific monoclonal antibodies selected by analysis after cloning.

The portion of IEF gel pH 8.4 to 8.5 from IEF of a CF serum pool had successfully produced a polyclonal antiserum in guinea pigs and there was no reason to believe that this same immunogen would not produce antibodies in mice.

The technique of immunisation with immunoprecipitins has been used with great success to produce high titre polyclonal antisera against several serum proteins (Kroll, 1981). The immune-complex formed from RIE of CF serum against anti-IEF is a partially purified source of CFAg. Well washed immunoprecipitins will contain guinea pig Ig as a major contaminant but few other proteins and should contain sufficient CFAg to produce antibodies.

CML granulocyte lysate is an abundant source of CFAg. It was considered that the lysate contained sufficient CFAg, in relation to other proteins, to be suitable for use in immunising mice without further purification.

b) Immunisation.

Initial immunisation of antigen is given sucutaneously. The IEF homogenate and RIE precipitins are insoluble forms of antigen and will ellicit a strong antibody response. The CML granulocyte lysate is a soluble form of antigen and can be mixed with an immune system stimulant, such as Freund's adjuvant, which primes the immune system to recognise avidly any antigen injected with it.

The final boost of antigen is given intravenously and intraperitoneally 3-4 days prior to removal of the spleen to ensure that B cell clones are maximally stimulated. The intravenous immunisation ensures that a large number of B-cells will be stimulated simultaneously. These rapidly dividing cells grow best after fusion.

c) Primary screening.

Design of the primary screening system is crucial if specific monoclonal antibodies are to be identified. A poor screening method will fail to identify the hybridomas required. When dealing with hybridomas from an impure immunisation it is preferable to screen against an antigen source which does not have too many other proteins present in order to limit the number of irrelevant hybridomas showing a positive reaction. The simplest assays depend on the ability of a monoclonal antibody to bind to its antigen. A source of antigen can be immobilised onto PVC microtitre plates by incubating at an alkaline pH. The

hybridoma supernatants are incubated with the antigen and the excess washed off. The binding reaction can be detected by using an enzyme-labelled second antibody against the monoclonal antibody.

Hybridoma supernatants can be screened for immunological activity against any partially purified source of CFAg.

The CFAg positive fraction from Sephacryl S300 gel filtration contains several serum proteins (see Fig.4.6.) and it was thought that a negative control using the equivalent fraction from gel filtration of a normal serum pool would considerably reduce the number of positive hybridomas selected.

CFAg was immunopurified on a CNBr-sepharose-anti-IEF column and eluted with 50% ethylene glycol pH 11.0 (see Fig.4.7.). The immunopurified protein seemed very unstable and it could no longer be detected by RIE with anti-IEF after a short storage time. However it was possible that sufficient CFAg remained to be detectable in a more sensitive enzyme immunoassay system.

CML granulocyte lysate contained sufficient CFAg to be used directly without further purification.

d) **Secondary screening.**

CFAg is a protein defined by it's immunological reactivity with a polyclonal antiserum (anti-IEF). The secondary screening method must relate directly to this original definition. Immunodepletion of the CFAg

rocket from CF serum, using hybridoma supernatants, directly relates the immunospecificity of the monoclonal antibodies to the polyclonal antiserum.

5.2. Methods.

5.2.1. Immunisation of mice and production of hybrids.

The production of hybridomas began before the identification of granulocytes as a major source of CFAg and early immunisations used either the homogenised portion of IEF gel (as described for the production of anti-IEF; section 4.2.1.) or the homogenised precipitin peaks derived from RIE of CF serum. Later CML granulocyte lysate, (as described for the production of anti-CML; section 4.2.18.), was used. In all cases BALB/c mice were initially immunised subcutaneously with antigen preparation. After four weeks they were boosted with the same material intravenously and intraperitoneally. Four days later the spleen was removed and the cells hybridised with P3 NS1/Ag1-4 myeloma cells. The fused cells were plated and sub-cloned as described in van Heyningen et al. (1982). The immunisation protocols are summarised in Table 5.1.

5.2.2. Primary screening of hybridoma supernatants.

Primary screening of the culture supernatants was by enzyme-linked immunoabsorbent assay (ELISA). Supernatants from clones produced by immunisation with the IEF pH 8.4-8.5 homogenate were tested against the Sephacryl S300 gel filtration CFAg positive fraction and the equivalent fraction from normal serum; section 4.2.4.. Those with a stronger reaction against the CFAg positive fraction were

Table 5.1. Immunisation protocols for the production of monoclonal antibodies to CFAg.

Fusions 1-3	
Immunogen	The portion of IEF gel pH 8.4-8.5 from IEF of a CF serum pool. 1g of gel homogenised in 1ml of PBS.
Primary Immunisation	0.2ml of homogenate per mouse.
Secondary Immunisation	1.0ml of homogenate per mouse.
Fusions 4/5	
Immunogen	The precipitin peaks from CF serum pool RIE against anti-IEF. Rockets from 200 µl of serum homogenised in 1ml of PBS.
Primary Immunisation	0.25ml of homogenate, emulsified with an equal volume of Freund's complete adjuvant (FCA), per mouse.
Secondary Immunisation	0.25ml of homogenate, emulsified with an equal volume of Freund's incomplete adjuvant (FIA), per mouse.
Fusion 6	
Immunogen	CML granulocyte lysate 10^8cells per ml of PBS (3mg/ml protein).
Primary Immunisation	0.15mg of protein, emulsified with an equal volume of FCA, per mouse.
Secondary Immunisation	0.15mg of protein, emulsified with an equal volume of FIA, per mouse.

selected for further testing. The supernatants were also tested against ethylene glycol pH 11.0 immunopurified material; section 4.2.10.

50µl of diluted Sephacryl S300 fraction (1µg protein/ml) or ethylene glycol pH 11.0 immunopurified material (1µg protein/ml), in 0.1M glycine buffer pH 9.5, was incubated overnight at 4°C in PVC microtitre plates. The plates were washed in 0.9% sodium chloride containing 0.05% Tween 20 (saline/Tween). 50µl of hybridoma culture supernatant was added, incubated for 1 hr at RT and the excess washed off with saline/Tween. 50µl of peroxidase-conjugated sheep anti-mouse Ig (Serotec), diluted 1/1000 in PBS containing 0.1% BSA and 0.05% Tween 20 (diluent), was added and incubated for 1 hr at RT. After washing, the peroxidase was developed for 30 min by adding 100µl of a solution containing 20mg of o-phenylenediamine and 20µl of 100vol hydrogen peroxide in 100ml of 0.1M citrate buffer pH 5.0. The reaction was stopped by adding 50µl of 4M sulphuric acid and the plates read at 492nm on a Titretek Multiscan.

The culture supernatants from the clones resulting from the immunisations with RIE precipitins and CML granulocyte lysate were screened against CML granulocyte lysate dilute 1/1000 in 0.1M glycine buffer pH 9.5 and coated onto PVC plates. CML granulocyte lysate appeared to contain much higher levels of CFAg than any of the serum derived materials. The remainder of the procedure was as described above.

5.2.3. Secondary screening of hybridoma supernatants.

All culture supernatants positive in the primary screening were tested for specificity towards CFAg.

Immunodepletion of the antigen in CF serum was detected by RIE. 200µl of culture supernatant was incubated with 100µl of a 10% (v/v) concentration of solid phase donkey anti-mouse Ig (SAC-CEL, Wellcome) with mixing for 1 hr at RT. The SAC-CEL was pelleted by centrifugation and washed three times in saline/Tween. 20µl of CF serum pool was added to the pellet and incubated, with mixing, for 1 hr at RT. The mixture was centrifuged and the depleted serum run against anti-IEF by RIE.

5.2.4. Further screening of non-depleting antibodies.

Hybridomas, produced by immunisation with IEF pH 8.4-8.5 homogenate, which showed no immunodepletion of CFAg, but were positive in primary screening, were further tested to check whether the fusion had been successful and had produced secreting clones against other proteins.

The assay system devised used commercially available polyclonal antisera to bind proteins from CF serum. The hybridoma supernatant was then incubated with the antigen bound to polyclonal antisera. The presence mouse Ig detected by labelled second antibody indicating the specificity of the monoclonal antibody.

50µl of commercially available polyclonal antisera, anti-albumin, anti-IgG, anti-IgM, anti-a_2M,

anti-transferrin and anti-C3a, diluted 1/1000 in 0.1M glycine pH 9.5 was incubated, on PVC plates, overnight at 4°C. After washing, 50µl of CF serum pool, 1/100 and 1/1000 in diluent, was incubated for 2 hr at RT. 50µl of hybridoma culture supernatant was added to the washed plates and incubated for 2 hr at RT. The mouse Ig binding was detected as described above.

5.2.5. Subtyping of monoclonal supernatants.

50µl of sheep anti-mouse Ig (Dako) (1µg/ml in 0.1M glycine buffer pH 9.5) was incubated in microtitre plates at 4°C overnight. The plates were washed in saline/Tween and then blocked with 1% BSA in PBS for 1 hr at RT. Hybridoma supernatants diluted 1/500, 1/1000 and 1/2000 in 0.1% BSA/PBS were incubated for 2 hr at RT. After washing, peroxidase conjugated anti-IgG1 diluted 1/1000, anti-IgG2a diluted 1/1000, anti-IgG2b diluted 1/2000 and anti-IgM diluted 1/1000 (all from Serotec) ,in 0.1% BSA/PBS were incubated for 2 hr at RT. The peroxidase was developed as described above.

5.2.6. Radiolabelling of granulocyte lysate.

A CML granulocyte lysate was labelled by the chloramine-T method (Bolton, 1977).

CML granulocyte lysate containing 20µg of protein was mixed with 10µl of 0.25M potassium phosphate pH 7.5, 1mCi of sodium iodide and 10µl of chloramine-T (1.6mg/ml in 0.25M potassium phosphate pH 7.5). After mixing for 15

sec, 10µl of cysteine (0.56mg/ml in phosphate buffer) and 100µl of potassium iodide (10 mg/ml in phosphate buffer) was added.

A sepharose G50 column was equilibrated with PBS containing 0.1% BSA and 0.02% sodium azide. The iodinated material was loaded onto the column and fractions containing the labelled protein were collected. The labelled material was diluted in PBS containing 0.1% BSA to give $5x10^7$cpm/ml.

5.2.7. Dilution curves of immunodepleting monoclonal supernatants.

The labelled granulocyte lysate (1ml) was pre-absorbed with 4ml of 10% SAC-CEL donkey anti-mouse Ig, to remove any labelled material which binds non-specifically to the SAC-CEL. Serial 10-fold dilutions in diluent were incubated with pre-absorbed labelled lysate (1.7×10^5cpm) overnight at RT. 100µl of SAC-CEL donkey anti-mouse Ig was added and incubated for 2 hr at RT. The mixture was washed with 3ml of saline/Tween and the pellet counted.

5.2.8. Epitope analysis by competition binding.

All proteins are made up of several different antigenic fragments known as epitopes. Each monoclonal antibody detects one antigenic determinant of a protein. Therefore, monoclonal antibodies used to devise a two-site sandwich ELISA must detect different epitopes. Epitope

analysis is designed to define the antigenic determinant identified by each monoclonal antibody by comparing it's binding to a labelled antigen in competition with other antibodies.

PVC plates were coated with rabbit anti-mouse Ig (Dako) diluted 1/1000 in 0.1M glycine buffer, pH 9 5. After 2 hr incubation at RT the plates were washed and 50µl of monoclonal supernatant added and incubated for 1 hr at RT. After further washing 50µl of non-immune mouse serum diluted 1/200 in diluent was added, to block unoccupied antibody sites, and incubated for 1 hr at RT. A second monoclonal supernatant (50µl), pre-mixed for 30 min with SAC-CEL pre-absorbed labelled granulocyte lysate (35000cpm), was added and incubated overnight. After further washing individual wells of the plates were cut out and the bound label counted.

