FELINE LEUKAEMIA VIRUS-SPECIFIC PROTEINS

by

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DECLARATION

This work was done independently by the author apart from the following: lectin affinity chromatography and subsequent electrophoresis was done by Dr. J. Smart of the Imperial Cancer Research Fund laboratories, London. In addition, two experiments described in Chapter Seven were done in London by myself and Dr. Smart.

SUMMARY

This study was concerned with the polypeptides of feline leukaemia virus (FeLV), both the structural polypeptides of the virus particle and putative non-structural proteins which appear in FeLV-infected and transformed cells.

In the first part of the work (Chapter Two) it was established that virus purification and storage methods did not cause serious losses which might invalidate further characterisation. At the same time, the extent to which purified virus preparations are contaminated with host cell proteins was investigated.

The next chapter concerned the structural location of virus polypeptides within the virus particle, which was examined by protease digestion, enzymic radio-iodination and generation of sub-viral fractions. The results were similar to published findings for murine leukaemia viruses, at least with respect to the major surface glycoprotein and the major core protein.

Comparison of the polypeptides of different FeLV strains, documented in Chapter Four, revealed essentially no detectable differences in the molecular size of the low molecular weight virion proteins. However, the major glycoprotein showed considerable strain to strain variation. Although this did not correspond to the virus subgroup (A, B or C) it did seem to be a stable property for a given virus strain, which may relate to the serotypic specificities of the glycoprotein.

Purification of FeLV polypeptides and immunisation of heterologous species was the subject of study in Chapter Five. Highly reactive antisera to two virus polypeptides, p27 and p15, were raised and were utilised in immune precipitation studies to detect immunologically related molecules in purified virus and in infected cells. Attempts to raise antisera to FeLV glycoproteins, which would be expected to neutralise viral infectivity, were largely unsuccessful. A more promising approach to this problem is suggested.

Finally, in Chapters Six and Seven, the cell-membrane glycoproteins of cultured cells, both uninfected and infected with FeLV, were examined, and an attempt was made to elucidate the biochemical nature of the feline oncornavirus associated cell membrane antigen (FOCMA). In the course of these

studies, a number of interesting observations were made regarding the intracellular processing and shedding into culture medium of molecules related to virion structural proteins. However, the cat sera which were expected to react with FOCMA did not precipitate any additional molecule when compared to control sera. Possible reasons for this observation are discussed.

ABBREVIATIONS

AMV	Avian myeloblastosis virus.
bis	N, N' methylene-bis-acrylamide.
c81	Clone 81.
cpm	Counts per minute.
DOC	Sodium deoxycholate.
DTT	Dithiothreitol.
EDTA	Ethylenediamine tetraacetic acid.
EFB	Eagle's minimal essential medium. Glasgow modification, containing 10% foetal bovine serum.
eFOV	Endogenous feline oncornavirus.
FBS	Foetal bovine serum.
FeLV	Feline leukaemia virus.
FeSV	Feline sarcoma virus.
FOCMA	Feline oncornavirus-associated cell membrane antigen.
GuHCl	Guanidine hydrochloride.
Ki-MSV	Kirsten strain, murine sarcoma virus.
M_r	Apparent molecular weight (migration relative to standard proteins).
MSV	Murine sarcoma virus.
MuLV	Murine leukaemia virus.
NDC	NP-40/DOC detergent mixture.
NP40	Nonidet-P40.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Dulbecco's phosphate buffered saline 'A'.
R-MuLV	Rauscher murine leukaemia virus.
RSV	Rous sarcoma virus.
SDS	Sodium dodecyl sulphate.
TEMED	N, N, N, N. tetramethylethylenediamine.
Tris	Tris (hydroxymethyl) methylamine.
TS	Tris saline buffer

NOTE ON NOMENCLATURE

Throughout the thesis, the terms oncornavirus and RNA tumour virus are used interchangeably. Recommendations have recently been made by the International Committee on the Taxonomy of Viruses (ICTV) on this group of viruses and the designation of FeLV in the new system would be as a species within the genus "Type C oncovirus".

The thesis includes many SDS polyacrylamide gel analyses. The migration of proteins under this system is expressed as M_r (apparent molecular weight) which is the correct term since molecular weight is estimated by comparison with proteins of known molecular weight. To avoid excessive repetition, proteins are designated as e.g. 10,000 daltons, as if synonymous with 10,000 M_r, although the dalton is a measure of molecular mass.

GENERAL INTRODUCTION

Oncornaviruses are members of a widely distributed group of animal viruses, many of which induce tumours in their hosts of origin. Apart from their structural similarity, an important shared property is the ability to integrate the viral genome into host cell genetic material, which gives rise to persistent infections. In addition some oncornaviruses are inherited as normal chromosomal genes.

The first representatives of the group were discovered when it was demonstrated that chicken leukaemias (Ellerman and Bang, 1908) and sarcomas (Rous, 1911) could be transmitted by cell-free filtrates. For many years, the chicken was regarded as unique in this respect until the first mammalian leukaemia virus was demonstrated in 1951, when Gross showed that filtrates of tumours from a mouse strain (AKR) with a high incidence of leukaemia could transmit the disease to a low incidence strain. This discovery was followed by other reports of virus induced mouse leukaemias (Friend, 1957; Rauscher, 1962) and sarcomas (Moloney, 1960). Subsequently Jarrett et al. (1964) showed that cell-free homogenates from cat lymphosarcoma tissue could induce lymphosarcoma when inoculated into neonatal cats, and that there was an associated replicating C-type* virus. FeLV was then demonstrated in spontaneous cases of the disease (Laird et al., 1967; Rickard et al., 1967; Kawakami et al., 1967).

Many species have since been shown to carry oncogenic C-type viruses, from fish to higher primates. However, in many cases viruses are detected which are endogenous in their host species: that is, integrated into host chromosomes of the germ line and inherited in a Mendelian fashion. Such viruses often have no established role in disease. Apart from the "conventional" FeLV, the domestic cat carries an endogenous virus, which was discovered when human rhabdomyosarcoma cells were inoculated intracerebrally into cats (McAllister et al., 1972). It was originally believed that this RD 114 virus might be of human origin. However further studies demonstrated that this was an endogenous feline oncornavirus (eFOV) which was antigenically unrelated to the already known FeLV (Fischinger et al., 1973; Livingston and Todaro, 1973). The subject of endogenous mammalian RNA tumour viruses has been reviewed recently by Aaronson and Stephenson (1976).

* see page 4.

The existence of complete endogenous viral genomes in many cells and their activation by agents which include carcinogens led to the "virogene-oncogene" theory of Huebner and Todaro (1969), which proposed that each cell carries viral information (a virogene) which includes an "oncogene" responsible for cell transformation. Release of these genes from cellular regulatory mechanisms might result in virus expression and tumour growth. However, in cats, wild mice and chickens, where the epidemiology of leukaemia has been fairly well investigated, it appears that horizontal or congenital transmission are of prime importance in inducing disease, (Jarrett et al., 1973c; Hardy et al., 1973; Gardner, personal communication; Purchase and Burmester, 1972). This contrasts with transmission in "covert form" suggested in the virogene-oncogene theory.

The usual replication cycle of RNA tumour viruses can be briefly summarised as follows: virus surface components interact with specific receptors on the cell surface, allowing penetration. The viral genetic information, which is borne in the virion on a single stranded RNA molecule, is converted into double stranded circular DNA by the RNA-dependent DNA polymerase or reverse transcriptase enzyme. The next step is the integration of this "provirus" into host chromosomal DNA. In a permissive cell, the viral genes are then expressed. Messenger RNA is produced which codes for virus structural proteins and reverse transcriptase. These are assembled along with viral RNA under the host plasma membrane where virions bud outward without noticeable cytopathic effect.

Temin's protovirus hypothesis (1971) proposed that RNA tumour viruses have evolved from normal cellular genes involved in the transfer of genetic information from DNA to RNA and back to DNA, mediated by reverse transcriptase. Temin also suggested that this "reverse flow" of information could occur normally during differentiation. This theory may be supported by the observation that a virus-coded virion structural component, the gp70 of mouse leukaemia virus, has been shown to be representative of a family of related molecules, some being virion envelope components, some free serum proteins and some expressed selectively on various differentiated cells (Elder et al., 1977b). Also, viruses can be selected which have acquired host genes and hence new transformation properties. Mouse or avian sarcoma viruses have been produced by recombination of leukaemia viruses with host cell genetic material (Scolnick

et al., 1976; Hanafusa et al., 1977).

Structure of feline oncornaviruses

In electron microscope studies of thin sections of infected cells, FeLV and eFOV appear to be typical C-type particles as defined by Bernhard (1958). In the extracellular "immature" form or in budding virions, three concentric electron dense layers are visible. The apparent size of these structures varies considerably with different preparation methods (Sarkar et al., 1975). However, with carefully standardised techniques, it can be shown that eFOV particles are 5 to 10% smaller than those of FeLV. The measured diameters are 120 nm. for the outer membrane of FeLV, 90 nm. for the intermediate structure and 70 nm. for the dense ring of the inner core or nucleoid. The comparative diameters for eFOV are 110 nm., 80 nm., and 50 nm, respectively. (H.M. Laird, personal communication). In the mature particles, only the outer envelope and an electron dense core are seen. The intermediate "membrane" is no longer apparent. This maturation process has recently been attributed to cleavage of a precursor polypeptide in MuLV (Yoshinaka and Luftig, 1977). The relative infectivity of immature and mature particles has not yet been established, although rapidly harvested virus seems more infectious, with an optimum of 20 min for RSV (Smith, 1974).

A-type particles (Bernhard, 1958) have not been described in FeLV studies, either in tissue culture or virus infected tissues. However, a recent paper by Calafat et al. (1977), describes these structures in feline mammary carcinomas, although their detection was not related to expression of FeLV of eFOV antigens, leading the authors to suggest that the cat harbours another, as yet undefined oncornavirus.

FeLV and eFOV particles show surface projections, particularly in the immature form. It was originally believed that MuLV and FeLV lacked these surface structures since they are much less prominent than those of avian leukosis virus or mouse mammary tumour virus.

Genome of FeLV

The genome of FeLV is similar to that of other C-type viruses. It is a single stranded RNA molecule which sediments rapidly (70 S) in neutral sucrose gradients (Jarrett et al., 1971) which was thought to correspond to a molecular weight of $10 - 12 \times 10^6$. In addition to this high molecular weight RNA, minor

species of 35S and 4-5S can also be isolated from virions. The 70S molecule can be dissociated by denaturation with dimethyl sulphoxide or heat into ∿ 35S subunits. It was believed until recently that the 70S RNA was a complex of 3 to 4 35S subunits linked by hydrogen bonding (Whalley, 1973). It was demonstrated for RSV that the 35S was a precursor to 70S RNA (Cheung et al., 1972; Canaani et al., 1973). More recent work with the more thoroughly investigated avian and murine leukaemia viruses have shown that 70S RNA corresponds to a molecular weight of 6×10^6 (Delius et al., 1974; Riggin et al., 1975) while the 35S subunit is approximately 3×10^6 daltons in size, indicating that the native molecule is composed of only two subunits. Studies on 70S RNA, the 3' ends of which were marked with SV40 DNA have indicated that the viral RNA consists of two subunits joined together at their 5' ends in a "dimer linkage structure"; in addition, the monomers both contained a loop structure close to the middle of the molecule (Kung et al., 1976). The 35S subunits of various FeLV isolates were shown to differ in size, with FeLV-A smaller than FeLV-B or C (Whalley, 1973). The native RNA of eFOV is smaller than that of FeLV, sedimenting at ∿ 60S (Whalley, unpublished data; East et al., 1973).

It has been shown for a number of RNA tumour viruses that the 35S subunit has similarities to eukaryotic mRNAs. There is a cap structure at the 5' end, a poly-A track at the 3' end and several methylated adenosines (Furuichi et al., 1975). A recent study with FeLV found methylated adenosines, but failed to demonstrate a cap structure (Thomason et al., 1976). However, these authors also report a lower sedimentation coefficient (50 - 60S) for the native RNA and do not rule out the possibility of artefactual endonuclease hydrolysis. They used a different strain of FeLV from the earlier study of Jarrett et al. (1971).

Three separate lines of investigation have indicated that the RNA tumour virus genome is diploid, i.e., both 35S subunits are identical : firstly, RNA-DNA hybridisation kinetics (Baluda et al., 1974); secondly, analysis of unique oligonucleotides after digestion of 70S RNA with ribonuclease T (Billeter et al., 1974; Beemon et al., 1976); and thirdly, genetic analysis (McCarter, 1977).

The coding potential of RNA of 3×10^6 molecular weight is approximately 300,000 daltons of protein. Baltimore (1974) proposed four genetic functions for the RNA tumour viruses which would account for most of the coding potential of the genome: gag for the group-specific antigens or internal viral polypeptides, pol for the reverse transcriptase, env for the envelope glycoprotein and onc or

src for a cell transforming protein. All RNA tumour viruses capable of independent replication would carry gag, pol and env. The onc gene or src gene of avian and murine sarcoma viruses can be demonstrated by the existence of mutants which lack these functions (Wyke, 1975; Vogt, 1977). No onc gene has yet been attributed to FeLV. RSV can carry all four functions in one virus particle, but in this property it is unusual. All the mammalian sarcoma viruses identified so far appear to be defective. The onc gene in these viruses would appear to be carried at the expense of some other function, often the env function (Hartley and Rowe, 1966; Sarma and Log, 1977).

Nomenclature of RNA tumour virus structural polypeptides

In 1974, a consistent nomenclature was established for oncornavirus structural polypeptides (August et al., 1974). According to this convention the polypeptides are designated p (protein) or gp (glycoprotein) followed by their molecular weight in thousands. The molecular weights of the smaller components (10,000 - 30,000) are to be estimated by gel filtration in the presence of 6 molar guanidine hydrochloride (GuHCl), while SDS-PAGE should be used for larger components. In addition, the structural location of a polypeptide in the virion can be specified in parentheses (E for envelope, C for core, N for nucleoprotein). The authors recognise that accepting the GuHCl gel filtration estimate for p10, 12, 15 and 30 gives different results from comparison with various globular proteins under SDS-PAGE. The convention also ignores the anomalous migration of glycoproteins with regard to molecular weight, under SDS PAGE. Although this system of nomenclature was a great improvement over the previous confusing variety of systems, it has encountered a number of problems. Firstly, small env gene products have been discovered in MuLV strains. These components, p15(E) and p12(E), migrate in the void volume under GuHCl gel filtration, due to their hydrophobic nature. Hence SDS PAGE must be used to estimate their molecular weight (Ihle et al., 1975; Ikeda et al., 1975). A more serious problem is that, even within a species, virus polypeptides of a given molecular weight do not correspond when antigenic or ionic properties are compared. For instance, Rauscher MuLV p15 appears to correspond to Friend MuLV p12 (Strand and August, 1977). This can create confusion, particularly with viruses like FeLV which are less completely characterised than MuLV isolates.

Gene products of FeLV

The gene products of RNA tumour viruses have only recently been established by in vitro translation of viral RNA (Von der Helm and Duesberg, 1975; Salden et al., 1976; Kerr et al., 1976). Until then, it could only be established that a virion protein or viral function was virus coded by the isolation of mutants. Thus, the transformation defective avian sarcoma viruses (Duesberg and Vogt, 1973) or the env gene delection of Bryan strain RSV (Duesberg et al., 1975) demonstrated that these functions were carried by the viral genome. In most cases, however, it could only be inferred that a protein was virus-coded by its antigenic expression in a variety of host cells, and its absence from uninfected controls (Strand and August, 1976). This was certainly the case for FeLV when this study began in 1974. A further difficulty in studying oncornavirus gene expression is that unlike many other viruses, these agents do not shut off host protein synthesis. Thus, intracellular virus protein synthesis can be detected only when the proteins are selected by techniques such as immunoprecipitation.

The processing of RNA tumour virus gene products into virion proteins appears to involve post-translational cleavage of precursor polypeptides (Vogt and Eisenman, 1973; Van Zaane et al., 1975; Arcement et al., 1976). This has been shown for the gag gene of FeLV (Okasinski and Velicer, 1977). The FeLV structural polypeptides which have already been described are gp70, p27, p15, p12 and p10 (Bolognesi et al., 1974). Khan and Stephenson (1977) demonstrated immunological cross-reactivity between polypeptides of Rauscher MuLV and FeLV. They showed that the elution pattern of these cross-reactive antigens under GuHCl gel filtration corresponded closely and concluded that R-MuLV and FeLV had a common progenitor. This was further supported by the similar N-terminal amino acid sequence of FeLV p27 and R-MuLV p30 (Oroszlan et al., 1975). It seems reasonable, therefore, to draw comparisons with the better characterised R-MuLV when discussing the viral proteins individually.

gag-gene polypeptides of FeLV

It has been shown that FeLV p27, p15, p12 and p10 are derived from a common precursor (Okasinski and Velicer, 1977). However, the order of the polypeptides on the precursor is the subject of controversy. Okasinski and Velicer, from pactamycin ordering studies, deduced the sequence NH_2 - p12 -

p15 - p10 - p30 - COOH. However, Khan and Stephenson (1977), on discovering the cross-reactivity between R-MuLV and FeLV, suggested that both viruses would show the same precursor sequence. This has been well characterised for R-MuLV as NH_2 - p15 - p12 - p30 - p10 - COOH (Barbacid et al., 1977).

p27. This is the major protein of the oncornavirus core (Bolognesi et al., 1974). It carries interspecies antigenic determinants (shared by viruses of different species), group-specific determinants (shared by viruses of one species) and type-specific determinants (unique to an individual virus strain) (Green et al., 1973). Biochemical characterisation of this molecule from a range of RNA tumour viruses has confirmed the immunological evidence for their relatedness (Buchhagen et al., 1975; Oroszlan et al., 1975). In MuLV, p30 is not expressed on the virion surface (Hunsmann et al., 1976; Schwarz et al., 1976). Similar observations for FeLV p27 are reported below. However, MuLV p30 and FeLV p27 are expressed on the surface of infected cells (Yoshiki et al., 1974). It has recently been suggested that this expression may be in the form of a gag precursor (Snyder et al., 1977; Ledbetter and Nowinski, 1977). The pI of FeLV p27 is 7.5 (Khan and Stephenson, 1977).

p15. This is the "subgroup" specific antigen for R-MuLV. It carries the FMR (Friend, Moloney, Rauscher MuLV) cell surface antigen (Friedman et al., 1974). For FeLV, evidence is presented below that p15 is not expressed on the virion envelope, although it is not associated with the core either. Its location could thus be termed "extra-core". FeLV p15, like p27, is close to neutral in overall charge, (pI = 7.6; Khan and Stephenson, 1977).

p12. MuLV p12 carries type-specific determinants and is useful in the classification of endogenous viruses (Stephenson et al., 1975). It has been shown that FeLV p12 is phosphorylated (Pal et al., 1975), and can bind to its homologous RNA with a high degree of specificity (Sen et al., 1977). Hence, p12 would be expected to be located in the viral core. This is discussed below. Bolognesi et al. (1973) showed that a polypeptide of F-MuLV* which migrated as p12 in SDS-PAGE was present on the virion surface. In the light of recent evidence, this may have been a small env subgene product. FeLV p12 is a relatively acidic molecule with a pI of $\sim$5.4 so that its binding to RNA would not be by a simple acid-base or histone-like affinity.

p10. In MuLV, p10 shows a high degree of homology between isolates (Buchhagen et al., 1975). It is a basic protein (FeLV p10, pI = 9.1) (Khan and

* Friend murine leukaemia virus

Stephenson, 1977) and is found in the viral core, in strong association with the nucleic acid (Bolognesi et al., 1973). It binds to single stranded DNA cellulose columns (Davis et al., 1976). Most authors have found p10 to be poorly antigenic, but the strongest reactivity appears to be group specific (Barbacid et al., 1977).

gag precursors in virions

Okasinski and Velicer (1977) have reported a p70 precursor of p27 in FeLV virions. Such precursors have also been demonstrated in FeLV pseudotypes of Moloney MSV (Oskarsson et al., 1975) and in R-MuLV (Jamjoom et al., 1975). The latter authors have suggested that the viral RNA catalyses the processing of the gag precursor and that gag-pr 65 is present in a small fraction of virions lacking RNA.

env-gene products of FeLV

In MuLV it has been shown that gp70 is derived from a precursor in which it is linked to a small protein, designated p15(E) (Famulari et al., 1976; Arcement et al., 1976). The surface spike of avian leukosis virus has been shown to consist of two glycoproteins (Bolognesi et al., 1972a), gp85 and gp37, which are disulphide linked in virions (Leamnson and Halpern, 1976). This type of linkage has also been demonstrated recently for MuLV gp70 and p15(E) (Leamnson et al., 1977). Another minor component of R-MuLV, gp45, has recently been shown to have most of the reactivities of MuLV gp70, but to differ in its lack of interspecies reactivity, and in its greatly reduced glycosylation compared to gp70 (Charman et al., 1977).

For FeLV, the only established envelope glycoprotein is gp70 (Bolognesi et al., 1974). The possible existence of minor env-gene products is discussed below. In MuLV, the gp70 carries type, group and interspecies antigenic determinants (Strand and August, 1974). Thus, anti-Friend MuLV gp70 serum reacts with FeLV by virtue of its interspecies activity. This has been exploited to inhibit viraemia in FeLV-infected cats (de Noronha et al., 1977). It is reported below that the electrophoretic mobility of FeLV gp70 varies considerably and the biological significance of this difference is discussed.

The viral envelope determines properties such as host range, interference and serological specificity. All FeLV strains which have so far been isolated can be placed in one of three subgroups A, B or C (Sarma and Log, 1971).

This classification, which is analogous to that described for avian leukosis virus (Vogt and Ishizaki, 1966) is based on the phenomenon of viral interference: infected cells are resistant to superinfection with viruses of the same subgroup, while viruses of any other subgroup may successfully infect.

There is an unusual pattern in the frequency of FeLV field isolations. FeLV-A is present in all known isolates. FeLV-B is also present in about half of these isolations, and about 1% of isolates contain FeLV-C, either with FeLV-A (FeLV-AC) or with FeLV-A and B (FeLV-ABC) (Jarrett, et al., 1978). However, FeLV-B and C have been separated and grown independently in cell culture (Sarma and Log, 1973).

FeLV-A strains are usually restricted to growth in feline cells, while FeLV-B or C have a wider host range (Jarrett et al., 1973a). It can be demonstrated that this property, along with viral interference, is an envelope determined property, by infection of cells with pseudotype particles which carry defective sarcoma virus genomes and the envelope specificity of any FeLV subgroup (Fischinger et al., 1974; Sarma and Log, 1973).

The surface components of the virus also act as receptors for neutralising antibodies. The gp70 molecule seems to be the main target for those antibodies, since antisera to purified gp70 can neutralise viruses (Steeves et al., 1974). Antibodies to minor components, such as host antigens or p15(E) seem to require serum complement to successfully neutralise (Aupoix and Vigier, 1975; Fischinger et al., 1976).

Feline oncornavirus-associated cell membrane antigen (FOCMA).

Anti-FOCMA antibody is detected by indirect immunofluorescence (Riggs, 1971) on a feline lymphoblastoid cell line, FL74 (Theilen et al., 1969).

This antibody was observed after inoculation of cats with feline sarcoma virus (FeSV) and its presence was found to be associated with tumour regression (Essex et al., 1971). Anti-FOCMA antibody is also associated with FeLV infection (Jarrett et al., 1973b) and it has been suggested that FOCMA acts as a target for natural immunosurveillance against leukaemia development (Essex et al., 1975). Anti-FOCMA antibodies were found in 50% of urban Glasgow cats and in 6% of rural cats (Rogerson et al., 1975) giving further evidence for the horizontal transmission of FeLV (Jarrett, 1976). An experimental vaccine has been developed which is based on whole FL74 cells. This raises a high level of anti-

FOCMA antibody in inoculated cats, which are resistant to subsequent challenge with a normally pathogenic dose of FeLV (Jarrett et al., 1974; Jarrett, 1975)

The molecular nature of FOCMA is as yet unclear. The FL74 cell line on which FOCMA is detected releases FeLV of all subgroups in large quantities. However, the presence of virus neutralising antibodies in cats was found to be unrelated to the presence of FeLV (Jarrett et al., 1973b) or to the regression of FeSV induced sarcomas (Schaller et al., 1975). It was also claimed that absorption of cat sera with FeLV of all 3 subgroups failed to remove anti-FOCMA antibody (Essex, 1975). A recent study (Stephenson et al., 1977a) demonstrated a lack of correlation between anti-FOCMA antibody and anti-gp 70 or antip30 as measured by radioimmunoassay. This was particularly striking for viraemic cat sera, which often had antibodies to FOCMA, but never to gp70. The authors attributed this to a state of antigen excess of virion antigens. Essex originally described FOCMA expression on FeLV infected cells of heterologous species (Essex et al., 1972). However, since his redefinition of FOCMA as a non-virion antigen, he and his co-workers now suggest that the expression of this antigen is limited to FeSV transformed cells in vitro and in vivo, and FeLV induced feline tumour cells (Hardy et al., 1977). These authors also find FOCMA on virus negative lymphosarcoma tissues, but fail to find the antigen on FeLV infected normal blood lymphocytes, even in a cat with lymphosarcoma. They conclude that FOCMA is a tumour specific antigen which is induced by FeLV infection and is probably FeSV coded.

Very recently, a FeSV transformed non-producer cell line has been characterised (Sliski et al., 1977). This line, which does not release infectious FeLV, expresses FOCMA in the absence of detectable gp70 or p30. Immuno-precipitation studies with this cell line showed that anti-FOCMA serum reacts with a protein of 65,000 molecular weight and a precursor of 85,000 daltons which also contains FeLV p15 and p12 antigenic determinants (Stephenson et al., 1977b). It should be noted that a goat anti-FeLV serum also precipitated the 65,000 molecule to some extent. The 85,000 precursor also appears in FeSV virions when rescued from the non-producer mink cells with helper viruses other than FeLV (Sherr et al. in press).

On the other hand, a number of workers have found evidence suggesting that FOCMA may be a virion antigen. It has been shown that cats have a high incidence of antibodies to FeLV-C, although this virus is rare in nature (Russell,

1977). This neutralising antibody is present even when cats are viraemic with FeLV-A or AB. Thus, the survey of Stephenson et al. (1977a) might have failed to detect some type specific anti-FeLV gp70, since these authors used gp70 from FeLV-AB for immunoassay. Calafat (personal communication) demonstrated by immunoferritin studies that cat sera reacted with the FL74 virion envelope even when the cats were persistently viraemic. This may be due to the large amount of FeLV-C envelope antigen on the FL74 surface (Russell, 1977). Calafat also claims that anti-FOCMA activity in viraemic cat sera could be absorbed with purified FL74 virus, in contradiction of the results of Essex (1975). Attempts by Russell (1977) to correlate anti-FeLV-C neutralising antibodies with anti-FOCMA activity were unsuccessful except in certain categories of cat, notably virus-negative leukaemic cats. However, a cross-reactive determinant between FeLV-C and FOCMA could not be ruled out. Ruscetti et al. (1976) described an FL74 surface antigen, "p85", which was detected by immunoprecipitation after lactoperoxidase-catalysed ^{125}I labelling. This molecule had gp70 determinants since purified gp70 could block its precipitation. These authors suggested that FOCMA was a virus coded antigen which was closely related to gp70. However, they have recently modified this interpretation (Ruscetti and Parks 1977) since it appears that they were unable to precipitate p85 with viraemic cat sera, which are claimed to be specific for FOCMA (Stephenson et al., 1977a). These workers maintain that a high incidence of anti-p85 reactivity in cats would make this an important determinant of immunity to FeLV, even if it cannot be defined as "FOCMA" according to the criteria of Essex.

My results, presented below, do not show that FOCMA is a virion antigen. Gag proteins and their precursors in FL74 cells may be detected and gp70 and a "p85", like that of Ruscetti et al., may be demonstrated in FL74 cell culture medium and on the cell surface with virus neutralising serum. However, a viraemic cat anti-FOCMA serum, and two anti-FOCMA sera without virus neutralising activity failed to precipitate any molecule which was not detected by control sera, despite our experimental techniques being very similar to those of Stephenson et al. (1977b) who demonstrated a cross-reactive antigen in FeSV transformed mink cells.

At present the molecular nature of FOCMA in the cell line on which it was defined (FL74) is still unknown. It seems possible from the published evidence that Stephenson et al. (1977b) and Sherr et al. (in press) have

characterised a gene product of FeSV, which cross-reacts with FOCMA. It also seems possible from the evidence of Russell (1977) that FeLV-C carries or induces a determinant of FOCMA. These results are supported by the results of M.B. Gardner (personal communication) who claims to be able to absorb anti-FOCMA with FeLV-C infected feline embryo fibroblasts. It will be interesting to determine which FeLV genes are encoded in the 25 - 50% of the genome which has been shown to be endogenous to cat cells (Okabe et al., 1976; Levin et al., 1976; Niman et al., 1977).

CHAPTER ONE

GENERAL MATERIALS AND METHODS

Cells

Feline embryo cells (FE) of the FEA strain (Jarrett et al., 1973a), uninfected or infected with FeLV, were used between passages 9 and 40. The FER strain, which spontaneously releases eFOV, was used in a similar manner.

FL74 cells are feline lymphoblastoid cells which continuously release FeLV-ABC/KT. They originated from a lymphoid tumour in the kidney of a kitten infected with FeLV (Theilen et al., 1969) and are the cells used to detect anti-FOCMA antibodies by indirect immunofluorescence (Riggs, 1971). F422 cells are also lymphoblastoid cells, established by Rickard (1969) from a thymic tumour of a kitten, and these release FeLV-A/Rickard. They were obtained from Dr. M. Essex.

Clone 81 (c81) cells derived from the Crandell feline kidney cell line (CCC) contain the genome of Moloney MSV and were obtained from Dr. P.J. Fischinger (Fischinger et al., 1974).

CT45S cells are canine thymocytes originally isolated by Dr. J. Mitchell and obtained from Dr. M. Essex.

Human embryonic lung (Flow 2000) cells were obtained from Flow Laboratories Ltd., Irvine.

Media

Monolayer cell lines (FEA, FER, c81, Flow 2000) were maintained in Eagle's minimal essential medium (MEM) - Glasgow modification (Macpherson and Stoker, 1962) which was prepared at the Institute of Virology, University of Glasgow. This was supplemented with 10% foetal bovine serum (FBS) (Gibco Biocult Ltd.) and the complete medium is referred to as EFB.

Suspension cultures (FL74,F422, CT45S) were grown in medium consisting of equal proportions of Liebovitz L-15 and McCoy's 5A media (Gibco Biocult Ltd.) supplemented with 15% FBS. Special media for radioactive labelling are detailed in the text.

Cell culture

Monolayer cells were maintained either in 8 ounce glass bottles or 2.5 litre rotating bottles and were subcultured by removal from the glass after 2 rinses with 0.01% trypsin in 0.02% EDTA. Resuspended cells were transferred

to fresh bottles. The culture vessels were flushed with 5% carbon dioxide in air before sealing. For radioactive labelling or for virus assay, monolayer cells were grown in 9 cm or 5 cm diameter polystyrene plates (Nunclon) and were maintained in an atmosphere of 5% carbon dioxide in air in an incubator.

Suspension cells were grown in stationary cultures at densities of 5×10^5 - 3×10^6 per ml. They were generally subcultured every three days : the cells were pelleted by low speed centrifugation (500 x g, 5 min) and were resuspended in fresh medium.

