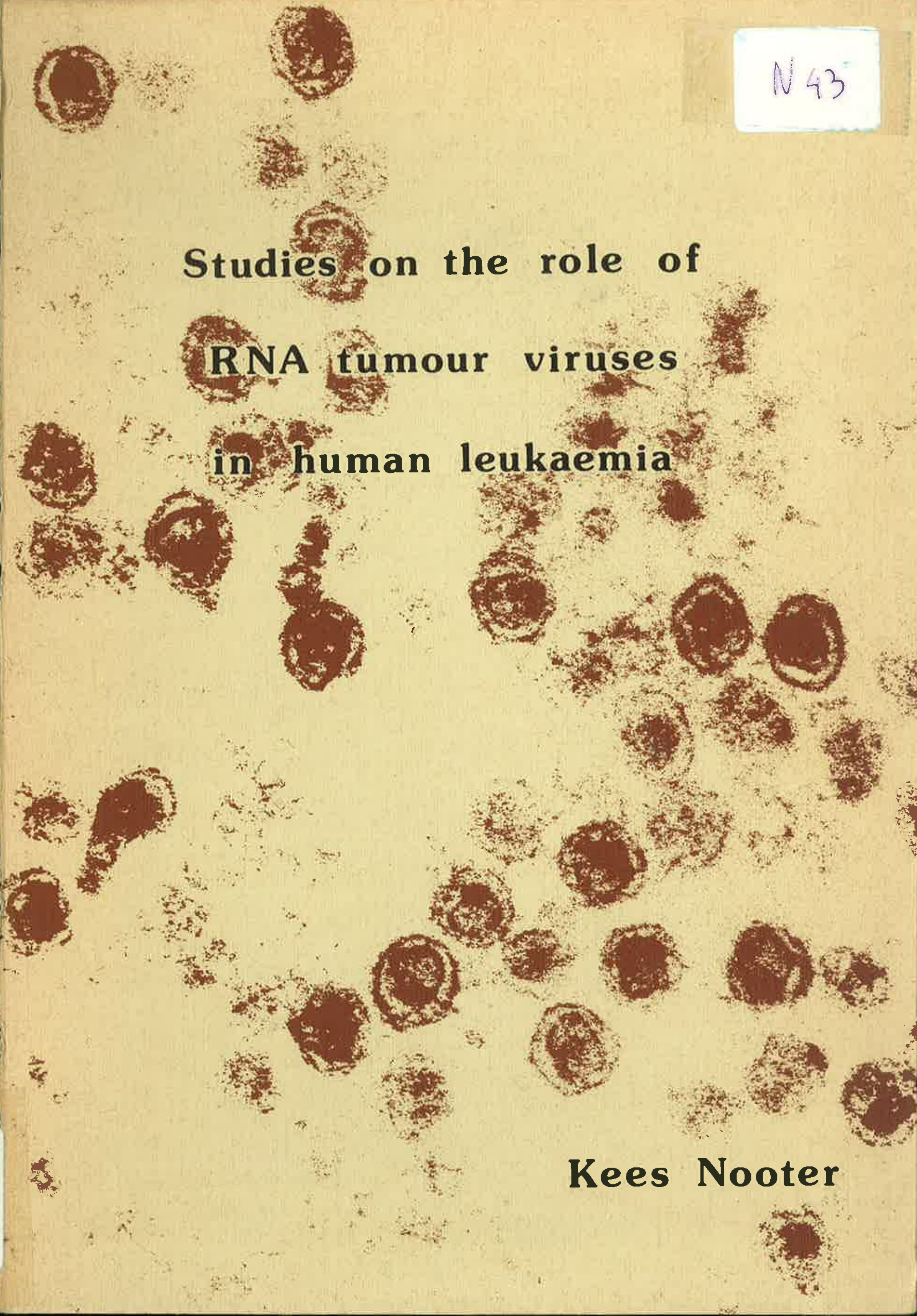
Studies on the role of RNA tumour viruses in human leukaemia



Kees Nooter

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2288 GJ RIJSWIJK, The Netherlands

STELLINGEN

-1-

Oncogene C-type virussen zijn geïsoleerd uit twee subhumane primaten species, het wolaapje en de gibbon. Aan de hand van de beschikbare gegevens is het onwaarschijnlijk te veronderstellen dat deze virussen onder natuurlijke omstandigheden deze apensoorten als gastheer hebben. Veeleer komt de mens hiervoor in aanmerking.

Kawakami, T.G., Sun, L. en McDowell, T.S.
In: Advanc. Comp. Leuk. Res., Elsevier/North-Holland, Biomedical Press, p. 33 (1978).

-2-

Het model van partiele provirus integratie zoals beschreven bij inductie van tumoren bij ratten en muizen door een vogelsarcoomvirus, verdient in het licht van een mogelijke virale etiologie van kanker bij de mens meer aandacht.

Shevlyaghin, V.Ya., Kusnetzova, N.N. en Biryulina, T.I., Neoplasma 24, 5 (1977).
Svoboda, J., Popovic, M., Sainerova, H., Mach, O., Shoyab, M. en Baluda, M.A.
Int. J. Cancer 19, 851 (1977).

-3-

Het aantonen van een RNA-afhankelijk DNA-polymerase en van een positieve simultane detectietest in menselijk tumormateriaal hebben niet bijgedragen tot de geloofwaardigheid van het bestaan van menselijke oncornavirussen.