5.2.9. Immunoprecipitation of labelled antigen recognised by monoclonal supernatants and guinea pig anti-CML.

Radiolabelled granulocyte lysate was pre-absorbed with an equal volume of Protein-A sepharose (Pharmacia), 10% w/v in PBS, to remove any non-specific binding of the labelled proteins to the protein-A sepharose, before use in immunoprecipitation.

100µl of a 10% (w/v)mixture of Protein-A sepharose was washed three times in saline/Tween before incubation with 200µl of a mixture containing equal volumes of all

positive monoclonal supernatants, for 2 hr at RT. A mixture of anti-a-fetoprotein monoclonal supernatants was used as a negative control. The sepharose was washed a further three times before the addition of 50µl of Protein-A pre-absorbed ^{125}I labelled CML lysate ($5x10^5$cpm) and incubation overnight at 4^oC. The pellet was washed three times with 0.1M Tris buffer pH 6.8 containing 0.5M lithium chloride and 1% (v/v) mercaptoethanol. The immunoprecipitated label was eluted by boiling in sample buffer and run on 15% polyacrylamide SDS gels as described earlier (section 4.2.5.).

Immunoprecipitation using guinea pig anti-CML was as above, except that 20µl of the polyclonal antiserum replaced the monoclonal supernatant. Non-immune guinea pig serum was used as a negative control.

The electrophoresed gel was fixed in 50% ethanol, 10% acetic acid (v/v) in distilled water for 30 min. The gel was then vacuum dried onto 3MM filter paper and exposed onto pre-flashed x-ray film.

5.2.10. Production of ascites.

Ascitic fluid was produced by growing subclones of hybridomas as ascitic tumours in BALB/c mice pretreated with pristane.

5.2.11. Purification of ascitic fluid antibodies.

Protein content of ascitic fluid was determined by the method of Lowry et al. (1951).

All purification steps were at 4°C. Ascitic fluid diluted to 10 mg/ml protein in PBS was added to an equal volume of saturated ammonium sulphate and mixed overnight. The mixture was centrifuged and the precipitate washed twice with cold saturated ammonium sulphate. The precipitate was resuspended in PBS and dialysed, with three changes, against PBS, overnight. The protein concentration of the dialysed material was measured.

The PBS in 2.5ml of dialysed ascites was exchanged in a PD10 column (Sephadex G25 Pharmacia) with 0.05M 2-(N-morpholino) ethane-sulfonic acid (MES) containing 0.005M sodium chloride pH 6.0 (start buffer).

The Ig was further purified by use of a cation exchange column adapted for use with fast protein liquid chromatography equipment (FPLC, Pharmacia).

A Mono S HR 5/5 column was equilibrated with start buffer. The ascites, in start buffer (4ml) was loaded onto the column at a flow rate of 0.5ml/min. After 8 min the bound Ig was eluted in a gradient formed by adding 0.05M MES with 1M sodium chloride up to a concentration of 25% (v/v) in start buffer. Protein remaining bound to the column was eluted with 100% 0.05M MES with 1M sodium chloride.

The fractions containing the specific Ig were identified by screening against CML granulocyte lysate,

diluted 1/1000 in 0.1M glycine buffer pH 9.5, coated onto plates, as previously described.

5.2.12. Biotinylation of ascites Ig.

The purified Ig was dialysed against 0.2M borate buffer pH 8.0 and diluted to a protein concentration of 1mg/ml in the same buffer. 100µl of N-hydroxysuccinimidobiotin (1mg/ml in dimethylsulphoxide) was added to 1ml of Ig. After 4 hr at RT the mixture was dialysed against PBS with 0.02% (w/v) sodium azide and stored in small aliquots at -70^{o}C until use.

5.2.13 Development of a sandwich assay.

Development of a two-site sandwich assay requires two antibodies which recognise different epitopes of an antigen. The first antibody must retain its immunological function either when bound directly to a solid-phase (PVC plates) or indirectly to an anti-mouse Ig previously bound to a solid phase. A suitably diluted antigen source incubated with the first antibody can be detected by a labelled second antibody. The amount of second antibody bound is proportional to the amount of antigen present. Levels of antigen can be determined by comparing with a standard curve.

The purified ascites were all tested in a sandwich assay. The best antibody pair was selected by biotinylating each antibody and testing against each possible coating antibody in an ELISA using CML

granulocyte lysate as an antigen source. By comparing the dilution curves of CML granulocyte lysate in each of these pairs, the pair giving the greatest range of detection of antigen was chosen as the basis of the assay. These were CF356 as coating antibody and biotinylated CF344 as second antibody.

PVC plates were coated with 50µl of ascites CF356 diluted 1/1000 in 0.1M glycine pH 9.5 and incubated for 2 hr at RT. Plates were washed and then blocked with 0.5% (w/v) BSA in PBS. 50µl of a preparation of CML granulocyte lysate in doubling dilutions from 1/5000 to 1/640000 was used as an antigen standard. Serum pools, from CF homozygotes, heterozygotes and normal controls were tested to determine the range and sensitivity of the assay system. Serum samples diluted 1/100, 1/500, 1/1000 and 1/2000 or diluted standards were incubated overnight at $4^{o}C$. After washing, 50µl of biotinylated CF344, 1/1000 in diluent, was added and incubated for 1 hr at RT. After further washing streptavidin-peroxidase (Amersham), 1/1000 in diluent, was added for 30 min at $35^{o}C$. Peroxidase was developed as described previously.

5.3. Results.

5.3.1. Screening.

The results of the fusions attempted to produce monoclonal antibodies to CFAg are summarised in Table 5.2..

In the primary screening of hybridomas, produced in fusions from immunisation with IEF homogenate, against sephacryl S300 fractions all supernatants which showed a higher level in the CFAg positive fractions than in the normal were considered positive. Also all supernatants which reacted with the ethylene glycol pH 11.0 immunopurified material were treated as positive.The three IEF homogenate fusions failed to produce any specific antibodies to CFAg as defined by the immunodepletion of antigen from CF serum in RIE.

However, further testing against common serum antigens identified forty-two antibodies to albumin, twenty-two to IgG, one to transferrin and one to a_2M. The remaining twenty-six supernatants were positive when tested against the sephacryl S300 fraction, from CF serum, but their specificity was not identified. Twenty-two supernatants were positive against the immunopurified material: these were all antibodies to IgG.

Immunisation with RIE immunoprecipitins produced four specific antibodies and immunisation with CML granulocyte lysate produced six. Immunodepletion of CFAg in CF serum by these ten monoclonal antibodies is shown in Fig.5.1..

Table 5.2. Outcomes of fusions attempted to produce monoclonal antibodies to CFAg.

Immunogen	Number of fusions	Number of wells tested	Number Positive, Primary Screening	Number Positive, Secondary Screening
IEF homogenate	3	1437	92	0
RIE precipitins	2	1018	84	4
CML lysate	1	352	75	6

Fig.5.1. Immunodepletion of CFAg from serum using monoclonal supernatants.

Immunodepletion of the rocket formed by RIE of CF serum against anti-IEF. A reduced or depleted rocket indicates a monoclonal antibody against the same protein as anti-IEF.
Non-specific antibodies in wells 2,3,5,10, and 11. Specific antibodies in wells 1 (CF31), 4 (CF145), 6 (CF297), 7 (CF320), 8 (CF344), 9 (CF345), 12 (CF557), 13 (CF46), 14 (CF285) and 15 (CF356). The negative control with an irrelevant monoclonal (anti-aFP) in well 16. CF serum sample in well 17.

5.3.2. Subtypes of monoclonal supernatants.

Subtyping of the 10 monoclonal antibody supernatants identified seven to be IgG1, two to be IgG2b and one to be IgM.

5.3.3. Dilution curves.

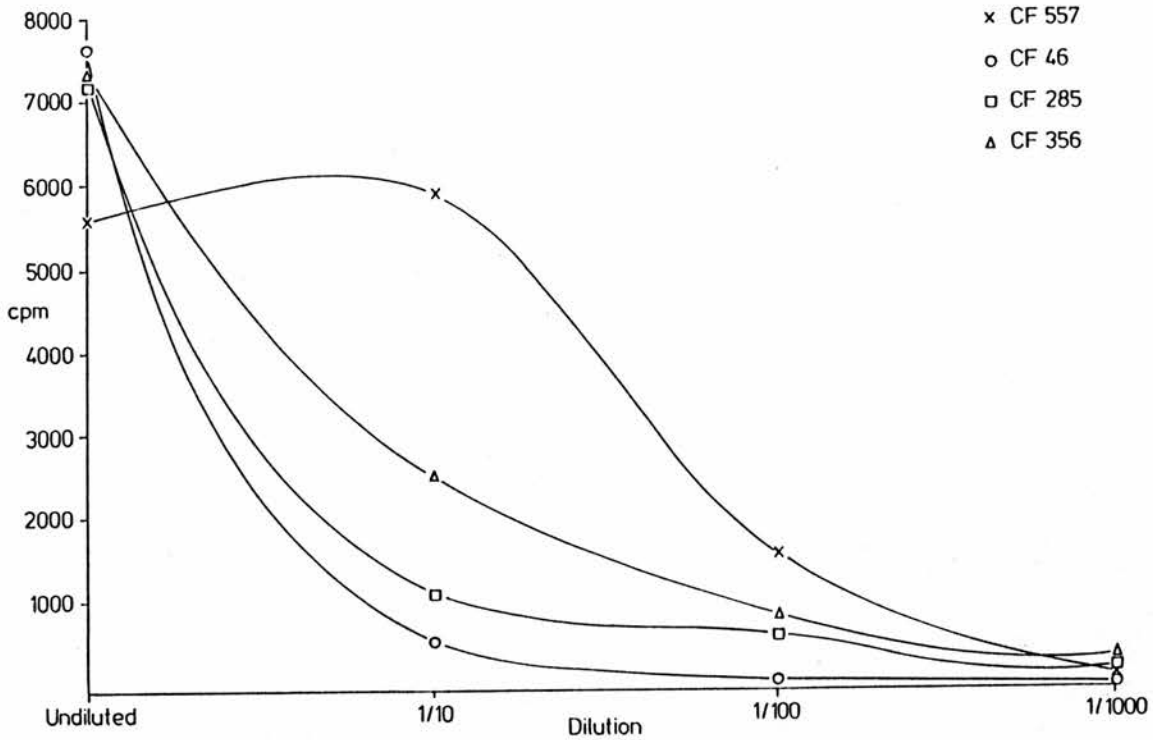
All the immunodepleting supernatants were tested against labelled CML granulocyte lysate by dilution analysis. The resulting dilution curves gave a crude estimate of the affinity ranking for each antibody (van Heyningen et al. 1983). The curves are shown in Fig.5.2.. All the monoclonals, except for CF297, exhibited a maximum binding of approximately 3-4% of the total label added. CF297 had a maximum binding of only 0.5% indicating that it was very weak and therefore unsuitable for further studies.

5.3.4. Epitope analysis.

The very similar binding curves of the nine monoclonal antibodies allowed epitope analysis to be performed. The percentage binding of labelled CML granulocyte lysate to an immobilised first antibody in the presence of a competing second antibody is shown in Table 5.3.. The observed antigen level in the absence of a second antibody is taken as 100%. There were at least two epitope groups.

Fig.5.2. Dilution curves of monoclonal antibodies against labelled CML granulocyte lysate.

a) Dilution curves of monoclonals produced from immunisation with rocket immunoprecipitins from CF serum against anti-IEF.
b) Dilution curves of monoclonal produced from immunisation with CML granulocyte lysate.