Viruses

FeLV of subgroups A, B and C, and eFOV, were used. The standard members of each subgroup were FeLV-A/Glasgow-1, FeLV-B/Sarma and FeLV-C/Sarma (Jarrett et al., 1973a; Sarma and Log, 1973). For parts of the work these viruses were used as cloned stocks prepared after 3 or 4 cycles of selecting virus from two-fold terminal dilutions. At each cycle the viruses were grown in FEA cells for 3 weeks and were then detected by a microinterference test (Russell and Jarrett, 1976). The origin and description of the other isolates of FeLV which were used are listed in Table 1.

Assay of FeLV

FeLV was assayed by focus formation in c81 cells (Fischinger et al., 1974). The titre of FeLV obtained by the use of c81 cells was expressed as focus inducing units (FIU) per ml. The c81 assay method was as follows: 3×10^5 FEA cells and 3×10^4 c81 cells were seeded onto 5 cm plates. The following day FeLV in 1 ml of EFB containing 4 μg/ml polybrene was adsorbed for 90 min and was then replaced by 4 ml of fresh EFB. That medium was changed 3 - 4 days later and foci were counted under the microscope at 6 - 7 days after infection.

Virus purification

This is the subject of study in Chapter Two.

(i) Clarification. Culture fluids were collected, cooled to $4^{\circ}C$, and spun at 10,000 x g for 10 min. If the material had to be stored, it was stored at this stage: at $-20^{\circ}C$ for periods of up to a month, and at $-70^{\circ}C$ if longer storage was necessary.

TABLE 1.

Virus isolates used in this study.

Subgroup	Country of origin	Designation
A	UK	Glasgow-1
	USA	Rickard (F422)
	USA	Boston-1
B	USA	Sarma
	USA	Boston-1
C	USA	Sarma
ABC	USA	KT*
eFOV	UK	FER

* Kawakami - Theilen

(ii) Ammonium sulphate precipitation. When FeLV was purified from large volumes of culture fluid the virus was concentrated first by precipitation with ammonium sulphate. Equal volumes of culture fluid and saturated ammonium sulphate (pH 7.0) were mixed and incubated at $4^{o}C$ for 30 min. The mixture was then centrifuged at 10,000 x g for 10 min, and the precipitate was resuspended in TS buffer (0.1 M. NaCl, 0.1 M Tris-HCl, 0.001 M. EDTA, pH 7.4).

(iii) Density gradient centrifugation. Further purification was achieved by ultracentrifugation in discontinuous and continuous sucrose gradients. For discontinuous gradients, a typical method was to overlay 1 ml of 50% sucrose in TS with 5 ml of 20% sucrose in TS, in a Beckman SW41 rotor tube. The remainder of the tube was filled with the virus sample, and this was centrifuged at 36,000 rpm for 60 min, after which time the virus banded at the interface of the two sucrose solutions. Appropriate volumes were used for other rotors. A cycle of centrifugation on a preformed linear 20 - 50% sucrose gradient was always used, with or without prior discontinuous gradient centrifugation. Gradients were formed either directly with a Buchler gradient mixer, or by overlaying 20% sucrose on 50% sucrose and leaving the tube overnight at $4^{o}C$. In each case the virus band was removed by puncturing the base of the tube and collecting fractions, either by visualising the light-scattering virus band in a strong light, or by drop collection from a Buchler gradient collector attached to a peristaltic pump. Virus-containing fractions were distinguished either by refractive index measurement (since FeLV bands at density of approximately 1.16 $g.cm^{-3}$ in sucrose; Jarrett et al., 1971) or by estimation of radioactivity.

(iv) Pelleting. As a final step in virus concentration, purified virus was diluted in TS buffer and pelleted by ultracentrifugation in a Beckman SW50.1 rotor at 40,000 rpm for 60 min. The sides of the tubes were dried and the virus pellet was resuspended for further analysis or stored at $-70^{o}C$ until use. For some experiments, where soluble tissue culture fluids were studied, an initial virus pelleting step was used before sucrose gradient centrifugation.

Radioactive labelling

Radioisotopes were obtained from the Radiochemical Centre, Amersham. Isotopes used were L(4,5 - ^{3}H) leucine (40 - 60,000 m Ci/m.mol), L(U-^{14}C) leucine (>270 mCi/m.mol), D(6 - ^{3}H) glucosamine hydrochloride (> 10,000 mCi/m.mol), and iodine - 125 (100 mCi/ml). Specific labelling media and conditions

are specified in the text.

Chemicals

Unless otherwise specified, chemicals were "analar" grade, purchased from British Drug Houses (BDH) or Sigma Chemical Company, London.

Scintillation counting

Samples for scintillation counting were prepared in a number of ways. For dual label counting (^{3}H and ^{14}C) it was necessary to dry the samples. Such samples were precipitated with 5% trichloroacetic acid (TCA) washed with 5% TCA and collected on Whatman GF/C filters; or were dropped directly onto filter papers. The filters were dried in an oven before placing in scintillation vials. Dry samples were counted in a toluene based scintillation fluid: toluene with 0.4% 2, 5 diphenyloxazole (PPO) and 0.04% 1, 4 di - 2 - (5 phenyloxazolyl - benzene (POPOP).

Samples containing water were made up to 1 ml volume with water in scintillation vials, to which 6 ml of a xylene based scintillator (NE260, Nuclear Enterprises, Edinburgh) was added.

Samples were counted in an Intertechnique SL40 scintillation counter.

Protein estimation

Protein estimation was done according to the method of Lowry et al. (1954).

SDS polyacrylamide gel electrophoresis

Two different methods for SDS PAGE were used during the study.

Method 1.

The first method was as described by Jarrett (1973) using a phosphate buffered system. Gels were mixed to give a final concentration of 7.5% to 10% acrylamide (BDH, electrophoretic grade), 0.375% bis (N, N methylene-bis-acrylamide), 0.1 M. sodium phosphate buffer (pH 7.4) 0.1% SDS (BDH, electrophoretic grade) and 0.5 M urea. The last ingredients to be added were ammonium persulphate to 0.07% and TEMED (N, N, N, N-tetramethylethylenediamine) to 0.125%, which initiated the polymerisation. The gel was then poured into siliconised glass tubes (10 cm long, 6 mm internal diameter) and a layer of water was added to keep a flat meniscus on the gel until polymerisation was complete.

These gels were used immediately or stored in a humified chamber at $4^{o}C$ until use. Samples were solubilised for this method by boiling for 3 minutes in solubilising buffer which contained 1% SDS, 1% dithiothreitol (DTT), 0.8 M urea, 10% (w/w) sucrose, and bromophenol blue (0.001%). After cooling, the samples were layered on top of the gels which were held in a Shandon Southern electrophoresis tank. Both top and bottom reservoirs contained the same buffer solution (0.01 M sodium phosphate (pH 7.4), 0.5 M urea and 0.1% SDS). The gels were run at a constant current of 2 mA per gel overnight. They were then stained or sliced for radioactive counting.

Method 2.

The second method was that of Laemmli (1970). This is a discontinuous system, involving a stacking gel which concentrates the sample, and a separating gel. The separating gel was cast with fixed concentrations of acrylamide ranging from 7.5% to 15%. Bis-acrylamide was always present such that the ratio of acrylamide to bis was 30: 0.8. Other ingredients were 0.375 M Tris-HCl buffer (pH 8.8) 0.1% SDS, 0.1% ammonium persulphate, and 0.025% TEMED. A layer of 5% ethanol was carefully pipetted onto the gel surface and left until the separating gel polymerised. This recipe was used for slab gels of various dimensions (70 mm x 70 mm x 3 mm, 70 x 70 x 1 mm or 90 x 150 x 1.5 mm). In each case, enough space was left for a "stacker" gel approximately 10 mm in length. The stacker gel contained 3 to 5% acrylamide, bis-acrylamide (30: 0.8), 0.125 M Tris/HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 0.025% TEMED. Teflon or perspex "combs" were used to produce sample wells of the desired size in the stacker gel. Solubilising buffer for this method contained 0.0625 M Tris/HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol and bromophenol blue (0.001%). This was made up as a double strength mixture which was added to an equal volume of sample, before boiling for 3 minutes. After layering on to the gel, the samples were run at a constant voltage of 40V during "stacking", after which the potential was increased to 100V. Gels were run until the tracker dye was approximately 1 cm from the end of the gel and were then removed for staining or autoradiography.

Staining of gels

Protein was visualised by staining with Coomassie blue R. This was done initially by the method of Weber and Osborn (1969) using a fix-stain solution

of 0.25% Coomassie blue in a 5: 5: 1 methanol : water : acetic acid mixture for 1 hour, followed by destain solution (5% v/v methanol, 7.5% v/v acetic acid in water). Later, a modified method was used in which gels were left in fix-stain solution of 0.0125% Coomassie blue in a 10: 8: 1 water : methanol : acetic acid mixture, overnight. This was followed by destain solution as before. The advantages of this method were that protein bands could be visualised before destaining, and that destaining by diffusion was very much faster.

Glycoproteins were visualised by periodic acid-schiff (PAS) staining. The gels were fixed in 12% trichloroacetic acid for 10 minutes, and left in 15% acetic acid overnight. They were then soaked in 1% periodic acid/3% acetic acid for 90 minutes, rinsed exhaustively with water until all traces of acid were removed, and then stained with fresh Schiff's reagent for 40 min. The gels were destained and stored in 5% acetic acid.

Autoradiography

Gels for autoradiography were dried under vacuum at 80° - 100°C onto hard filter paper (Whatman grade 182). For fluorography, which was necessary to detect tritium-labelled proteins, the gels were first dehydrated by soaking in dimethyl sulphoxide (DMSO), then impregnated with 2, 5 diphenyloxazole (PPO) by soaking for 3 hours in a 22% solution of PPO in DMSO. The DMSO was removed by soaking in water, and the gels were dried as described above. This method is as described by Bonner and Laskey (1974).

The dried gels were clamped in close contact with medical X-ray film (Kodak XH-1 or Kodirex) which had been pre-exposed to a short flash of light which makes the response of the film to radioactivity linear (Laskey and Mills, 1975). The films were exposed to the gels at -70°C for periods ranging from 3 days to 4 weeks depending on the amounts of radioactivity in the sample. The films were developed in a 50% solution of bromophen (Ilford) for up to 5 min and fixed in Ilford Hypam rapid fixer.

Gel slicing

Rod gels were sliced for radioactive counting using a Mickle gel slicer. The gels were first frozen and then cut into 1 mm slices. Each slice was placed in a scintillation vial, to which 0.5 ml of NCS solubiliser (Nuclear Chicago) was added. The vials were sealed and left at 37°C overnight. Six ml of toluene-based scintillation fluid was then added to each vial and the samples were counted

for radioactivity as previously described.

Immune precipitation

Protein A bearing Staphylococcus aureus cells were used to harvest immune complexes. Typically, 20 µl of antiserum was added to 0.5 - 1 ml of soluble antigen preparations. After overnight incubation at 4°C, 50 µl of a 10% suspension of fixed bacteria were added. After incubation for a further hour, the tertiary antigen-antibody-bacterial complexes were harvested by low speed centrifugation (2,000 x g, 15 min) and washed three times in buffer. The antigen-antibody complexes were removed and solubilised with electrophoresis sample buffer (usually 100 µl).

The bacteria were Staphylococcus aureus Cowan I strain (NCTC 8530). They were grown overnight at 37°C in tryptone broth in an orbital shaking incubator. The cells were harvested by centrifugation at 2,500 x g in a MSE 6L centrifuge. They were washed once in PBS (pH 7.2) with 0.2% sodium azide (PBS-azide) and resuspended to a 10%(w/v) suspension in PBS with 1.5% formaldehyde. The suspension was stirred at room temperature for 90 min and then washed, resuspended in PBS-azide, incubated at 80°C for 2.5 min and then immediately cooled in a melting ice bath. The bacteria were recentrifuged and resuspended to a 10% (w/v) suspension in PBS-azide. The suspension was stored at 4°C for immediate use, or -70°C for longer storage. Before use the bacteria were washed twice in the appropriate buffer.

The method of growing the bacteria and their use in immunoprecipitation was essentially as described by Kessler (1975) and Cullen and Schwartz (1976).

The bacteria were checked for their ability to bind immunoglobulin from various species by incubating 20 µl of serum with 50 µl of bacterial suspension in a total volume of 1 ml TS buffer. After a 2 hour incubation period at 4°C, the complexes were collected by low speed centrifugation and were washed and prepared for electrophoresis as described. The recovered antibody preparations were analysed on an 11% polyacrylamide gel (Laemmli) and the proteins visualised by staining with Coomassie blue R. The results are presented in Fig. 1.

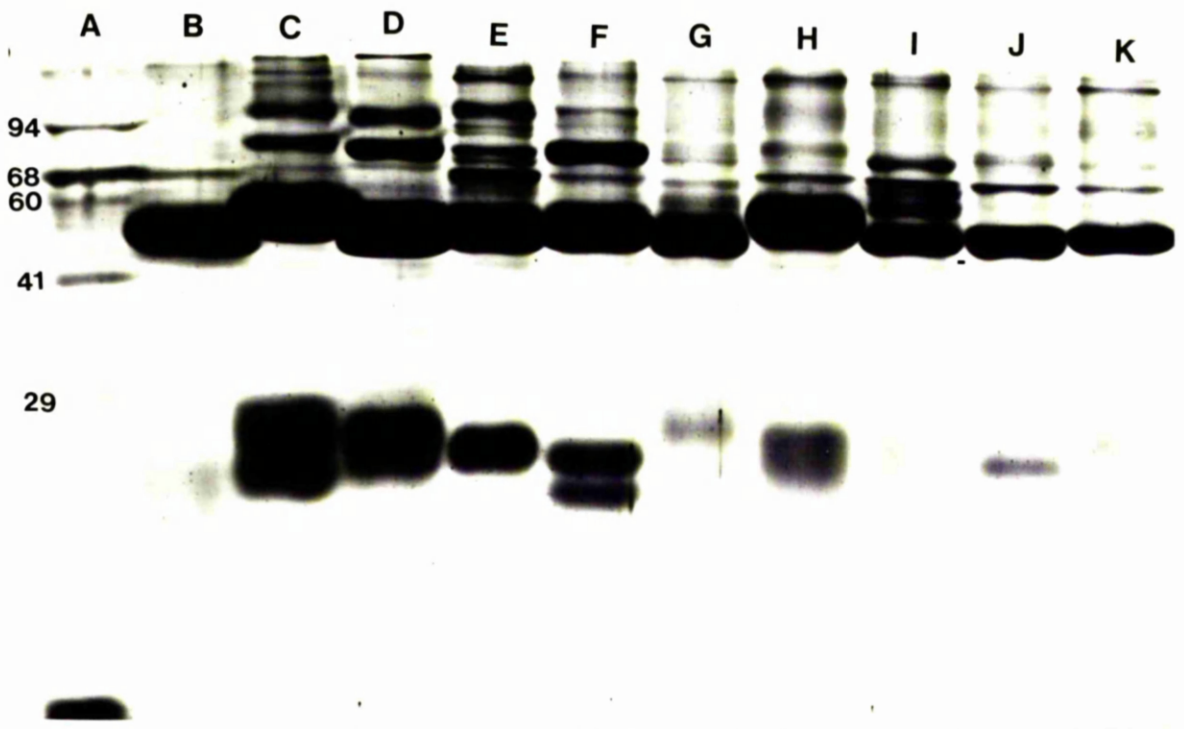


FIGURE 1

Serum proteins which bind to Staphylococcus aureus

Proteins eluted from the fixed bacteria were separated on a 10% polyacrylamide gel.

Lane A shows standard molecular weight marker proteins: phosphorylase a (94,000), bovine serum albumin (68,000), catalase (60,000), alcohol dehydrogenase (41,000), carbonic anhydrase (29,000). Lane B rabbit serum, C cat serum, D dog serum, E human serum, F pig serum, G horse serum, H bovine serum, I sheep serum, J rat serum, K goat serum.

CHAPTER TWO

PURIFICATION OF FeLV

INTRODUCTION

For detailed study of the biochemical and antigenic properties of any virus it is important to ensure that purification procedures yield preparations sufficiently free from contamination with cellular and growth medium components, while leaving virus structure and infectivity intact. This task is often difficult since cells release a multitude of constituents, some of which may closely resemble the virus in size and shape. Virus particles may also aggregate, or adsorb medium components, either before or during the isolation procedure. These problems will be aggravated by virus instability or low virus yield. Finally, a quantitative virus infectivity assay must be available to follow the effects of the various treatments (Knight, 1974).

The enveloped RNA tumour viruses present particular difficulties, since they mature by budding at the cell membrane, acquiring their outer membrane along with many cellular components. Thus, de Thé (1964), reported that avian myeloblastosis virus (AMV) carried ATPase only when grown in cells which carried the enzyme. Ishizaki et al. (1973) showed that guinea pigs immunised intracerebrally with AMV produced antiserum which reacted with a virion-surface antigen distinct from known virus-specific antigens, concluding that the viral membrane was represented mainly by host cell material. Aupoix and Vigier (1975) reported inactivation of avian sarcoma viruses by antiserum to chick embryo cells in the presence of guinea pig complement. Recently, Bubbers and Lilly (1977) have reported the selective incorporation of H-2 antigens into Friend MuLV particles. Cellular actin can also become incorporated into virions, and it has been suggested that this may have a role in virus budding (Damsky et al., 1977).

Techniques for virus purification may result in loss of virion components, particularly loosely attached surface proteins. Thus, Strand and August (1976) reported that one cycle of freezing and thawing released 98% of MuLV glycoproteins. Witter et al. (1973) have also found that centrifugation of MuLV in sucrose gradients results in considerable loss of surface spikes and hence glycoprotein. In fact, these authors have used the "osmotic shock" of suspending virus in 30% sucrose solutions as a first step in the purification of MuLV gp70.

In view of the above information it seemed important to ensure that the purification techniques to be used in this study did not result in gross release

of virion components, particularly gp70. Host cell components in virus preparations were also studied.

MATERIALS AND METHODS

1. The effect of purification methods on FeLV-A/Glasgow-1 proteins

FEA cells, infected with FeLV-A/Glasgow-1, were seeded onto four 9 cm petri dishes at 1 x 10^6 cells per plate. After overnight incubation, they were labelled with L(4, 5-^{3}H) leucine (250 μCi/plate) in leucine-free MEM supplemented with 10% foetal bovine serum. After 24 hours the culture fluid was collected, cooled to 4°C, and centrifuged at 10,000 x g for 10 min. The supernatant fluid (36 ml) was split into six fractions, and the aliquots were treated as listed in Table 2.

Sucrose gradients were continuous 20 - 50% gradients which were centrifuged for 2 hours (Beckman SW41 rotor, 36,000 rpm). Virus was then concentrated by pelleting or immunoprecipitation. Fractions 2 and 3 were frozen and thawed by placing in a -70°C deep freeze for 30 min and then thawing in a water-bath at 37°C. Ammonium sulphate precipitation was as previously described. Direct immunoprecipitation of fraction 4 was carried out by incubating the culture fluid for 2 hours at room temperature with 200 μl goat anti-FeLV serum (raised to whole FeLV-A/Glasgow-1) and then for a further one hour after adding 200 μl of a 10% suspension of fixed S. aureus. For fraction 5, the precipitation was carried out in approximately 1 ml of sucrose-banded virus suspension. The final step for each aliquot was either pelleting or immunoprecipitation followed by resuspension in 100 μl of electrophoresis sample buffer (Laemmli). Aliquots 1 - 5 were then compared by electrophoresis on SDS polyacrylamide slab gels, as described. Proteins were visualised by fluorography.

2. The effect of purification methods on FeLV-B/Boston-1 proteins.

FEA cells, infected with FeLV-B/Boston-1, were seeded onto four 9 cm plates at 1 x 10^6 cells per plate. One similar plate of uninfected FEA cells was also used. Radioactive labelling was carried out as in Experiment 1. After overnight growth, the medium was removed and 500 μCi (4, 5 - ^{3}H) leucine in leucine-free EFB was added to each plate. Culture fluid was harvested and clarified, as in Chapter One. The virus-containing supernatant was split into six aliquots, and, along with the uninfected cell culture fluid, subjected to various treatments as listed in Table 3 .

The details of the various methods were as in the first experiment except that for immunoprecipitation the amount of serum added was reduced to 100 µl. For "mock immunoprecipitation", only the fixed bacteria (100 µl, 10% suspension) were added.

3. The effect of purification methods on FeLV-A/Glasgow-1 infectivity.

Culture medium was harvested from rapidly growing cultures of FEA cells infected with FeLV-A/Glasgow-1 the medium of which had been changed 24 hr previously. The harvested fluid was clarified, split into six aliquots of 5 ml, and subjected to various treatments as listed in Table 4.

Treatments were performed exactly as described in the first experiment in this chapter, except that, after any treatment which concentrated the virus samples, this was reconstituted to the original 5 ml volume with fresh tissue culture medium (EFB). Assay of FeLV infectivity on c81 cells was as described in Chapter One.

4. Host cell proteins in FeLV preparations

4a. Electrophoresis of uninfected FEA cell culture supernatant proteins. A 24 hr. harvest (500 ml) of tissue culture fluid was collected from rapidly dividing FEA cells. This was processed as if for virus purification as described in Chapter One. The steps employed were clarification, ammonium sulphate precipitation and sucrose density gradient centrifugation. Fractions were collected from the gradient, the densities calculated from refractive index measurement, and protein content analysed by SDS-PAGE on an 11% polyacrylamide gel which was stained with Coomassie blue.

4b. Electrophoretic comparison of FeLV and FEA cell proteins. FeLV and uninfected FEA culture fluid were radioactively labelled with ^{3}H leucine as described in Chapter Four. Samples were electrophoresed on an 8% polyacrylamide gel and proteins visualised by fluorography.

RESULTS

1. The effect of purification methods on FeLV-A/Glasgow-1 proteins.

1a. Yield of radioactivity. The yield of leucine counts as listed in Table 2 was in the range 70 - 100% compared to "controls" 1 and 6, apart from fraction 4, the direct immunoprecipitate, which showed a yield of around 30%.

1b. Protein composition. This is presented in Fig. 2. Lanes a to e correspond to fractions 1 - 5. There were no detectable differences in lanes a to c. However, lane d showed a marked reduction in the p27 band with no apparent reduction in gp70. Other minor changes were the disappearance of a high molecular weight band and the appearance of several new species from 30,000 - 55,000 apparent molecular weight (M_r). There may also have been a relative increase in a band at 21,000 M_r and a relative decrease in material at the tracker dye front. Lane e also lacked the high molecular weight band which was absent in lane d, but in other respects was similar to lanes a to c.

2. The effect of purification methods on FeLV-B/Boston-1 proteins

2a. Yield of radioactivity. These results showed some striking differences from those obtained with FeLV-A. It appeared that direct immunoprecipitation (fraction 4)gave the highest virus yield. However, the reason for this is clear when the protein composition is examined. It did appear in this experiment that ammonium sulphate precipitation (fraction 3) gave a much lower yield. This is discussed below.

2b. Protein composition. These results are presented in Fig. 3 . One striking difference from the results of Experiment 1 was the appearance of a heavily labelled high molecular weight band which non-specifically bound to the fixed bacteria (since it was present in fraction 6). Unfortunately, the "control" fraction (immunoprecipitation from uninfected cells) could not be compared: this sample was accidentally overheated and the antibody seemed to have been degraded since no heavy chain band was visible on the stained gel before fluorography. It seems probable that the labelled antigens had been degraded also. The 30 - 55,000 molecular weight bands seen in Experiment 1 were resolved in this experiment. These are seen in fractions 4 and 5S and represent soluble antigens which were left in the supernatant after virus pelleting. Fraction 5S shows a considerable amount of "soluble" gp70. Ammonium sulphate

TABLE 2

Treatments of FeLV-A/Glasgow-1 tested for effect on protein composition and yield of radioactive proteins

Fraction	Treatment *	^{3}H leucine cpm in 10 µl (10%) of fraction
1	Sucrose gradient. Pelleted.	41,200
2	Frozen and thawed. Sucrose gradient. Pelleted.	37,800
3	Frozen and thawed. Precipitated with ammonium sulphate. Sucrose gradient. Pelleted.	36,700
4	Immunoprecipitated.	15,300
5	Sucrose gradient. Immunoprecipitated.	33,400
6	As fraction 1.	46,700

* Details of treatments are described in the text.

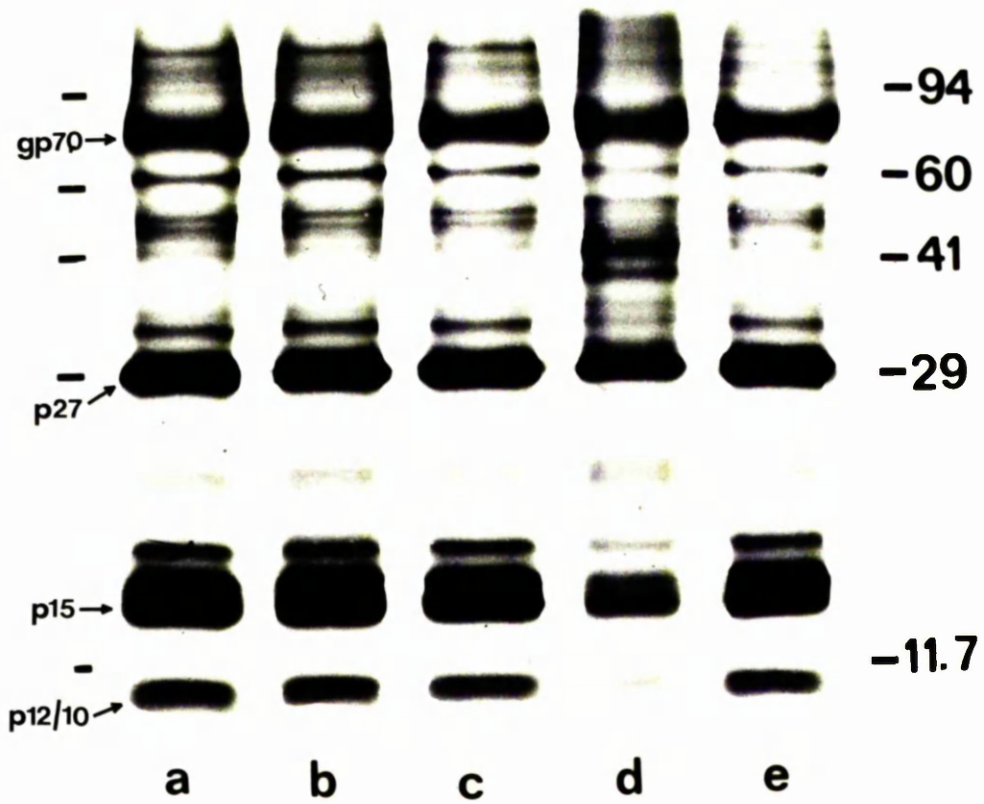


FIGURE 2

Effect of purification methods on FeLV-A/Glasgow-1 proteins

SDS-polyacrylamide gel fluorograph of the polypeptides of the FeLV-A/Glasgow-1 after various treatments (see Table 2).

Lane a	:	fraction 1
Lane b	:	fraction 2
Lane c	:	fraction 3
Lane d	:	fraction 4
Lane e	:	fraction 5

Standard protein markers are phosphorylase a (94,000), catalase (60,000), alcohol dehydrogenase (41,000), carbonic anhydrase (29,000) and cytochrome c (11,700).

TABLE 3

Treatments of FeLV-B/Boston-1 tested for effect on protein composition and yield of radioactive proteins

Fraction	Treatments	^{3}H leucine cpm in 10µl (10%) of fraction
1	Sucrose gradient. Pelleted.	22,000
2	Frozen and thawed. Sucrose gradient. Pelleted.	21,000
3	Frozen and thawed. Precipitated with ammonium sulphate. Sucrose gradient. Pelleted.	12,000
4	Immunoprecipitated.	46,000
5P	Pelleted.* Sucrose gradient. Pelleted.	23,000
5S	Supernatant fluid of 5P.* Immuno-precipitated.	12,000
6	Mock immunoprecipitated[a].	10,000
Control[b]	Immunoprecipitated.	12,000

a No antiserum. Fixed S. aureus only.

b Performed on uninfected FEA cell culture supernatant fluid.

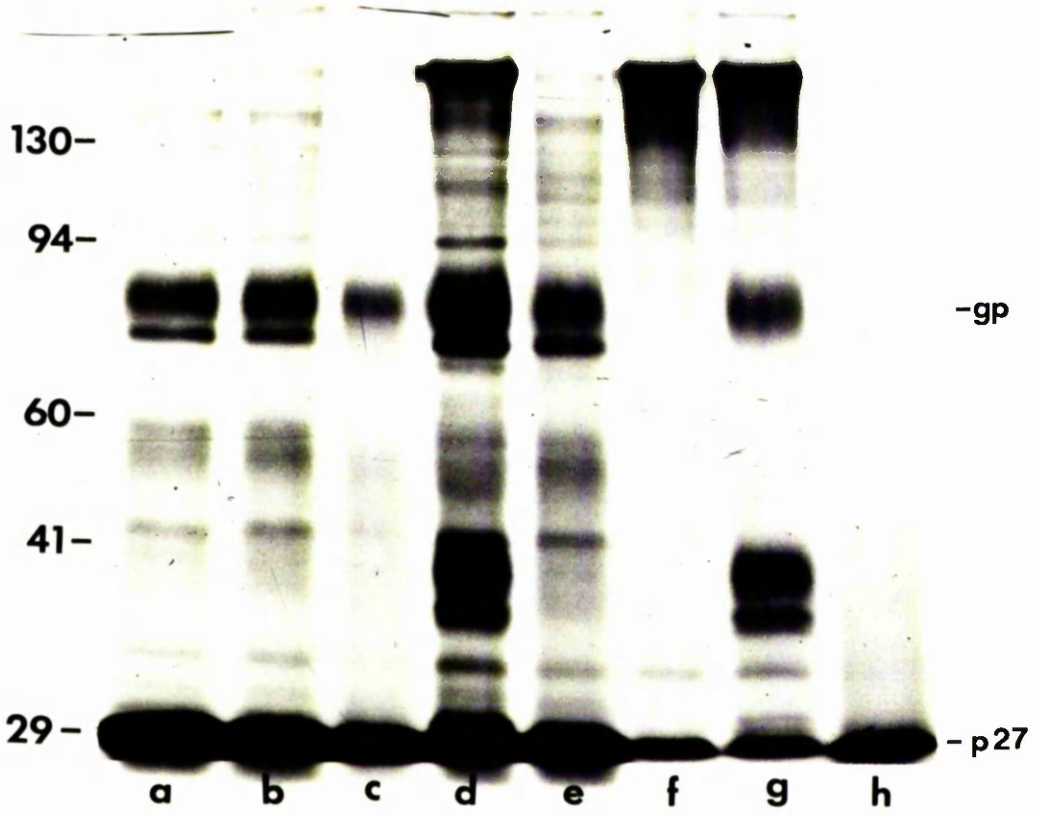


FIGURE 3

Effect of purification methods on FeLV-B proteins

SDS polyacrylamide gel fluorograph of FeLV-B/Boston-1 and FEA cell culture fluid proteins after various treatments (see Table 3).

Lane a : fraction 1
Lane b : fraction 2
Lane c : fraction 3
Lane d : fraction 4
Lane e : fraction 5P
Lane f : fraction 6
Lane g : fraction 5S
Lane h : control

precipitated virus (fraction 3) showed a reduced yield in this experiment. It is not clear whether the disappearance of minor bands is a quantitative or a qualitative difference.