-4-

Celdifferentiatie is de precipiterende factor voor de oncogene werking van leukemievirussen.

-5-

Er is geen reden om aan de hand van de tot nu toe gepubliceerde resultaten te veronderstellen dat de haemopoëtische stamcel de doelwitcel is van het Rauscher muizeleukemievirus.

-6-

De postulering van een "luk"-gen naar analogie van het "src"-gen bij vogelsarcoomvirussen als genetische informatie voor oncogene transformatie van haemopoëtische cellen door muizeleukemievirussen ligt voor de hand. De tot nu toe beschikbare gegevens laten eerder een verdenking uitgaan naar het virale envelop gen als oncogene informatie.

Elder, J.H., Gautsch, J.W., Jensen, F.C., Lerner,R.A., Hartley, J.W. en Rowe, W.P.
Proc. nat. Acad. Sci. USA 74, 4676, (1977).
Troxler, D.H., Lowy, D., Howk, R., Young, H. en Scolnick, E.M. Proc. nat. Acad. Sci. USA 74, 4671, (1977).

-7-

Het is niet waarschijnlijk dat bij laboratoriummuizen met een lage melkkliertumor-incidentie, virussen een rol spelen bij het ontstaan van dit soort tumoren door excessieve hormoonbehandelingen.

Michalides, R., Van Deemster, L., Nusse, R., Röpcke, G. en Boot, L.
J. Virol. 27, 551 (1978).

-8-

Dat in virussen behorend tot de paramyxogroep de fusiefaktor identiek is aan het kleinste der twee virale glycoproteinen, volgt niet dwingend uit de proeven van Homma, Scheid en Nagai.

Homma, M. en Ohuchi, M., J. Virol. 12, 1457, (1973).
Scheid, A. en Choppin, P.W., Virology 57, 475, (1974).
Nagai, Y, Klenk, H.-D. en Rott, R., Virology 72, 494, (1976).

-9-

Waarschijnlijk is het enzym "terminaal deoxynucleotidyl transferase" (nucleosidetrifosfaat: DNA deoxy-nucleotidylexotransferase, E.C. 2.7.7.31) een marker voor differentiatie van T-cellen. Hoewel dit enzym als regel in grote hoeveelheden kan worden aangetoond in perifere bloedcellen van patienten met acute lymfatische leukemie, heeft het geen diagnostische waarde.

-10-

Het optreden van kanker bij de mens moet grotendeels worden toegeschreven aan milieufaktoren. In de gezondheidszorg moet daarom een hogere prioriteit aan preventieve maatregelen worden gegeven bij de bestrijding van deze ziekte.

Stellingen behorende bij het proefschrift
"Studies on the role of RNA tumour viruses in human leukaemia"

Kees Nooter
Leiden, 21 februari 1979

Studies on the role of RNA tumour viruses in human leukaemia

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN
DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE
LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. D.J. KUENEN, HOOGLERAAR IN DE
FACULTEIT DER WISKUNDE EN NATUURWETENSCHAPPEN, VOLGENS BESLUIT VAN HET COLLEGE
VAN DEKANEN TE VERDEDIGEN OP WOENSDAG,
21 FEBRUARI 1979 TE KLOKKE 14.15 UUR

DOOR

Kees Nooter

GEBOREN TE AMSTERDAM IN 1946

W.D. MEINEMA B.V. - DELFT

CONTENTS

ABBREVIATIONS

ASV - avian sarcoma virus
BaEV - baboon endogenous virus
cpm - counts per minute
EBV - Epstein-Barr virus
FCS - fetal calf serum
FeLV - feline leukaemia virus
FITC - fluorescein isothiocyanate
FOCMA - feline oncornavirus associated cell membrane antigen
GALV - gibbon ape leukaemia virus
IFA - immunofluorescence assay
Ig - immunoglobulin
MSV - murine sarcoma virus
MuLV - murine leukaemia virus
MuMTV - murine mammary tumour virus
M-PMV - Mason-Pfizer monkey virus
NP cells - nonproducer cells
PBS - phosphate buffered saline
PHA - phytohaemagglutinin
R-MuLV - Rauscher murine leukaemia virus
RSV - Rous sarcoma virus
SiSV - woolly monkey (simian) sarcoma-leukaemia virus
SLE - systemic lupus erythematosus
S-MuLV - Soule murine leukaemia virus
TMP - thymidine monophosphate

"The essential feature of a strategy of discovery lies in determining the sequence of choice of problems to solve. Now it is in fact very much more difficult to see a problem than to find a solution for it. The former requires imagination, the latter only ingenuity"