8000
7000
6000
5000
4000
3000
2000
1000
cpm
Undiluted
1/10
1/100
1/1000
Dilution
x CF 557
o CF 46
□ CF 285
△ CF 356

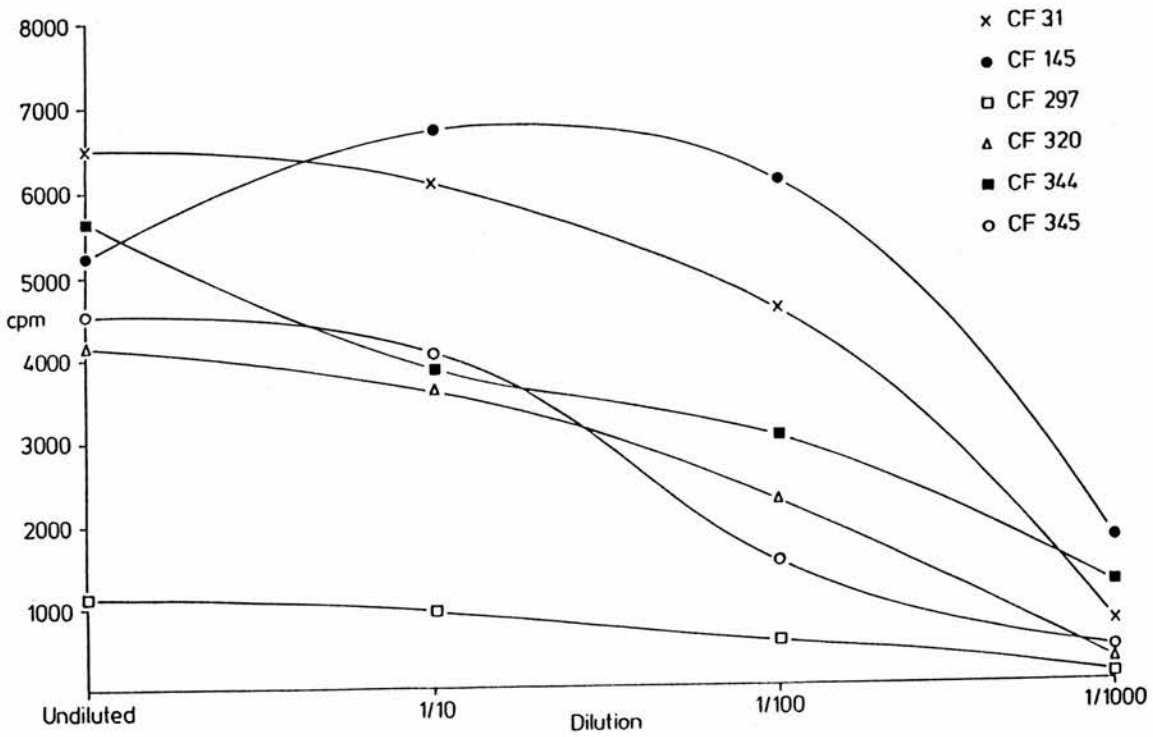

8000
7000
6000
5000
4000
3000
2000
1000
cpm
Undiluted
1/10
1/100
1/1000
Dilution
x CF 31
● CF 145
□ CF 297
△ CF 320
■ CF 344
o CF 345

Table 5.3. Epitope analysis: percentage binding of labelled granulocyte lysate, preincubated with a competing second antibody, to immobilised first antibody. Replacement of second antibody by PBS establishes 100% binding.

Second antibody	First antibody								
	31	145	320	344	345	557	46	285	356
31	4	82	35	53	0	0	16	0	0
145	77	0	62	0	85	73	86	76	86
320	78	87	6	129	105	116	144	103	103
344	50	29	21	8	39	49	111	71	60
345	9	76	95	51	0	7	29	0	9
557	17	73	100	56	0	0	15	0	2
46	81	101	91	53	32	0	46	63	63
285	37	105	58	46	0	4	39	0	19
356	66	126	125	103	23	31	47	26	30

1 : CF31, CF46, CF285, CF345, CF356, CF557.

2 : CF145, CF344.

Uncertain : CF320

5.3.5. Immunoprecipitation.

Immunoprecipitation of the labelled antigen reacting with monoclonal antibodies and with guinea pig antiserum is shown in Fig.5.3..

Two specific bands, MW 14kd and 11.5kd, are precipitated with monoclonal supernatants. These same bands are precipitated with anti-CML along with extra bands at 21kd and 9.5kd.

5.3.6. Purification of monoclonal antibodies.

Use of ammonium sulphate precipitation in conjunction with cation exchange chromatography rapidly produces material suitable for use in immunoassay. These purified antibodies were either used directly as coating antibody or biotinylated before use as second antibody. An example of a chromatography profile (CF344) and the identified antibody fractions is shown in Fig.5.4..

5.3.7. Sandwich assay.

The pair of antibodies giving the widest range of detection of antigen in CML granulocyte lysate was chosen as the basis for development of a two-site sandwich assay. These were CF356 as coating antibody and biotinylated

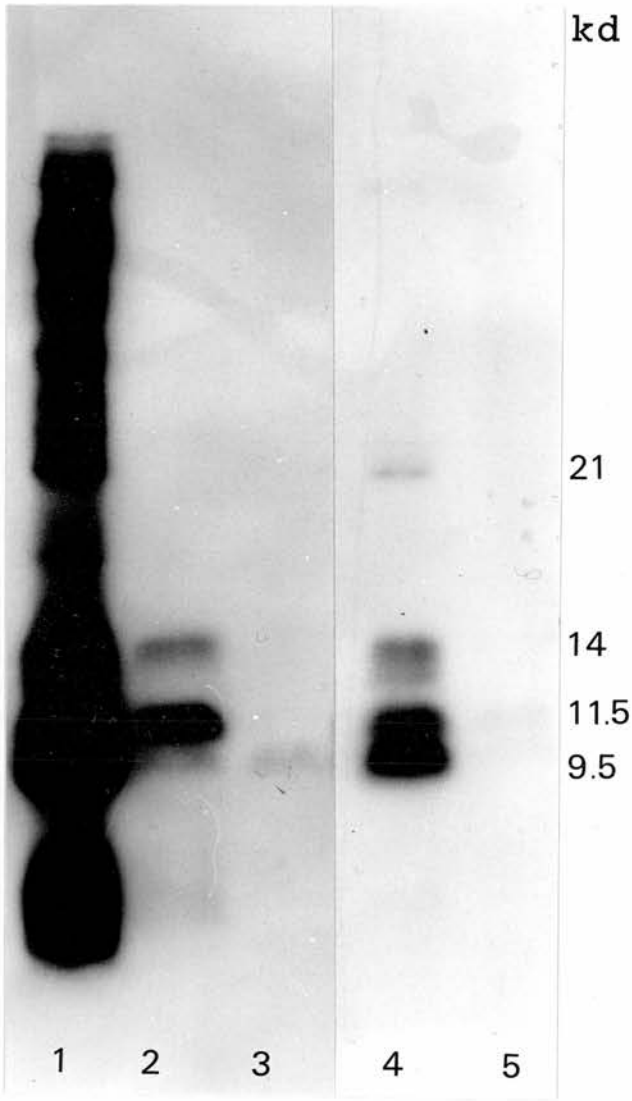
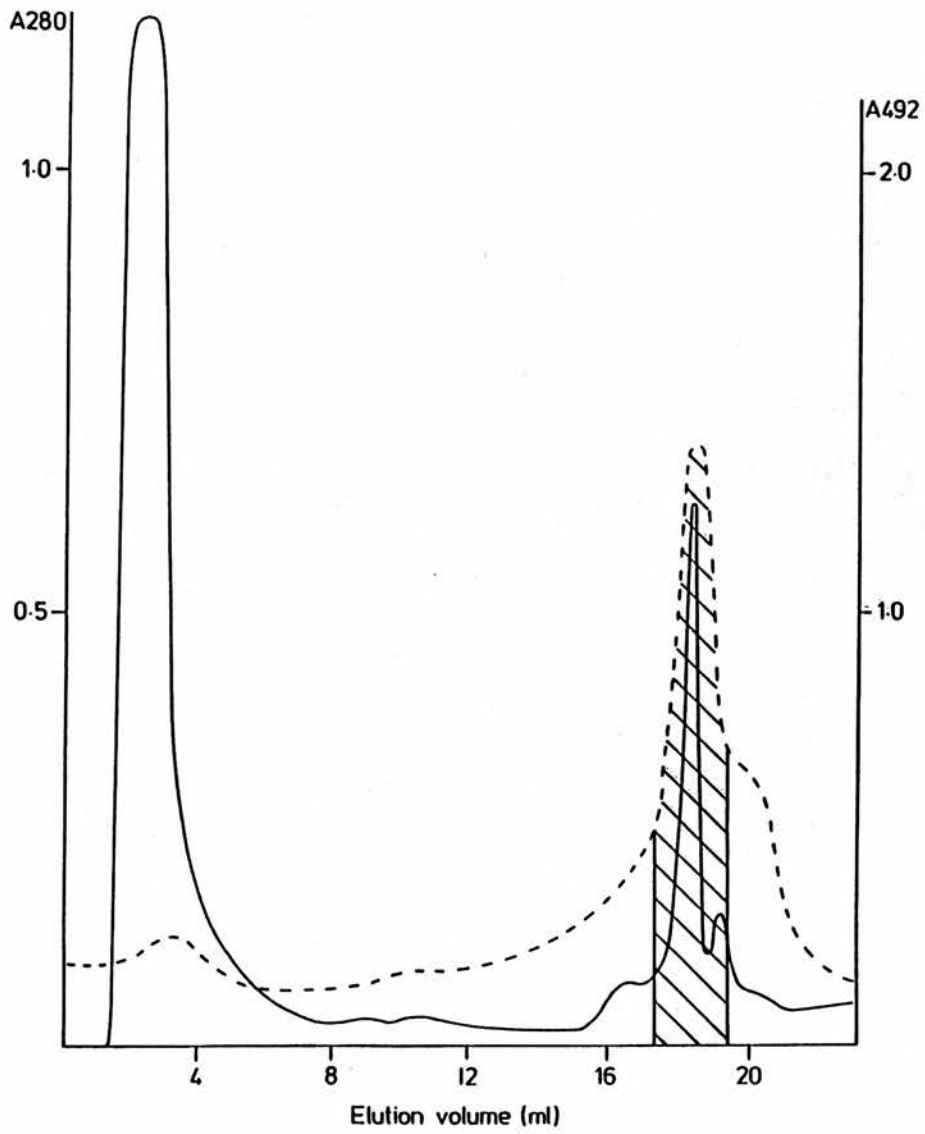


Fig.5.3. Immunoprecipitation of proteins from radiolabelled CML granulocyte lysate reacting with monoclonal and polyclonal antibodies against CFAg.

1, CML granulocyte lysate; 2, Mixed monoclonal supernatants against CFAg (CF145, CF344, CF557 and CF356 v/v); 3, Monoclonal supernatant against aFP (AFP161); 4, Guinea pig anti-CML; 5, Non-immune guinea pig serum. The MW's of immunoprecipitated proteins are indicated.

Fig.5.4. Purification of monoclonal ascites Ig, from CF344, by cation-exchange chromatography.

The solid line shows the protein absorbance profile at A280. The dashed line shows the immunoreactivity against CML granulocyte lysate detected by peroxidase-conjugated anti-mouse Ig at A492. The shaded area indicates the fractions pooled as purified Ig.


A280
1·0
0·5
A492
2·0
1·0
4
8
12
16
20
Elution volume (ml)

CF344 as second antibody. CML granulocyte lysate was used as a standard in all assays. The antigen present in 10^8 cells/ml was given an arbitrary value of 10^7 units (U)/ml.

The standard curve covers a range of 62.5-1000U as shown in Fig.5.5.. Serial dilutions of serum pools gave readings parallel to the standard curve. Antigen concentration in the CF homozygote pool was greater than in the heterozygote serum pool which was greater than the normal pool. The dilution curves are shown in Fig.5.6..

Fig.5.5. Standard curve of CML granulocyte lysate for the two-site sandwich ELISA of CFAg.