3. The effect of purification methods on FeLV-A/Glasgow-1 infectivity.

The results are presented in Table 4.

4. Host cell proteins in FeLV preparations.

4a. Electrophoresis of uninfected FEA cell culture supernatant proteins.

It was discovered on analysing FEA culture fluids by centrifugation through sucrose gradients that the cells released a large range of proteins which would contaminate virus preparations, since they had similar density and sedimentation rates to FeLV. This is shown in Fig. 4 . However, since FEA plasma-membrane proteins were also being studied (see Chapter Six) it was immediately obvious that not all plasma membrane components were represented in this cell "exfoliate". In particular, the glycoproteins were overrepresented. In FEA cells this is mainly a 145,000 - 160,000 M_r doublet. This was also noted for other cell types, such as CT45S canine thymocytes which shows a very distinctive membrane glycoprotein content (see Chapter Six; virus preparations from these cells showed so much cellular glycoprotein that even the viral p27 was obscured (see Fig. 10).

4b. Electrophoretic comparison of FeLV and FEA cell proteins. When FEA "exfoliate" was compared directly with virus preparations, another interesting observation was made (Fig. 5). Although FEA "exfoliate" proteins comigrated with proteins in virus preparations, there were marked quantitative differences. These are highlighted in a diagrammatic representation of the fluorograph (Fig. 5b). The yield of radioactive protein (^{3}H leucine cpm) from the FEA cell culture fluid was 10% of that of the FeLV preparations. The loading of material on the gel in Figure 5 is thus biased so that the FEA cell proteins could be visualised more clearly. This is reflected in the fact that some major species in the FEA cell protein preparation were barely seen in the FeLV preparations. On the other hand two polypeptides, marked with 1 and 2 in Figure 5b were clearly seen in the virus preparations. Unfortunately, the 74,000 M_r band was obscured by the viral gp70 in the photographic prints of Figure 5a. However, the original X-ray fluorographs clearly distinguished these species and this is emphasised in Figure 5b. The bands marked "gp" are of similar migration properties to the

TABLE 4

Treatments of FeLV-A/Glasgow-1 and their effect on viral infectivity.

Fraction	Treatment[Δ]	Titre *	% recovery of infectivity
1	Held at 4°C	3.4×10^5	100
2	Precipitated with ammonium sulphate.	2.2×10^5	65
3	Frozen and thawed.	1.8×10^5	52
4	Frozen and thawed. Ammonium sulphate precipitated.	2.0×10^5	59
5	Pelleted.	1.6×10^5	47
6	Sucrose gradient.	7×10^4	21

* Average of two replicate plates with a countable number of foci (5-40 per plate).

Δ Between treatments and before dilution for assay, all samples were held at 4°C.

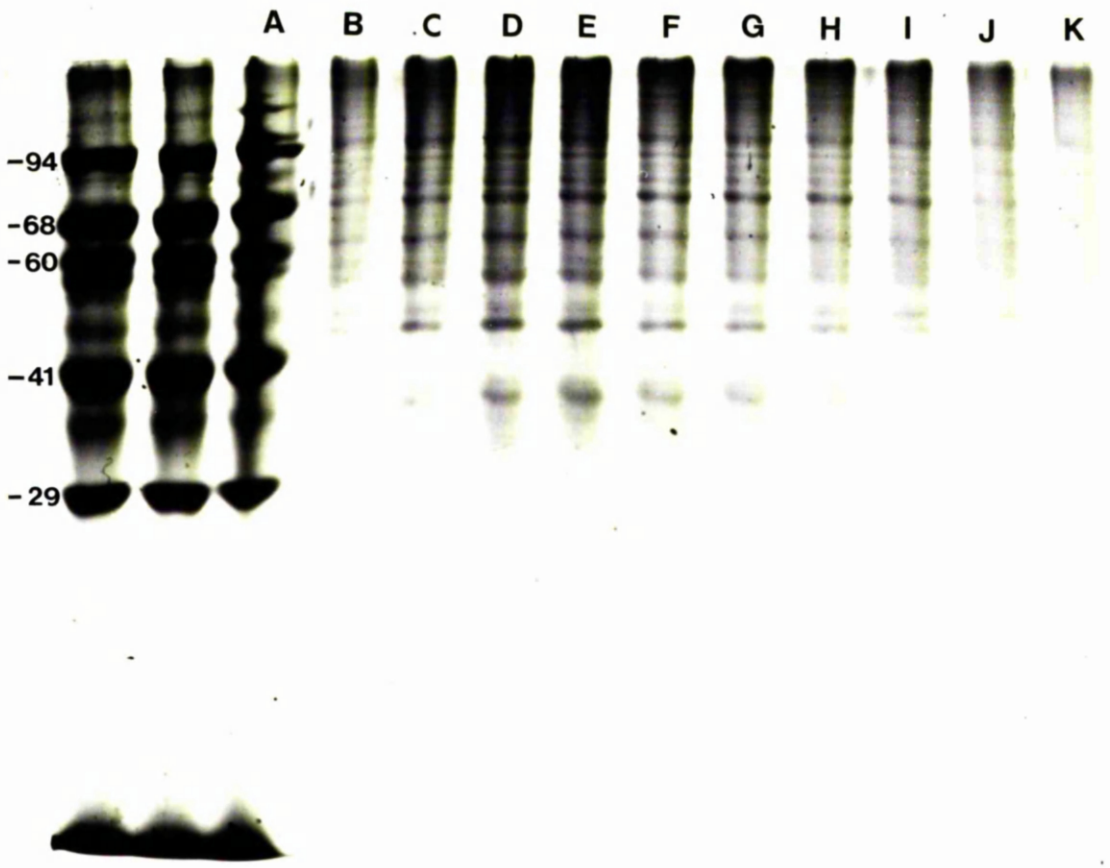


FIGURE 4

Proteins purified from FEA cell culture supernatant

SDS PAGE analysis of sucrose density gradient purified FEA cell culture proteins. Samples were run on an 11% acrylamide gel and proteins were visualised by staining with Coomassie blue R. The key below refers to the density of the fractions chosen for electrophoresis.

Lane A Standard marker proteins
Lane B 1.207 $g.cm^{-3}$
Lane C 1.199 $g.cm^{-3}$
Lane D 1.189 $g.cm^{-3}$
Lane E 1.177 $g.cm^{-3}$
Lane F 1.163 $g.cm^{-3}$
Lane G 1.152 $g.cm^{-3}$
Lane H 1.139 $g.cm^{-3}$
Lane I 1.129 $g.cm^{-3}$
Lane J 1.101 $g.cm^{-3}$
Lane K 1.082 $g.cm^{-3}$

Figure 5a shows a SDS PAGE fluorograph of ^{3}H-leucine labelled proteins separated on an 8% acrylamide gel.

Lane a 10,000 cpm of fractionated FEA proteins

Lane b 25,000 cpm of FeLV-A/Boston-1 proteins

Lane c 25,000 cpm of FeLV-AB/Glasgow-1 proteins

Figure 5b is a diagrammatic representation of Fig. 5a.

FIGURE 5

Comparison of purified FeLV and uninfected FEA cell culture supernatant proteins

FIGURE 5a

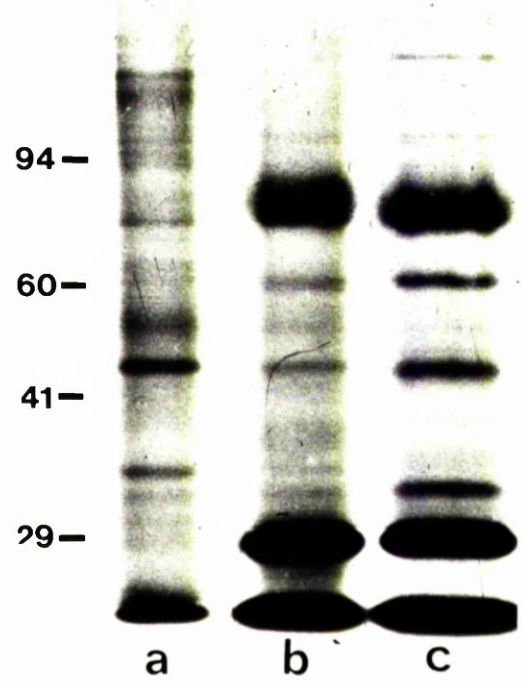
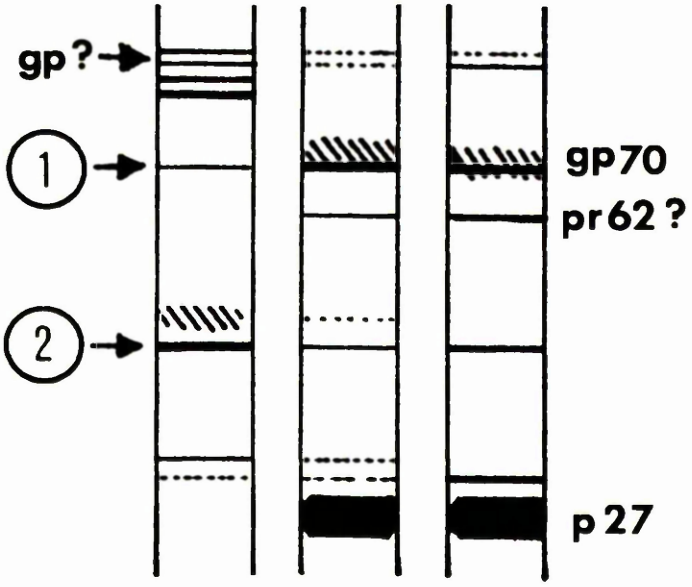


FIGURE 5b



FEA cell membrane glycoproteins shown in Chapter Six and detected in FEA-grown FeLV preparations in Chapter Four.

DISCUSSION

In the first experiment, direct immunoprecipitation of virus gave a reduced overall yield of radioactive counts, and much less label in the major core protein, p27, but a similar amount of labelling of gp70. This could have been due to the effect of sucrose of removing virus surface spikes (Witter et al., 1973). However, another reason might be that direct immunoprecipitation, as in Fig. 2(d) brings down soluble gp70. This was tested in the second experiment by including a precipitate from culture medium from which virus had been removed by ultracentrifugation. Nevertheless, there is no serious loss of gp70 after any of the treatments used. This is in marked contrast to the results of Strand and August (1976) who reported a 98% loss of gp70 from one freeze-thaw step. I would suggest that this difference could be explained by the fact that these authors were freezing very large quantities of highly purified virus, whereas this experiment involved freezing whole culture medium, which may have a considerable protective effect. The loss of a high molecular weight band in immunoprecipitated virus (lanes d and e) suggests that this is a non-virion component which merely copurifies with virus on sucrose gradients. The significance of minor bands of 30,000 to 55,000 M_r is unclear: these may be highly labile virus components, or may be soluble cellular antigens recognised by the goat anti-FeLV serum. A relative reduction in the material at the tracker dye front of lane d is more difficult to explain, but it could be explained if the low amount of radioactivity in this band leaves it in the range where the response of the X-ray film to light is not linear (Laskey and Mills, 1975).

The second experiment again demonstrated that treatments such as sucrose density gradient centrifugation and freeze-thawing had no serious effect on FeLV protein composition. However, ammonium sulphate precipitated virus showed a considerably reduced yield and the apparent loss of minor bands. The reason for this is unknown. It is possible that precipitation activated a protease which digested the minor components, but it is also possible that these bands are present and are not seen because of the non-linear response of the X-ray film to very low levels of light. Pre-exposure of films was not examined quantitatively, and it is not known whether in this experiment the "fog" increment was as great as the level required to make the response of the film to light completely proportional (Laskey and Mills, 1975).

Fraction 5S showed that tissue culture fluid is an abundant source of soluble gp70. This has also been reported for Friend MuLV by Bolognesi et al. (1975) . Direct immunoprecipitation appears to yield gp70 free of any other component of similar molecular weight so that culture fluids might be usefully extracted to yield the purified antigen. Fraction 5S demonstrated that the extra components seen in fraction 4 are also soluble tissue culture fluid proteins. However, since the uninfected FEA cell control was degraded and no more goat anti-FeLV serum was available, it was not possible to decide whether these molecules were specific for virus infection or were normal cell antigens. This would be worth investigating with other antisera.

None of the treatments caused a dramatic loss of FeLV infectivity, since in every case the measured infectivity was within an order of magnitude of the control. The lowest yield of infectivity was after sucrose density gradient centrifugation. From the results of the two previous experiments this could not be explained by loss of surface glycoprotein. Presumably the rapid change in osmotic pressure on centrifugation through sucrose has some other effect on the integrity of the virus particles. One possibility would be that the loss of integrity of the virion outer membrane would allow access to proteases or nucleases. The results in this Chapter demonstrate no gross change in protein composition after sucrose gradient centrifugation. However, loss of reverse transcriptase activity or degradation of viral RNA should also result in loss of infectivity.

Although none of the infectivity losses were sufficient to justify a search for new purification methods, it is still possible that additive or multiplicative effects of sequential treatments would result in much greater losses. This was tested for salt precipitation and freeze/thawing in fraction 4. Surprisingly, the final yield was as high as that after either one of the treatments alone. This may represent the destruction of a sub-fraction of virus particles which are labile to both treatments, leaving a resistant residual fraction. Alternatively, the results may be explained by the inherent imprecision of manipulating small quantities of virus and diluting for assay. To resolve this, the experiment would have to be repeated with a sufficiently large number of replicate samples to allow statistical analysis. Such an exercise might be confounded, unfortunately, by the fact that virus strains, and even individual harvests of one virus strain, vary markedly in their stability (Smith, 1974; Strand and August, 1976).

Strand and August (1976) have reported that over 50 polypeptides are detectable in Rauscher MuLV preparations by high resolution polyacrylamide gel electrophoresis. In view of recent estimates of the limited coding potential of the RNA tumour viruses, it seemed likely that the great majority of these bands were host cell-coded. On applying this technique to FeLV, similar results were recorded. Although FEA "exfoliate" proteins comigrated with proteins in virus preparations, there were marked quantitative differences.

This could be explained if the membrane exfoliate composition varied widely from time to time, or if it was grossly affected by virus infection. However, it could mean that these cell components are specifically incorporated into virus particles and may have an important role in virus budding or virion structure. This was suggested by Damsky et al. (1977) on finding cellular actin (43,000 molecular weight; see Fig. 5) to be overrepresented in MuMTV preparations when compared to virus-free vesicles. Another possibility is that there are virus-coded proteins (e.g. gag precursors) which have similar migration properties to certain host membrane components. Some criterion other than migration in SDS-PAGE would have to be used to decide between these possibilities. In collaboration with Dr. J. Smart, Imperial Cancer Research Fund, London, tryptic peptide digests of these molecules are being analysed. Since antisera to virion proteins p^{27} and p15 were available, it was decided to test whether any of the vesicle components were immunologically related to these virus-coded proteins. This is described in Chapter Five.

CHAPTER THREE

STRUCTURAL LOCATION OF FeLV PROTEINS

INTRODUCTION

The structural location of a virion component is of interest since it may indicate its role in viral function. Thus, surface location would suggest that a protein might be involved in the initial interaction between virus and its host cell and might also be implicated as a target for virus-neutralising antibodies. Internal proteins, on the other hand, may be expected to play a different role: this might be purely structural such as a protein which would, by virtue of its affinity for itself or other molecules, maintain the shape and structural integrity of the virus particle. Alternatively internal proteins could have a role in stabilising the viral nucleic acid or some enzymic activity important for viral infectivity. It should be emphasised that internal virion components, if they are expressed on the infected cell surface, may also be determinants of immunity to virus infection.

Approaches to establishing surface location for a virion polypeptide include removing surface components with enzymes and surface labelling with ^{125}I. These techniques are facilitated with enveloped viruses by the fact that the virion outer membrane represents a permeability barrier to proteolytic enzymes or lactoperoxidase respectively.

Convincing evidence now exists that the RNA tumour virus glycoproteins are located on the surface of the virion. Bromelain treatment of RSV (Rifkin and Compans, 1971) and Friend MuLV (Witter et al., 1973) removed the virion glycoproteins and the surface spikes or knobs as seen in the electron microscope. Further evidence that the surface spike of the avian leukosis/sarcoma viruses consists of the virion glycoproteins comes from the isolation of "rosettes" of material morphologically resembling the surface spike and containing mainly gp85 and gp37 (Bolognesi et al., 1972a). Both surface spikes and glycoproteins are absent from non-infectious strains of RSV when they are grown in the absence of helper virus in chick helper factor negative cells (De Giuli et al., 1975). The spike of MuMTV has been attributed to the major virion glycoprotein (gp55) in a similar way (Cardiff et al., 1974). In all the experiments mentioned above, viral infectivity was greatly reduced by digesting the surface glycoproteins and, where measured, the ability of virus particles to absorb neutralising antibodies was abolished.

Lactoperoxidase-catalysed ^{125}I labelling of MuMTV labelled almost exclusively gp55, suggesting that the outer membrane is represented mainly by this glycoprotein (Damsky et al., 1977). Similar results have been obtained with Moloney MSV (MuLV) (Witte and Weissman, 1974) and Rauscher MuLV (McLellan and August, 1976). A much more complex pattern was recorded by Panem and Kirsten (1975) with Kirsten MSV (MuLV) who found 14 envelope proteins including 3 glycoproteins by this method. This is discussed below with reference to results with FeLV.

In a limited number of cases, sufficiently pure preparations of MuLV gp70 have been obtained to raise highly specific antiserum and to show that this has virus neutralising activity (Steeves et al., 1974; Hunsmann et al., 1975). The surface location of MuLV p15(E) was also suggested by virus neutralising activity of anti-p15(E) serum in the presence of the complement (Fischinger et al., 1976) although its inaccessibility to surface labelling and its hydrophobic nature have led workers to believe that this molecule is firmly embedded in the viral outer membrane, and has some anchoring function for the spike proteins.

There is less agreement about the proteins of viral cores. These structures have been isolated by centrifuging viruses through layers of surfactants such as Sterox SL or non-ionic detergents such as Triton X-100 or Nonidet P-40; or by pretreatment with these agents. There is some doubt as to whether these treatments entirely remove lipid, as it has been reported that core structure is destroyed by phospholipase-C treatment (Bolognesi et al., 1972b). Cores of FeLV were found to band at a density of 1.27 $g.cm^{-3}$ in sucrose density gradients. As reported below, core preparations contained a large number of proteins, although they were found to lack p15 and have considerably reduced amounts of gp70.

1. Surface proteins of FeLV detected by lactoperoxidase-catalysed ^{125}I labelling.

A sample of 600 µg of sucrose gradient purified FeLV-A/Rickard (F422 was labelled with ^{125}I by the lactoperoxidase method (Marchalonis, 1969). To virus in 1 ml of TS buffer was added 1 ml reaction mixture containing 25 µl of ^{125}I (250 µCi), 7.5 µg lactoperoxidase (grade B, Calbiochem : 122 I.U. per mg) and TS buffer. The labelling reaction was started by adding 20 µl of H_2O_2 (6% diluted 1:1000 in TS buffer). This addition was repeated at 10 and 20 minutes later. After 30 minutes the reaction mixture was transferred to dialysis tubing and dialysed overnight against TS buffer at 4^oC. Virus was then separated by sucrose density gradient centrifugation. About 4×10^6 cpm of virus (approximately 30 µg protein) was pelleted and resuspended in 100 µl electrophoresis sample buffer (Laemmli). Virus proteins were separated on an 11% polyacrylamide gel and labelled bands were detected by autoradiography.

2. Identification of FeLV surface components by proteolytic digestion.

FeLV from FL74 cells was purified as described in Chapter 1. A virus suspension (1 ml) containing 1 mg protein, as measured by the method of Lowry et al. (1954), was dialysed into TS buffer supplemented with 20 mM β-mercaptoethanol. The sample was then split into two fractions: one was diluted with an equal volume of the same buffer while the other was diluted in buffer containing 1 mg/ml bromelain (Sigma). After overnight incubation at 37^oC, virus was purified from the fractions by sucrose density gradient centrifugation and pelleting, and prepared for electrophoresis as in Chapter One. The samples were electrophoresed in a phosphate buffered gel system (Method 1), overnight at 2mA/gel. Proteins were visualised by staining with Coomassie blue R and absorbance profiles were compared on a Kipp and Zonen densitometer.

3. Isolation of FeLV cores and analysis of their polypeptide composition.

The method described here is based on that of Bolognesi et al. (1973). One mg of sucrose gradient purified FL74 virus (FeLV-ABC/KT) was sedimented through two detergent layers on top of a gradient consisting of

1 ml of 70% sucrose overlayed with a linear 20 - 60% sucrose gradient. All sucrose solutions were in TS buffer and contained 20 mM DTT. The top detergent layer consisted of 5% sucrose in 0.5 ml TS buffer containing 1% Sterox SL and 0.1 M DTT. The layer beneath was in 10% sucrose (also in 0.5 ml TS buffer) containing 0.25% NP40 and 0.1 M DTT. After centrifugation at 10,000 rpm for 15 minutes in a Beckman SW50.1 rotor, the speed was increased to 45,000 rpm for a period of 90 minutes. After this time two sharp light-scattering bands were visible. One corresponded to the expected core density, 1.27 g.cm^{-3} and the other to the reported density for disrupted envelope material, 1.08 g.cm^{-3}. These bands, and an intermediate density region corresponding to whole virus, were collected, diluted, and pelleted. The proteins were then solubilised for electrophoresis as described in Chapter One. Proteins were separated on an 11% polyacrylamide gel (Laemmli) and visualised by staining with Coomassie blue R.

RESULTS

1. Surface proteins of FeLV detected by lactoperoxidase-catalysed ^{125}I labelling.

The results are shown in Fig. 6. The major labelled protein was at 70,000 M_r. A minor, well-resolved band was also visible at 62,000 daltons and a diffuse band at approximately 21,000 M_r. Apart from low molecular weight material migrating ahead of the tracker dye, there was also strongly labelled material at the front of unresolved molecules. The stained gel, before autoradiography, showed that p10 and p12 migrated with this unresolved material. A trace of label is visible in association with p27 and a protein at 45,000 M_r. Finally, a very high molecular weight band (> 200,000 daltons) was visualised.

2. Identification of FeLV surface components by proteolytic digestion.

The results are shown in Fig. 7. Two high molecular weight bands (80,000; 72,000) are greatly reduced in the bromelain treated virus. A minor band at 37,000 also disappeared while a new band at approximately 12,000 was seen, and a shoulder on the p27 peak at 26,000 increased. Another minor change was the disappearance of a shoulder from the high molecular weight side of the p15 peak. All other bands appeared to be unaltered by the treatment.

3. Isolation of FeLV cores and analysis of their polypeptide composition.

The results are shown in Fig. 8 and summarised in Table 5. The "envelope" fraction consisted almost entirely of material of 70,000 molecular weight. The core fraction showed a reduction in staining material at 70,000 M_r relative to whole virus, while viral p15 was virtually undetectable in any of the three fractions. Other minor differences are considered in Discussion.

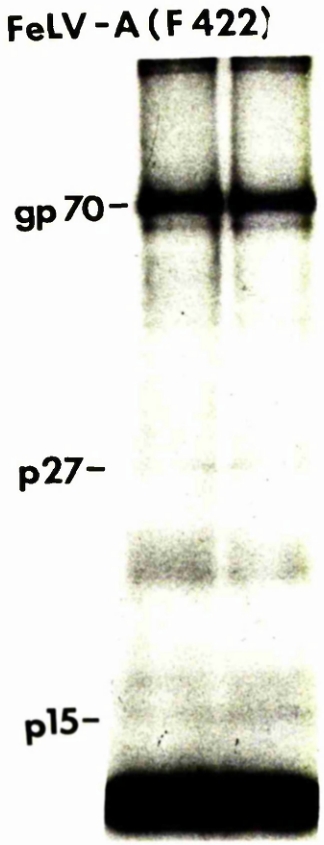


FIGURE 6

Lactoperoxidase-catalysed ^{125}I labelling of FeLV surface proteins

SDS polyacrylamide gel (11%) autoradiograph of ^{125}I labelled FeLV-A/Rickard (F422). Around 200,000 cpm of virus was applied to each well. The migration positions of p27 and p15 are marked. These were clearly visible on the stained gel before autoradiography.

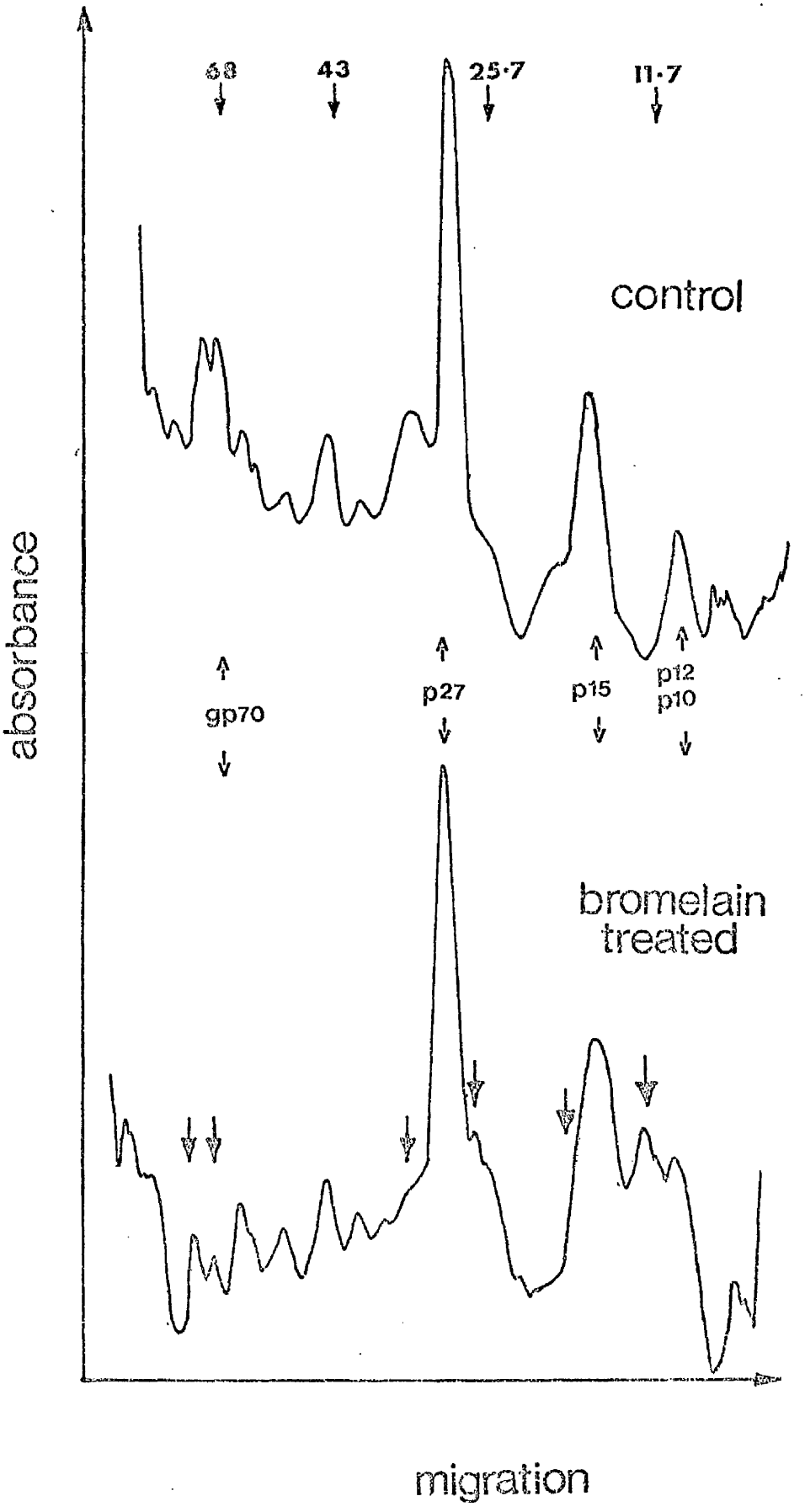
FIGURE 7

Bromelain digestion of FeLV surface components

Shows densitometer traces of SDS PAGE separated virus proteins with and without bromelain treatment. Numbers at the top refer to migration positions of standard marker proteins on a gel run in parallel. These were bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen (25,700) and cytochrome C (11,700).

Also, arrows on the lower part of the figure denote bands which are altered after bromelain treatment.

FIGURE 7


68
43
25·7
11·7
control
absorbance
gp70
p27
p15
p12
p10
bromelain
treated
migration

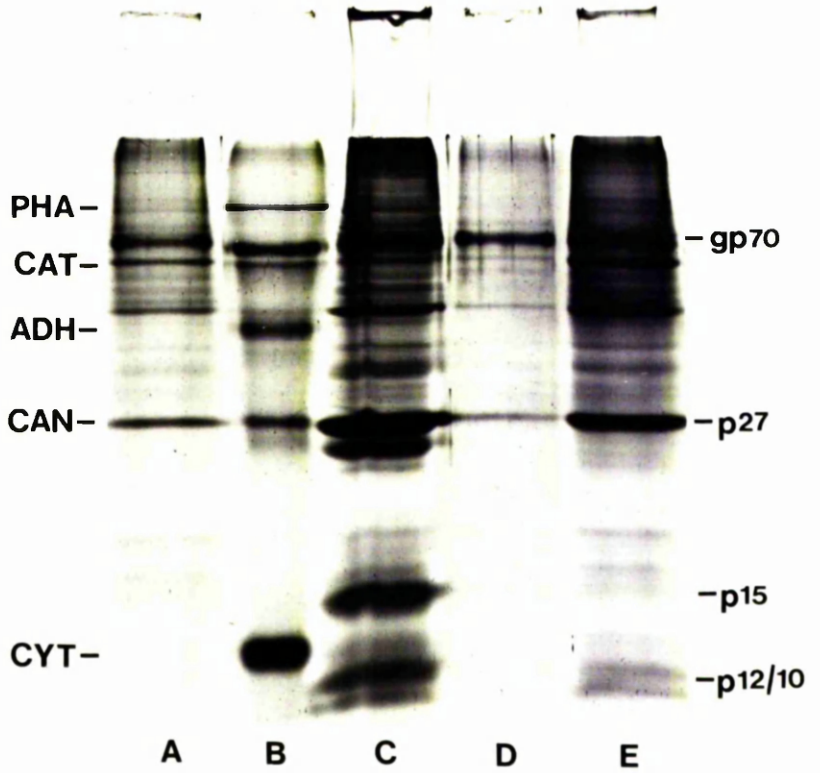


FIGURE 8

Comparison of sub-viral fractions of FeLV

FeLV-ABC/KT was disrupted by centrifugation through layers of detergents (Sterox SL, NP40). Three density bands from the sucrose gradients were collected and analysed on SDS PAGE (11% gel). Proteins were visualised by staining with Coomassie blue.

Lane A Intermediate density region (1.16 $g.cm^{-3}$)

Lane B Standard marker proteins. Phosphorylase a (PHA) 94,000 daltons, catalase (CAT) 60,000 daltons alcohol dehydrogenase (ADH) 41,000 daltons, carbonic anhydrase (CAN) 29,000 daltons, cytochrome c (CYT) 11,700 daltons

Lane C Control virus.

Lane D Envelope fraction (1.08 $g.cm^{-3}$).

Lane E Core fraction (1.26 $g.cm^{-3}$).