Bernal, 1971
Science in History, Vol. I, p. 39

Aan mijn ouders

CHAPTER I

INTRODUCTION

1.1. TYPE C RNA TUMOUR VIRUSES

RNA tumour viruses are classified in the group of retroviruses, whose main characteristic is the presence of an RNA-directed DNA polymerase. Mature virions consist of RNA (1 %), protein (60-70 %), lipid (20-30 %) and carbohydrate (2 %) (Beard, 1963). The viral genome and the viral polymerase are embedded within viral structural proteins and together form the electron-dense core. The core is surrounded by an envelope consisting of lipids and glycoproteins. The envelope is partly built up of host cell constituents during the process of virus budding from the cell membrane. The mature virion has a roughly spherical shape with a diameter of approximately 100 nm. The density of the particles is between 1.15 - 1.18 g per ml in a sucrose gradient. The RNA tumour viruses are referred to in the literature as retroviruses (showing reverse transcriptase activity) or as oncornaviruses (because of their oncogenic activity); for most isolates, however, evidence for oncogenicity is lacking. They are divided into types A, B, C, and D mainly on the basis of electron microscopic morphology (Bernhard, 1960; Dalton et al., 1975). The murine intracytoplasmic type A particles are most likely precursors of the type B mouse mammary tumour virus (Sarkar and Whittington, 1977). The prototype of type D viruses is the Mason-Pfizer monkey virus. The naturally occurring leukaemia and sarcoma viruses are classified as type C. They now appear to be ubiquitous in nature and have been isolated from a wide variety of animal species, among which are fishes, reptiles, birds, lower mammals and primates. Many of these isolates have not been shown to be oncogenic either in vivo or in vitro. For that reason we will refer to the type C RNA tumour viruses as type C retroviruses. The genetic information of the viral RNA can be either endogenous or exogenous to the host. This means that the base sequence homology of the viral genome with sequences of normal host cell DNA can vary from complete homology to none. Endogenous viruses have been found among others in mice, cats and primates and are transmitted genetically through the germ lines.

1.1.1. Genome of type C viruses

The genome of the mature type C retroviruses consists of a single-stranded RNA with a sedimentation coefficient of 60-70 S. It is built up of two identical 30-40S subunits joined together at their 5'-ends by hydrogen bonds (Dube et al., 1976; Kung et al., 1976; Bender et al., 1978).

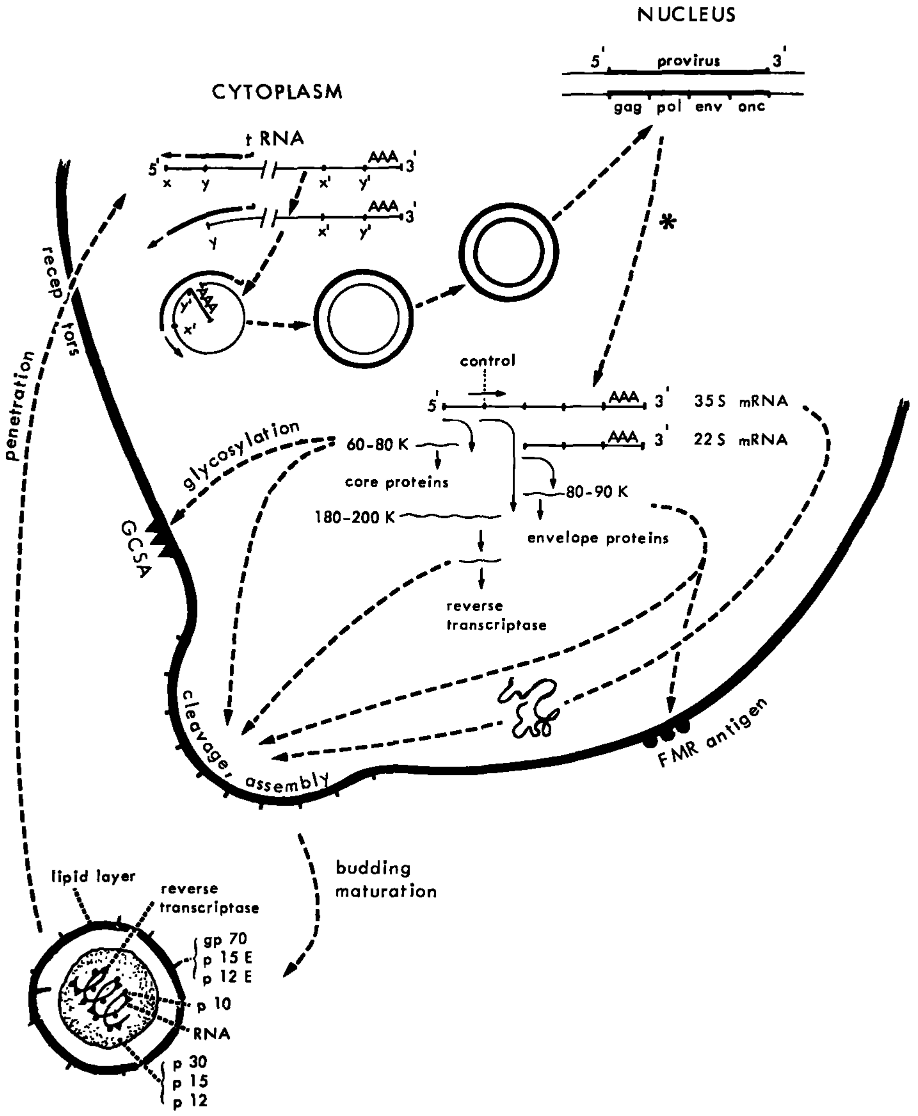

Figure 1: Life cycle of murine type C leukaemia viruses.
For explanation, see text. The hypothetical life cycle is based on models proposed by Jamjoom et al. (1977), Taylor (1977), Bolognesi et al. (1978), Collett and Faras (1978), Eisenman and Vogt (1978)*. There is some evidence that the subgenomic mRNAS are produced by nuclear processing of a viral-genomic precursor mRNA rather than by direct transcription of proviral DNA (Stacey and Hanafusa, 1978).