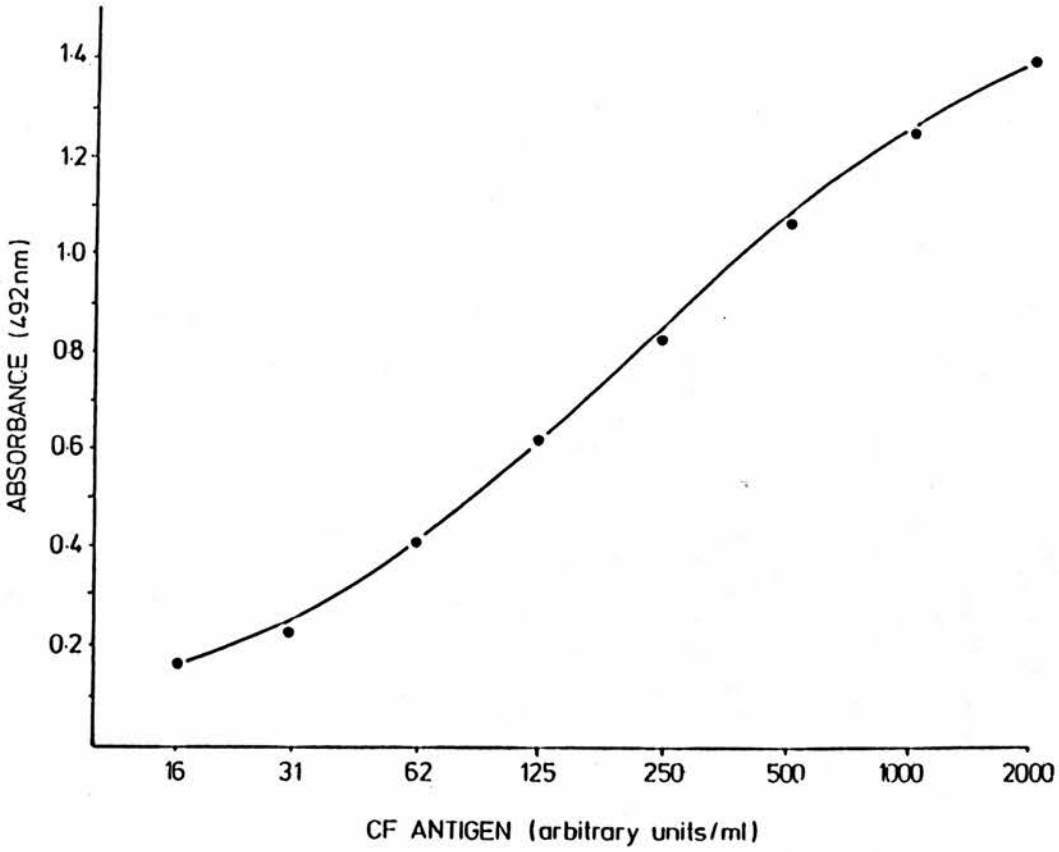
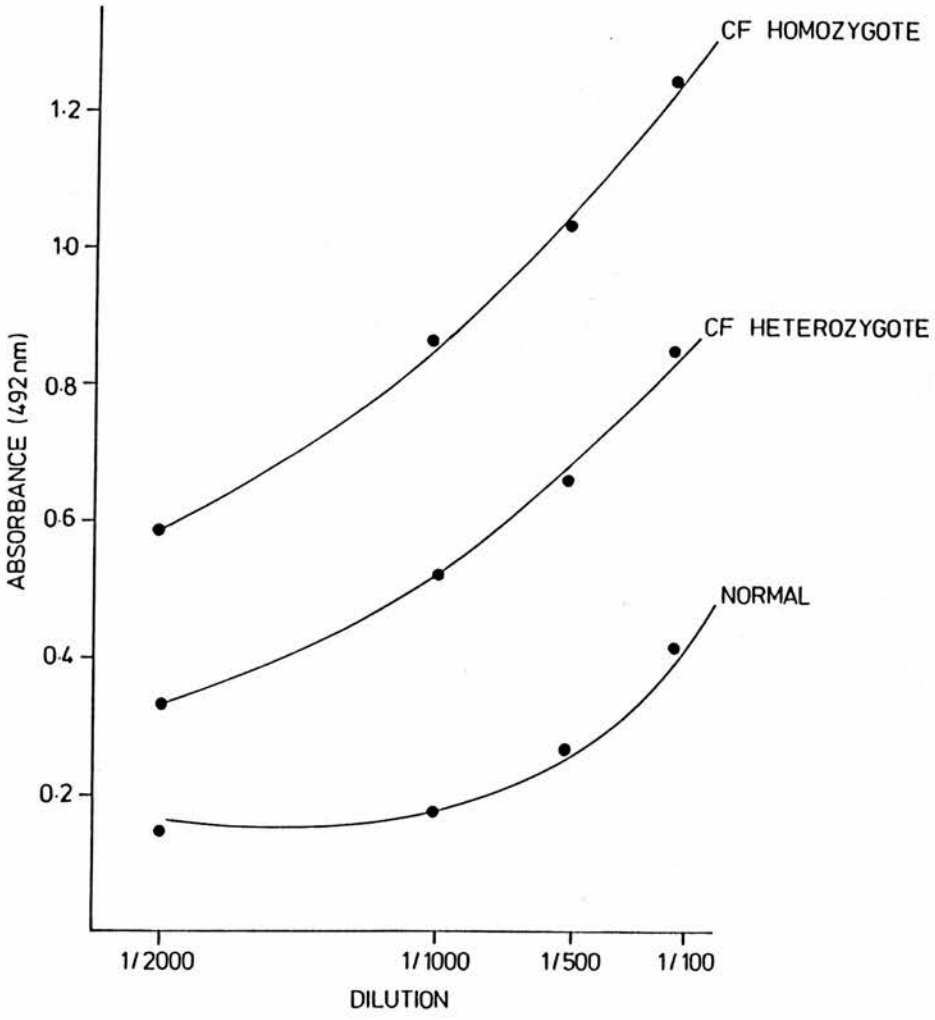

ABSORBANCE (492nm)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
16
31
62
125
250
500
1000
2000
CF ANTIGEN (arbitrary units/ml)

Fig. 5.6. Dilution curves of serum samples in a two-site sandwich ELISA of CFAg.


CF HOMOZYGOTE
CF HETEROZYGOTE
NORMAL
ABSORBANCE (492 nm)
1·2
1·0
0·8
0·6
0·4
0·2
1/2000
1/1000
1/500
1/100
DILUTION

5.4. Discussion.

By immunising mice with the IEF homogenate, pH 8.4-8.5 from a CF serum pool, it was possible to produce hybridomas. However, screening of these hybridoma supernatants revealed no antibodies with apparent immunoreactivity against CFAg, as determined by immunodepletion of anti-IEF rockets. Instead there were monoclonal antibodies against albumin, IgG, transferrin and a_2M. The CFAg positive fractions from sephacryl S300 contain several serum proteins of which CFAg is probably a relatively minor component. The CFAg present must have been largely competed out by other irrelevant proteins when the fractions were bound to plates. Therefore, any monoclonal antibodies against CFAg would have had only a few molecules of protein to bind to and would have been regarded as negative in the primary screening system. The material derived from immunopurification of CF serum on a CNBr-sepharose anti-IEF column which was used in screening of hybridoma supernatants had very little CFAg present as determined by RIE. The amount of CFAg bound to plates may have been too low to be detected in the screening assay. All the antibodies which reacted with this material turned out to be anti-IgG, which was probably due to cross-reaction with small, but detectable, amounts of guinea pig IgG leaching from the solid-phase.

Identification of granulocytes as the tissue source

of CFAg provided a more abundant source of the protein which led to a successful primary screening procedure being devised. Screening of supernatants against CML granulocyte lysate identified monoclonal antibodies against CFAg. Immunisation with RIE precipitins produced four stable hybridomas and immunisation with CML granulocyte lysate produced six stable hybridomas. The specifity of these monoclonals was established by their ability to immunodeplete CF serum of CFAg, as detected by RIE.

SDS-polyacrylamide gel analysis of immunoprecipitated labelled antigen showed that these monoclonals bound specifically to proteins of MW 14kd and 11.5kd. Anti-CML bound to proteins of the same MW, plus extra bands at MW 21kd and 9.5kd. The extra bands detected by the anti-CML are probably due to it being a polyclonal antiserum produced against a crude preparation of CML granulocyte lysate, and therefore reacting with more than one protein present in the labelled material.

Identification of at least two epitope groups allowed for the setting up of a two-site sandwich ELISA which could be used for the quantitation of CFAg in serum samples. Preliminary testing of the assay using CF homozygote, CF heterozygote and normal serum pools showed an elevated level of CFAg in the CF homozygote, an intermediate level in the CF heterozygote and a low level in the normal, indicating that the antibody is useful over a wide range of CFAg levels.

The production of monoclonal antibodies combined with the identification of granulocytes as a rich source of CFAg paved the way for the purification of the protein and ultimately cloning of the gene.

CHAPTER SIX

CLINICAL STUDIES

6.1. Introduction.

6.1.1. Assay of CFAg in serum samples.

A two-site ELISA assay system was developed for CFAg. A preliminary series of serum samples (Series 1) was assayed for CFAg using anti-CML RIE in parallel with the two-site sandwich ELISA. These assays were firstly to ascertain whether previous observations of an apparent link in levels of CFAg to expression of the CF gene (Manson and Brock, 1980; Bullock et al., 1982) were still demonstrable using anti-CML in place of anti-IEF and secondly, to check that the levels of CFAg measured by RIE and ELISA were comparable (Hayward et al. 1986).

A second larger series of different serum samples (Series 2), which included disease controls, was assayed for CFAg, lactoferrin (Lf) and C-reactive protein (CRP) by ELISA (Hayward et al.).

CFAg is a granulocyte derived serum protein. Therefore, any raised level of CFAg observed in sera from CF homozygotes and CF heterozygotes compared with normal individuals may be directly attributed either to raised granulocyte numbers or granulocyte damage due to recurrent infection. In an attempt to ascertain whether this was indeed correct the serum level of Lf, a granulocyte protein found in the specific granules, was measured and used to 'correct' CFAg values. The level of CRP, an acute phase reactant, was used as an indication of acute inflammation caused by infection (Pepys and Baltz, 1983).

These proteins were also measured in a series of disease controls. The white cell and granulocyte counts of CF homozygotes, CF heterozygotes and disease controls were also considered in analysis.

6.2. Methods.

6.2.1. Serum samples.

Serum samples were collected into glass tubes (Seward) and immediately cooled to 4°C and allowed to clot for 16-24 hr before centrifugation at 4°C. The samples were aliquotted into plastic tubes and stored at -70°C until use.

Series 1: 37 CF homozygotes, 17 obligate CF heterozygotes and 14 normal healthy controls.

Series 2: 50 CF homozygotes, 34 obligate CF heterozygotes 60 normal healthy controls and 25 disease controls

The disorders of the disease controls are described in Table 6.1..

6.2.2. Assay of CFAg.

In series 1 CFAg was assayed by RIE using 3% anti-CML with a 20µl serum sample and by ELISA as described in section 5.2.13.. In series 2 CFAg was measured by ELISA.

6.2.3. Assay of Lf.

Monoclonal antibodies to Lf were produced as described by Chung et al.(1985). A two-site sandwich assay was carried out by coating PVC plates with ascitic fluid from monoclonal antibody B92, diluted 1/1000 in 0.1M glycine, pH 9.5, and incubating for 2 hr at RT. Plates were washed and blocked, before addition of serum samples undiluted, and diluted 1/10, 1/50 and 1/100 in diluent. Lf

Table 6.1. Characteristics of the disease control group.

Disorder	Number
Chronic asthma	4
Acute asthma	9
Pulmonary eosinophilia	3
Sarcoidosis	2
Chronic bronchitis with emphysema	1
Chronic bronchitis with pulmonary fibrosis	1
Severe bronchiectasis	1
Mild bronchiectasis	2
Bronchial carcinoma	2

standard was purchased from Sigma. After overnight incubation at 4°C, biotinylated second monoclonal antibody, B97, diluted 1/1000, was added and incubated for 1 hr at RT. The remainder of the assay was as described for CFAg (section 5.2.13.).

6.2.4. Assay of CRP.

CRP was assayed by a modification of a two-site sandwich ELISA described by Salonen (1982).

PVC plates were coated with 50µl of polyclonal anti-CRP (Dako), diluted 1/1000 in 0.1M glycine pH 9.5, and incubated for 2 hr at RT. Plates were washed and blocked , before addition of serum samples diluted 1/100, 1/500, 1/1000 and 1/2000 in diluent. CRP standard serum was purchased from Behringwerke. After overnight incubation at 4°C, peroxidase-conjugated polyclonal second antibody, anti-CRP (Dako), diluted 1/1000 , was added and incubated for 1 hr at RT. The peroxidase was developed as described earlier (section 5.2.2.).