TABLE 5

Distribution of proteins in sub-viral fractions

Molecular weight ($\times 10^{-3}$)	Core	Intermediate	Envelope
70	+	++	++++
62	+++	+++	tr
46	++	+++	tr
40	++	+	-
37	++	+	-
29	+++	++	tr
18.5	++	++	tr
16.5	++	++	tr
14.5	-	-	-
<11.5	++	-	-

++++)
+++)
++) Representation in sub-viral fraction compared to whole virus. ++ denotes similar proportions to whole virus.
+)
tr)
-)

DISCUSSION

Enzymic iodination clearly demonstrated that the major virion surface component is a 70,000 M_r protein. This corresponds to the results of Witte and Weissman (1974) for Moloney MuLV and Damsky et al. (1977) for MuMTV. The identity of other labelled components is unknown, although the 62,000 M_r band could conceivably be a gag-precursor expressed on the virion surface. Immunoprecipitation with anti p27 serum might resolve this question. The diffuse band at approximately 21,000 daltons may correspond to the minor protein in this size range reported by Graves and Velicer (1974) in FeLV-A Rickard F422 preparations. A band in the same molecular weight was seen in FeLV-A/Glasgow-1 (see Chapter Two) but material comigrating with this band was shed by uninfected FEA cells, suggesting that this is a contaminating cellular component. The very low efficiency of labelling of p27 and a protein at 45,000 M_r suggests that these are not surface components; likewise, p15 is not labelled. However, it must be borne in mind that surface iodination requires a tyrosine residue to be accessible to the large lactoperoxidase enzyme (80,000 molecular weight)(Marchalonis, 1969). Antibody to p15(E) will bind to the surface of MuLV (Ihle et al., 1976) but Witte and Weissman (1974) did not record labelling of any low molecular component in Moloney MuLV, even after digestion of the virion surface proteins. The most likely explanation is that MuLV p15(E) lacks surface tyrosine residues. For the same reason, this experiment may have failed to detect some surface components. However, some material migrating with the tracker dye front was labelled in this experiment and it is possible that this is a small env-gene product analagous to MuLV p15(E).

A greater number of components were recognised as surface components by protease digestion than by iodone labelling. Bromelain is a relatively non-specific protease (Murachi and Neurath, 1960) and hence does not require the side-chain of one particular amino acid to be exposed in order to achieve digestion.

Two bromelain-sensitive components were seen in the 70 - 80,000 M_r range. If all the analyses of FL74 virus presented in this work and elsewhere are compared, it can be seen that the detection of the higher molecular weight component varies considerably from time to time. Since this cell line is not cloned, subpopulations may exist which demonstrate reduced synthesis or

virion-incorporation of the higher molecular weight component. Another bromelain sensitive component was seen at 37,000 M_r. This may be another virion surface component. However, if cell membrane vesicles copurify with virions, these may also have enzyme-sensitive surface components. This possibility must be considered for the > 15,000 M_r component also.

After enzyme treatment, a new peak is seen at 13,000 M_r and there is an increase in a peak at 25,000 M_r. These may represent "core" polypeptides which are resistant to further enzyme digestion.

The results of sub-viral fractionation provided further evidence that gp70 is associated with the viral envelope, since it could easily be removed by centrifugation through detergent layers. The "envelope" fraction consisted almost entirely of material at 70,000 M_r and thus this procedure might be useful as a first step in the purification of gp70.

Viral p27, and some low molecular weight protein migrating at the tracker dye front (p10 or p12?) were associated mainly with the core fraction. These include bands at 18,500 and 16,500 M_r. A molecule migrating at 62,000 M_r was associated strongly with the core and intermediate fractions. This is possibly an uncleaved gag-precursor. Jamjoom et al. (1976) have suggested that a Rauscher MuLV gag-precursor of 65,000 daltons is present in virions lacking RNA. The observations recorded here would not be consistent with this since cores lacking RNA would not be expected to band at such a high density. However, the 62,000 M_r molecule may have become artifactually adsorbed to the cores during disruption. As discussed in Chapter Four, the gag-precursor specificity of the 62,000 M_r protein could be established with antisera to p27 and p15. In a similar way, the 40,000 and 37,000 dalton proteins which are found predominatly in the core fraction, could be analysed immunologically.

The 46,000 M_r species, which is found predominantly in the intermediate layer, is found in uninfected cell culture fluids (see Chapter Two). Also, the 70,000 Mr species seen in the intermediate layer may be the non-glycosylated species (Chapter Four) which is also found in uninfected cell extracts (Chapter Two).

Viral p15 could not be found in any of the fractions isolated here. It is possible that this molecule remained in the detergent layers rather than enter the gradient.

CHAPTER FOUR

POLYPEPTIDE COMPOSITION OF DIFFERENT STRAINS OF FeLV

INTRODUCTION

Strains of FeLV vary in their host range and interference properties, which are determined by the virion envelope (see Chapter One). Another difference between FeLV strains is the diseases which they induce. For instance, FeLV-C strains cause severe aplastic anaemia when inoculated into kittens. This is in contrast to FeLV-A or FeLV-AB strains which induce a later neoplastic disease (Mackey et al., 1975; Jarrett et al., 1978). Differences in oncogenicity of isolates of avian leukosis virus are well documented (Purchase et al., 1977) and murine leukaemia viruses also vary widely in the spectrum of disease they induce. Examples are the thymic lymphoma of AKR MuLV (Gross, 1970) and the erythroblastosis and late erythroleukaemia of Friend MuLV (Steeves, 1975). No gene responsible for transformation analagous to the src gene of the sarcoma viruses has been attributed to the "weakly transforming" leukaemia viruses. It is not clear whether this is due merely to the difficulties involved in studying these viruses, since their transforming effects have been observed only in vivo and the latent period before clinically apparent disease is often long. In view of these problems, it seemed important to characterise the virion structural antigens of the various FeLV strains, in the event that these influence pathogenicity or oncogenicity.

At the time this study began very little information was available on type-specific antigens of FeLV. One report (Green et al., 1973) claimed that the gs-1 antigen of FeLV, which has been identified on p27, carried type-specific as well as group and interspecies determinants. It has also been shown indirectly that FeLV gp70 has interspecies reactivity (Strand and August, 1974). Pal et al., (1976) have reported charge heterogeneity of FeLV p30, p12 and p10. Variation in isoelectric point of the viral p30 has recently been suggested as a useful marker to distinguish murine xenotropic from ecotropic viruses (Chuat et al., 1978). Hopkins et al. (1977) have found that N-B tropism differences in MuLV strains are associated with a slight difference in p30 mobility in SDS polyacrylamide gels.

A study of the serum neutralisation of FeLV strains was in progress in parallel with the work presented in this thesis (Russell, 1977) which revealed that some antisera were highly type or subgroup specific in their neutralisation while others showed a broad spectrum of reactivity. An interesting observation was that all strains of FeLV-A tested were of the same serotype: they responded

in an identical manner to a range of antisera, including one raised to a cloned isolate of FeLV-A. By contrast, FeLV-B and FeLV-C showed antigenic heterogeneity, even though only four isolates of FeLV-C were available for testing.

Evidence has emerged that ecotropic MuLV strains can recombine with endogenous xenotropic viruses and produce viruses with expanded host range and increased transformation potential (Elder et al., 1977a; Troxler et al., 1977). These have been shown to be env gene recombinants, and this has raised the possibility that the major viral glycoprotein may be crucially involved in leukaemia virus transformation, although concomitant rescue of a distinct transforming gene by recombining MuLV has not been ruled out. It is shown in this chapter that the major glycoprotein of FeLV varies in apparent molecular weight in SDS PAGE. It is possible that a range of gp70 molecules exists in FeLV strains and in normal cat cells in a similar fashion to the mouse system (Elder et al., 1977b).

MATERIALS AND METHODS

1. Comparison of FeLV-A, B and C by coelectrophoresis

FeLV-A/Glasgow-1, FeLV-B/Sarma and FeLV-C/Sarma were grown in FEA cells. FeLV-A and C were labelled with L (4, 5 - ^{3}H) leucine by plating infected cells at 1 x 10^{6} cells per 9 cm plate and replacing the EFB medium after 24 hours growth with leucine-free Eagle's medium for 1 hour. After this time label was added (100 μCi/ml) in EFB with 10% normal levels of leucine. After a further 24 hours, the culture fluid was harvested and virus was purified as described in Chapter One. FeLV-B was labelled with L(U - ^{14}C) leucine in a similar manner, except that label was added at 10 μCi/ml. Purified viruses were prepared for electrophoresis as described in Chapter One (Method 1). Proteins were separated on 7.5% polyacrylamide gels and radioactive bands were detected by slicing gels into 1 mm slices followed by scintillation counting.

2. Comparison of FeLV and eFOV on SDS-polyacrylamide slab gels.

Sucrose gradient purified virus samples (∼ 50 μg protein) of FeLV-ABC/KT, FeLV-B/Sarma and eFOV/FER were compared by electrophoresis on a slab gel (Laemmli) with an acrylamide monomer concentration of 11%. Proteins were visualised by staining with Coomassie blue R250.

3. Comparison of the major glycoprotein of FeLV strains by lectin affinity chromatography and SDS polyacrylamide gel electrophoresis.

The viruses used in this study were produced in the experiment involving infected cell labelling which is described in Chapter Six. ^{3}H-leucine labelled virus was purified from culture fluids by discontinuous and continuous density gradient centrifugation. The purified virus pellets were dissolved in 1% DOC and centrifuged at 100,000 x g for 60 min. The supernatant fluid was fractionated on lectin affinity columns and the fractions were lyophilised and electrophoresed as described for cell membrane preparations in Chapter Six.

4. Further comparison of FeLV glycoproteins by SDS PAGE.

All viruses were grown in FEA cells and labelled for 48 hours with L(4, 5 - ^{3}H) leucine at 30 μCi/ml in EFB with 10% of the normal leucine concentration. Virus-containing and uninfected culture fluids were harvested and purified by discontinuous and continuous sucrose density gradient centrifugation. Virus proteins were separated by SDS PAGE (Laemmli) and visualised by fluorography.

RESULTS

1. Comparison of FeLV-A, B and C by coelectrophoresis.

Results are shown in Fig. 9. Fig. 9a shows a comparison of ^{3}H leucine labelled FeLV-A and ^{14}C leucine-labelled FeLV-B, while Fig. 9b shows ^{3}H leucine FeLV-C and ^{14}C leucine-FeLV-B. No differences could be detected in the migration of the three major peaks corresponding to p27, p15 and p10/12 respectively (Bolognesi et al , 1974). The resolution of this system was not sufficient to assess with any confidence the minor differences seen in the high molecular weight range.

2. Comparison of FeLV and eFOV on SDS-polyacrylamide slab gels.

This result (Fig. 10) shows that the SDS PAGE technique can differentiate one RNA tumour virus from another. Again, the p27, p15 and p12/p10 bands of two different FeLV strains (FeLV-ABC/KT and FeLV-B/Sarma) had identical mobilities, while eFOV showed a higher molecular weight "p28" and a clearly distinguishable pattern of low molecular weight proteins.

3. Comparison of the major glycoprotein of FeLV strains by lectin affinity chromatography and SDS-polyacrylamide gel electrophoresis.

Fig. 11 shows a SDS PAGE separation of cloned FeLV-A/Glasgow-1 produced by FEA cells which was fractionated by lectin affinity. Proteins which failed to bind to either lectin (lane e,f)exhibited molecular weights of approximately 74,000, 44,000 and 30,000. Lanes g and h revealed that three virion glycoproteins of approximately 160,000, 145,000 and 80,000 daltons bind equally well to both LcH and RCA_1. The analysis was repeated on an uncloned stock of FeLV-A/Glasgow-1, (Fig. 12) giving an apparent 2 to 4-fold enhancement of the 80,000 dalton component relative to the other two glycoproteins.

A cloned stock of FeLV-B/Sarma produced by FEA cells was examined in a similar manner (Fig. 13). As with FeLV-A, three major proteins which failed to bind to the lectin columns were observed at approximately 74,000, 44,000 and 30,000 daltons. Inspection of lanes c to k revealed only two major glycoproteins at approximately 160,000 and 145,000 daltons, both of which were specifically bound by LcH and RCA_1. This analysis was also repeated with an uncloned stock of FeLV-B/Sarma and these results are shown in Fig. 14. As before the major components which failed to bind to the lectins migrated at

FIGURE 9

Comparison of FeLV-A, B and C
by coelectrophoresis on SDS polyacrylamide gels.

Figure 9a shows a separation of FeLV-C/Sarma and FeLV-B/Sarma, labelled with ^{3}H-leucine and ^{14}C-leucine respectively on a 7.5% acrylamide gel (sodium phosphate buffer system). The gel was cut into 1 mm slices which were analysed for ^{3}H and ^{14}C by scintillation counting.

Figure 9b shows a similar separation of FeLV-B/Sarma and FeLV-A/Glasgow-1.

FIGURE 9a

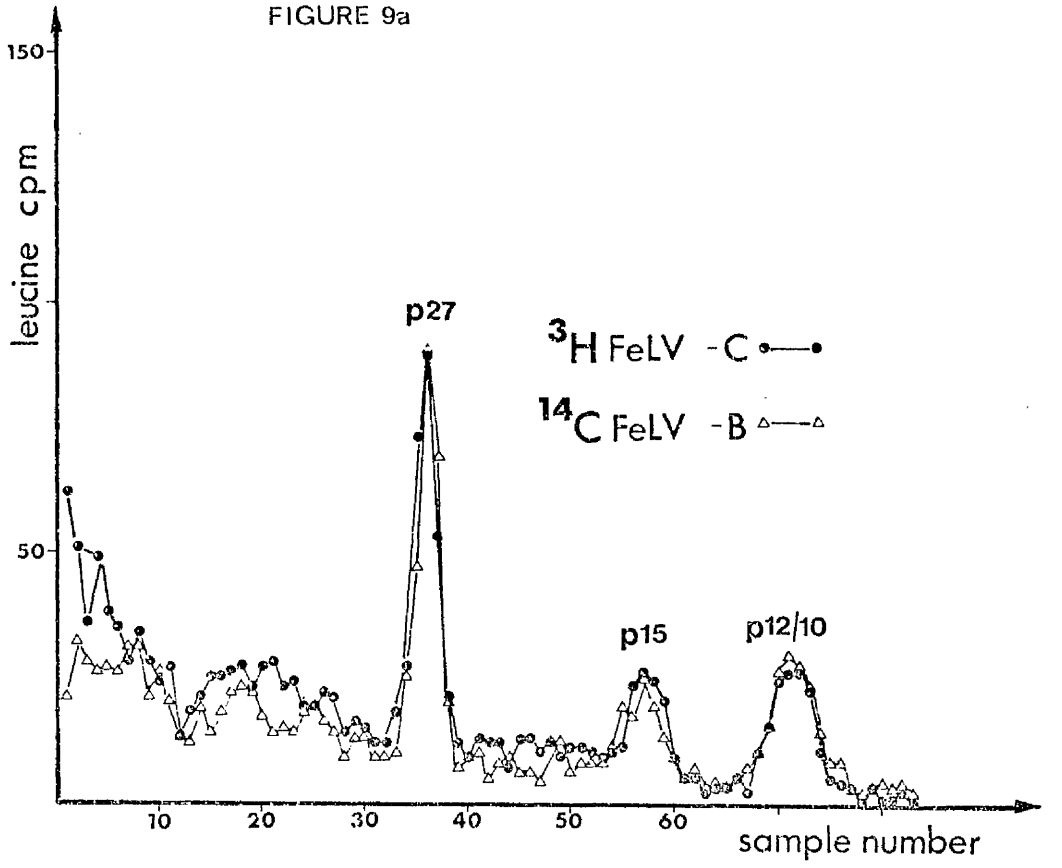
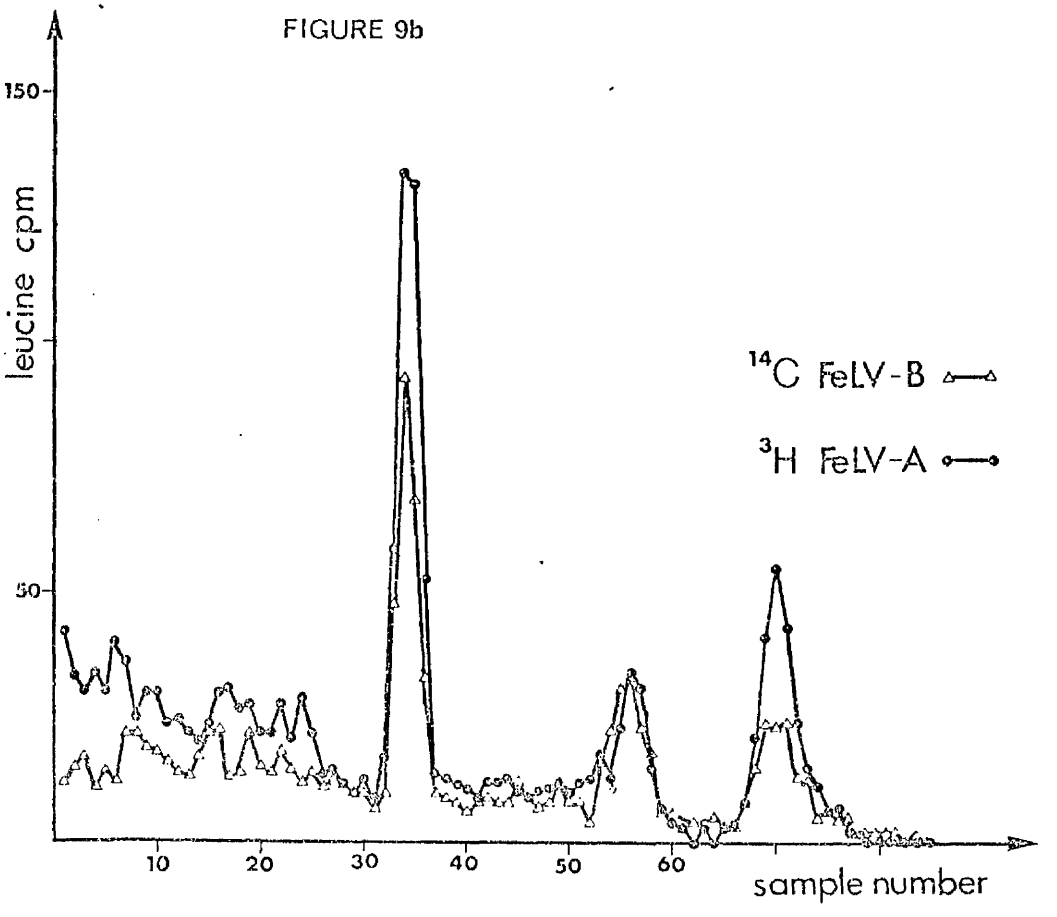


FIGURE 9b



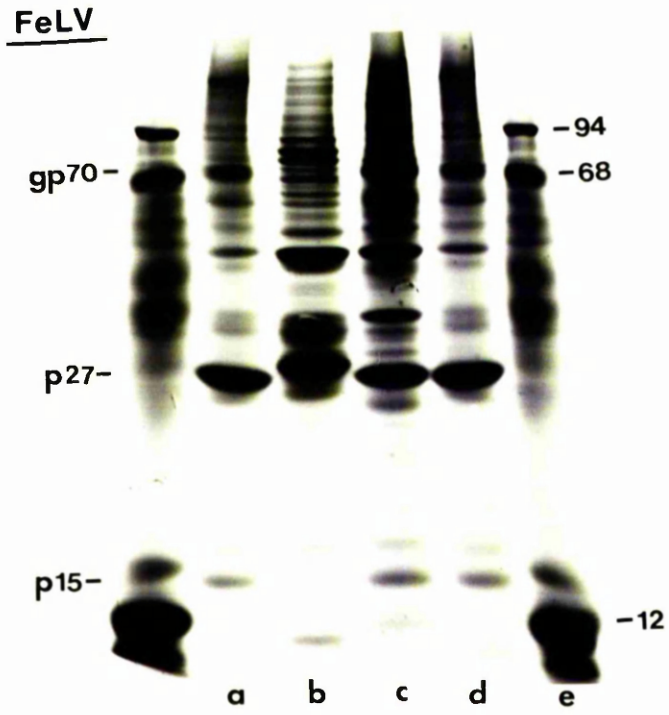


FIGURE 10

Comparison of FeLV and eFOV on SDS PAGE

Virus proteins (c. 50 µg) were separated on an 11% slab gel, and visualised by staining with Coomassie blue.

Lane a FeLV-ABC/KT purified from FL74 cells.

Lane b eFOV grown in CT45S cells.

Lane c FeLV-B/Sarma grown in FEA cells.

Lane d As lane a.

Lane e Standard marker proteins. Phosphorylase a (94,000 daltons), bovine serum albumin (68,000) and cytochrome c (11,700).

Migration positions of FeLV proteins gp70, p27 and p15 are marked as on the left of the figure.

FIGURE 11

Glycosylation of cloned FeLV-A/Glasgow-1 proteins examined by lectin affinity chromatography

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled components of cloned FeLV-A/Glasgow-1 after fractionation on LcH and RCA_I affinity columns. Monomer concentration was 10%. Marker proteins are β-galactosidase (130,000 daltons), phosphorylase-a (94,000), catalase (60,000), alcohol dehydrogenase (41,000) and carbonic anhydrase (29,000).

Lane a Original virus preparation.

Lane b 1% DOC soluble components (100,000 x g supernatant).

Lane c Components which failed to bind to LcH.

Lane d Components which failed to bind to RCA_I.

Lane e Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane f Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane g LcH bound components.

Lane h RCA_I bound components.

Lane i Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane j Components which failed to bind to LcH and subsequently bound to RCA_I.

FIGURE 12

Glycosylation of uncloned FeLV-A/Glasgow-1 proteins examined by lectin affinity chromatography

As Figure 11, except that uncloned FeLV-A/Glasgow-1 was used, and monomer concentration of gel was 8%.

Lane a Original virus preparation.

Lane b Components which failed to bind to LcH.

Lane c Components which failed to bind to RCA_I.

Lane d Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane e Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane f LcH bound components.

Lane g RCA_I bound components.

Lane h Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane i Components which failed to bind to LcH and subsequently bound to RCA_I.

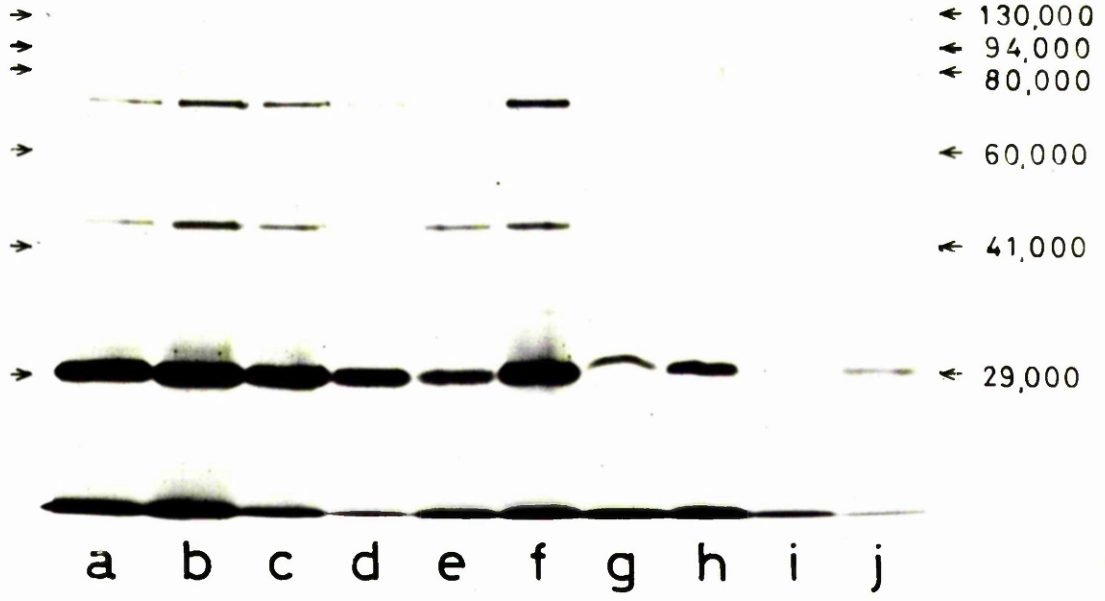


FIGURE 11

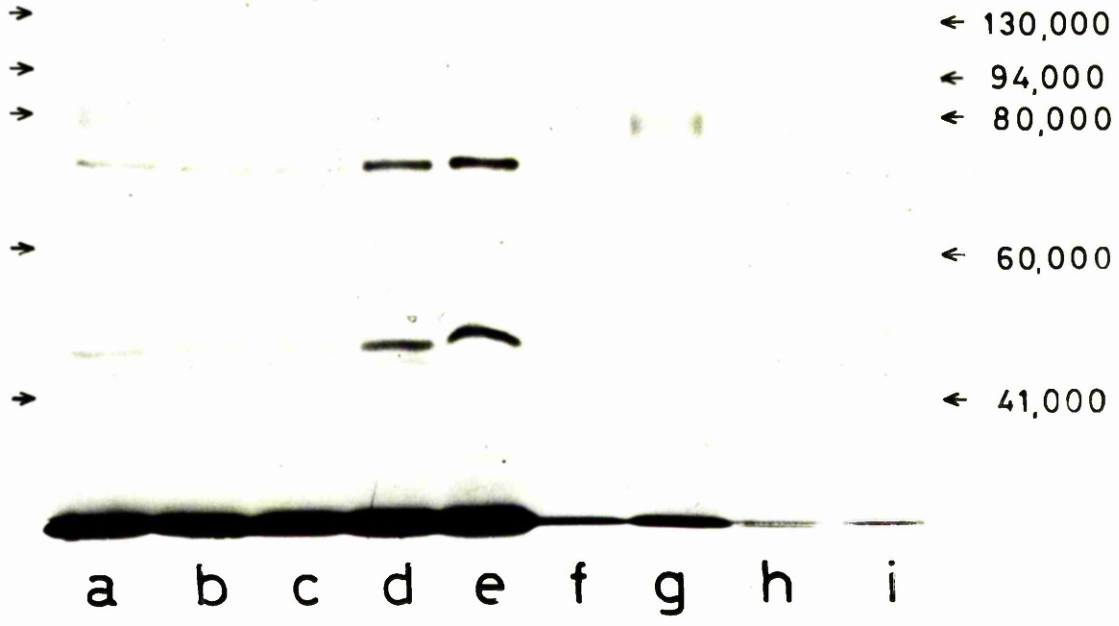


FIGURE 12

FIGURE 13

Glycosylation of cloned FeLV-B/Sarma proteins examined by lectin affinity chromatography

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled components of cloned FeLV-B/Sarma after fractionation on LcH and RCA_I affinity columns. Monomer concentration was 10%. Marker proteins are as in Figure 11.

Lane a Original virus preparation.

Lane b 1% DOC soluble components (100, 000 x g supernatant).

Lane c Components which failed to bind to LcH.

Lane d Components which failed to bind to RCA_I.

Lane e Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane f Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane g LcH bound components.

Lane h RCA_I bound components.

Lane i Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane j Components which failed to bind to LcH and subsequently bound to RCA_I.

FIGURE 14

Glycosylation of uncloned FeLV-B/Sarma proteins examined by lectin affinity chromatography

As Figure 13, except that uncloned FeLV-B/Sarma was used.

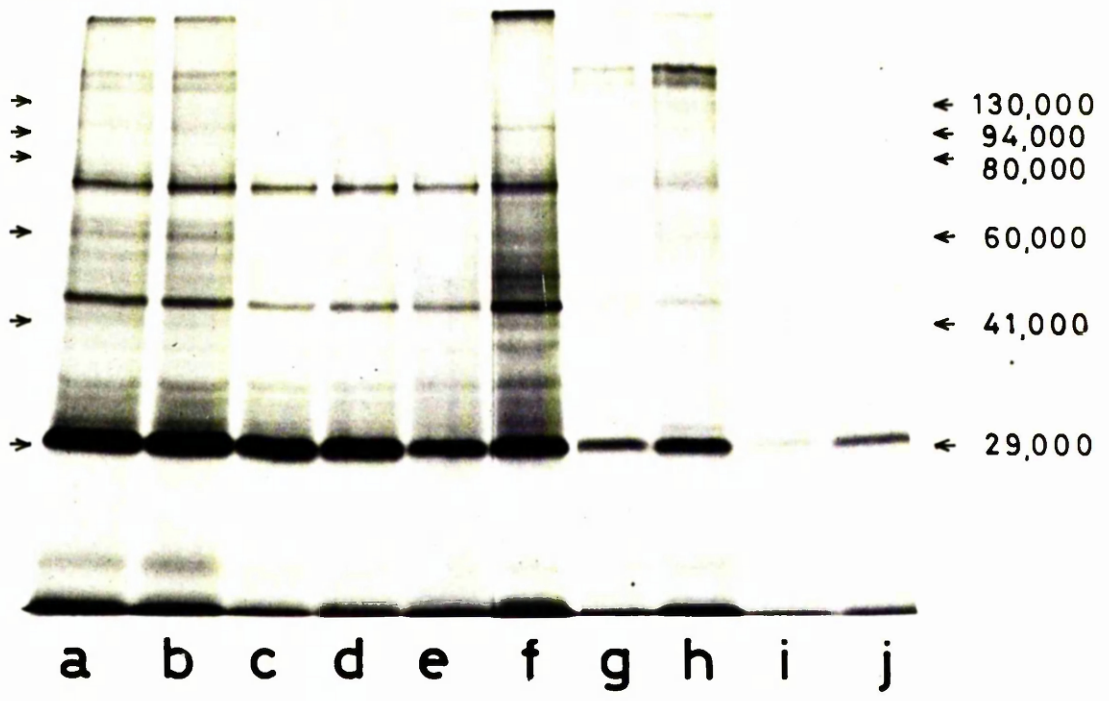


FIGURE 13

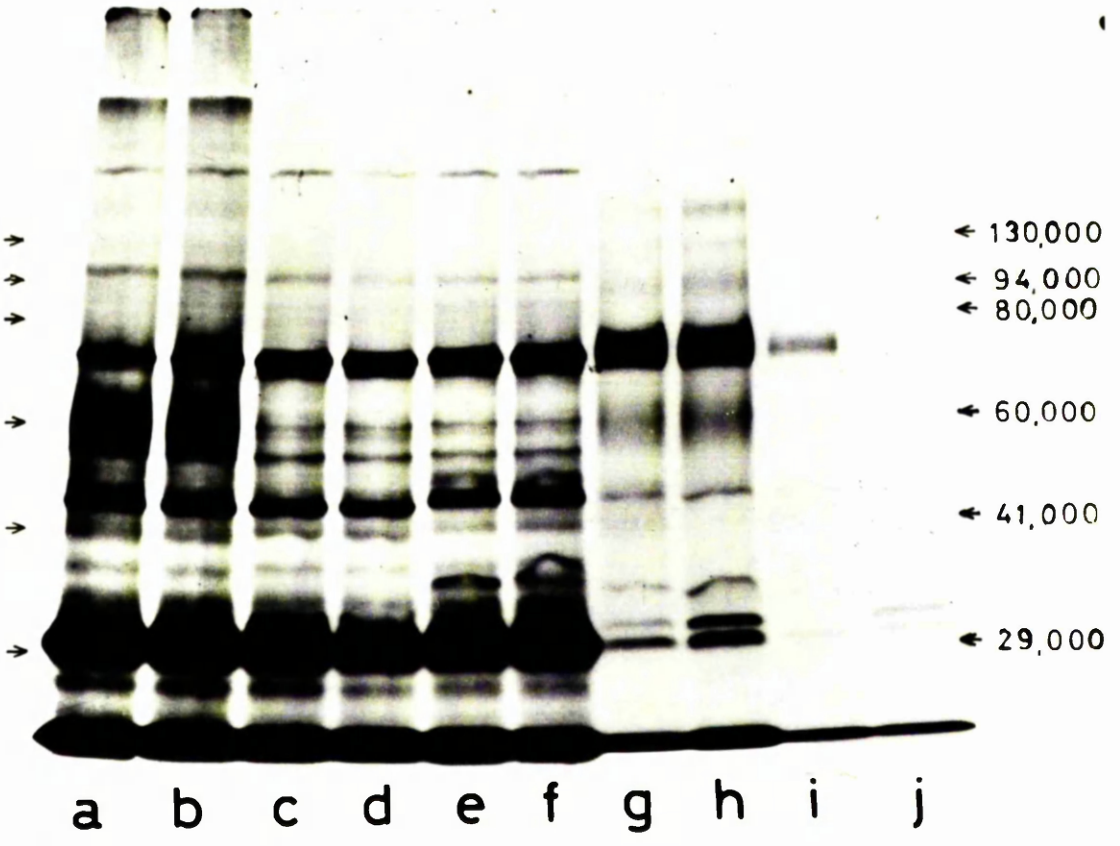


FIGURE 14

approximately 74,000, 44,000 and 30,000 daltons. However,unlike the results shown in Fig. 13, the FeLV-B virus obtained from this infection of feline fibroblasts contained only one major glycoprotein at approximately 75,000 daltons.