Each subunit has a sequence complexity of about 3 x 10^6 dalton (Duesberg et al., 1974; Weissman et al., 1974; Beemon et al., 1976). One subunit is about 9000 nucleotides long and has a typical messenger RNA structure: a 5'-terminal cap structure (Furuichi et al., 1975; Rose et al., 1976) and a polyadenylated 3'-end (Rho and Green, 1974; Wang and Duesberg, 1974). The viral genome can code for approximately 250,000 -300,000 dalton of protein and 4 different genes have been postulated within a single subunit (Baltimore, 1974): 5'-cap-gag-pol-env-onc-poly(A)-3', in which gag stands for the gene coding for the group specific antigens, pol for the gene coding for the viral polymerase, env for the gene coding for the envelope protein and onc for the gene coding for the putative transformation inducing protein. The genes are mapped in this order on the avian type C viral genome (Coffin and Billeter, 1976; Joho et al., 1976; Wang et al., 1976) Avian type C viruses are similar to mammalian type C viruses in many aspects; therefore, the mapping order of mammalian and avian type C viral genes is most likely analogous. Our discussion here will mainly concentrate on the murine viruses, but most aspects of biosynthesis, function of viral proteins and viral replication mechanisms are also valid for other mammalian and avian retroviruses.

1.1.2. Viral structural proteins

The gag-gene of murine type C viruses codes for a 65,000 - 80,000 molecular weight protein (with group-specific antigenic determinants) which is cleaved to structural proteins with molecular weights of 30,000 (p30), 15,000 (p15), 12,000 (p12) and 10,000 (p10) (Van Zaane et al., 1976; Shapiro et al., 1976; Arcement et al., 1977) (Fig. 1). The gag-proteins are located within the viral core (Oroszlan et al., 1970; Bolognesi et al., 1973; Fleissner and Tress, 1973). The intracistronic mapping within the gag-gene is (5')-p15-p12-p30-p10 (3') (Reijnolds and Stephenson, 1977). The major structural protein of the mammalian type C retroviruses is p30. This polypeptide contains mainly group-specific (shared by viruses of one species) and interspecies-specific (shared by viruses of different species) antigenic determinants (Parks and Scolnick, 1972). Major determinants of immunologic type specificity (unique to an individual virus strain) are localized on polypeptides of low molecular weight, especially p12 and p15 (Parks et al., 1975), although the envelope glycoproteins also contain type specificity (Strand and August, 1974a). Murine leukaemia virus p12 is phosphorylated (Pal and Roy-Burman, 1975) (for some other viruses, the phosphorylated protein is p15) and has been shown to specifically bind 35S RNA (Sen et al., 1976). The p12 polypeptide is supposed to act as a ligand between the polymerase and homologous viral RNA. The p10 polypeptide is found in ribonucleoprotein complexes (Fleissner and Tress, 1973). The Gross cell surface antigen (GCSA) produced by cells infected with Gross murine leukaemia virus appears to be the glycosylated form of the gag-precursor protein (Ledbetter and Nowinski, 1977).

1.1.3. Viral polymerase

The pol-gene codes for the viral RNA-directed DNA polymerase (reverse transcriptase). The 35S viral genomic RNA is most likely the mRNA for both gag-and pol-gene products (Murphy and Arlinghaus, 1978; Murphy et al., 1978). A large (180,000 - 200,000 dalton) precursor protein with both gag- and polymerase-antigens has been isolated (Jamjoom et al., 1977). It has been proposed that the large precursor results from an occasional read-through translation of the large messenger (Murphy et al., 1978; Philipson et al., 1978). From this initial translation product of the gag and pol regions, mature reverse transcriptase with a molecular weight of approximately 70,000 - 80,000 dalton is formed (Moelling, 1974). Mammalian type C virus reverse transcriptase has interspecies, group and type specificity (Krakower and Aaronson, 1978a). Reverse transcriptase is the only core protein of which the function is understood. It transcribes the viral RNA into DNA which becomes integrated into the chromosomal DNA of the cell. From this proviral DNA, new viral RNA which can act either as viral mRNA (that can be translated) or as viral genomic RNA (that can be packed into a virion particle) can be transcribed. Thus reverse transcriptase is required for the productive infection of cells by retroviruses (Linial and Mason, 1973) and for the initiation of transformation but not for the maintenance of the virally-transformed cell state (Ting et al., 1972). Besides RNA-directed DNA and DNA-directed DNA polymerase activity (Temin and Baltimore, 1972), the molecule possesses an RNAse H activity (Moelling, 1974; Collett et al., 1978) which also plays a role in viral replication.