6.2.5. White cell and neutrophil counts.

These were carried out by routine methods in the Haematology laboratories of either the Western General Hospital or the Royal Hospital for Sick Children, Edinburgh.

6.3. Results.

6.3.1. Series 1: Comparison of RIE and ELISA in the assay of CFAg.

In Series 1 the CFAg concentration was measured by RIE using anti-CML and ELISA in sera from 37 CF homozygotes, 17 obligate heterozygotes and 14 normal healthy controls. The results are shown in Figs.6.1. and 6.2..

The relationship of the values of CFAg measured by RIE and ELISA was examined using the Spearman rank correlation test (Table 6.2.). Highly significant correlations were found in all three groups of samples. The difference between the three groups of samples was tested for significance in both assays by using the Median test of significance (Table 6.2.). Both RIE and ELISA showed significant differences between the median values of all three groups of serum samples. In the more sensitive ELISA the differences were more significant than in RIE ($p<0.001$ compared with $p<0.02$).

6.3.2. Series 2: CFAg, Lf and CRP values.

Sera from 50 CF homozygotes, 34 CF heterozygotes, 60 healthy controls and 25 disease controls were assayed for CFAg , Lf and CRP. The results are shown in Figs.6.3-6.5.. For each protein measured there was considerable overlap between the groups, although the median value of each was greater for CF homozygotes than CF heterozygotes, which

Fig.6.1. Series 1: Levels of CFAg in serum samples, measured by RIE.

CF homozygotes (CF), CF heterozygotes (HZ) and normal controls (N). Median values of each group are indicated by the solid line.

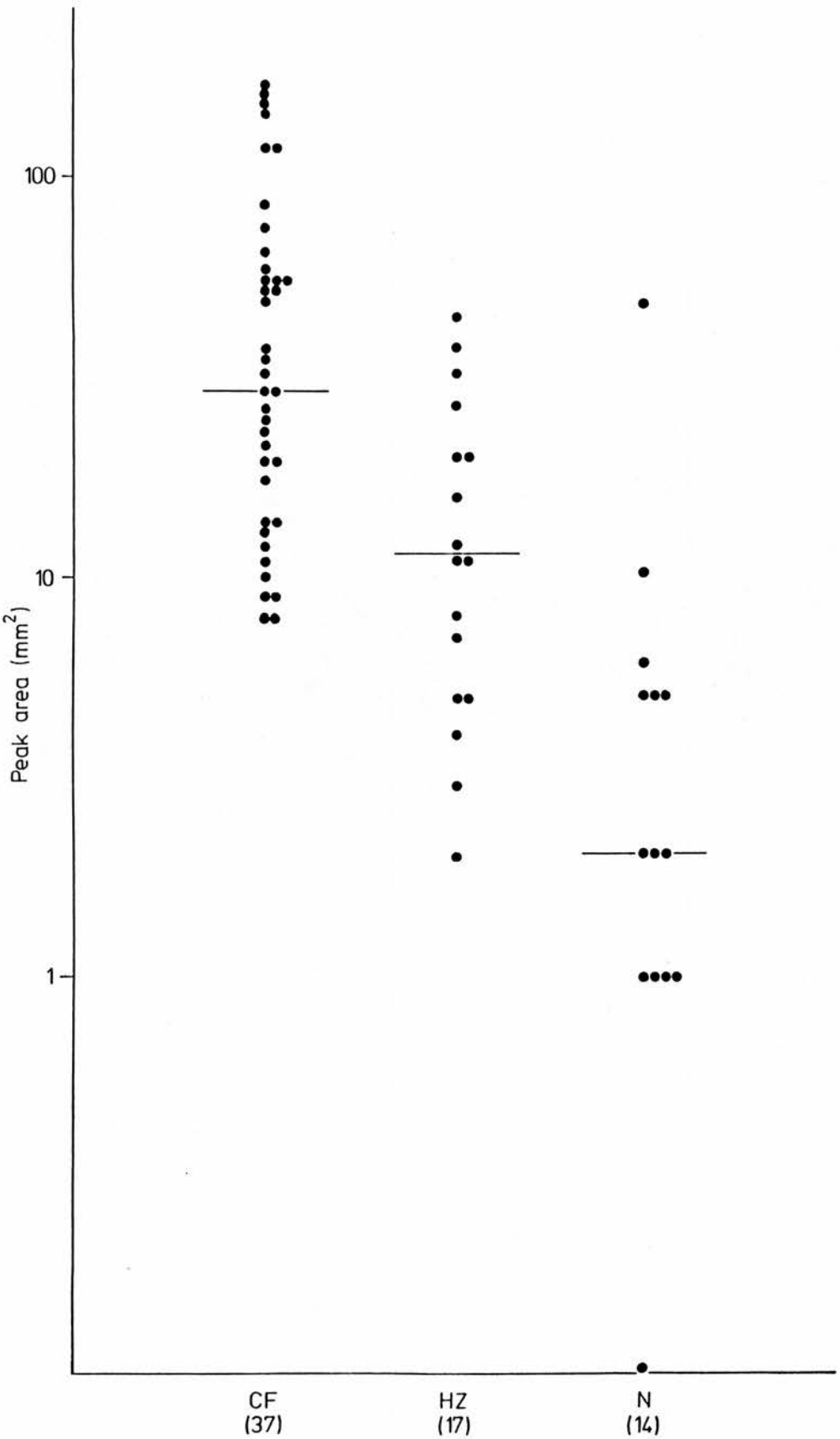

100
10
1
Peak area (mm²)
CF
(37)
HZ
(17)
N
(14)

Fig.6.2. **Series 1:Levels of CFAg in serum samples, measured by ELISA.**

CF homozygotes (CF), CF heterozygotes (HZ) and normal controls (N), measured by ELISA.Median values of each group are indicated by the solid line.

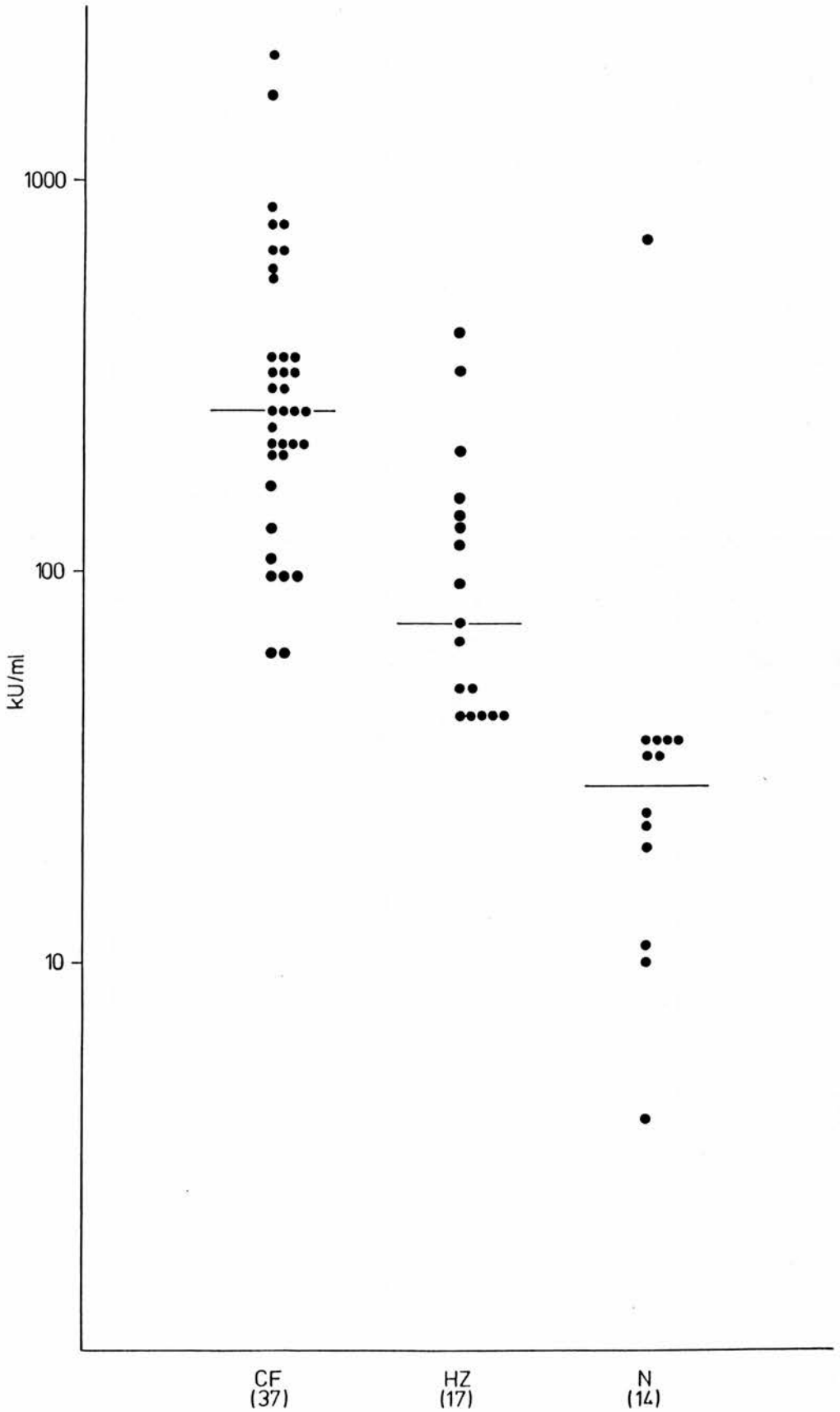

1000
100
10
kU/ml
CF
(37)
HZ
(17)
N
(14)

Table 6.2. The median values and correlations of RIE and ELISA of serum samples.

Sample	No.	RIE mm	ELISA KU	Correlation coefficient
Normal	14	2	22	0.9782
CF heterozygotes	17	11.5	72	0.7168
CF homozygotes	37	30	250	0.7371

Median test of significance.

RIE	Normal/CF heterozygote	$p<0.02$
	CF heterozygote/CF homozygote	$p<0.02$
ELISA	Normal/CF heterozygote	$p<0.001$
	CF heterozygote/CF homozygote	$p<0.001$

Fig.6.3. Series 2: Levels of CFAg in serum samples.

CF homozygotes (CF), CF heterozygotes (HZ), normal controls (N), and disease controls (DC) measured by ELISA. Median values of each group are indicated by the solid line.