A fluorograph of lectin fractionated ^{3}H-leucine labelled components of FeLV-C/Sarma produced by FEA cells is shown in Fig. 15. The unbound species were similar to those seen with FeLV-A and FeLV-B. The lectin bound virion glycoproteins were composed of a major glycoprotein at 69,000 M_r, as well as lesser amounts of glycoproteins at approximately 160,000, 145,000, 135,000 and 62,000-65,000 daltons.

Fig. 16 shows an analysis of virus produced by feline lymphoblastoid (FL74) cells (FeLV-ABC/KT). Lane b again demonstrated unbound components at 74,000, 44,000 and 30,000 daltons, while lanes c to f demonstrated a 69,000 M_r component which bound to both lectins and two lesser glycoproteins of approximately 41,000 and 35,000 daltons which were specifically bound to the LcH lectin but not to the RCA_1 lectin.

The endogenous feline oncornavirus (eFOV) was also analysed in this comparative study. A line of feline fibroblasts which spontaneously release this virus (FER) was used. The analysis is shown in Fig. 17. The major glycoprotein of this virus migrated at approximately 85,000 daltons. Traces of glycoproteins at 160,000, 145,000, 135,000 and 62,000-65,000 M_r were also observed. All of the glycosylated proteins were bound effectively to both lectins. It should be noted that this fibroblast-produced virus contained essentially no protein at 74,000 daltons.

4. Further comparison of FeLV glycoproteins by SDS polyacrylamide gel electrophoresis.

The results are shown in Fig. 18. The glycoproteins were all diffuse bands, the resolution of which was obscured in some strains by the presence of the non-glycosylated 74,000 molecular weight band. However, an estimate of the mean M_r value for each major virion glycoprotein is given in Table 6.

In addition, a diffuse band was seen in the FeLV-B/Boston-1 preparation at 37,000 M_r, which may represent a minor virion glycoprotein. Other bands which vary from strain to strain were observed at 60,000-65,000 daltons and 34,000 daltons.

FIGURE 15

Glycosylation of FeLV-C/Sarma proteins examined by lectin affinity chromatography

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled components of FeLV-C/Sarma after fractionation on LcH and RCA_I affinity columns. Monomer concentration was 8%. Marker proteins are as in Figure 11.

Lane a Original virus preparation.

Lane b Components which failed to bind to LcH.

Lane c Components which failed to bind to RCA_I.

Lane d Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane e Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane f LcH bound components.

Lane g RCA_I bound components.

Lane h Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane i Components which failed to bind to LcH and subsequently bound to RCA_I.

FIGURE 16

Glycosylation of FeLV-ABC/KT proteins examined by lectin affinity chromatography

As Figure 15, except that FeLV-ABC/KT was used, and monomer concentration of gel was 10%.

Lane a Original virus preparation.

Lane b 1% DOC soluble components (100, 000 x g supernatant).

Lane c Components which failed to bind to LcH.

Lane d Components which failed to bind to RCA_I.

Lane e LcH bound components.

Lane f RCA_I bound components.

Lane g Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane h Components which failed to bind to LcH and subsequently bound to RCA_I.

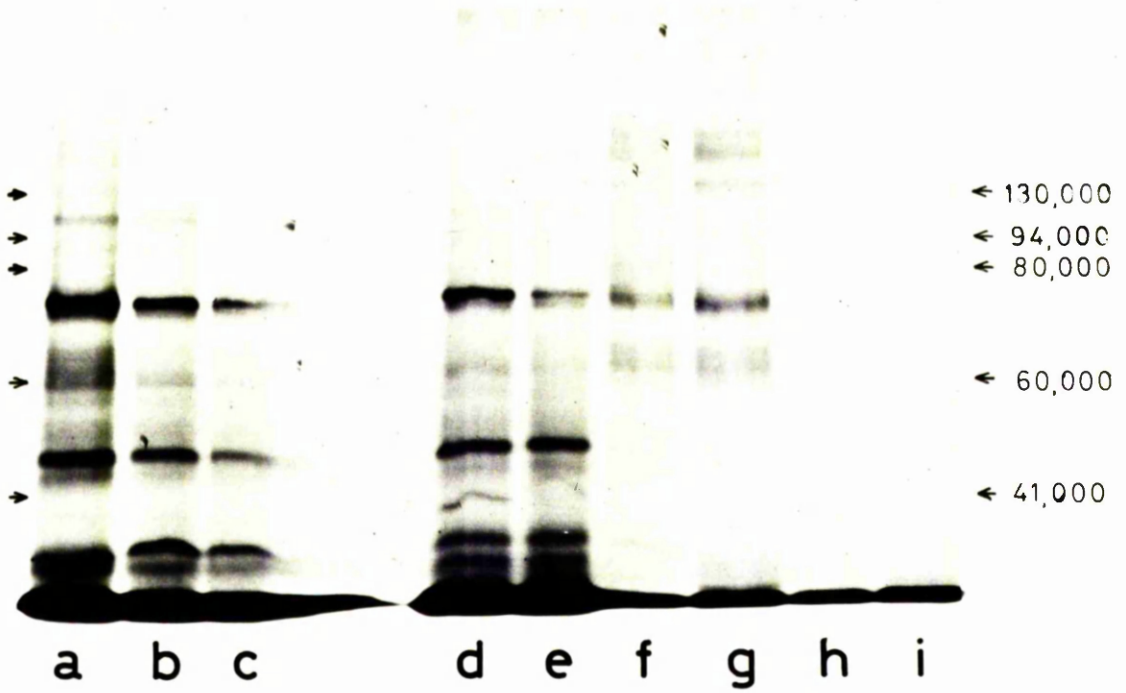


FIGURE 15

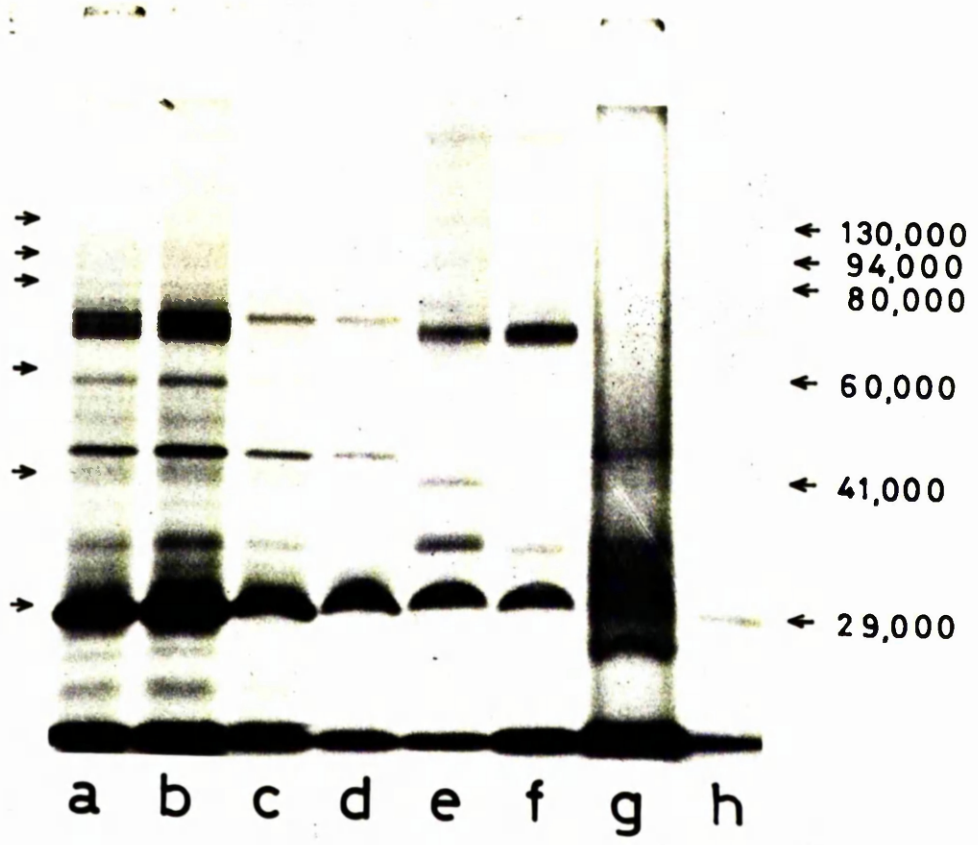


FIGURE 16

FIGURE 17

Glycosylation of endogenous feline oncornavirus proteins examined by lectin affinity chromatography

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled components of endogenous feline oncornavirus after fractionation on LcH and RCA_I affinity columns. Monomer concentration was 8%. Marker proteins are as in Figure 11.

Lane a Original virus preparation.

Lane b Components which failed to bind to LcH.

Lane c Components which failed to bind to RCA_I.

Lane d Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane e Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane f LcH bound components.

Lane g RCA_I bound components.

Lane h Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane i Components which failed to bind to LcH and subsequently bound to RCA_I.

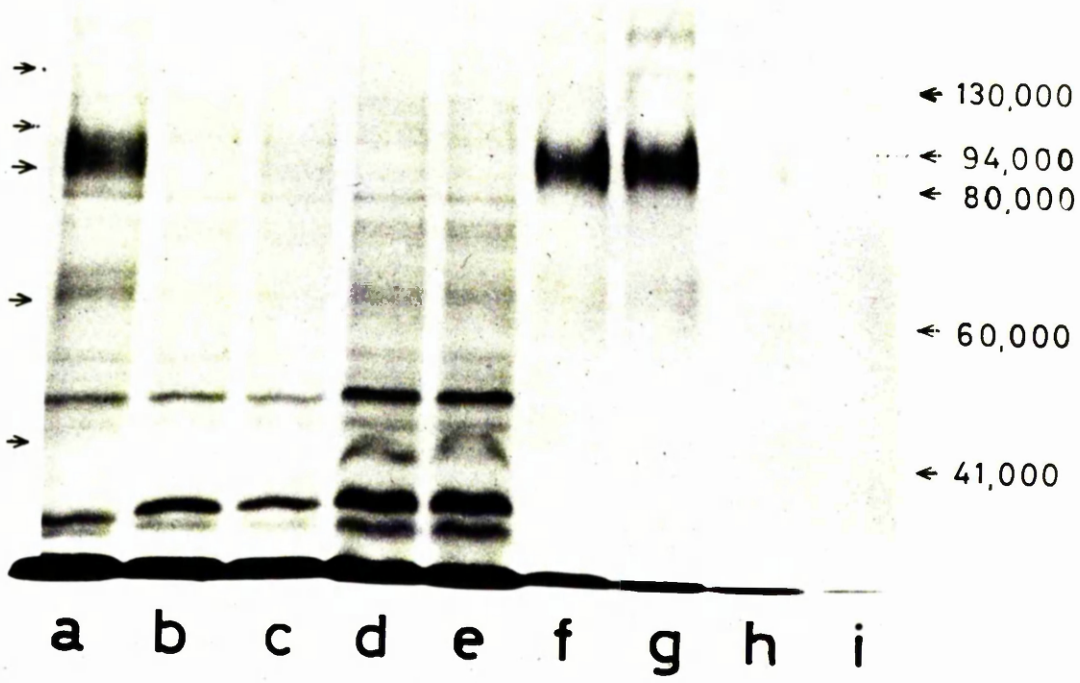


FIGURE 17

FIGURE 18

Comparison of gp70 of various FeLV strains on SDS PAGE

Proteins of various FeLV strains (25,000 cpm ^{3}H-leucine per virus preparation) and FEA cell culture supernatant (10,000 cpm ^{3}H-leucine) were separated on SDS PAGE and visualised by fluorography. Monomer concentration was 8%.

Lane a FEA cell culture supernatant proteins.

Lane b FeLV-A/Boston-1.

Lane c FeLV-A/Glasgow-1 (contaminated with unknown FeLV-B).

Lane d FeLV-A/Glasgow-1 (cloned).

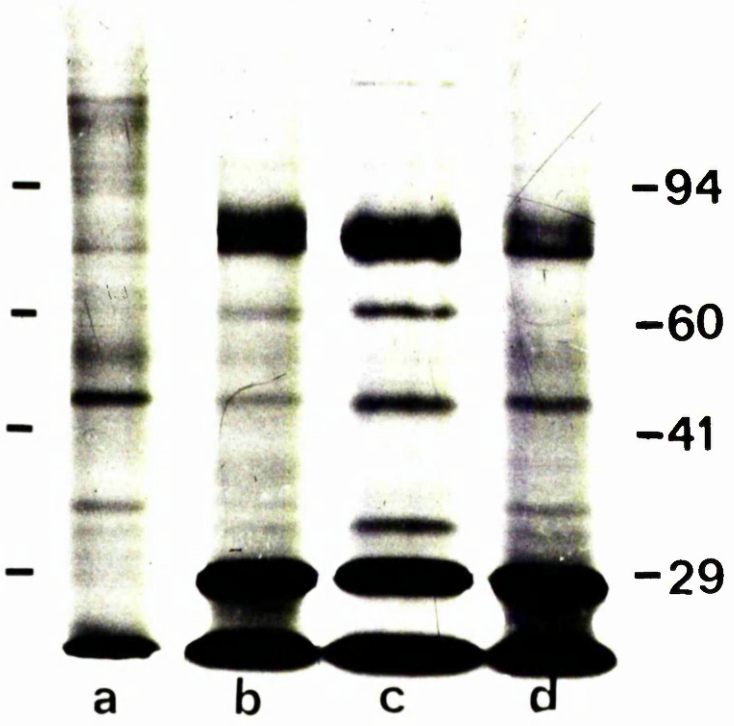
Lane e FeLV-B/Boston-1.

Lane f FeLV-B/Sarma (cloned).

Lane g FeLV-C/Sarma.

Lane h FeLV-B/Sarma (uncloned).

FIGURE 18



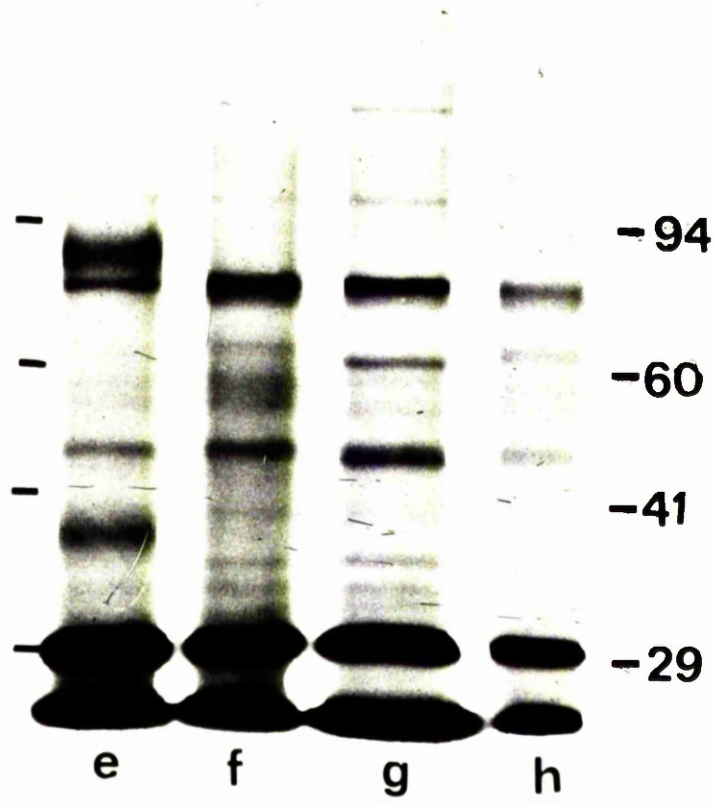


TABLE 6

Comparison on SDS PAGE of apparent molecular weights of gp70 of different FeLV strains

FeLV strain	Apparent molecular weight	
A/Glasgow-1 (cloned)	80,000	(80,000) * +
AB/Glasgow-1[Δ]	76,000	
A/Boston-1	78,000	
B/Sarma	76,000	
B/Sarma (cloned)	74,000	(75,000) *
B/Boston-1	82,000	(82,000) +
C/Sarma	72,000	(69,000) *

Δ contaminated with unknown FeLV-B.

* values recorded in Experiment 2 in parentheses.

\+ values recorded in Chapter Two in parentheses.

DISCUSSION

The results of the first experiment showed that there were no major differences in the migration in SDS PAGE of the low molecular polypeptides of various FeLV strains. However, the resolution of this system involving gel slicing is relatively poor and no conclusions could be drawn regarding the high molecular weight range (> 40,000 daltons).

Further analysis on slab gels confirmed the observation that p27, p15 and p12/p10 bands had identical electrophoretic mobility from different FeLV strains. In Fig. 10, a preparation of eFOV/FER is included to demonstrate the ability of SDS PAGE to discriminate one oncornavirus from another.

Lectin affinity chromatography and SDS PAGE demonstrated marked differences in the apparent molecular weights of the major glycoproteins of various FeLV strains. The significance of this finding is discussed in Chapter Eight. Some preparations contained very little detectable glycoprotein in the expected molecular weight range. From further analyses, it can be concluded that this is not a reproducible finding for a given virus subgroup, but is more likely to be an artifact of virus purification or simply experimental variation. Although the effect of purification techniques were analysed in Chapter Two and found to be negligible for the two viruses tested, it is possible that some virus preparations will have more labile surface glycoproteins. Similar results have been noted for Rauscher MuLV by Strand and August (1976). However, it was of interest that in the FeLV-B preparations which showed no detectable gp75, the two bands at 160,000 and 145,000 daltons were enhanced. These are FEA cell membrane glycoproteins (see Chapter Six). As discussed in Chapter Two, the presence of some cellular components in virus preparations is quantitatively greater than can be accounted for by plasma membrane exfoliate contaminants. Preparations of FeLV-B from CT45S canine thymocytes showed almost equal amounts of plasma membrane and virus proteins (data not shown) but this cell line seemed to release much larger quantities of such material, compared to the other cell lines used. Minor glycoprotein bands in FeLV-ABC/KT at 41,000 and 35,000 M_r are probably normal cell glycoproteins, but since no uninfected control is available for FL74 cells, this could not be verified.

The presence of only one "gp70" band in FeLV-ABC/KT requires consideration since FEA-grown FeLV-A, B and C demonstrate such heterogeneity of molecular weight. Titration of FL74 virus reveals that its specific infectivity is 10^{-4} to 10^{-6} times that of FeLV-A grown in FEA cells (Jarrett , 1975). Of the three components in FL74 cells, FeLV-A has the highest infectivity titre (data not shown). Absorption of neutralising antisera with FL74 cell extracts revealed that FeLV-C neutralising activity is removed far more efficiently than FeLV-A neutralising activity (Russell, 1977). Also, inoculation of FeLV-ABC/KT into cats raised antisera which neutralised FeLV-C to higher titres than FeLV-A. These results, together with the observation that FeLV-C and FL74 virion glycoproteins are of similar apparent molecular weight, suggest that the majority of the FL74 virus envelope material may be subgroup C specific. As discussed in Chapter Three, FL74 (FeLV-ABC/KT) virus contains variable amounts of an additional component of 80 to 85,000 molecular weight, and a molecule of similar molecular size is precipitated from FL74 cell fractions by cat sera which neutralise FeLV (see Chapter Seven).

Lectin affinity chromatography characterised the glycoproteins of various FeLV strains and demonstrated differences in their migration in SDS PAGE. In the final experiment in this Chapter these observations were confirmed and extended to further strains to assess the possible relationship of virus subgroup to glycoprotein molecular weight.

The M_r values recorded here for FeLV glycoproteins agree well with those previously recorded (see Table 6) showing that this is a stable property for a virus strain grown in a particular cell. The variation in molecular weight does not relate to the subgroup of the virus: FeLV-B strains showed a wide range of M_r values, from 74,000 to 82,000. However, it is of interest to note that FeLV-B/Boston-i, unlike other FeLV-B strains tested, shows a high degree of cross-neutralisation with FeLV-A isolates (Russell, 1977) as well as a similar molecular weight for the major glycoprotein. This raises the possibility that the serotype of FeLV strains may correlate with the M_r value of the gp70, but more isolates must be tested before this conclusion can be confirmed.

Migration of glycoproteins under SDS PAGE can be anomalous due to reduced SDS binding relative to simple proteins and hence M_r values for glycoproteins must be interpreted with caution (Weber and Osborn, 1969). However, Sefton (1976) has examined this question with glycoproteins of various enveloped viruses grown in different cells. His conclusions were that the type of sugar residues added to the glycoprotein varied from virus to virus grown in the same cells, and varied in preparations of a single virus grown in different cells. However, the apparent size of the glycoprotein of a particular virus was very similar even when grown in different cells, suggesting that the specificity and extent of glycosylation were functions of the primary structure of the nascent glycoprotein. He also made the relevant point that although comigration of two unrelated glycoproteins could occur fortuitously, an apparent molecular weight difference does indicate real differences between two species.

Attempts to investigate some of these propositions with FeLV were made by growing two viruses of distinct glycoprotein molecule weight in the same cells and by growing a single virus in a number of different cell lines and examining the effect on the M_r of the virion glycoproteins. Unfortunately, the experiment was unfruitful due to poor resolution on the gel, but it seems worth repeating, and would probably be easier to interpret if a carbohydrate label was used.

There is little information on murine leukaemia and avian leukosis viruses to compare with the observations discussed here. However, it seems that small differences in M_r of gp85 between avian leukosis isolates relate to the serotype rather than to the subgroup of the virus (M. Hayman, personal communication). MuLV gp70s have been compared by tryptic peptide mapping (Elder et al., 1977b) and it has emerged from these studies that there is a "multi-gene family" of MuLV glycoproteins, mouse serum glycoproteins and differentiation antigens which show different degrees of relatedness in their peptide maps. Intriguingly, recently isolated viruses from preleukaemic AKR mice, and the spleen focus-forming virus in Friend MuLV preparations, have been shown to be env gene combinants by this technique (Elder et al., 1977a; Troxler et al., 1977). A comparison of the tryptic peptide digests of FeLV glycoproteins which should give more information regarding the relatedness of virion glycoproteins than molecular size can, is in progress.

An interesting exploitation of the apparent molecular size differences recorded here would be in the study of phenotypic mixing in FeLV strains. This is probably of biological significance for FeLV since FeLV-B may be defective for contact transmission between cats unless in a phenotypic mixture with FeLV-A (O. Jarrett, personal communication). Coprecipitation with subgroup specific antisera of the two distinguishable glycoproteins by virtue of their expression on the same virion could be used to quantitate the envelope "mosaic" particles which are thought to exist in these phenotypic mixtures (Vogt, 1967).

CHAPTER FIVE

PURIFICATION OF FeLV PROTEINS AND PROPERTIES OF ANTISERA TO FeLV

INTRODUCTION

There are a number of reasons for wishing to raise antisera to purified viral antigens. Firstly, these antisera can be used to detect antigen expression in the tissues of infected animals and normal animals in the case of RNA tumour viruses. The antisera may also be employed to examine the cellular location and the intracellular synthesis and processing of virus polypeptides.

Thus, murine leukaemia virus proteins have been shown to be expressed in mouse tissues in the absence of whole virus production (Aaronson and Stephenson, 1976). A similar situation exists in the chicken. Although all chicken cells were demonstrated to contain genetic information related to RAV-(O) (Varmus et al., 1972), some chick embryonic fibroblast cultures express the viral glycoprotein (gp85) in the absence of whole virus production (Hanafusa et al., 1973). Using radioimmunoassay with specific antisera, one line (line 6) has been shown to express viral proteins p27 and p19, but not p15 (Smith et al., 1976). This is thought to reflect an incomplete provirus in these cells.

The use of specific antisera to virion polypeptides in immune precipitation has revealed that the proteins of RNA tumour viruses are first synthesised as covalently linked polyproteins and then cleaved to yield the processed virion proteins (Vogt and Eisenman, 1973; Van Zaane et al., 1975; Arcement et al., 1976; Okasinski and Velicer, 1976; Famulari et al., 1976.

A feature which has emerged from the study of purified oncornavirus antigens is that individual molecules, notably gp70 and p30, reveal multiple antigenic determinants (Strand and August, 1974; 1975) which can be exploited to assess relatedness between RNA tumour viruses.

PURIFICATION OF FeLV PROTEINS

1. Guanidine hydrochloride gel filtration

This technique can be used for polypeptide molecular weight estimation, since in the presence of 6M guanidine hydrochloride (GuHCl) and reducing agents, many proteins behave as randomly coiled chains (Fish et al., 1969; Mann and Fish, 1972). As such it has been applied to RNA tumour viruses (Fleissner, 1971; Nowinski et al., 1972). However, it can also be used preparatively (Green and Bolognesi, 1974) since GuHCl denaturation is often reversible on removal of the salt.

Pharmacia columns (K16/100 or K15/90) were filled with Sepharose 6B or CL 6B in 6M GuHCl, 0.02M sodium phosphate buffer pH 6.8. After the gel had settled, columns were run at a flow rate of 1.5 ml/hr for Sepharose 6B or 5 ml/hr for Sepharose CL 6B for two days before use for protein separation. Also, the buffer was made 0.01M with dithiothreitol (DTT) and run for 24 hours before use.

2. Separation of FeLV-ABC/KT proteins on 6M GuHCl gel filtration columns

FeLV-ABC/KT was harvested from FL74 cells by collecting culture fluid two days after subculture of cells, which was then clarified and stored at -70°C. Such fluids contained up to 5 mg per litre of viral protein. Between 5 and 20 mg of sucrose gradient purified FL74 virus was mixed with purified ^{3}H-leucine-labelled tracer virus (10^{5} to 10^{6} cpm). The virus sample, in sodium phosphate buffer (0.02M, pH 6.8) was made 8M with guanidine hydrochloride, to 2% (w/v) β-mercaptoethanol, to 0.01M with dithiothreitol (DTT), and was adjusted to a final volume of 1ml. The sample was held at 37°C overnight, and boiled for three minutes before applying to the column. Fractions of 1ml were collected and protein-containing fractions were detected by scintillation counting of 50 µl aliquots from each sample.

A separation of FeLV-ABC/KT on a Sepharose CL 6B column is shown in Fig. 19a. This profile was compared to the SDS PAGE method by pooling peak samples A, B, C and D, dialysing them against dilute buffer (Tris-HCl pH 6.8, 0.001 M, 2% β-mercaptoethanol), concentrating with Carbowax, and electrophoresing aliquots on polyacrylamide gels (Laemmli). This is shown in Fig. 19b. This separation was inferior to subsequent runs using

FIGURE 19

Comparison of 6M GuHCl gel filtration and SDS PAGE separation of FeLV proteins

Figure 19a shows separation of 10 mg of FeLV-ABC/KT proteins (c.10^5 cpm ^{3}H-leucine) on a Sepharose CL6B, 0.01 M. DTT and 0.02M sodium phosphate pH 6.8. Protein-containing fractions were detected by scintillation counting of 50 µl samples.

Figure 19b shows the analysis of pooled and concentrated fractions from Fig. 19a on SDS PAGE. The samples were separated on an 11% gel, and proteins were visualised by Coomassie blue staining. V denotes whole virus (50 µg and 25 µg of FeLV-ABC/KT protein). A, B, C and D correspond to the four peak fractions in Fig. 19a.

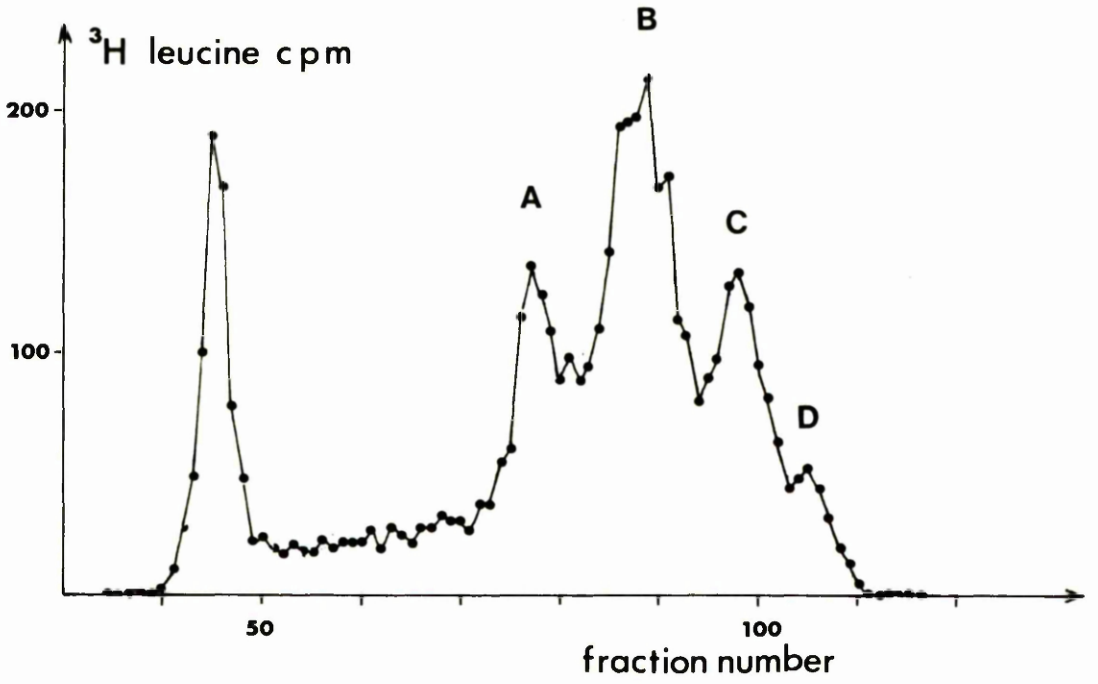


FIGURE 19a

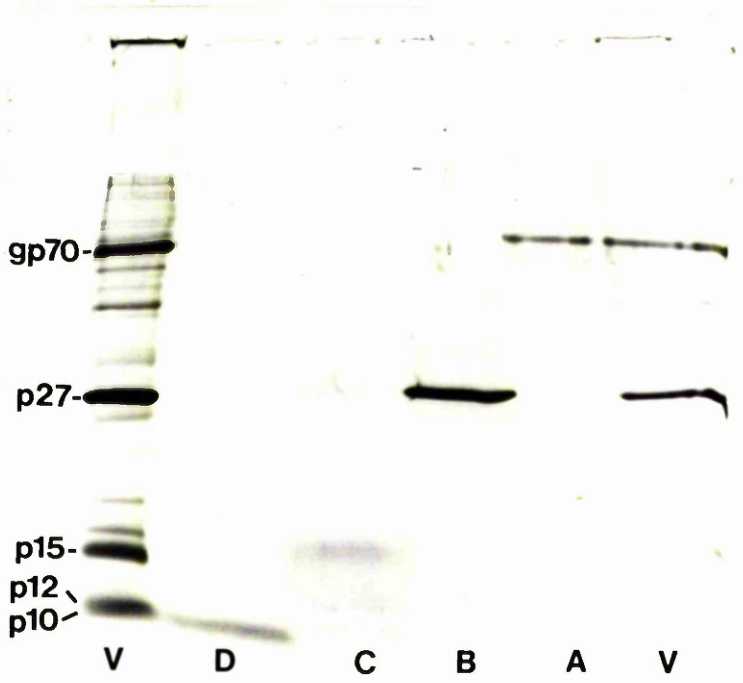


FIGURE 19b

Sepharose 6B columns which had a more suitable elution profile (Fig. 20a). Low molecular weight polypeptides p10 and p12 were not resolved, however, and only p27 and p15 could be obtained in a sufficiently homogenous state for immunisation. Fractions containing p27 and p15 were concentrated and recycled on Sepharose 6B (Fig. 20b and 20c) before final dialysis into 0.02 M sodium phosphate buffer pH 6.8 and reconcentration to a suitable volume for rabbit inoculation.