1.1.4. Viral envelope proteins

The env-gene codes for the envelope proteins. The major envelope glycoprotein of murine type C viruses with a molecular weight of approximately 70,000 dalton (gp70) is obtained by cleavage from a 80,000-90,000 molecular weight glycosylated polypeptide (Shapiro et al., 1976; Van Zaane et al., 1976; Karshin et al., 1977). The other cleavage products of this precursor are p15E and p12E. The envelope proteins gp70, p15E and p12E are located on the surface of murine leukaemia viruses (Montelaro et al., 1978) and provide the virion surface with "spikes" (Bolognesi et al., 1978). The viral envelope proteins determine to a great extent the biological properties of the virions. They are involved in virus-host interactions, e.g., virus neutralization by antiviral antibodies (Hunsmann et al., 1974), and virus penetration of the cell (DeLarco and Todaro, 1976). These proteins manifest all 3 classes of antigenic determinants (Strand and August, 1974a). The FMR cell surface antigen which is found on the surface of cells infected with Friend, Moloney or Rauscher murine leukaemia viruses is shown to be an antigenic determinant of the env-gene product (Nowinski et al., 1978). Although the viral genome is a polycistronic single stranded RNA the expression of the different viral genes is noncoordinate, which is realized by the existence of separate messengers. In the murine system, besides the 35S mRNA for gag and pol products, a 22S mRNA is reported to code for env-gene products (Van Zaane et al., 1977; Fan and Verma, 1978).

1.1.5. Viral transforming proteins

All oncogenic RNA viruses are thought to have a hypothetical onc-gene coding for transforming proteins. Convincing evidence for this hypothesis is provided by research on the sarcomagenic avian viruses. There, a single viral gene (referred to as src) is responsible for sarcomagenic transformation *in vitro* and *in vivo* (Stehelin et al., 1976). The avian leukaemia viruses lack this genetic src-information, which apparently is not essential for viral replication. A viral gene responsible for leukaemogenesis, a luk-gene, has not yet been identified.

Cells producing avian sarcoma viruses contain 3 size classes of virus-specific messengers: a 38S mRNA which is the template for core proteins and reverse transcriptase; a 28S mRNA which codes for envelope proteins; and a 22S mRNA which mainly contains biochemically defined src-information (Hayward, 1977; Weiss et al., 1977). The gag-pol, env, and src genes are located near the 5'-end of the different mRNAs and most likely only these regions are translated.

The identification of the src gene product and its possible role in transformation is presently a highlight in tumour virology and most progress is made in the avian virus system; src-specific translation products with molecular weights of 18,000, 25,000 and 60,000 dalton encoded by approximately 20S messengers have been claimed by different groups (Beemon and Hunter, 1977; Kamine et al., 1978). *In vitro* translation of viral RNA from transformation-defective deletion mutants of avian sarcoma virus did not yield such polypeptides. Another approach to the identification of a putative transformation-specific protein induced by an avian sarcoma virus (ASV) is that of Brugge and Erikson (1977). Sera from rabbits bearing tumours induced by ASV were shown to specifically precipitate a 60,000 dalton protein in cell lysates from ASV transformed chicken and hamster cells (Brugge et al., 1978). This protein was absent in the mature virion. Arguments for transformation specificity of the 60,000 dalton protein are: 1) it is not present in cells infected with transformation-defective mutants of ASV (Purchio et al., 1978); 2) the 60,000 dalton protein cannot be precipitated with antisera to structural proteins of ASV; and 3) the 60,000 dalton protein obtained by *in vitro* translation of viral RNA appeared to be antigenically and biochemically identical with the immune precipitable protein present in ASV transformed cells (Purchio et al., 1978). Recent results showed a protein kinase (ATP: protein phosphotransferase) activity for the src-gene product (Erikson et al., 1978; Collett and Erikson, 1978), suggesting that ASV may transform cells by aberrant phosphorylation of cellular proteins.

1.1.6. Viral life cycle

Little is known about the mechanism of assembly of type C particles. The various proteins are probably transported to the cell surface where cleavage of precursor proteins, packaging of polypeptides and RNA and subsequent budding of virions from the cell membrane takes place (Fig. 1). Complete virion maturation takes place only after budding is completed. A mature particle can infect susceptible cells by a mechanism in which specific receptors for the viral envelope proteins are involved (DeLarco and Todaro, 1976). After penetration, the viral RNA is transcribed into DNA (RNA-directed DNA polymerase activity), the RNA template is subsequently removed from the transcription product (RNase H activity) and the DNA molecule is made double-stranded (DNA-directed DNA polymerase activity). The proviral DNA is then transported to the cell nucleus where it becomes integrated into the host genome (Shank and Varmus, 1978). Transcription of the integrated proviral DNA is carried out by cellular polymerase (Rymo et al., 1974). Much new information on the mechanism of proviral DNA formation has recently become available. The synthesis of cDNA on viral template RNA is initiated on a tRNA primer molecule (Dahlberg et al., 1974) hydrogen bonded near the 5'-end of the genome (Taylor and Illmensee, 1975). Transcription proceeds to the 5' terminus, reinitiates at the 3'-end of the RNA and copies the remaining part of the viral genome (Junghans et al., 1977). This "jumping over" of reverse transcriptase is likely to be brought about by a circularization of the viral genome. The viral genomes of avian and mammalian type C viruses are terminally redundant at their extreme 5'- and 3'-ends (Haseltine et al., 1977; Coffin et al., 1978). These redundant sequences provide "sticky-ends" for circularization of the viral RNA genome. The reverse transcriptase-associated RNase H activity removes the viral 5'-end RNA sequences (Collett et al., 1978), facilitating the association of the 5'-terminally located cDNA transcripts with the complementary redundant sequences at the 3'-end of the viral RNA.

In subsequent steps, the DNA is assumed to be be converted into a double-stranded form which becomes integrated into the cellular chromosomes (for a review on provirus intermediate forms and provirus integration, see Weinberg, 1977).