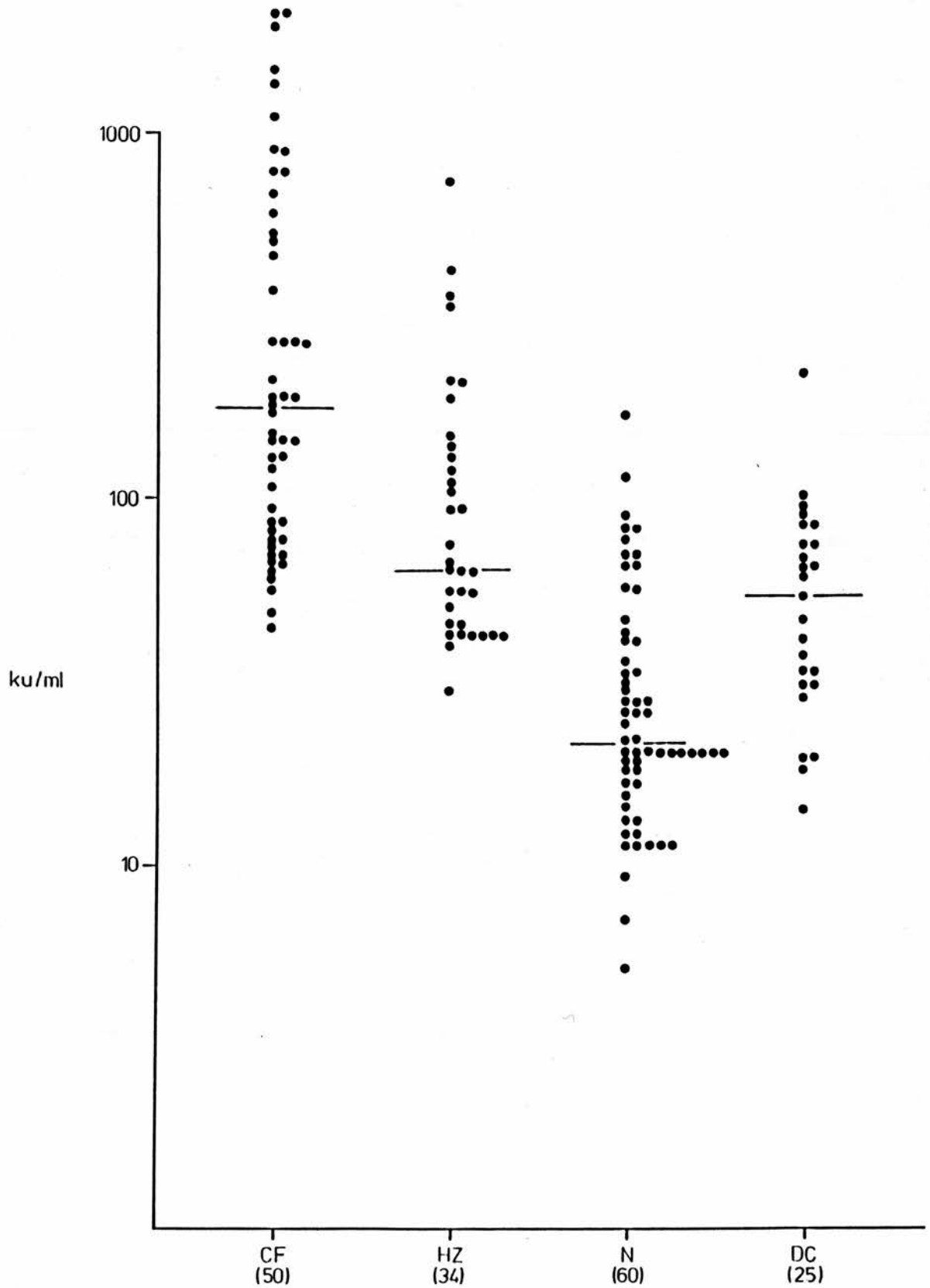

1000
100
10
ku/ml
CF
(50)
HZ
(34)
N
(60)
DC
(25)

Fig.6.4. Series 2: Levels of Lf in serum samples.

CF homozygotes (CF), CF heterozygotes (HZ), normal controls (N), and disease controls (DC) measured by ELISA. Median values of each group are indicated by the solid line.

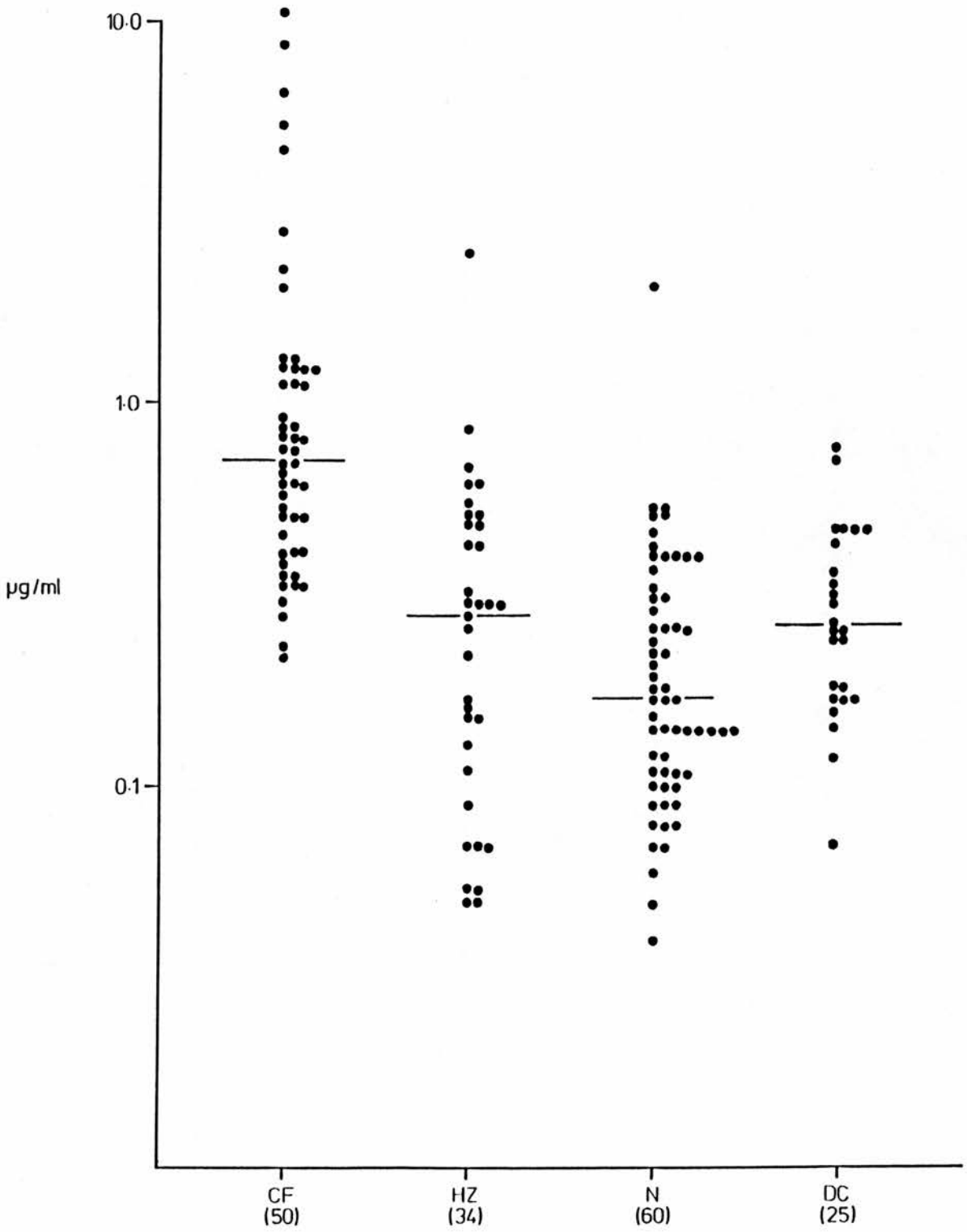
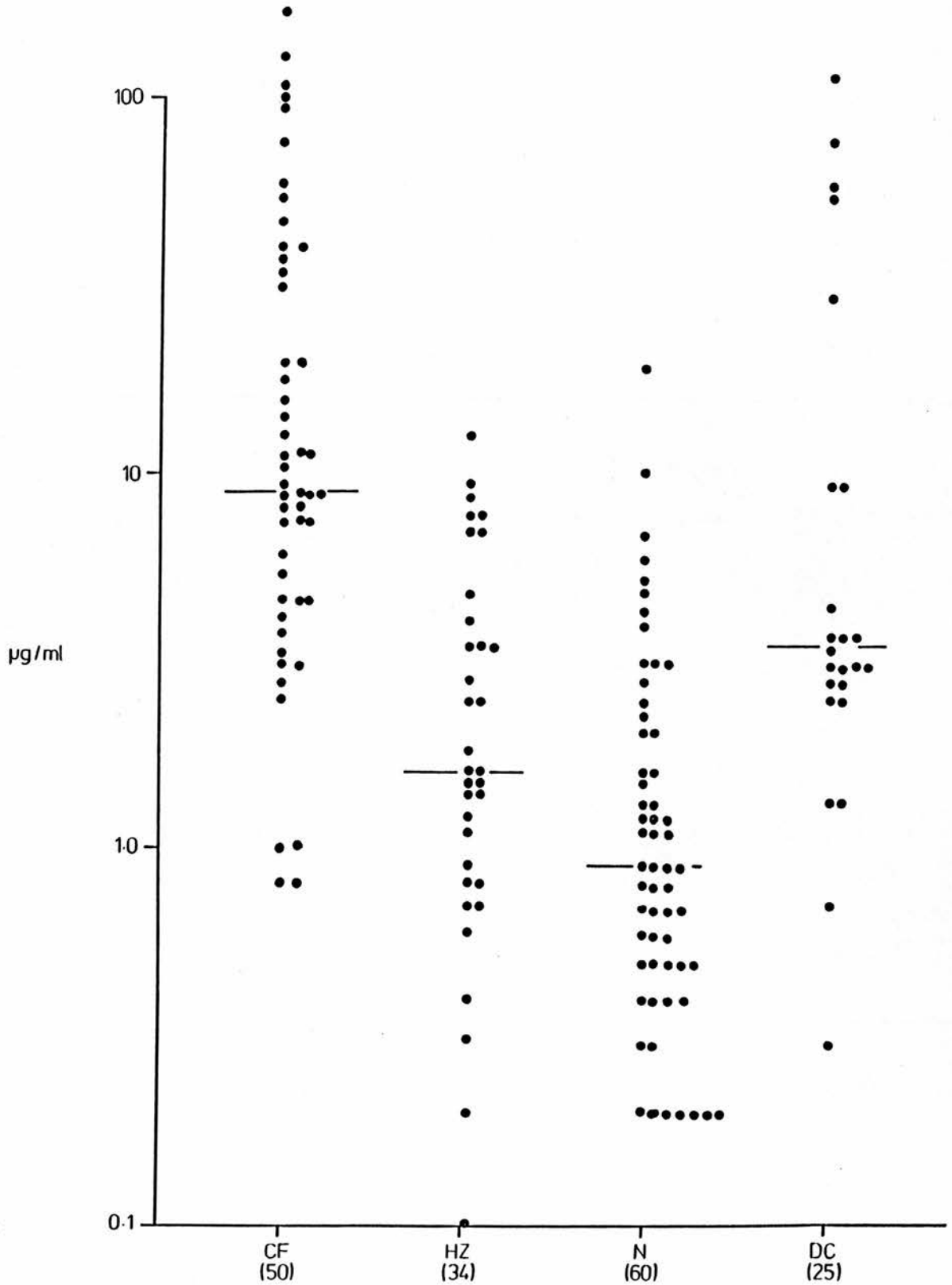

10·0
1·0
0·1
µg/ml
CF
(50)
HZ
(34)
N
(60)
DC
(25)

Fig.6.5. **Series 2: Levels of CRP in serum samples.**

CF homozygotes (CF), CF heterozygotes (HZ), normal controls (N), and disease controls (DC) measured by ELISA. Median values of each group are indicated by the solid line.


100
10
1·0
0·1
µg/ml
CF
(50)
HZ
(34)
N
(60)
DC
(25)

in turn was greater than normal controls. The disease control group median values were greater than the normal control group.

The ratio of CFAg to Lf and of CFAg to CRP was calculated for each sample. Ratios of the samples in the four groups are plotted in Figs.6.6. and 6.7. The ratios for the CF heterozygotes were the highest in both sets of data.

The relationship of the values of CFAg, Lf and CRP in each sample was examined using the Spearman rank correlation test. Significant correlations were found for each pair of proteins in CF homozygotes (Table 6.3.). Significant differences were also found between CFAg and Lf in CF heterozygotes and in normal controls, but not in disease controls.

The difference between the four groups of samples was tested for significance by comparing the median values of CFAg, Lf, and CRP, and the median values of the ratios of CFAg/Lf and CFAg/CRP. Results are shown in Table 6.4..

In all assays the same normal and CF homozygote serum samples were incorporated as serum standards to assess the variability of each assay system. The results are summarised in Table 6.5..

Fig.6.6. **Series 2: Ratios of CFAg to Lf in serum samples.**

CF homozygotes (CF), CF heterozygotes (HZ), normal controls (N), and disease controls (DC) measured by ELISA. Median values of each group are indicated by the solid line.