Electophoretic analysis of the "void volume" fraction from another separation is shown in Fig. 21. This consisted of high molecular weight material with a trace of p27. This is discussed in relation to the possible existence of a FeLV p15(E) in Chapter Eight.

3. Glycoprotein purification for immunisation

GuHCl gel filtration yielded gp70 fractions of insufficient purity for immunisation. The purification regime chosen for this purpose involved lectin affinity chromatography (with ricin RCA_1 lectin; see Chapter Six) followed by molecular size separation on SDS polyacrylamide gels.

Two litres of culture fluid harvested from cells infected with both FeLV-A/Glasgow-1 and FeLV-C/Sarma were concentrated by ammonium sulphate precipitation and sucrose density gradient centrifugation to yield 1.5 mg and 1.4 mg of virus proteins respectively. These samples were dissolved in 1% DOC and fractions binding to RCA_1 lectin were collected and lyophilised as described in Chapter Six. These fractions were solubilised and electrophoresed on SDS polyacrylamide gels (Laemmli) which were cast using a mould which produced a single sample application well 80 millimetres long. After electrophoresis a sample strip ($\sim$ 2 mm) was cut along each gel and stained with Coomassie blue R. Staining material in the region of 80,000 daltons for FeLV-A and 69,000 daltons for FeLV-C was located and aligned with the unstained glycoprotein bands. These were minced with fine scissors and liquefied by extrusion through a syringe before preparing for immunisation.

FIGURE 20

Purification of FeLV p27 and p15 by gel filtration in 6M GuHCl

Figure 20a shows a separation of FeLV-ABC/KT (20 mg virus protein) on a Sepharose 6B column (95 x 1.6 cm). The buffer contained 6M guanidine hydrochloride, 0.01M dithiothreitol and 0.02M sodium phosphate pH 6.8. Protein-containing fractions were detected by scintillation counting of 50 µl samples of the fractions, since ^{3}H-leucine labelled tracer virus was added to the virus sample.

Figure 20b and 20c show separation of the p27 and p15 peaks after dialysis and concentration. These recycled preparations were used for immunisation of rabbits.

FIGURE 20a

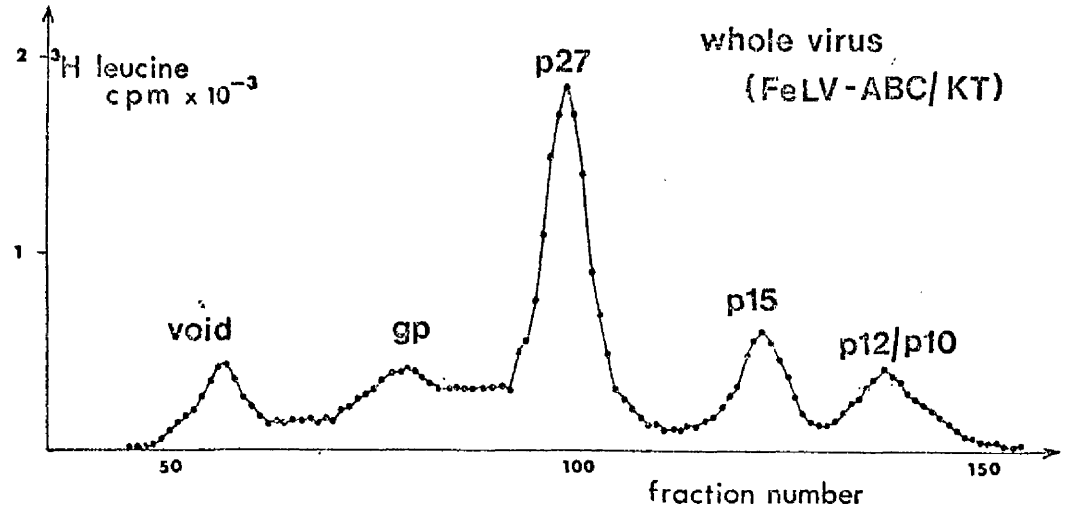


FIGURE 20b

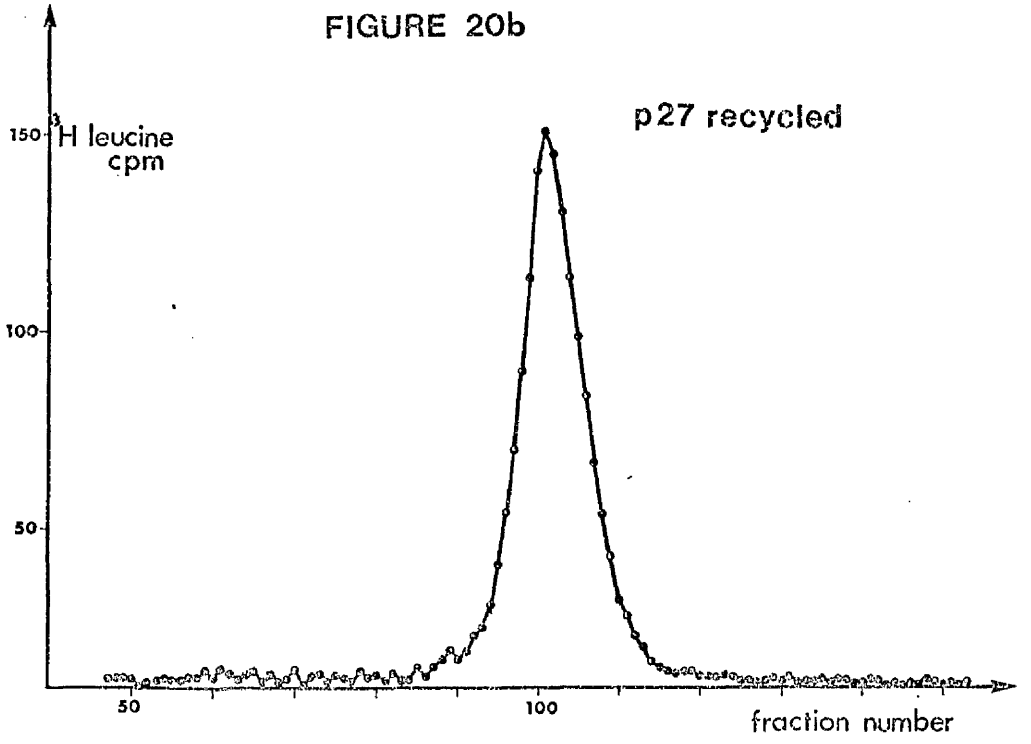
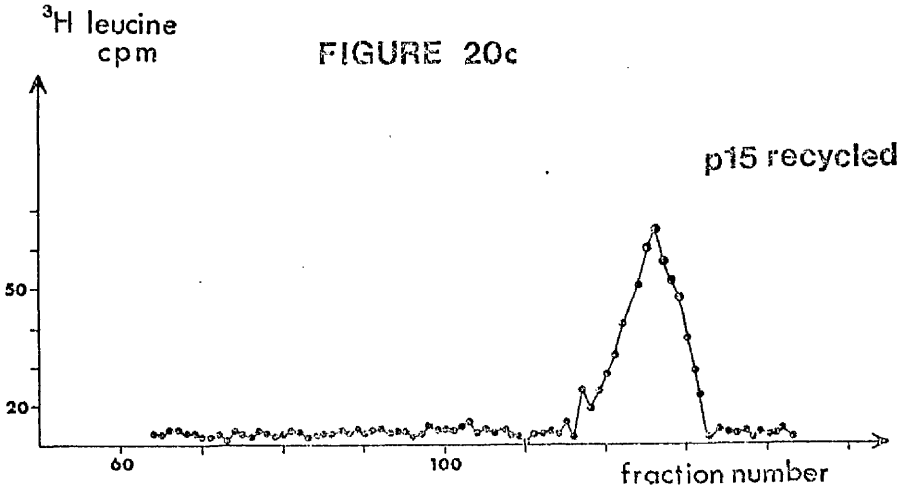


FIGURE 20c



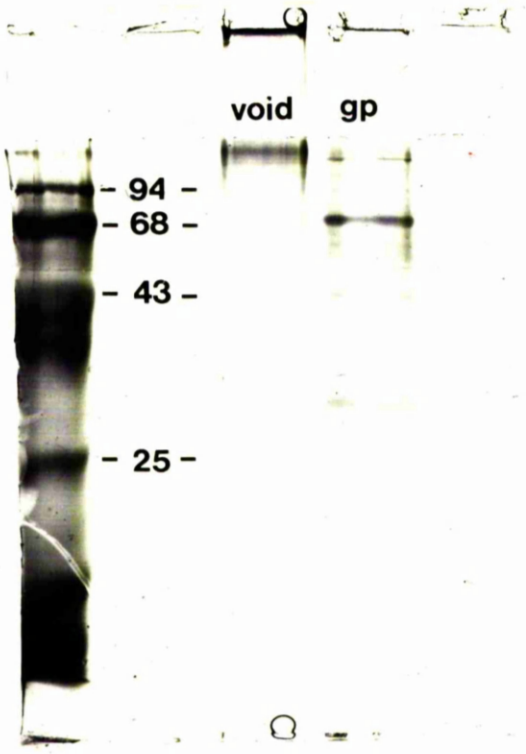


FIGURE 21

Analysis of 'void volume' fraction of FeLV on 6M GuHCl gel filtration

Pooled, dialysed and concentrated fractions from the 6M GuHCl-Sepharose CL 6B column (see Fig. 19) were analysed on an 11% acrylamide gel. Proteins were visualised by staining with Coomassie blue. "Void" and "gp" refer to the void volume and gp70-containing peaks from the column respectively. The standard protein markers were phosphorylase a (94,000), bovine serum albumin (68,000), ovalbumin (43,000) and chymotrypsinogen (25,700).

IMMUNISATION OF RABBITS WITH FeLV PROTEINS

Protein samples in buffer (p27, p15) or buffer/acrylamide suspensions (gp70 FeLV-A, FeLV-C) were added to an equal volume of Freund's Complete Adjuvant (Miles Laboratories Ltd) and mixed until a stable suspension formed. Half of each inoculum was administered in a primary immunisation, with a "booster" injection given 28 days later. At inoculation, half of the material (usually $\sim$ 0.5 ml) was injected intramuscularly into each hind leg of the animal. Estimates of the total protein adminstered at each inoculation were made by comparing the residual content of radioisotope to the original virus sample on which a protein estimation had also been made. Thus, each rabbit received 100 µg p27, 50 µg p15 or 50 µg gp70 at each inoculation. The rabbits were young adults, either New Zealand White (A662, A656) or Dutch black and white (A701, A626, gpA, gpC). They were bled for antiserum from the ear vein, at 7 and 14 days after the booster inoculation.

PROPERTIES OF ANTISERA TO FeLV PROTEINS

1. Ouchterlony gel diffusion

Rabbit antisera to FeLV p27 and p15 gave strong precipitin lines in Ouchterlony gel diffusion tests, which were done in Hyland pattern - C immunodiffusion plates (Travenol Laboratories Ltd). Figure 22 shows the reaction of an anti-p27 serum (A701) and an anti-p15 serum (A626) with purified FeLV-ABC/KT (50 µg/ml protein) which was disrupted with 0.5% NP40 in PBS. The two antisera showed no detectable cross-reactivity. The plate shown in Fig. 22 was photographed 24 hours after adding the reagents, when only one precipitin line was seen between the virus well and each antiserum well. Further incubation (to 5 days) resolved the p15 - FeLV precipitate into two closely apposed lines.

The two anti-FeLV glycoprotein sera showed no reactivity in this test.

2. Immunoprecipitation and SDS-polyacrylamide gel electrophoresis.

2a. FeLV-A/Glasgow-1. FEA cells infected with FeLV-A/Glasgow-1 were radiolabelled with ^{3}H-leucine at 10 µCi/ml for 24 hours. The culture fluid was harvested and clarified and virus was purified by sucrose density gradient centrifugation as described. The purified virus was solubilised with 0.5% NP40 and 0.5% DOC in TS buffer. Aliquots containing 5 x 10^{4} cpm of ^{3}H leucine were reacted with 20 µl volumes of various antisera as detailed in Table 7. The total reaction volume was one millilitre. After overnight incubation at 4^{o}C, the immune complexes were collected by incubation for a further hour with 50 µl of fixed Staphylococcus aureus suspension. The bacteria were washed three times with cold TS buffer before removing and dissociating the antigen-antibody complexes by addition of electrophoresis sample buffer (Laemmli). Electrophoresis was performed as described, on a 10% polyacrylamide gel.

The results are shown in Fig. 23. The control fraction with no serum (lane K) showed clear precipitation of FeLV p27, and some traces of other proteins seen in purified virus (lane F). Also, the specific pathogen free cat serum showed precipitation of a number of the resolved virion proteins with the possible exception of p15. The anti-p27 (lane G) and anti-p15 (lane E) sera gave greatly enhanced yield of their homologous antigens when compared to the other antisera. Two cat sera (lanes H and I) were used in experiments described in

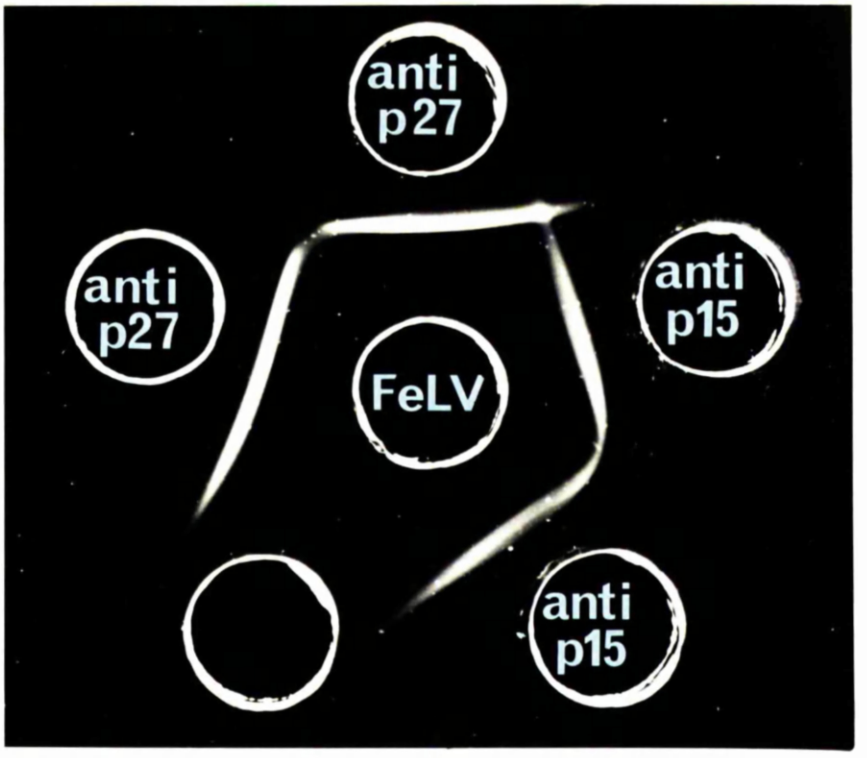


FIGURE 22

Reactivities of antisera to FeLV-ABC/KT proteins in immunodiffusion

Antisera to p27 (A 701) and p15 (A 626) from FeLV-ABC/KT were tested against purified, detergent-disrupted FeLV-ABC/KT. 5 µl of antiserum was applied to each well. The plate was incubated at $4^{o}C$ for 24 hrs before this photograph was taken.

TABLE 7

Sera used for immune precipitation studies with purified FeLV.

Serum	Species	Reactivity/specificity
A 701	Rabbit	Raised to 6M GuHCl purified FeLV p27.
A 656	Rabbit	Raised to 6M GuHCl purified FeLV p27.
A 626	Rabbit	Raised to 6M GuHCl purified FeLV p15.
A 662	Rabbit	Raised to 6M GuHCl purified FeLV p15.
gpA	Rabbit	Raised to SDS PAGE purified FeLV-A gp70.
gpC	Rabbit	Raised to SDS PAGE purified FeLV-C gp70.
a-BNS	Rabbit	Raised to whole bovine serum.
a-FEA	Rabbit	Raised to whole feline embryo cells (FEA).
831	Cat	Normal cat
SPF	Cat	Normal cat. Specific pathogen-free.
Q71	Cat	Neutralises FeLV (titres : 1/64 FeLV-A. 1/16 FeLV-C). Infected by contact with FeLV-excreting cat. Immune.
854	Cat	Neutralises FeLV (FeLV-A and C; not titrated). Vaccinated with live FL74 cells. Challenged with FeLV-A. Immune.
C10	Cat	Viraemic with FeLV-A. Indirect membrane immunofluorescence titre 1/256. Experimental infection.
V115	Cat	History unknown. No FeLV neutralising activity. Indirect membrane immunofluorescence titre 1/128.
a-gp70	Goat	Raised to purified FeLV gp70. The gift of Dr. M. Strand, Albert Einstein Institute, New York.

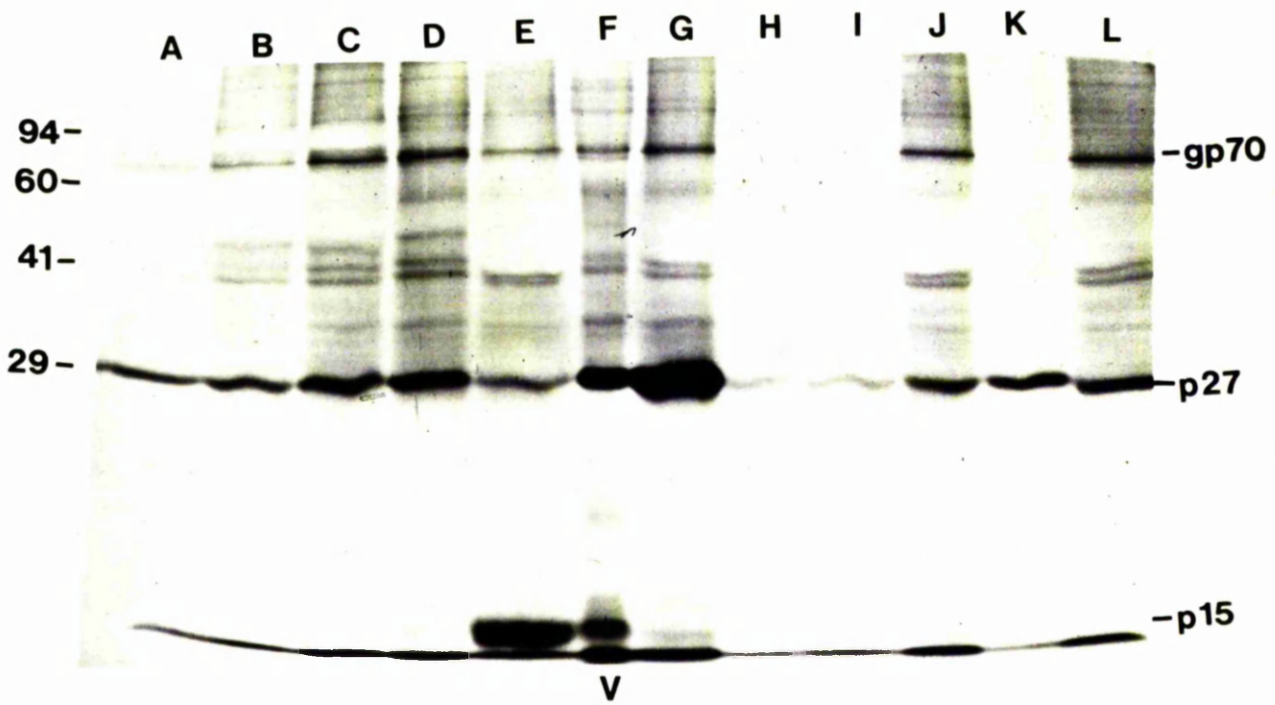


FIGURE 23

Reactivities of antisera to FeLV-A/Glasgow-1 proteins analysed by immune precipitation and SDS PAGE

Immune precipitates from FeLV-A/Glasgow-1 which bound to fixed S. aureus cells were analysed on a 10% acrylamide gel. Proteins, which were labelled with ^{3}H leucine were visualised by fluorography. Details of antisera are listed in Table 7.

Lane A SPF serum precipitate.

Lane B 854 serum precipitate.

Lane C Q71 serum precipitate.

Lane D Goat anti gp70 serum precipitate.

Lane E A 626 serum precipitate.

Lane F Control virus (25,000 cpm FeLV-A/Glasgow-1).

Lane G A 701 serum precipitate.

Lane H C10 serum precipitate.

Lane I V115 serum precipitate.

Lane J gpA serum precipitate.

Lane K No antiserum. Bacteria only.

Lane L gpC serum precipitate.

Chapter Seven to identify the feline oncornavirus-associated cell membrane antigen showed even less precipitated material than "negative" control sera. Two cat sera which had neutralising activity for FeLV showed precipitation of an additional band at 75,000 - 80,000 M_r which is presumably the FeLV gp70. However, this band almost comigrated with the non-glycosylated 74,000 M_r species which is described in Chapter Four. For this reason, it was not clear whether the goat anti-FeLV gp70 serum (lane D) also precipitated this species. In addition, the apparently specific precipitation of a molecule of approximately 50,000 daltons in lanes B, C and D may have been masked in other samples by the presence of a considerable excess of IgG heavy chain which is of lower molecular weight in the rabbit sera than in the cat sera. There was no clear difference between the serum precipitates from the rabbits which had been inoculated with FeLV-A gp70 (lane J) and FeLV-C gp70 (lane L). However, neither showed precipitation of the band which was tentatively identified as gp70 from its appearance in lanes B and C.

2b. FeLV-ABC/KT (FL74). This immune precipitation experiment was carried out exactly as before, except that fifty micrograms of purified bovine serum albumin (Sigma) was added to each reaction mixture in an attempt to prevent the artefactual precipitation seen in the first experiment. Also, a rabbit antiserum to normal bovine serum was included to discover whether the presence of large antigen-antibody complexes enhanced these artefacts. Each reaction mixture contained 10^5 cpm of ^{3}H-leucine labelled FeLV-ABC/KT.

Fig. 24 shows the immune precipitates separated on a 12% polyacrylamide gel. Again, the rabbit anti-p27 sera (A701 lane j; A656 lane h) and anti-p15 sera (A626, lane g; A662, lane precipitated large quantities of their homologous antigens. However, the same pattern of precipitation of other molecules ($\sim$80,000, $\sim$ 70,000, $\sim$40-50,000 and 17,000 daltons) as with control sera (lanes b and c) was seen. The rabbit anti-bovine serum showed only a very faint background of these molecules (lane e). The cat sera with FeLV neutralising activity (lanes d and f) showed additional precipitated bands at 130,000, 82,000 and 17,000 daltons. However, the control cat serum (lane f) also showed these bands. The band at $\sim$ 24,000 daltons visible in lanes b, d, e and f was probably FeLV p27 whose migration was affected by the presence of immunoglobulin light chain (molecular weight 25,000) on the gel. The presumed p27 band in lane b could be seen to "tail" up to the viral p27 band in lane a.

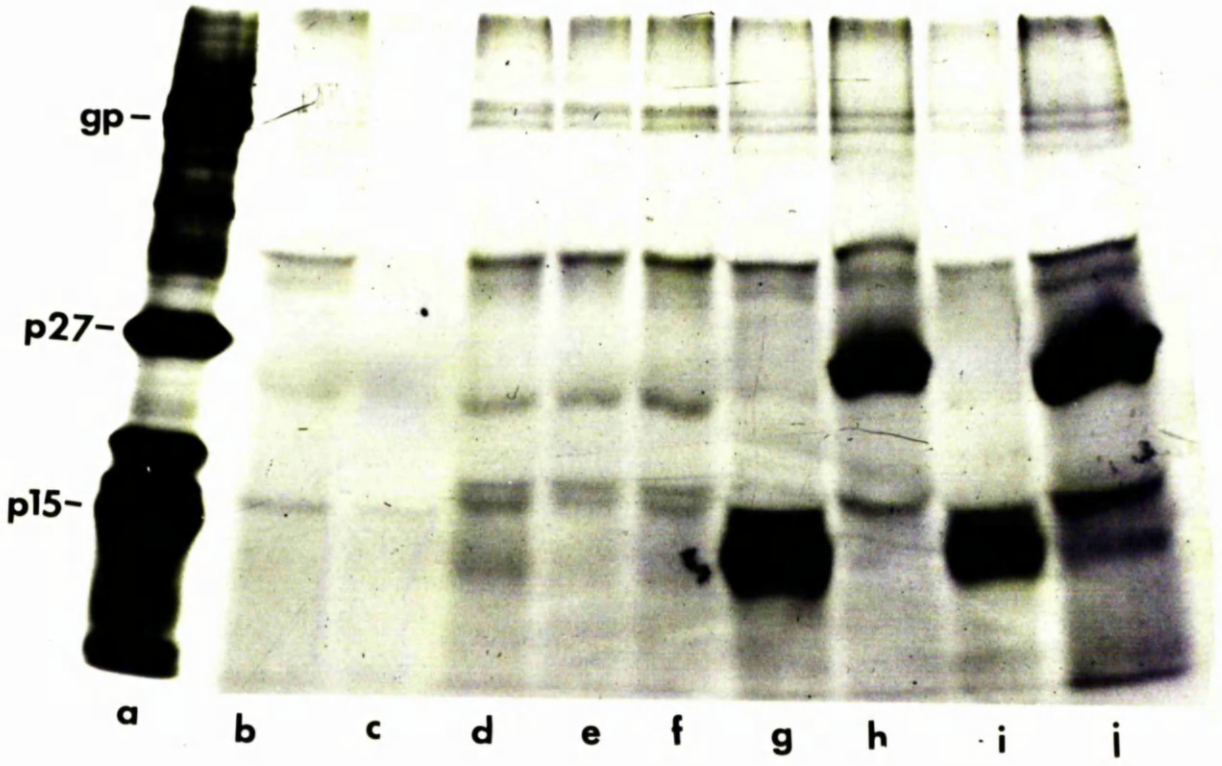


FIGURE 24

Reactivities of antisera to FeLV-ABC/KT proteins analysed by immune precipitation and SDS PAGE

Immune precipitates from FeLV-ABC/KT which bound to fixed S. aureus cells were analysed on a 12% polyacrylamide gel. Proteins, which were labelled with ^{3}H leucine, were visualised by fluorography. Details of antisera are given in Table 7.

Lane a Control virus (50,000 cpm FeLV-ABC/KT).
Lane b Anti FEA serum precipitate.
Lane c Anti BNS serum precipitate.
Lane d 854 serum precipitate.
Lane e 831 serum precipitate.
Lane f Q71 serum precipitate.
Lane g A 626 serum precipitate.
Lane h A 656 serum precipitate.
Lane i A 662 serum precipitate.
Lane j A 701 serum precipitate.

2c. Whole cell extract. The reactivities of the antisera to p27 and p15 were tested against detergent lysates of FL74 cells. The experimental procedures and results are presented and discussed in Chapter Seven. Briefly, the antisera precipitated their homologous antigens and a series of higher molecular bands which are presumably gag-precursor polypeptides. Minor bands unique to each serum were also seen.

DISCUSSION

The guanidine hydrochloride gel filtration technique proved to be very useful for separating the low molecular weight FeLV proteins, p27 and p15. The correspondence of the GuHCl elution order and migration order on SDS polyacrylamide gels was as reported by other workers (Green et al., 1973; Green and Bolognesi, 1974; Graves and Velicer, 1974). However, p10 and p12 were not resolved in this study. Graves and Velicer (1974) reported that FeLV p12 had a molecular weight of 11,000 estimated by the GuHCl technique, and could be resolved from p10 on 8% agarose gel columns (Bio-gel 1.5M). The agarose content of Sepharose 6B used in this work was 6%. However, even with 8% agarose gels, it would not be expected that p10 and p12 could be obtained entirely free from cross-contamination. In Graves and Velicer's study, p10 did not successfully immunise rabbits as detected by immunodiffusion tests. Thus, their anti-p12 serum may have appeared to be monospecific due to the low immunogenicity of p10 and the insensitivity of the immunodiffusion test.

The gp70 fraction from the agarose column was heavily contaminated with other components. In view of recent data (Jamjoom et al., 1975; Okasinski and Velicer, 1976) this fraction might have been expected to contain gag-precursor molecules in addition to normal cell proteins. Thus, any antiserum raised to this fraction would have multiple reactivities. Also, renaturation from 6M guanidine hydrochloride might be less efficient for the glycoproteins which have sugar side-chains which may prevent correct refolding of the peptide backbone. Regaining native conformation would also be less likely if this is dependent on a number of disulphide cross-links, which are reduced by the presence of dithiothreitol in the column buffer. Rauscher MuLV gp70 has an estimated 19 cysteine amino acids, suggesting a large number of such cross-links (Marquadt et al., 1977). The method used to purify gp70 in this study was clearly not suitable since immunogenicity appeared to be lost in the process. The rabbit antisera to SDS-PAGE purified FeLV-A and FeLV-C gp70 did not neutralise the virus (P.H. Russell, personal communication) and did not appear to precipitate gp70. Again, SDS-denaturation and reduction of disulphide bonds may have been irreversible. Denatured proteins have been found to be much less, if at all, immunogenic (Crumpton, 1973). A suggested explanation is the high strength of binding needed to induce B-cell differentiation and the "molecular rigidity" which this requires. Suitable purification regimes for p10, p12 and

gp70 are discussed later.