1.2. PRIMATE TYPE C RETROVIRUSES

The presence of type C retroviruses, either endogenous or exogenous to the host of origin, is not restricted to lower mammals and birds. Infectious type C viruses have also been isolated from Old and New World primates (Table 1).

1.2.1. Woolly monkey and gibbon ape viruses

The first of this series of isolates was made from a naturally occurring fibrosarcoma of a male woolly monkey (Lagothrix sp.) house pet (Theilen et al., 1971). When placed into tissue culture, the fibrosarcoma released type C retroviruses which could be transmitted to a variety of other mammalian cell lines. The presence of a transforming virus (simian sarcoma virus) in this isolate was shown by transformation in vitro and the induction of tumours in newborn marmosets (Wolfe et al., 1971). This transforming virus appeared to be replication-defective; a nontransforming replication competent helper virus, simian sarcoma-associated virus, was found in excess in the virus stock (Wolfe et al., 1972a; Scolnick and Parks, 1973). Although no leukaemogenic activity has yet been reported for the woolly monkey type C helper virus it is mostly referred to as the woolly monkey leukaemia virus. We will refer to this sarcoma-leukaemia virus complex as SiSV.

The second primate virus, closely resembling the helper component of SiSV, was isolated in widely different geographical locations and at different times. Type C viruses were electron microscopically found to be associated with generalized lymphosarcomas in an Old World primate species, the white-handed gibbon (Hylobates lar), in a colony at the San Francisco Medical Center (California) (Kawakami et al., 1972; Snyder et al., 1973). The animals in this colony had been repeatedly exposed to ionizing radiation for diagnostic skeletal survey in a study on the ageing of the spinal column. Tumour cell cultures obtained from the leukaemic gibbon produced infectious nontransforming type C particles referred to as gibbon ape leukaemia virus (GALV)-SFMC (Kawakami et al., 1972).

In the SEATO Laboratory gibbon colony in Bangkok (Thailand), five spontaneous cases of granulocytic leukaemia have been reported (DePaoli et al., 1973). The gibbons in the colony were used for malaria research and many were experimentally infected with Plasmodium falciparum by inoculation with human malarial blood. All diseased animals belonged to the experimentally treated group. Tumour samples received from one case of granulocytic leukaemia contained type C virus which was propagated in vitro (Kawakami and Buckley, 1974). This isolate is referred to as GALV-SEATO.

Another isolate (GALV-H) was obtained from an adult gibbon with acute lymphoblastic leukaemia belonging to a colony used for behavioural studies on Hall's Island (Bermuda) (Gallo et al., 1978).

Gibbon type C retroviruses are not found solely in association with lymphosarcoma and leukaemias. Three isolates (GBr-1, -2 and -3) have been obtained by cocultivation of normal gibbon brain tissues with permissive host cell lines (Todaro et al., 1975). Two of the three primates providing

Table 1

PRIMATE TYPE C VIRAL ISOLATES

	Viral isolate	Endo[a]/ exo	Oncogenicity[b]
Prosimians			
Tree shrew (Tupaia belangeri)	+	endo	-
New World monkeys			
Owl monkey (Aotus trivirgatus)	+	endo	-
Woolly monkey (Lagothrix sp.)	+	exo	+
Old World monkeys			
Stumptail macaque (Macaca arctoides)	+	endo	-
Gelada monkey (Theropithecus gelada)	+	endo	-
Baboon (Papio spp.)	+	endo	-
Apes and man			
Gibbon ape (Hylobates lar)	+	exo	+
Chimpanzee (Pan spp.)	-		
Gorilla (Gorilla spp.)	-		
Orangutan (Pongo spp.)	-		
Man (Homo sapiens)	$\pm^{c}$	exo	+

[a] The isolated virus is either endogenous or exogenous for the host of origin

[b] Oncogenicity shown by transformation *in vitro* or tumour induction *in vivo*

[c] Still controversial.

the brain tissues were previously inoculated with brain extracts of human patients with the progressive neurologic disease Kuru (Gajdusek and Zigas, 1957). The other ape was an uninoculated control cage-mate. The monkeys were housed in a colony in Louisiana and died of pneumonia 4 months after inoculation.

Apparently, virus infection of gibbons is not restricted to these few isolated cases. Without developing clinical signs of disease, many captive gibbons are naturally exposed to infectious type C viruses as determined by specific humoral antibodies and viraemia (Kawakami et al., 1973; 1977; Charman et al., 1975).

The gibbon ape and woolly monkey viruses must be acquired by horizontal infection, since GALV and SiSV ^{3}H-DNA probes do not hybridize to normal cellular DNA of the gibbon and the woolly monkey nor to that of other primates, e.g., marmosets (*Callithrix spp.*), rhesus monkeys (*Macaca mulatta*), chimpanzees (*Pan spp.*) and humans (Scolnick et al., 1974; Benveniste et al., 1974b). On the basis of immunological and molecular hybridization analysis, SiSV and the gibbon isolates form a closely related group of exogenous primate type C retroviruses. This group of viruses can replicate *in vitro* to high titres in, among others, human, rhesus monkey, dog, cat, rat, mink and bat, but not in mouse cells. These viruses are related to the extent that they cannot be distinguished from each other by either reverse transcriptase inhibition experiments or by competition radioimmunoassays for group-specific p30-antigens (Scolnick et al., 1972; Parks et al., 1973). However, type-specific differentiation can be made on the basis of a p12 assay (Tronick et al., 1975). Antigenic determinants of the p12 polypeptides of the SFMC and SEATO isolates are more closely related to each other than to those of the p12 of SiSV. This relationship is also reflected in nucleic acid sequence homology. The ^{3}H-DNA GALV-SFMC probe hybridizes well to GALV-SEATO RNA (83 % homology) and to a lesser extent to RNA of SiSV (70 % homology) and the brain isolates (55 % homology) (Todaro et al., 1975).