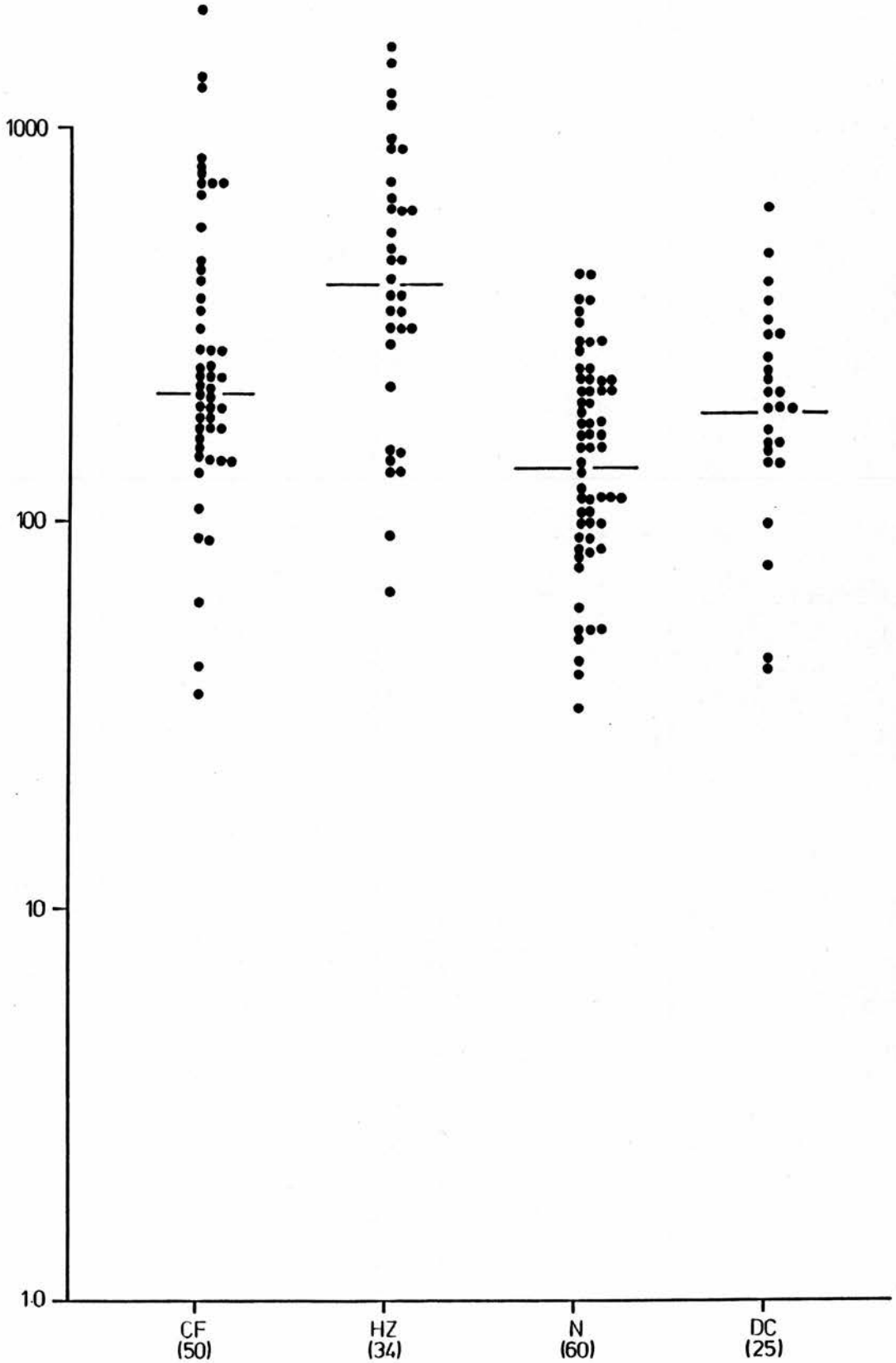

1000
100
10
1.0
CF
(50)
HZ
(34)
N
(60)
DC
(25)

Fig.6.7. **Series 2: Ratios of CFAg to CRP in serum samples.**

CF homozygotes (CF), CF heterozygotes (HZ), normal controls (N), and disease controls (DC) measured by ELISA. Median values of each group are indicated by the solid line.

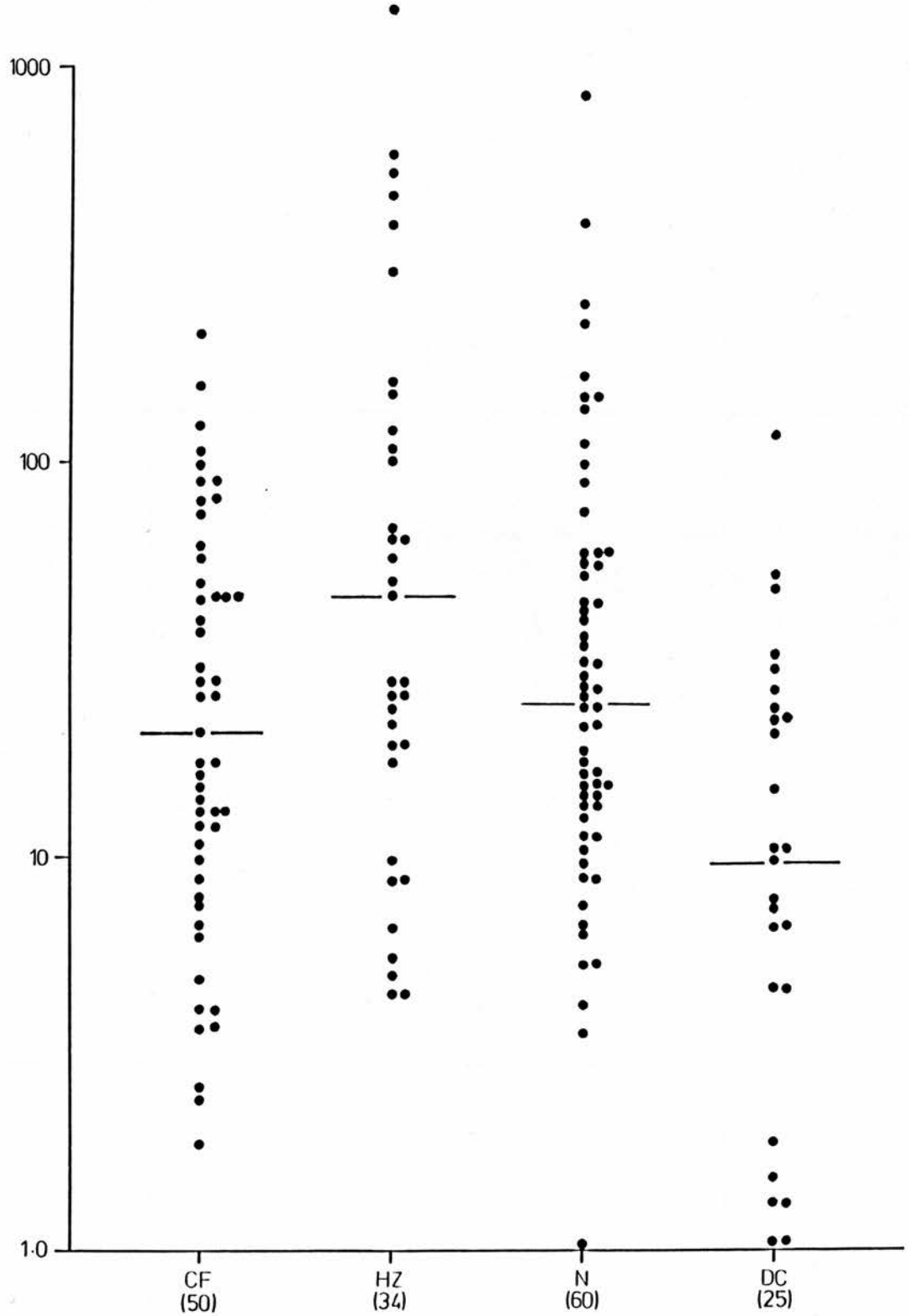

1000
100
10
1·0
CF
(50)
HZ
(34)
N
(60)
DC
(25)

Table 6.3. Significance of correlations between CFAg, Lf, and CRP in individual samples from the four different groups of serum samples. (Spearman rank test).

	CF homozygotes	CF heterozygotes	Normal controls	Disease controls
CFAg vs Lf	$p < 0.001$	$p < 0.01$	$p < 0.001$	NS*
CFAg vs CRP	$p < 0.01$	NS	NS	NS
Lf vs CRP	$p < 0.01$	NS	NS	NS

*NS, not significant

Table 6.4. Significance of differences between the mean values in the data in Figs.6.3.- 6.7. (Median test).

Pairs tested	CFAg	Lf	CRP	CFAg/Lf	CFAg/CRP
CF vs HZ	p <0.01	p <0.001	p <0.001	p <0.001	p <0.05
CF vs N	p <0.001	p <0.001	p <0.001	NS	NS
CF vs DC	p <0.001	p <0.001	p <0.05	NS	NS
HZ vs N	p <0.001	NS	p <0.02	p <0.001	NS
HZ vs DC	NS	NS	p <0.05	p <0.001	p <0.02
N vs DC	p < 0.001	NS	P <0.001	NS	NS

Table 6.5. Co-efficients of variation of serum standards in assay systems.

	Number of Repeats	CFAg RIE(mm^2) Mean and SD	CV%	CFAg ELISA(KU) Mean and SD	CV%
Series 1					
Normal serum	8	0	0	23.4 ± 2.3	10
CF serum	8	36 ± 4	11	240 ± 20.4	9

		CFAg ELISA(KU) Mean and SD	CV%	Lf µg/ml Mean and SD	CV%	CRP µg/ml Mean and SD	CV%
Series 2							
Normal serum	10	21.9 ± 2.0	9	0.40 ± 0.26	7	0.9 ± 0.18	20
CF serum	10	221 ± 15	7	0.46 ± 0.26	6	15.5 ± 1.29	8

6.3.3. White cell and neutrophil counts.

The white cell (12.6 ± 4.1 x 10^9/l) and neutrophil counts (9.0 ± 3.7 x 10^9/l) are elevated in CF homozygotes. The CF heterozygote and disease control groups all had white cell and neutrophil counts within the normal range; (white cells : 4.0-11.0 x 10^9/l; neutrophils : 2.0-7.5 x 10^9/l).

6.4. Discussion.

This study confirms earlier observations using polyclonal antisera (Manson and Brock, 1980; Bullock et al., 1982) that the level of CFAg is elevated in CF homozygotes, at an intermediate level in CF heterozygotes and is low in normal controls.

In Series 1, measurement of CFAg by RIE and ELISA showed highly significant correlations in all three groups of samples. The medians of all three groups were also significantly different but there is a considerable overlap of the RIE peak values in all three groups. In the ELISA there is overlap between the CF homozygote and CF heterozygote samples, But only 1 of the 14 normal values is in the CF range (Fig.6.2.). The ELISA appears to improve the discrimination of the three CF genotypes compared with peak area measurement in RIE.

The presence of CFAg in serum originates in granulocytes (van Heyningen et al., 1985). These cells are fragile and can release their contents during blood collection which explains the care which was necessary in both blood collection and handling previously observed (Bullock et al., 1982). The granulocyte turnover in normal individuals is very rapid, since a normal adult releases 10^{11} neutrophils into the circulation daily (Baggiolini, 1980). The short time spent by these cells in the blood means that granulocyte proteins are cleared very quickly.

Measurement of another granulocyte protein should allow the damage taking place *in vivo* and *in vitro* to be assessed. Lf, an iron-binding protein present in the secondary or specific granules of granulocytes (Baggiolini, 1980; Lash *et al*. 1983) was chosen as it is thought to reflect granulocyte activation (Lash *et al*., 1983). By measuring Lf simultaneously with CFAg in serum it should be possible to 'correct' for cell damage.

CRP is an indicator of tissue damage *in vivo*, which may cause undue elevation of granulocyte proteins (Pepys and Baltz, 1983). The level of this protein was also measured in each serum sample.