Results presented in Figs. 23 and 24 failed to resolve the question raised in Chapters Two and Four, as to whether the non-lectin-binding virion components of 44,000 and 74,000 daltons are trapped cell proteins or virus-coded molecules such as gag-precursors. The problem encountered here was the non-specific trapping of proteins of these molecular weights in precipitates from virus lysates incubated with control sera. FeLV p27 also appeared, in varying amounts, in every precipitate, suggesting non-specific trapping. However, the binding of this antigen to its homologous antisera did give much greater yields of p27 in the immune precipitates. Other workers have encountered similar difficulties in purifying RNA tumour virus proteins and establishing the specificity of antisera to them. Eisenman and Vogt (1978) have recently stated: "Oncoviruses are notorious for their ability to retain host cell nucleic acids and proteins as contaminants ..... In addition, it appears that the detergent lysis and incubation conditions (usually non-ionic detergent, 37^{o} C) often used in immune precipitation experiments, may favour the polymerisation and co-precipitation of other host cell proteins such as actin and a polypeptide that co-migrates with myosin". Future modifications which would help reduce this "background" precipitation might be : (1) Short incubation times for immune precipitation e.g. 15 minutes at 0^{o}C. This might reduce sensitivity but should improve the apparent specificity. (2) Minimising the amount of antiserum added e.g. 1-2 µl instead of 20 µl. (3) Addition of a large excess of unlabelled disrupted cell protein, although if not coupled with low temperatures and short incubation it is possible that this could aggravate the problems. Also, any antigen which cross-reacted with normal cell proteins, which could include endogenous oncornavirus products, might be undetected. If these modifications still do not allow the identification of the 44,000 and 74,000 dalton proteins, then analysis of tryptic peptide digests would seem to be the method of choice.

CHAPTER SIX

GLYCOPROTEINS OF FeLV-INFECTED CELL MEMBRANES

INTRODUCTION

Interactions between cell surfaces seem to be of prime importance in cell recognition and differentiation. The major histocompatibility gene complex, for instance, is crucially involved in most, if not all, cell-mediated immune functions (Bodmer, 1972). Histocompatibility gene products have been characterised as transmembrane glycoproteins (Walsh and Crumpton, 1977). Recently, it has been suggested that non-glycosylated membrane proteins are exposed only on the inner surface, and that all membrane carbohydrate is associated with the external surface, whether complexed to lipid or protein (Rothman and Lenard, 1977). This has been found to be true of virus glycoproteins as well as normal cell antigens.

The aim of this work was to examine the effect of FeLV infection on the composition of host cell membranes, with particular reference to the glycoproteins. The study involved no immunological selection, and was intended to be followed by immunoprecipitation studies. Thus, only gross changes in membrane composition were measured, selecting glycoprotein fractions by affinity chromatography with immobilised plant lectins.

MATERIALS AND METHODS

Cells.

FEA, FL74 and CT45S cells were grown as described in Chapter One. When the fibroblasts had reached about 70% confluence, the medium was removed and replaced with 5 ml of fresh medium containing leucine at 10% of its normal level, 10% FBS, and a total of 5 mCi of ^{3}H-leucine for 24 hours. Usually, a total of 10 plates (9 cm) was used for each experiment. Suspension cultures were labelled in complete medium at 25 μCi ^{3}H leucine per ml of medium (total of 5 mCi) for 24 hours.

Membrane preparation

Medium was poured from the plates and cells were washed twice with cold PBS before harvesting by scraping into cold PBS. Suspension cultures were washed in a similar manner and were harvested by low speed centrifugation (1000 x g, 5 min). The pellets were resuspended in 10^{-3}M $ZnCl_2$ and stirred at room temperature for 10 minutes. The cells were disrupted by equilibration for 10 minutes at 800 psi in a cold nitrogen pressure homogeniser (Atizan) followed by a sudden return of the cell suspension to atmospheric pressure. Large membrane fragments and nuclei were pelleted at 2,500 x g for 10 min at 4^{o}C. The pellet was resuspended in the chilled aqueous two phase polymer system (polyethylene glycol 6000 - dextran T-500) described by Brunette and Till (1971). Phase separation was achieved by centrifugation at 2500 - 4000 x g for 20 min at 4^{o}C in a swinging bucket rotor (Beckman JS-13). Nuclei and some of the more dense membranes pelleted while the less dense membranes floated to the interface between the two phases. The two phases (including the interface) were remixed and recentrifuged twice to remove any nuclei or more dense membranes trapped at the interface. The interface band was removed and diluted 5 - 10 fold with cold H_2O and pelleted at 10,000 x g for 10 min. The pellet of "plasma membranes" was resuspended in 5 ml of 2% sodium deoxycholate (DOC) and frozen at -70^{o}C.

Affinity Chromatography

Plant lectins were isolated and covalently attached to cyanogen bromide activated Sepharose 4B as described by Hayman and Crumpton (1972). The lectins were from *Lens culinaris* (LcH) specific for α-D-mannopyranosyl-like

carbohydrate residues, and from Ricinus communis (RCA_I), specific for β-D-galactopyranosyl-like residues. Lectin density was 1 mg/ml of Sepharose 4B.

The 2% DOC-plasma membrane suspension was thawed, diluted with an equal volume of cold H_2O, and then sonicated for 30 seconds with a Branson Microtip Sonicator, power setting 4. The solution was then centrifuged at 100,000 x g for 1 hour at 4°C and the resulting supernatant, containing the "soluble" plasma membrane components was applied to the appropriate affinity column (25 ml of settled beads in a 2.5 cm diameter column). The sample was eluted from the column until the absorbance at 280 nm of the eluate returned to base line. The specifically bound glycoproteins were then eluted with 0.3M - methyl-D-mannose (LcH) or 0.3M D-galactose (RCA) in 1% DOC. The specifically eluted peaks were then dialyzed overnight at 4°C against at least 20 volumes of 1% DOC. All samples were frozen at -20°C and then lyophilized. The dry residue was extracted with absolute ethanol at -20°C for at least 24 hours. The precipitated proteins were pelleted at 14,000 x g for 20 min and washed once with cold absolute ethanol. The final pellet was fully dried under vacuum before dissolving in the sample buffer for SDS polyacrylamide slab gel electrophoresis. After chromatography of each sample the columns were washed with about 2 volumes of H_2O and then with 1 volume of 80% saturated ammonium sulphate. Immediately prior to each new chromatographic run the column was washed with 2 volumes of H_2O, followed by approximately 1.5 volumes of 1% DOC before application of the sample.

Fig. 25 shows a SDS PAGE analysis of the lectin affinity fractionated components of uninfected FEA fibroblasts. The glycoproteins at 180,000, 160,000 and 145,000 M_r bind well to both LcH and RCA_1; those at 83,000, 74,000 and 50,000 M_r appear to bind preferentially to the LcH lectin. Direct comparison of plasma membrane components of uninfected and FeLV-A/ Glasgow-1 infected FEA cells (Fig. 26) revealed essentially no differences between the proteins and glycoproteins of the normal and infected cells, with the possible exception of an approximately twofold increase (on infection) of the 83,000 dalton component, which binds only to LcH, and the almost imperceptible appearance of a new glycoprotein at 80,000 M_r, which binds well to both lectins.

In contrast to the FEA cells, FL74 cells (Fig. 27) showed an abundance of material of the same molecular weight and binding specificity as the viral glycoprotein (see Chapter Four) (i.e. 69,000 M_r) binding equally well to both LcH and RCA_1. In addition bands were visible at 71,000, 41,000, 35,000 and 30,000 M_r which bound preferentially to LcH. Other glycoproteins which bound to both lectins were observed at 210,000 and 180,000 M_r.

No non-infected control cell is available for FL74 cells, since this line, and others like it, have been established from FeLV-positive cases of feline leukaemia. However, examination of lymphoid cells from another species, CT45S canine thymocytes (Fig. 28), showed a strikingly similar glycoprotein pattern to FL74 cells. Glycoproteins which bound to both LcH and RCA_1 were observed at 94,000, 41,000 and 30,000 to 35,000 M_r. When CT45S cells were infected with FeLV-B, no detectable difference was observed in the glycoprotein pattern.

FIGURE 25

Glycoproteins of FEA cell membranes detected by lectin affinity chromatography

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled plasma membrane components of feline embryonic fibroblasts (FEA) after fractionation on LcH and RCA_I affinity columns. Monomer concentration was 10%. Marker proteins are β-galactosidase (130,000 daltons), phosphorylase-a (94,000), catalase (60,000), alcohol dehydrogenase (41,000) and carbonic anhydrase (29,000).

Lane a Original plasma membrane preparation.

Lane b Components which failed to bind to LcH.

Lane c Components which failed to bind to RCA_I.

Lane d Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane e Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane f LcH bound components.

Lane g RCA_I bound components.

Lane h Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane i Components which failed to bind to LcH and subsequently bound to RCA_I.

FIGURE 26

Comparison of cell membrane glycoproteins of uninfected and FeLV-A/Glasgow-1 infected FEA cells

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled plasma membrane components of uninfected and FeLV-A/Glasgow-1 infected FEA cells. Monomer concentration was 8%. Marker proteins are as Figure 25.

Lane a Original plasma membrane components (FEA).

Lane b Original plasma membrane components (FEA + FeLV-A).

Lane c LcH bound components (FEA).

Lane d LcH bound components (FEA + FeLV-A).

Lane e RCA_I bound components (FEA).

Lane f RCA_I bound components (FEA + FeLV-A).

Lane g Components which failed to bind to RCA_I but subsequently bound to LcH (FEA).

Lane h Components which failed to bind to RCA_I but subsequently bound to LcH (FEA + FeLV-A).

Lane i Components which failed to bind to LcH but subsequently bound to RCA_I (FEA).

Lane j Components which failed to bind to LcH but subsequently bound to RCA_I (FEA + FeLV-A).

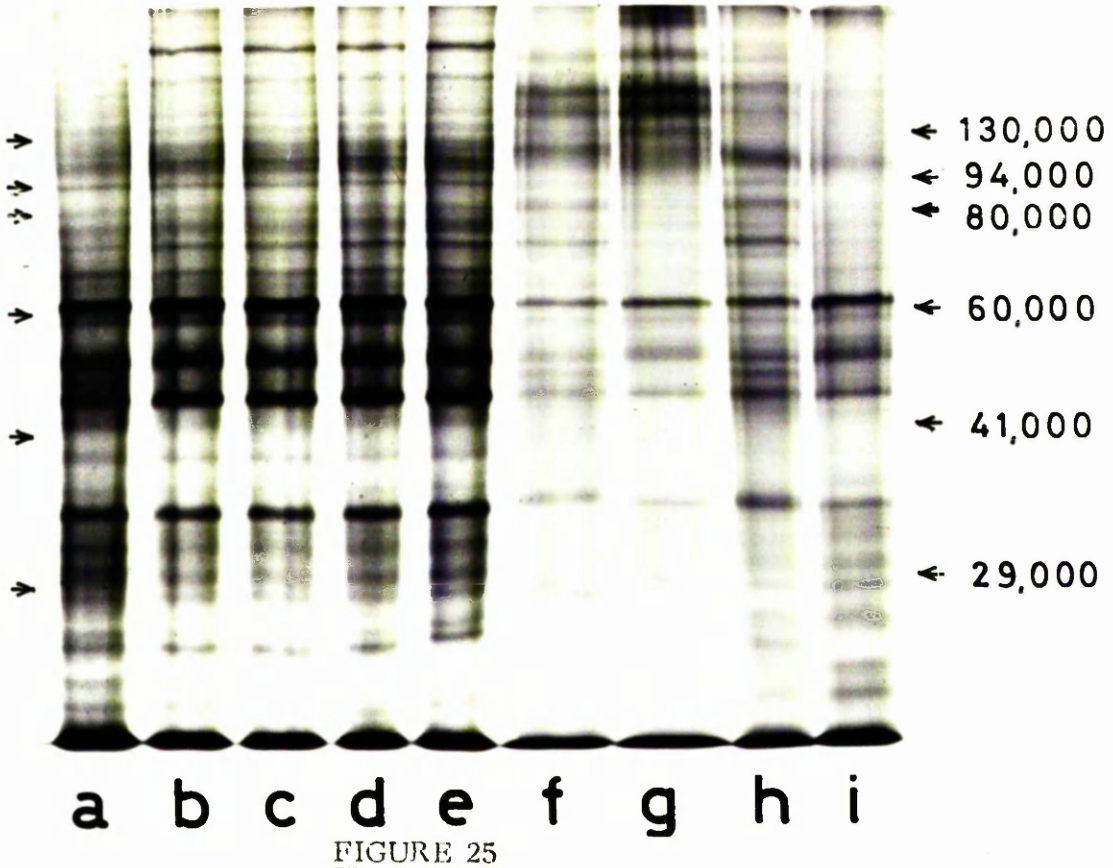


FIGURE 25

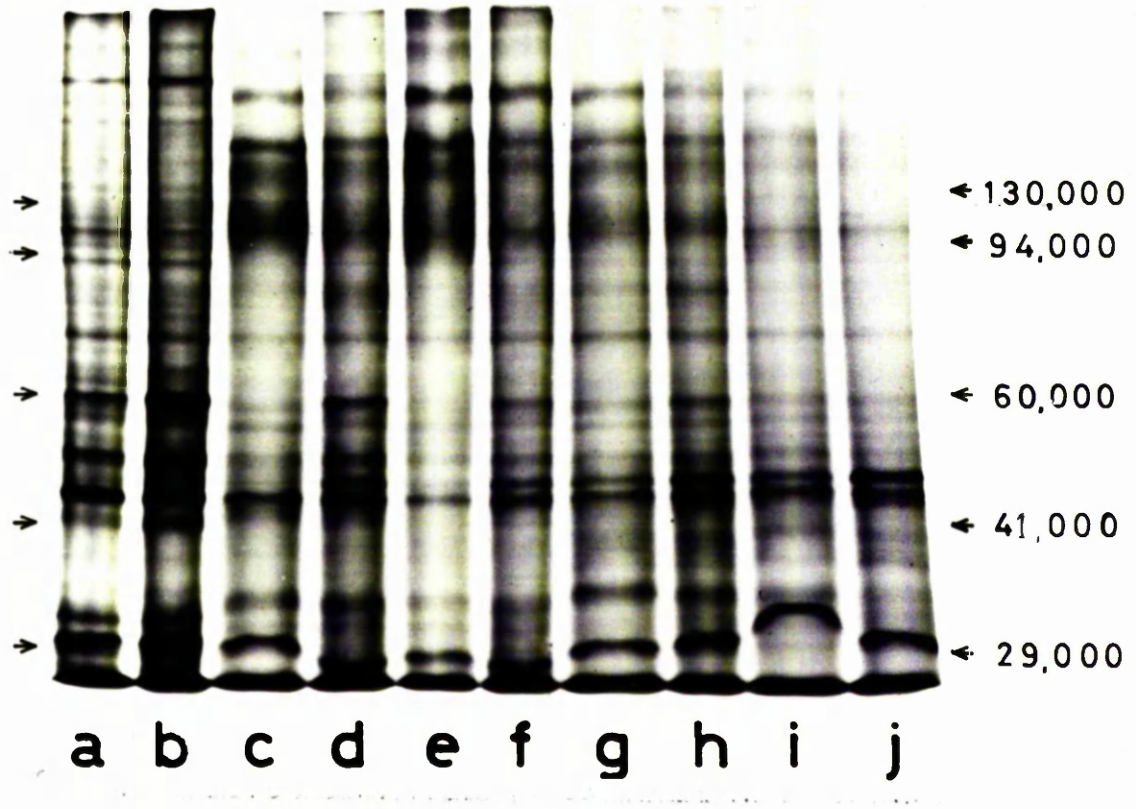


FIGURE 26

FIGURE 27

Glycoproteins of FL74 cell membranes detected by lectin affinity chromatography

Fluorograph of SDS PAGE separated ^{3}H-leucine labelled plasma membrane components of FL74 cells after fractionation on LcH and RCA_I affinity columns. Monomer concentration was 10%. Marker proteins are as in Figure 25.

Lane a Original virus preparation.

Lane b 1% DOC soluble components (100, 000 x g supernatant).

Lane c Components which failed to bind to LcH.

Lane d Components which failed to bind to RCA_I.

Lane e Components which failed to bind to RCA_I and subsequently failed to bind to LcH.

Lane f Components which failed to bind to LcH and subsequently failed to bind to RCA_I.

Lane g LcH bound components.

Lane h RCA_I bound components.

Lane i Components which failed to bind to RCA_I and subsequently bound to LcH.

Lane j Components which failed to bind to LcH and subsequently bound to RCA_I.

FIGURE 28

Glycoproteins of CT45S cell membranes detected by lectin affinity chromatography

As Figure 25, except that plasma membranes of CT45S cells were used.

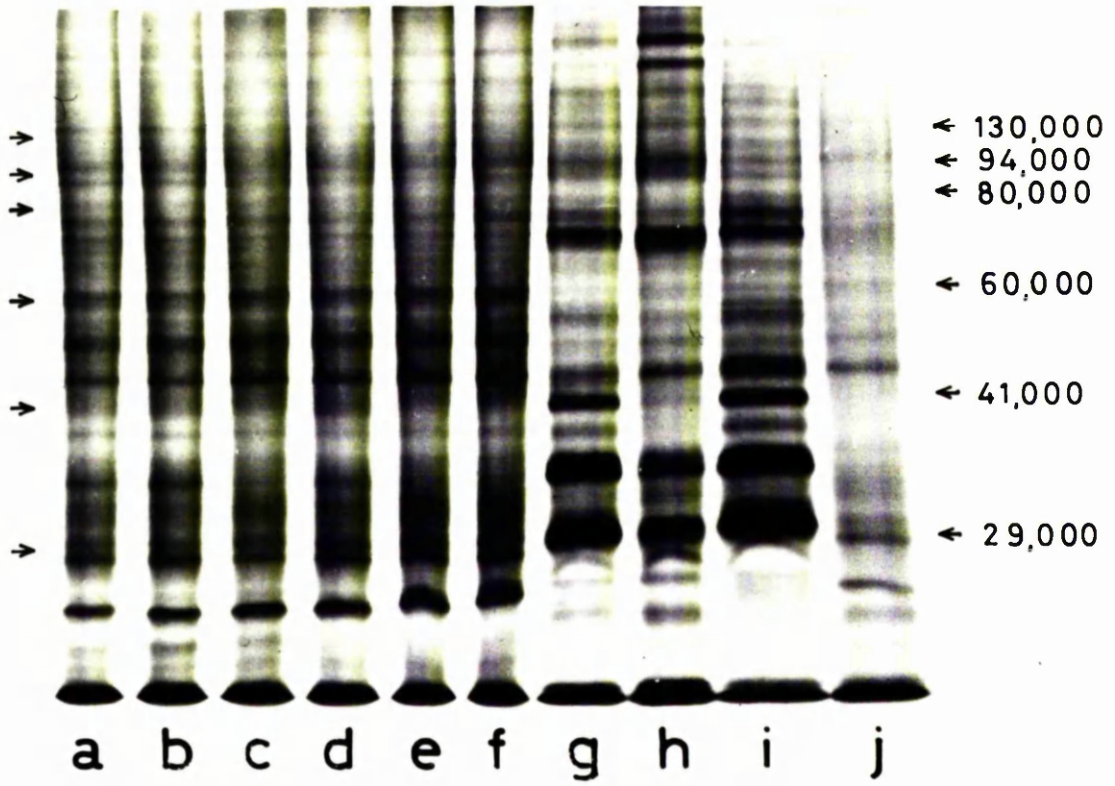


FIGURE 27

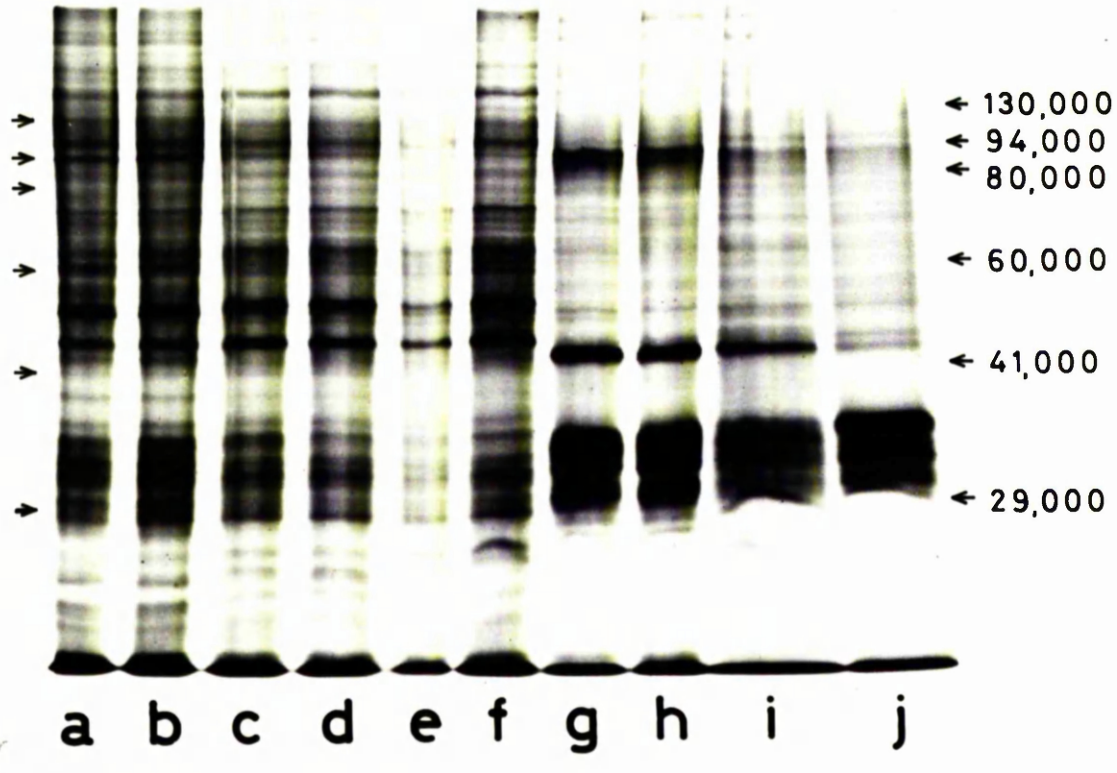


FIGURE 28

DISCUSSION

This study demonstrated a striking difference in the membrane glycoprotein composition of various differentiated cells. The 145,000/160,000 M_r doublet appears to be characteristic of feline embryonic fibroblasts, since it has been detected in virus preparations from both the FEA cells shown in Results, and from another such line, FER.

The strong, distinctive glycoprotein pattern observed with the FL74 and CT45S cell lines is a frequent feature of lymphoid cells from a range of species (J. Smart, personal communication). However, although the overall glycoprotein pattern is similar there are differences in apparent molecular weight and lectin binding specificities which distinguish the two cell lines. The glycoproteins of these cell lines are presumably differentiation antigens, analagous to the Θ (Thy-1) and Ly lymphocyte antigens of the mouse, (Boyse and Old, 1969) and other gene products expressed specifically on lymphocytes.

The FL74 cells are considered to have originated from T-lymphocytes, since they carry the characteristic erythrocyte rosetting marker, while the CT45S cells carry the EAC (erythrocyte-antibody-complement) rosette markers characteristic of B-lymphocytes, although they do not have detectable surface immunoglobulin. However, the normal canine thymus contains large numbers (80%) of cells revealing similar markers to the CT45S cells (Krakowka et al., 1977).

A possibly important difference between the FL74 and CT45S cells was the occurrence in the FL74 membranes of bands of similar apparent molecular weight to the major FeLV virion glycoprotein (see Chapter Four). Three such bands were observed; one at 69,000 M_r which bound to both lectins and two at 71,000 and 76,000 M_r which bound only to LcH. The FL74 virus major glycoprotein is a molecule of 69,000 M_r which binds to both LcH and RCA_1. On infection with FeLV-B, no change could be detected in the CT45S membrane glycoprotein composition. However, virus purified from the cells in this experiment also failed to demonstrate significant amounts of glycoprotein in the expected molecular weight range. This cast doubt on the observations recorded for FeLV-B in CT45S cells since subsequent experiments revealed normal quantities of gp70 in FeLV-B grown in FEA cells. Attempts to repeat the CT45S studies with FeLV-B and FeLV-C infection were hampered due to contamination

problems. The detection of what is assumed to be viral gp70 in FL74 membranes, while it is barely detectable in FEA cells could be explained by a quantitative difference in virus yield. FL74 cells generally release ten times as many virus particles as FEA cells, as judged by electron microscopic examination and by protein assay of purified virus preparations. Alternatively, the membrane glycoprotein species may be normal T lymphocyte antigens which show a fortuitous molecular weight similarity to FL74 virus glycoprotein. This should be resolved by comparing tryptic peptide digests of the membrane and virus glycoproteins. These experiments are in progress.

An interesting possibility is that the membrane glycoproteins are indeed "normal" differentiation antigens of feline T lymphocytes, but are also related to the virus gp70, in an analogous fashion to the G_{ix} antigen which is expressed only as a thymocyte-specific alloantigen in low leukaemia mouse strains, and is a constituent of MuLV gp70 (Obata et al., 1975; Tung et al., 1975). A hypothesis might thus be advanced to explain some of the curious observations regarding FeLV-A and FeLV-C. Neutralising antibodies to FeLV-C occur frequently in the cat population although the FeLV-C virus itself is only rarely isolated. Anti-FeLV-C antibodies also occur after experimental infection with FeLV-A, although again FeLV-C is not isolated from these cats (Russell, 1977). FeLV-C gp70 might be an endogenous gp70-like molecule coded for by the FeLV-related endogenous sequences in normal cat DNA (Okabe et al., 1976). Its expression on differentiated cells at certain stages of development might give rise to anti-C antibodies, and very rare recombination with superinfecting FeLV-A give rise to complete FeLV-C. This might be more probable in cells where FeLV-C gp70 mRNA is produced. The occurrence of anti-FeLV-C antibodies after infection with FeLV-A might be explained by the existence of a group-specific determinant shared by FeLV-A and FeLV-C. However, antibody to FeLV-C is detectable in the sera of many cats which are viraemic with FeLV-A. Such cats very rarely show any trace of anti-FeLV-A neutralising activity (Russell and Jarrett, 1978; Stephenson et al., 1977b). An alternative explanation is that FeLV-A infection breaks tolerance to an endogenous antigen (FeLV-C gp70). This is a well-known phenomenon for other antigens (Weigle, 1961). Generation of a type-specific FeLV-C gp70 antiserum would be useful to examine this hypothesis by probing the tissues of normal or FeLV-A infected cats for expression of the antigen.

CHAPTER SEVEN

FELINE ONCORNAVIRUS-ASSOCIATED CELL MEMBRANE ANTIGEN (FOCMA)

INTRODUCTION

This topic is reviewed in detail in Chapter One. FOCMA is considered to be a virus-induced non-virion antigen, immunity to which will protect cats from leukaemia (Essex et al., 1975; Hardy et al., 1977). When this study commenced no information was available on the molecular nature of FOCMA. Since antisera were available which were deemed to react with FOCMA in the absence of detectable virus neutralising or anti-virion polypeptide activity, we chose immunoprecipitation of FOCMA-bearing cell fractions as the most promising approach to characterise this antigen.

The cell line used in these studies was the FL74 cell line on which FOCMA was defined (Essex et al., 1971). As discussed in Chapter One, this is a suspension culture of lymphoblastoid cells established from a leukaemic cat. It releases FeLV of subgroups A, B and C in large quantities, although the specific infectivity of this virus is low (Jarrett , 1975).

1. Immunoprecipitation of FeLV antigens from FL74 cell lysates.

FL74 cells were grown as described in Chapter One. Two bottles (20 ml medium, 6 x 10^5 cells/ml) were grown for 24 hours. Five mCi of (4, 5 - ^{3}H) leucine was then added, split between the two bottles. After a further 24 hours the cells were harvested by low speed centrifugation, washed three times with cold PBS, then extracted at 0°C for 15 min in 5 ml buffer containing 0.02 MTris-HCl (pH 7.5), 0.05 M.NaCl, and 0.5% Nonidet P-40 (NP40). The material was centrifuged at 10,000 x g for 10 min. Sodium deoxycholate (DOC) was added to the supernatant, to 0.5%. This was incubated for a further 15 min at 0°C, and centrifuged at 100,000 x g for 1 hour. The supernatant after this step will be termed the NDC-lysate. This extraction method was as described by Rohrschneider et al.,(1975).

The NDC-lysate was made up to 7 ml with cold lysis buffer and split into 1 ml lots. These were incubated overnight at 4°C with 20 µl amounts of various sera.

The immune complexes and free antibody were collected by incubation with 40 µl of fixed Staphylococcus aureus (SaCI) suspension for 1 hour, and centrifugation (2,000 x g, 10 min). The bacteria were washed three times with cold lysis buffer before removing and dissociating the antigen-antibody complexes by addition of 100 µl of electrophoresis sample buffer (Laemmli). The bacteria were again removed by low-speed centrifugation and the supernatants were boiled for electrophoresis. Samples were separated on a 10% polyacrylamide gel (Laemmli) and radiolabelled antigens were detected by fluorography.

2. Immunoprecipitation of FeLV antigens from FL74 culture supernatant.

The efficiency of amino-acid labelling is very low in Liebovitz-15/McCoy's 5A medium due to the high concentration of unlabelled amino acids in the Leibovitz medium. In an attempt to overcome this problem the FL74 cells were grown in Eagle's medium (EFB) supplemented with 50 µg/ml L-arginine. In this medium the cells retain viability and multiply, at least for the term of pilot experiments which lasted up to two weeks. The value of this finding was that it allowed the suspension cells to be grown in the readily available-leucine-free Eagle's medium (Gibco Biocult Ltd.), which was found to increase the efficiency of virus labelling by at least twenty-fold.

Three bottles were set up each containing 20 ml Eagle's medium with 10% of the normal levels of leucine, 15% FBS, 50 µg/ml L-arginine and 8 x 10^5 FL74 cells/ml. After 24 hours of growth, 5 ml (4, 5-^{3}H) leucine (5 mCi) was added to each bottle with an additional 1 ml FBS. After a further 24 hours, the cells were spun out of the medium at 500 x g for 5 min. The medium was then clarified by centrifuging at 10,000 x g for 10 min, and virus was pelleted by centrifugation in a Beckman SW27 rotor, at 24,000 rpm for 2 hours. Virus was further purified by sucrose density gradient centrifugation and was then pelleted. The yield of virus was 33 x 10^6 cpm (^{3}H) from 15 mCi of input label, which was an increase of around twenty-fold on previous experiments. The "virus-free" medium was aliquoted and fractions of 1 ml were incubated overnight at 4°C with 20 µl volumes of various sera as shown in Table 8.

Immune complexes were harvested with fixed bacteria as in the first experiment, except that the bacteria were washed in Tris-HCl (pH 6.8) buffer with no detergent. The samples were prepared for electrophoresis as in the first experiment, separated on a 10% polyacrylamide gel, and radioactive bands were visualised by fluorography.

3. Immunoprecipitation of FL74 cell surface antigens.

Conditions were chosen which followed as closely as possible the initial steps in the immunofluorescence test for anti-FOCMA antibodies (Essex et al., 1971). One 8 ounce bottle of FL74 cells was labelled, as described in the second experiment with 3 mCi of (^{3}H) leucine. The cells were harvested and washed three times with sterile PBS at room temperature. The cells (2 x 10^7) were resuspended in 20 ml of L-15/McCoy's 5A medium with 10% FBS. Aliquots of 5 ml were incubated with 40 µl volumes of various sera as listed in Table 8.