Todaro et al. (1975) made a division into subgroups on the basis of nucleic acid hybridization studies: subgroup A, SiSV; B, GALV-SFMC; C, GALV-SEATO and D, GALV-Br-1, -2 and -3. To which subgroup the GALV-H belongs is not yet known.

1.2.2. Endogenous primate type C viruses

Endogenous viruses not capable of infecting cells of the species of origin (xenotropic viruses) have been found in fowl (Crittenden et al., 1973), mice (Levy, 1973) and cats (Todaro et al., 1973b). This replication restriction for the heterologous species is also a characteristic of the various genetically transmitted baboon type C viruses. Consequently, most baboon endogenous virus (BaEV) isolates have been obtained by cocultivation of baboon tissues with heterologous cells, e.g., dog or human cells. From the species *Papio cynocephalus*, six isolates are characterized: the M28 isolate produced by feline sarcoma virus transformed baboon testicular cells (Todaro et al., 1973a), the M7 isolate obtained by cocultivation of baboon placenta with various mammalian host cell lines (human, rhesus, dog and bat lines) (Benveniste et al., 1974a) and the four BAB isolates produced by cocultivations of normal baboon lung, kidney and testicular cells (Todaro et al., 1974). Type C viral isolates have also been retrieved from the baboon species *P. hamadryas*, *P. papio* (PP-1-Lu-isolate) and *P. anubis* and from the closely related genus *Theropithecus* (TG-1-K-isolate) (Todaro et al., 1976). The *P. hamadryas* BILN isolate is released by a dog line cocultivated with inguinal lymph node cells of a lymphomatous baboon (Goldberg et al., 1974). This baboon was a member of the Sukhumi primate colony (Georgia, USSR) in which a spontaneous outbreak of leukaemia had been reported. An etiologic relationship between type C virus and leukaemia as suggested previously (Lapin, 1975) is not very likely here, because an oncogenic herpesvirus was recently isolated from baboons of this leukaemia colony (Rabin et al., 1977; Falk et al., 1978).

The baboon type C viruses are closely related to each other and form a distinct class of primate viruses. This is based on viral host range, immunological criteria and nucleic acid sequence homology. Baboon viruses grow well in human, horse, bat, dog, mink and rhesus monkey cells, but not in those of the cat, rat, mouse, rabbit and baboon (Benveniste et al., 1974a; Todaro et al., 1974; 1976). An exception is the PP-1-Lu isolate, which up to now has been shown to replicate only in rhesus monkey cells. The baboon isolates cannot be distinguished from each other by the reverse transcriptase inhibition test and the major group-specific protein (p30) assay (Sherr and Todaro, 1974a; Todaro et al., 1974), just as has been found for the GALV isolates. However, competition radioimmunoassay of the p15 polypeptide (which is comparable to the p12 of other virus groups) revealed type-specific antigenic differences among different baboon isolates (Stephenson et al., 1976). By nucleic acid hybridization studies, only small differences in genome composition could be detected among the isolates obtained from the different baboon species and the related species Theropithecus gelada (Todaro et al., 1976). The least homology (57 %) of an PP-1-Lu DNA probe was found with viral RNA of the Theropithecus isolate.

A virus related to the endogenous baboon virus is the RD114 cat virus which arose in a human cell line after passage through a fetal cat (McAllister et al., 1972). This endogenous virus for domestic cats (Fischinger et al., 1973; Livingston and Todaro, 1973) shows partial relationship to baboon viruses in antigenic determinants of p30 (Sherr and Todaro, 1974a; Stephenson et al., 1976), reverse transcriptase (Sherr et al., 1974a; Todaro et al., 1974) and genome nucleic acid homology (Benveniste et al., 1974a) (for a review on the evolutionary aspects of this relatedness, see Benveniste et al., 1975).

Baboon virus related nucleic acid sequences can be found in normal cellular DNA of all Old World monkeys, higher apes, e.g., chimpanzee and gibbon, and man (Benveniste and Todaro, 1974; 1976). However, under more stringent nucleic acid hybridization conditions, only a limited number of species, taxonomically related to baboons, show a significant amount of homology with baboon virus RNA (Benveniste et al., 1974b; Sherr et al., 1974b). Infectious type C viruses genetically related to baboon viruses, have been isolated only from the genera Papio and Theropithecus. These genera seem to be unique among primates in having a low level of suppression of endogenous virogene expression.