In Series 2 the observed values of CFAg, Lf and CRP in serum were compared in the four different groups. The values of CFAg in serum samples could not safely distinguish CF homozygotes and heterozygotes from normal controls and in the case of disease controls, with above normal levels of CFAg, it was even more difficult. When each individual sample was compared for ratios of CFAg/Lf or CFAg/CRP no clear separation was observed. Therefore, neither of these proteins improved the separation of CF homozygotes, CF heterozygotes and controls.

This study of CFAg in relation to Lf and CRP was devised to identify whether the observed levels of CFAg had any specificity for the CF gene or was purely a result of granulocyte proliferation or tissue damage due to infection. The data accumulated does not clarify the situation.

The results from CF homozygotes and disease controls are complicated by the fact that both groups have a variety of clinical manifestations. The identification of any possible specificity for CFAg in CF should therefore be considered in disease-free controls and CF heterozygotes.

The data for CF heterozygotes shows that whilst the level of CFAg is significantly elevated compared with normal controls there is no significant increase in the levels of either Lf or CRP (Table 6.4.). Consideration of the ratio of CFAg/Lf further emphasizes this point.

The observation of the elevated CFAg in heterozygotes can be explained by this being due to a CF gene-specific component. The CF homozygotes have a two component system, one contributed to by increased granulocyte turnover resulting from active infection and the other being gene specific. The disease controls have increased CFAg due to the increased active infection indicated by elevated CRP levels observed.

The results of these assays indicate that CFAg levels in serum cannot be used as a heterozygote screening test in a general population. However, the persistent observation of elevated CFAg in the disease-free CF heterozygotes suggests that there is some link between CFAg and CF gene status.

CHAPTER SEVEN

GENERAL DISCUSSION

The work described in this thesis was initiated by reported observations of a CF-specific protein which could be detected as a doublet band at pI 8.46±0.05 after IEF of CF homozygote and heterozygote serum (Wilson et al., 1973; 1975). This method involved careful collection of blood and very specific running and staining conditions in order for the doublet band (CFP) to be observed in the majority of CF homozygotes and heterozygotes, but not in the majority of healthy controls. The test was qualitative and was dependent for success on the observer being able to identify the band correctly. Many laboratories succeeded in identifying the doublet band, but with such varying degrees of success that in most cases the test was not considered sufficiently accurate to be used as a diagnostic or carrier test for CF.

A limited quantity of antiserum had been produced in guinea pigs by immunisation with the homogenised portion of IEF gel containing the doublet band (Manson and Brock, 1980). The quality of the antiserum was very variable and only small quantities suitable for serum testing were available. The antiserum was tested by RIE and showed quantitative differences dependent on CF genotype, a large peak in CF homozygotes, an intermediate peak in CF heterozygotes and a small or absent peak in healthy controls. The apparent gene dosage effect observed indicated the possible potential of using this protein in diagnosis and carrier detection of CF.

In order to test the potential of using the

polyclonal antiserum produced against the portion of IEF gel containing the doublet band, a large quantity of antiserum was required. This was accumulated by immunising a large number of guinea pigs. Only 40% of the guinea pigs immunised produced a specific antiserum (anti-IEF) which showed an apparent quantitative difference of a serum protein, measured by RIE, in CF homozygotes, CF heterozygotes and healthy controls. The amount of antiserum from each guinea pig was small, but by pooling batches of serum from several guinea pigs sufficient was available to test a series of serum samples. Two batches of antiserum, AS60 and AS82/83, were used to assay the protein in the serum from 14 CF homozygotes, 28 CF heterozygotes and 23 healthy controls, by RIE and IRMA (Bullock et al, 1982). Selection of arbitrary cut-offs for each assay system enabled 94% of the CF genotypes to be correctly identified.

Some characterisation of the serum protein was possible using anti-IEF. The protein had an apparent molecular weight of 51-60 kd as assessed by gel filtration. Immunodepletion of serum, using commercially available anti-IgG, followed by RIE showed no reduction of the antigen peak. Similarly, depletion of the serum with protein-A sepharose showed no reduction of immunological activity. These three characteristics of CFAg differed from reported properties of CFP (Wilson, 1982). Therefore, the protein identified by anti-IEF was named cystic fibrosis antigen (CFAg). Attempts to purify CFAg from CF

serum by immunopurification, using anti-IEF, were unsuccesful.

The use of anti-IEF showed discrimination between the three different CF genotypes, but the the quality and quantity of this antiserum was extremely difficult to control.

In a search for a rich source of CFAg, which would hopefully make antiserum production easier, a variety of easily accessible cells and body fluids were tested, using anti-IEF. The only material, other than serum, containing detectable quantities of CFAg was the cytoplasmic fraction of granulocytes. Very high levels of CFAg were found in granulocytes from CF homozygotes, CF heterozygotes, healthy normals and CML patients. Identification of CML granulocytes as an abundant source of CFAg enabled the production of a high titre antiserum in guinea pigs. This antiserum (anti-CML) showed complete identity with anti-IEF when tested by Ouchterlony double diffusion against both CF serum and CML granulocyte lysate.

A cellular source of CFAg allowed for the production of somatic cell hybrids between a mouse stem-cell line and CML granulocytes. Segregation analysis of the hybrids assigned the gene for CFAg to chromosome 1 (van Heyningen et al., 1985).

Development of an immunoassay for a CF-associated serum protein is an improvement on the observer dependent IEF method and appears to classify a larger number of serum samples correctly. However the immunoassay is not

very sensitive and will not detect low levels or small differences of CFAg accurately.

Hybridoma culture techniques allowed for the production of monoclonal antibodies against CFAg (Hayward et al., 1986). These antibodies were initially identified by screening against CML granulocyte lysate. Their immunospecificity was verified by their ability to immunodeplete the CFAg peak from CF serum, when tested against anti-IEF. The ten stable hybridomas produced were further identified as being against the same protein as anti-IEF and anti-CML by immunoprecipitation of antigen from radiolabelled CML granulocytes. SDS-polyacrylamide electrophoresis of the specific immunoprecipitates showed two common bands of MW 14kd and 11.5kd.

Selection of a suitable pair of monoclonal antibodies allowed for the development of a sensitive, reproducible two-site sandwich ELISA for CFAg. A standard curve was derived using dilutions of a CML granulocyte lysate given an arbitrary value for CFAg of 10^7U/ml of lysate (10^8cells/ml). The assay was sensitive over a wide range of serum dilutions and therefore was suitable for rapid, large scale measurement of CFAg.

Preliminary testing of the ELISA in a series of serum samples from 37 CF homozygotes, 17 CF heterozygotes and 14 healthy controls in comparison with RIE, using anti-CML, showed a highly significant correlation between both sets of results. There was overlap between the three groups of samples in both assays, but the median values were

significantly different.

The location of granulocytes as the source of CFAg indicated that there was a possibility that the raised levels of CFAg in serum from CF homozygotes and heterozygotes was due to granulocyte proliferation. This possiblity was tested for by measuring CFAg, by ELISA, in a second series of samples, consisting of 50 CF homozygotes, 34 CF heterozygotes, 60 healthy controls and 25 patients suffering from non-CF related respiratory disorders. Another granulocyte-derived serum protein, Lf, and the acute-phase reactant CRP were measured simultaneously (Hayward et al.).

In these assays it was impossible to distinguish the four groups of samples by measuring CFAg. The median values for the CF homozygotes compared with CF heterozygotes and CF heterozygotes compared with healthy controls were still significantly different, but the disease controls were not significantly different from the CF heterozygotes. Consideration of the ratios of CFAg/Lf and CFAg/CRP did not improve the discrimination of samples. However, the ratio of CFAg/Lf in the healthy CF heterozygotes was significantly greater than in the other three groups. The elevated level of CFAg in serum is not related to an increase in granulocyte number and may indicate a possible association between CFAg and the CF gene.

The production of monoclonal antibodies and the development of an ELISA has greatly improved the

reproducibility, accuracy and sensitivity of the detection of CFAg. The overlap of antigen values observed in earlier assays is still apparent and the reason for this must be due to biological, rather than technical, phenomena.

Once monoclonal antibodies were produced against CFAg it was thought worthwhile to purify the protein and determine its biochemical identity. While the assay system was being developed and assessed other members of our CF research group undertook the task of purifying and characterising CFAg.

The monoclonal antibodies to CFAg were used to immunopurify the protein (Novak et al.).

CFAg was found to be inducible in the pro-myelocytic cell line HL60 using either retinoic acid (RA) or dimethyl sulphoxide (DMSO) to cause differentiation along the myeloid pathway (Collins et al., 1977).

One monoclonal antibody (CF557) was used for immunoaffinity purification of CFAg from CF plasma, granulocytes and induced HL60 cells. Purified protein from all sources was found to be identical by amino-acid sequencing for at least the first 20 residues.

The molecular mass of the CFAg subunit has been defined as approximately 14kd by specific immunoprecipitation from ^{125}I-labelled CML granulocyte lysate. Using specific immunoprecipitation following the incorporation of ^{35}S-methionine into proteins in intact cells in culture, CFAg synthesis was shown in normal and

CML granulocytes and in differentiated HL60 cells, identifying three available sources of mRNA.

The partial amino-acid sequencing of CFAg allowed the synthesis of a highly redundant oligonucleotide probe which was able to isolate a cDNA clone (CFA 8-9) from a library constructed in /gt10 using mRNA prepared from CML granulocytes (Dorin et al., 1987).

The nucleotide sequence of CFA 8-9 encoded 83 amino-acids from the first amino-acid of the oligonucleotide to the C-terminal residue. The sequence of the first 11 amino-acids was derived from the protein sequencing. A relative molecular mass of 10,938 was deduced from the 94 amino-acid sequence.

Northern blot analysis on a variety of tissues and cell lines was carrried out to assess message size and abundance. mRNA from CML granulocyte samples contained very high levels of CFA 8-9 transcripts with a major band of approximately 550 base pairs (bp) and a minor band of approximately 1000 bp. Equivalent amounts of mRNA isolated from uninduced HL60 and from RA and DMSO induced HL60 showed no message in uninduced, low abundance in RA induced and high abundance in DMSO induced HL60 cells. mRNA from other malignant cell lines gave no detectable signal.

Southern blot analysis of somatic cell hybrids between WEHI-TG and human CML cells only showed a positive signal in the presence of chromosome 1. The availability of cell hybrids containing defined fragments of chromosome

1 allowed the gene locus to be localised to the region between bands q12 and q22 on the long arm of chromosome 1.

The deduced amino-acid sequence of cloned CFAg was compared with sequences in an NBRF database. Highly significant homology was obtained with the subunits of a brain associated calcium-binding protein, bovine S100a and S100b (Isobe and Okuyama, 1981) and bovine, porcine and rat Intestinal calcium-binding proteins (Fullmer and Wassermann, 1981). Two other calcium-binding proteins, porcine p11, a regulatory subunit of the protein complex which is a cellular target for tyrosine kinase (Gerke and Weber, 1985; Glenney and Tack, 1985) and 2A9, also known as calcyclin, a protein that shows cell cycle dependent expression in WI-38 cells (a strain of human diploid fibroblasts (Calabretta et al.,1986; Ferarri et al., 1987). Calcyclin is not detectable in quiescent fibroblasts, but can be stimulated to express in cells treated with serum, platelet-derived growth factor or epidermal growth factor. It can also be induced in a wide variety of other cells including acute myeloid leukaemia (AML) and CML. In some AML cells it appears to be deregulated and a possible role in cell-cycle regulation has been postulated for calcyclin. The gene for calcyclin has been mapped, by in situ hybridisation to the long arm of chromosome 1 q21-q25 (Ferrari et al., 1987). Calcyclin is the only homologous protein to CFAg which has, so far, been mapped to a chromosome. The close proximity to the two gene locations indicates a possible gene cluster for

this family of calcium-binding proteins.

Despite development of the best possible assay system and characterisation of the gene, it is still not possible to ascertain whether CFAg has a role in CF. The generally elevated level of CFAg observed in serum from healthy CF heterozygotes compared with healthy controls indicates some connection with expression of the CF gene but at present the physiolological or biochemical mechanism responsible for this is unknown.

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