After incubation at room temperature for 1 hour and subsequently at 0°C for a further 30 min, a threefold excess of washed, unlabelled FL74 cells was added and the mixtures were kept on ice for a further 15 min. The cells were then harvested by low speed centrifugation (500 x g, 5 min) and resuspended in 2 ml TS buffer with 0.5% NP40. After 15 min at 0°C, nuclei were pelleted by centrifugation at 2,000 x g for 10 min. The fixed bacterial suspension (100 µl) was then added to each lysate. This mixture was incubated for a further 1 hour at 4°C before washing the bacteria in TS buffer with 0.5% NP40 and finally in 0.01M Tris HCl buffer (pH 6.8) before preparing for electrophoresis as described in Experiment 1.

TABLE 8.

Sera used for immune precipitation studies with FL74 cell fractions

Serum	Species	IF titre *	VN titre *	Viraemia	Additional information
SPF	Cat	0	0	-	Specific pathogen-free.
831	Cat	0	0	-	
719	Cat	0	0	-	
Q71	Cat	8	64(A) 8(C)	-	Recovered from infection by contact with FeLV excreting cat.
Q89	Cat	8	64(A) 16(C)	-	Recovered from infection by contact with FeLV excreting cat.
854	Cat	256	32(A) 32(C)	-	Vaccinated with live FL74 cells. Challenged with FeLV-A. Immune.
V115	Cat	128	0	-	History unknown.
V150	Cat	128	0	-	History unknown.
C10	Cat	256	8(A) 0 (C)	+	Challenged FeLV-A.
NRS	Rabbit	ND$^{\nabla}$	ND	NA$^{\nabla}$	Normal rabbit serum.
A701	Rabbit	64	0	NA	Anti FeLV p27 (see Chapter Five).
A626	Rabbit	256	0	NA	Anti FeLV p15 (see Chapter Five).

* IF = indirect membrane immunofluorescence (Riggs, 1971).

VN = virus neutralisation (Russell and Jarrett, 1976).

Titres are given as reciprocal of highest dilution with positive result.

$^{\nabla}$NA = not applicable.

$^{\nabla}$ND = not done.

RESULTS

1. Immunoprecipitation of FeLV antigens from FL74 cell lysates.

The results are shown in Fig. 29. The rabbit antisera to GuHCl-purified FeLV p15 and p27 precipitated their homologous antigens and also a series of minor bands (lanes e and f). Some of these were of high molecular weight which corresponded in both precipitates (190,000, 75,000, 68,000, 56,000, 50,000 and 44,000) while others were unique to the anti p15 (< 12,000) or the anti p27 serum (175,000, 37,000, 33,000, 32,000, 15,500). The two "anti-FOCMA" sera (lanes a and b) precipitated a series of faint bands, but this pattern was identical to a control serum with no FOCMA immunofluorescence titre or virus neutralising activity (lane d). The virus neutralising serum showed no detectable precipitation.

2. Immunoprecipitation of FeLV antigens from FL74 culture supernatant.

The results are shown in Fig. 30. A background of precipitated bands at 50,000, 34,000, 31,000, 28,000, 25,000, 21,000 and 18,000 M_r was present with all sera tested, regardless of species of origin. It therefore seems probable that this precipitation was non-specific, these molecules sticking to IgG or the fixed bacteria in the absence of detergents.

No new bands were visible in the three "anti-FOCMA" sera (lanes d, g and h) compared to the controls (lanes a and b).

The most striking differences were observed with the virus neutralising antisera (lanes e and f). Both showed prominent bands at 80,000, 70,000 and 37,000 M_r and a minor band at 120,000.

3. Immunoprecipitation of FL74 cell surface antigens.

Fig. 31 shows separation of the immune precipitates on a 10% polyacrylamide gel. Radiolabelled FL74 virus was run on the same gel for direct comparison (lane a). All cell surface precipitates showed a large number of bands, with the most prominent at > 300,000, 150,000, 57,000, 43,000 and 29,000. There were quantitative and proportional differences in these bands between serum precipitates. The only detectable qualitative difference was the appearance of new bands in the virus-neutralising antiserum precipitate (lane b) at 76,000 and 85,000 M_r. These appeared to comigrate with bands from purified FeLV (lane a). The 76,000 M_r band was a major virion component,

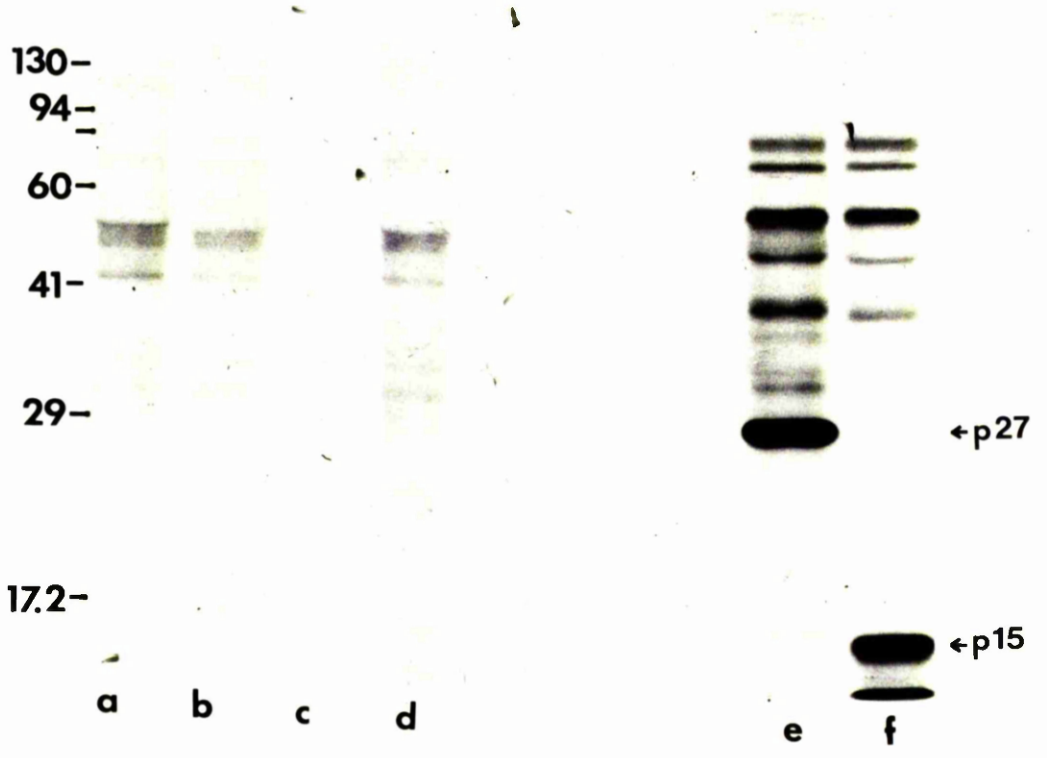


FIGURE 29

Reactivities of antisera to FL74 cell lysate proteins analysed by immune precipitation and SDS PAGE

FL74 cells, labelled with ^{3}H-leucine, were lysed with NP40 and DOC detergents. The lysates were incubated with various sera and the immune complexes which bound to fixed S. aureus cells were analysed on a 10% acrylamide gel. Proteins were visualised by fluorography. Details of sera are given in Table 8.

Lane a V115 serum precipitate.
Lane b V150 serum precipitate.
Lane c Q89 serum precipitate.
Lane d 719 serum precipitate.
Lane e A701 serum precipitate.
Lane f A626 serum precipitate.

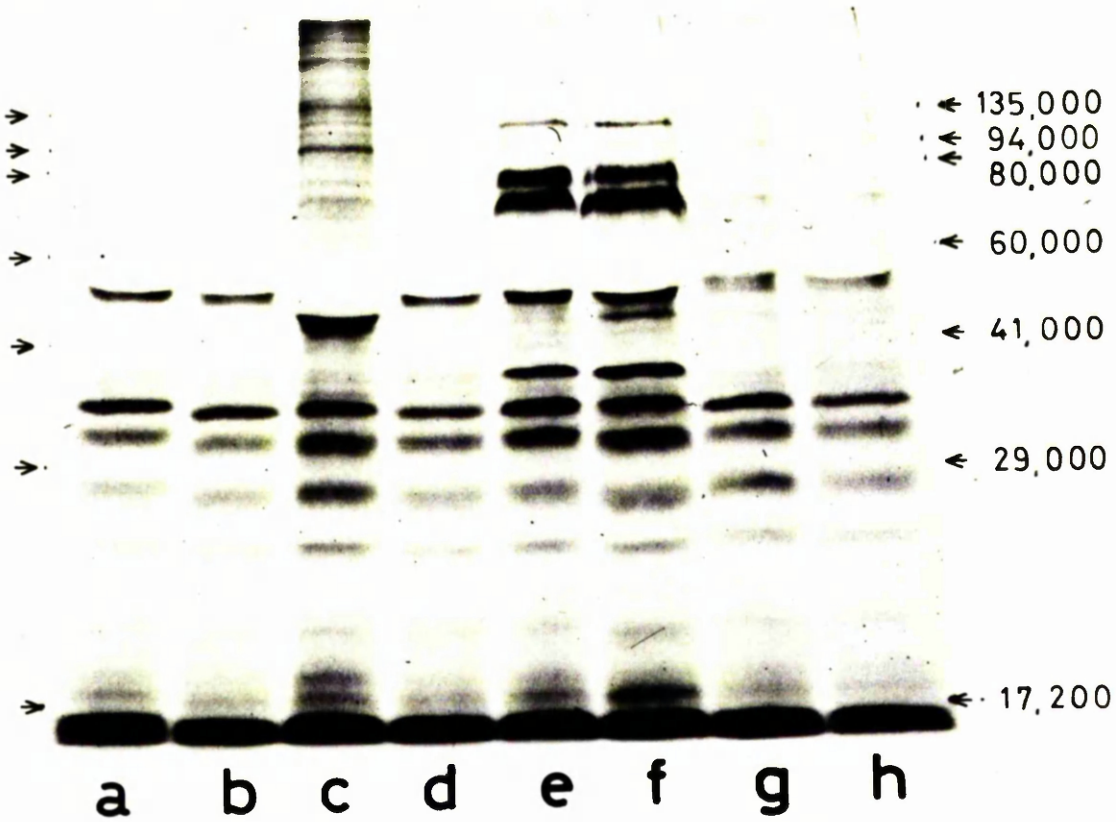


FIGURE 30

Reactivities of sera to FL74 cell culture supernatant proteins analysed by immune precipitation and SDS PAGE

Supernatant fluids from FL74 cells, which had been labelled with ^{3}H-leucine, were incubated with various antisera. Immune complexes were collected with fixed S. aureus cells and analysed on a 10% acrylamide gel. Proteins were visualised by fluorography. Details of sera are given in Table 8.

Lane a SPF serum precipitate.

Lane b 831 serum precipitate.

Lane c gpC serum precipitate. (see Table 7).

Lane d C10 serum precipitate.

Lane e Q71 serum precipitate.

Lane f 854 serum precipitate.

Lane g V115 serum precipitate.

Lane h V150 serum precipitate.

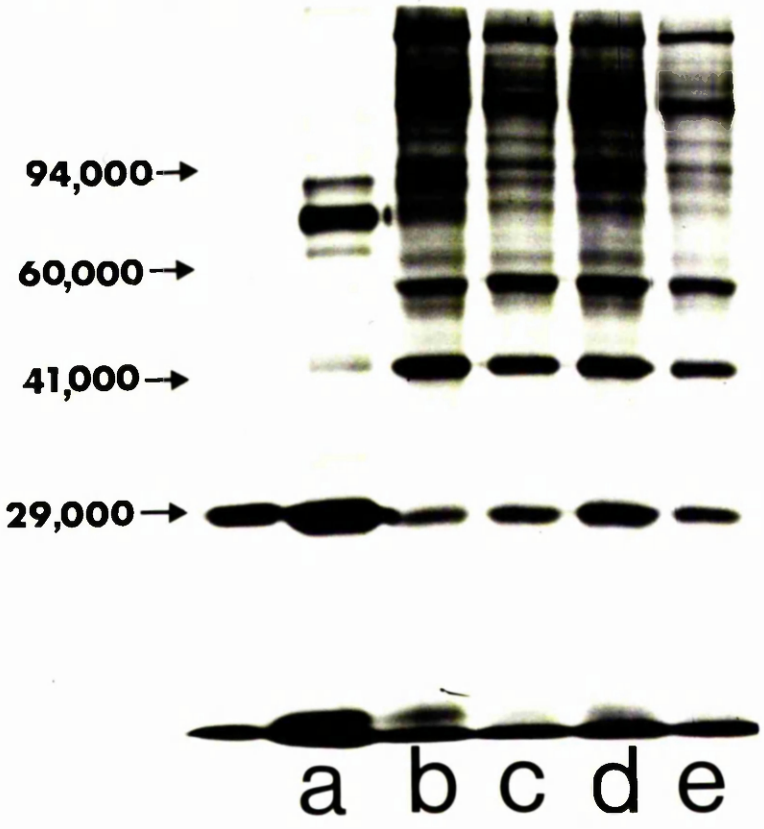


FIGURE 31

Reactivities of sera to FL74 cell surface proteins analysed by immune precipitation and SDS PAGE

FL74 cells were labelled with ^{3}H-leucine. Sera were incubated with live cells before detergent lysis and collection of immune complexes which were analysed on a 10% acrylamide gel. Proteins were visualised by fluorography. Details of sera are given in Table 8.

Lane a FeLV-ABC/KT (50,000 cpm).

Lane b Q71 serum precipitate.

Lane c C10 serum precipitate.

Lane d V115 serum precipitate.

Lane e SPF serum precipitate.

but the band at 85,000 M_r was faint. Another new band which was seen in the Q71 precipitate appeared to comigrate with FeLV p15.

DISCUSSION

The rabbit anti-p15 and anti-p27 sera precipitated their homologous antigens as well as a series of minor bands. The bands in common are presumably intracellular precursors to the gag proteins as has recently been published by others (Okasinski and Velicer, 1977). Some of the minor bands brought down by the anti-p27 serum may be minor antigens which contaminated the GuHCl purified p27 since they have molecular weights close to that of p27. It is not clear whether the faint band at 15,500 M_r was p15. It seemed to migrate slightly slower and corresponded to a band seen in the cat serum precipitates. The significance of the two high molecular weight bands is also unclear. They may represent gag-pol precursors, similar to those described by Jamjoom et al. (1975) although one would appear to lack p15 reactivity. This seems feasible since p15 may be at the N-terminal end of the gag-precursor (Khan and Stephenson, 1977). The anti-p15 serum also precipitated some material which migrated with the tracker dye. This could be p10 or p12 which contaminated the GuHCl p15 fraction (see Chapter Five).

The "anti-FOCMA" sera precipitated no additional band when compared to the control serum. The separation of gag-precursors and gag-proteins on the same gel suggested that the "FOCMA" specificity was not carried on these molecules. However it could not be ruled out that the lysis procedure failed to release the molecule, or that the "FOCMA" specificity was a detergent-sensitive conformational determinant of any of the virion proteins or precursors. The complete lack of detectable labelled antigens in the neutralising antiserum precipitate is difficult to explain. The stained gel showed the expected quantities of heavy and light antibody chains. Subsequent experiments have shown that the precipitation of gp70 by some cat sera is markedly inhibited by the presence of NP40 and DOC detergents. However, one might have expected to see the same background precipitation pattern as occurred with the other three cat sera.

In view of the failure to demonstrate any antigen which could be defined as FOCMA in NDC-lysates, it was decided to adopt other approaches to identify the antigen. Since FOCMA is a cell surface antigen, and many cell surface antigens are shed into the culture medium (see Chapter Two; and Bolognesi et al., 1975) it seemed that this might prove to be a rich source of FOCMA.

It would also be unnecessary to add detergents if the antigen were present in the culture supernatant in soluble form.

FL74 cell culture supernatants were thus analysed by immunoprecipitation. However, the "anti-FOCMA" sera again failed to precipitate any antigen which was not seen in control precipitates. The only striking difference was seen in precipitates of cat sera with FeLV-neutralising activity. Two major species were seen at 70,000 and 80,000 M_r. It seems most likely that the 70,000 species is the major virion glycoprotein. The 80,000 molecule may be analagous to the "p85" of Ruscetti and Parks (1977) which has since been reported. This is supported by the fact that a molecule with similar M_r was detected on the FL74 cell surface in the third experiment.

The identity of the other bands (120,000, 37,000 daltons) is unknown. The 37,000 band may be a minor glycoprotein or a gp70 cleavage product, but no such component was found in FL74 virions (see Chapter Five). If the precipitation could be blocked by excess purified gp70 this could resolve the question. Blocking could also be attempted with other virion proteins for both the 37,000 and the 120,000 dalton components.

The only detectable difference between the contact infected cat (lane e) and the FL74 vaccinated cat (lane f) serum precipitates was a band at 44,000 M_r. This is interesting since it is of similar molecular weight to the heavy chain of murine and human histocompatibility antigens (Henning et al., 1976). It would not be surprising if inoculation of whole cells into a non-syngeneic host raised antibodies to this class of molecules. The result with the rabbit anti-gp70 (lane c) was unexpected. No clear precipitation of gp70 was seen, but a large number of high molecular weight bands was brought down. Since this serum was not preabsorbed, this could represent merely naturally occurring rabbit antibodies which fortuitously react with the heterologous cells.

Since no novel antigen was demonstrable in immune precipitates with anti-FOCMA sera from NDC lysed cells or untreated culture supernatant, a third approach to the identification of FOCMA was made. It was considered that FOCMA may have a hydrophobic moiety which anchors it firmly in the cell membrane, explaining its absence from soluble fractions of culture supernatants. This might also explain the absence of the FOCMA specificity from NDC lysates: the hydrophobic molecule could become trapped in the micelles

which these detergents form in aqueous solutions. Alternatively, if FOCMA were a conformational antigen, interference with hydrophobic protein - protein interactions might destroy its activity. The rationale of the third experiment was that formation of the FOCMA-anti-FOCMA complex before cell lysis may stabilise conformational determinants or prevent loss of FOCMA into detergent micelles. Antibody was adsorbed to whole cells under similar conditions as in the initial stages of the indirect immunofluorescence test (Riggs, 1971) for FOCMA, and cells were lysed with the non-ionic detergent NP40.

The experiment did not characterise FOCMA, although virus neutralising antiserum was shown to react with new antigens, one of which comigrated with the major virion glycoprotein, one with FeLV p15 and another with a minor virion antigen of 85,000 molecular weight. The two high molecular weight antigens would seem likely to correspond to the two antigens of similar molecular weight which are shed into the culture medium (see Fig. 30). Ruscetti and Parks (1977) detected two components of 70,000 and 85,000 daltons by lactoperoxidase catalysed iodination of the FL74 cell surface. Precipitation of both molecules could be blocked by purified FeLV gp70.

Taking into account slight discrepancies between gels and buffer systems, it seems highly probable that this observation corresponds to the results presented here.

The identity of "p85" is unknown. Results presented in Chapter Four show that the apparent molecular weight of FeLV gp70 varies considerably from strain to strain of FeLV. It seems possible that p85 might represent glycoprotein of one subgroup while the 70,000 dalton component is from another virus. It should be added, however, that the 70,000 dalton component is present in virions in great excess over material at 85,000 daltons. Thus, one glycoprotein would have to be more efficiently incorporated into virions while both glycoproteins would be more or less equally represented on the cell surface and free in culture medium.

The demonstration of what appears to be FeLV p15 on the cell surface is interesting, since recent work with MuLV has suggested that cell surface p15 antigenic determinants are present mainly in high molecular weight gag-precursors (Ledbetter and Nowinski, 1977).

The technique of pretreatment of cells with antibody before lysis clearly requires some refinement. The high background of precipitation meant that FOCMA may have been undetected because it comigrated with one of the bands which was heavily represented in normal serum controls.

GENERAL DISCUSSION

This study was concerned with the polypeptides of feline leukaemia virus, both the structural polypeptides of the virus particle and putative non-structural proteins which appear in FeLV-infected and transformed cells.

Studies on the effect of purification methods on the protein composition and infectivity of FeLV did not show the dramatic losses reported by other workers (Witter et al., 1973; Strand and August, 1976). One possible explanation is that the virus strains used by these authors were much more labile and were sensitive to "osmotic shock" or freezing and thawing. However, a more likely explanation seems to be that these treatments were performed on highly purified and concentrated virus preparations. The presence of large quantities of serum protein and much greater dilution of virus may have protected against these deleterious effects in my studies.

Three lines of evidence have shown that gp70 is on the surface of FeLV: bromelain digestion, surface labelling with ^{125}I and fractionation of virus particles on sucrose density gradients all showed at least one molecule of approximately 70,000 molecular weight associated with the viral envelope. The p27 was defined as an internal virion component associated with the core. Although p15 did not appear to be a surface component, it could not be found in association with core fractions. It is possible that the hydrophobic, "lipophilic" nature of p15 (Green et al., 1973; Strand and August, 1976) caused it to remain in association with the lighter detergent layers in the fractionation experiments. Polypeptides p12 and p10 could not be resolved on the gel electrophoresis system used in these studies and so their subviral locationcould not be defined, although at least one of these polypeptides was associated with the viral core fractions. From recent evidence (Pal et al., 1975; Sen et al., 1977),p12 as well as p10 would be expected to be located in viral cores, in association with genomic RNA. An unresolved, but intriguing problem is the possible existence in FeLV of a molecule analogous to the p15(E) of MuLV. This is not merely an academic question since Ihle et al. (1976) have shown that naturally occurring antibodies in mice which have a broad spectrum of reactivity against MuLV strains are directed toward p15(E) which is serologically related in a range of viruses. Further more, p15(E) has been implicated as the receptor for binding of the serum complement component C1q (Bartholomew et al., 1978)which is the first step in the non-immune lysis of oncornaviruses by human sera (Welsh

et al., 1975). The present circumstantial evidence for the existence of a FeLV p15(E) is that antiserum to MuLV p15(E) will neutralise FeLV in the presence of complement (Fischinger et al., 1976). Also, evidence presented in Chapter Three showed a protease-sensitive molecule of approximately 17,000 daltons present in FeLV-ABC/KT. However, lactoperoxidase-catalysed iodination of surface proteins gave only a trace of label in two proteins in this molecular weight range. The more stringent requirement of this technique for exposed tyrosine residues may explain this discrepancy. Also, analysis of the 6M GuHCl gel filtration column void volume fraction, which is a rich source of MuLV p15(E) (Ikeda et al., 1975), showed no trace of any component of low molecular weight.

The best approach to resolve this question would seem to be immune precipitation analysis of FeLV with antiserum to MuLV p15(E). This should define the molecular size and allow comparison of the cross-reactive component in FeLV strains. This serum should also be used to examine the 80,000 - 85,000 dalton protein released into FL74 culture fluid and precipitable with cat sera which neutralise FeLV. Ruscetti and Parks (1977) have shown a molecule of this molecular size on the FL74 cell surface which has gp70 determinants. The anti-p15(E) serum might resolve whether this is a gp70 - p15(E) precursor. This would be interesting, since processing of the analagous env gene precursor polypeptide in avian sarcoma virus infected cells has been shown to take place intracellularly (Hayman, 1978). The expression of precursor polyproteins may lead to new cell surface antigenic determinants which could be defined as virus-induced non-virion antigens (Snyder et al., 1977; Tung et al., 1977). An alternative explanation for the existence of p85 may be that it is a "gp70" species distinct from that of 70,000 molecular weight. This seems possible from the observations reported in Chapter Five on the variable apparent size of this molecule.

The difference in apparent molecular weight of the FeLV glycoprotein (see Table 6) may be examined further to establish whether a relationship exists between the serological specificity, as detected by virus neutralisation tests, and SDS PAGE mobility. The results of Russell (1977) showed that FeLV-B/Boston-1 was related to FeLV-A/Glasgow-1 in neutralisation tests. This virus also has a glycoprotein of approximately 80,000 daltons, like

FeLV-A/Glasgow-1 and distinct from FeLV-B/Sarma. A particularly interesting virus in this respect is the FeLV-C/FS246 isolate which behaves identically to FeLV-A isolates when neutralised by a range of antisera.

The FeLV system may also be a useful model to study the role of host and virus factors in glycosylation of virus proteins since two molecules of similar function, FeLV-A and FeLV-C gp70, emerge from the same cells (FEA) with a marked difference in SDS PAGE mobility. This may be due to a difference in the polypeptide chain length of the two molecules, or to the existence of more, or longer, carbohydrate side chains on the FeLV-A gp70; or the observed difference may be due to a combination of both factors. Whalley (1973) found that the subunits of FeLV RNA showed apparent size differences on SDS PAGE. However, the size order (FeLV-A < FeLV/B < FeLV/C) was the inverse of that recorded for glycoprotein sizes (FeLV/A > FeLV/B > FeLV/C) here. If this is indeed reflected in polypeptide chain length, and the env-gene of FeLV-C is smaller than that of FeLV-A, this leaves the intriguing possibility that FeLV-C may carry some additional genetic information other than the gag, env and pol replication genes.

Marquadt et al. (1977) have purified the major glycoprotein of Rauscher MuLV and found that the apparent molecular weight in SDS PAGE varied according to the percentage of acrylamide in the gel. The relationship was that of an asymptotic curve, decreasing with increasing acrylamide concentration, and approaching a minimal value of 67,500 daltons. This phenomenon does not explain the results in Chapter Four, however, since various FeLV glycoproteins separated on the same gel still showed marked differences. However, since glycosylation affects proteins so that their molecular weights can be overestimated in SDS PAGE, it would seem that a comparison of tryptic peptide digests of FeLV gp70 species offers a better method of assessing relatedness. The demonstration of the "multi-gene family" of gp70s and related molecules in mouse leukaemia viruses and mouse tissues (Elder et al., 1977b) could be repeated in the FeLV system. There is some cause to believe that FeLV-C gp70 may be a normal differentiation antigen, or the product of a partial endogenous provirus (Russell, 1977) which is activated by FeLV-A infection. A type-specific radioimmunoassay for FeLV-C gp70 could be employed to investigate this possibility.

This study revealed no size differences in the low molecular weight gag-gene polypeptides of FeLV-A, B or C. Antisera were raised to p27 and p15 purified from FeLV-ABC/KT, so that these could not be used to search from subgroup-specific determinants on these molecules. There is a report in the literature (Green et al., 1973) of type-specific determinant(s) on FeLV p27 but these observations do not appear to have been extended. Results in this thesis would suggest that the gag-proteins of FeLV strains will be more closely interrelated than those of MuLV isolates.

Gel filtration in 6M guanidine hydrochloride allowed separation of quite pure and highly immunogenic preparations of FeLV p27 and p15. These two proteins showed no immunological cross-reactivity, suggesting no over-lapping sequences when they are cleaved from their common precursor polypeptide (Okasinski and Velicer, 1976). FeLV p10 and p12 could not be resolved on the Sepharose 6B column used in these studies. Since these two proteins have such different charge properties (Khan and Stephenson, 1977) a purification method which exploits this difference would be appropriate. Ion-exchange chromatography on phosphocellulose (P-11, Whatman) is the method described by Strand and August (1976) which efficiently resolved Rauscher MuLV p10 and p12. A combination of this technique with a molecular size separation method should allow purification of p10 and p12 near to homogeneity.

Recent studies where viral glycoproteins have been purified and used to raise specific neutralising antisera (Moennig et al., 1974; Hunsmann et al., 1974; Steeves et al., 1974; Marquadt et al., 1977) have avoided techniques which involved protein denaturation such as 6M GuHCl or SDS PAGE. Again, the best regime for purification of gp70 would be one which exploits a number of different properties of the molecule, such as size, charge properties or carbohydrate content. Hence, lectin affinity chromatography, phosphocellulose ion-exchange chromatography, and gel filtration on Sephadex, might be a useful combination. As with all protein purification regimes, increased purity must be weighed up against reduced final yield. This is particularly true of RNA tumour virus proteins, since large quantities of virus are very expensive to produce.

Analysis of FeLV-infected cell membrane glycoproteins as reported in Chapter Six revealed a number of interesting features. Although infection of feline embryonic fibroblast cells with FeLV showed almost no discernible effect on the total membrane glycoprotein composition, the membranes of transformed FeLV-producing lymphblastoid cells showed an abundance of a glycoprotein of the same apparent molecular weight and lectin binding specificity as the FeLV-ABC/KT and FeLV-C/Sarma gp70 molecules. In addition, two glycoproteins of 71,000 and 76,000 daltons were seen which bound only to the lentil lectin column. The possible relationship of these molecules to FeLV gp70 or gag proteins, or to the molecule with FOCMA determinants detected in FeSV-transformed mink cells (Stephenson et al., 1977b) could be investigated by analysis of tryptic peptide digests. It may also be fruitful to extend the work on cell membrane glycoproteins to other cell lines which are known to express FOCMA. Two other lymphoblastoid cell lines are available, F422 (Rickard et al., 1969) and 57176 (P. Rogerson, unpublished). F422 releases FeLV-A and 57176 releases FeLV-B. Both are established lines of transformed lymphoid cells, like FL74. A more telling comparison, however, might be with the "virus-negative" lymphoid tumour cell lines which have very recently been established by Hardy and his co-workers (W.D. Hardy Jr., personal communication) which express FOCMA in the absence of virus production. Also, a range of mink cell lines are available. Control cells could be compared to FeLV-infected and to FeSV transformed non-producer cells. This might establish the molecular nature of the FOCMA-reactive molecule as expressed in the mink cell membrane.

As reported in Chapter Seven, three separate studies have failed to demonstrate any FL74 cell antigen precipitated by anti-FOCMA sera which is not precipitated by control sera. However, there remain a number of possible explanations for this which suggest further experiments.

The NDC lysis method was used by Rohrschneider et al.(1975) to demonstrate an avian tumour-specific surface antigen (TSSA) in RSV-transformed cells. This was a glycoprotein of 100,000 M_r, and was unrelated to the major virion glycoprotein, gp85. However, this claim has not been substantiated by further work with other antisera and the gp100 was subsequently detected in non-transformed cells (Kurth, personal communication). Recent reports of successful demonstration of tumour-specific antigens by immune

precipitation have involved much stronger detergent lysis regimes. The initial step has involved both a non-ionic detergent (Triton X-100 or NP40) and a weakly ionic detergent (DOC), which apparently leave nuclei intact if used in appropriate time and concentration combinations, followed by addition of the strongly polar, and usually strongly denaturing detergents, SDS, to 0.1%. This apparently does not interfere with the immune precipitation step. Thus, Brugge and Frikson (1977) have reported the isolation of an avian sarcoma virus tumour-specific antigen, and Stephenson et al. (1977b) have claimed that a molecule cross-reactive with FOCMA can be found in FeSV-transformed mink cells. This modification certainly seems worth attempting with FL74 cells, although it is in contrast to most of the experimental modifications used here, which were designed to make extraction less destructive of native molecules.

A second possibility is that the FOCMA determinants in FL74 cells, unlike the FeSV-transformed mink cells, are highly sensitive to membrane disruption. The way to approach this problem would seem to be to refine, by subsequent addition of stronger detergents, the antibody pretreatment technique described here, in the hope that preformation of the FOCMA antibody complex would stabilise the antigen through the extraction procedures.

Finally, the problem may lie in the antisera which were chosen for this study. We have no information regarding the relative sensitivities of the indirect immunofluorescence test and our immunoprecipitation technique. The antisera could be checked by using them to repeat the experiments of Stephenson et al. (1977b) with FeSV-transformed mink cells. If the relative insensitivity of our technique is the problem, this might be remedied by first selecting the purified FL74 membrane fraction, as described in Chapter 6, and performing the experiment on this, since FOCMA may be relatively more plentiful in the plasma membrane than in the cytoplasm.

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