Recently, endogenous type C viruses were isolated with an extremely low frequency from two other primate species, stumptail and owl monkeys. Cocultivation of a continuous normal spleen cell line of a stumptail monkey (Macaca arctoides) with human cells resulted in the isolation of an endogenous stumptail monkey type C retrovirus (MAC-1-isolate) (Todaro et al., 1978b). The host range of this isolate differs from that of the baboon viruses. The MAC-1 virus replicates in human and cat cells, but not in cells from bats, dogs, mink, rhesus and owl monkeys. This new isolate is, in immunological(transcriptase inhibition and p30-assay) and nucleic acid hybridization tests, totally different from other primate viruses. There is no nucleic acid sequence homology with the baboon viruses and with cellular DNA of New World monkeys and man. Old World primates, on the contrary, harbour multiple copies of MAC-1 related sequences in their normal cellular DNA.

A third set of endogenous primate type C virogenes is found in the New World owl monkey (*Aotus trivirgatus*) and evolutionarily related species, among which are the spider monkey (*Ateles sp.*), the squirrel monkey (*Saimiri sp.*) and the woolly monkey (*Lagothrix sp.*) (Todaro et al., 1978a). A type C virus isolate (OMC-1) was detected in an owl monkey kidney cell line and could be transmitted to bat lung and cat embryo cells. According to immunological criteria and nucleic acid hybridization studies, this virus has no homology with other retrovirus isolates.

The recently isolated type C virus from the tree shrew (*Tupaia belangeri*) (Flügel et al., 1978), a primitive prosimian, is of special importance because prosimians are thought to bridge the gap between rodents and primates. Comparison of this endogenous *Tupaia* virus with other known rodent and primate type C viruses will be looked forward to with interest.

1.3. SEARCHING FOR HUMAN TYPE C RETROVIRUSES

1.3.1. Electron microscopy

Human tissue specimens, serum, milk, urine and faeces of normal individuals and patients with neoplasia have been extensively examined for the presence of electron microscopic virus particles. However, so far no convincing evidence has been obtained because: 1) very few reports deal with particles which can eventually represent human type C viruses (e.g., Dmochowski et al., 1967; Seman and Seman, 1968; Hirshaut et al., 1974); 2) no budding particles seem to be present; and 3) it is obvious that, in case of doubts, morphology is a poor criterion.

A new development is the electron microscopic observation of immature, mature and budding type C virions in placentas of normal marmosets (*Saguinis oedipus*) (Seman et al., 1975), rhesus monkeys (*Macaca mulatta*) (Schidlovsky and Ahmed, 1973), chimpanzees (*Pan spp.*) (Kalter et al., 1975a), the baboon (*Papio cynocephalus*) (Kalter et al., 1973a), the patas monkey (*Erythrocebus patas*) (Kalter et al., 1975b) and humans (Kalter et al., 1973b; Vernon et al., 1974; Dirksen and Levy, 1977). Whether these particles represent infectious viruses transmitted vertically from parent to offspring is not clear. Evidence for this has been obtained only for the baboon up to now. Benveniste et al. (1974a) isolated a baboon type C virus by cocultivation of baboon placenta with target cells permissive for virus replication. Nucleic acid hybridization studies demonstrated that the virus was of endogenous origin (Benveniste et al., 1974b) and that related sequences could be found in normal cellular DNA of evolutionarily related species, e.g., macaques. However, cocultivation of placenta tissues of other primate species or man did not result in virus isolation.

1.3.2. Reverse transcriptase

The detection of the viral enzyme reverse transcriptase (RNA-dependent DNA polymerase) (Temin and Mizutani, 1970; Baltimore, 1970) led to increased interest in the search for human type C retroviruses. Electronmicroscopic studies had already shown that human tumor material did not provide abundant evidence for the existence of human retroviruses. With the discovery of reverse transcriptase, a new molecular probe with which putative human retroviruses could be detected, even when these were only partially expressed, became available.

The first report came from Gallo et al. (1970), who showed an RNA-dependent DNA synthesis in cell extracts of three patients with acute lymphoblastic leukaemia. Rat liver RNA was used as a template; the reaction was RNase sensitive and the reaction product appeared to be DNA. Control cells were phytohaemagglutinin stimulated normal peripheral blood lymphocytes. This was the beginning of a series of experiments showing "biochemical" particles in human neoplasia. High speed pellet fractions isolated from white blood cells of patients with acute leukaemia exhibited endogenous DNA polymerase activity which was RNase sensitive, suggesting the involvement of an RNA in the reaction (Sarngadharan et al., 1972).

By chromatography with DEAE-cellulose, phosphocellulose and "Sephadex G-200", the enzyme was purified from the high speed pellets. A natural 70S RNA of an avian type C virus could be transcribed by the enzyme and the synthesized DNA appeared to be complementary to the template RNA. The molecular weight of the human tissue derived reverse transcriptase was found to be 70,000 dalton (Gallagher et al., 1974; Witkin et al., 1975). Besides the use of specific synthetic template-primer combinations (Goodman and Spiegelman, 1971; Bobrow et al., 1972; Robert et al., 1972; Lewis et al., 1974a; Gillespie et al., 1975), an immunological approach was applied in later studies, in order to discriminate satisfactorily between reverse transcriptase and the normal cellular DNA polymerases alpha, beta and gamma (Mayer et al., 1975) and terminal deoxynucleotidyl transferase which can be found in leukaemic cells (McCaffrey et al., 1973). Cytoplasmic "particles" were purified from high speed pellets of cell extracts by isopycnic gradient centrifugation.

The buoyant density of these particles was about 1.16 g per ml, a density characteristic for retroviruses. Preparations of such biochemical particles do not contain conspicuous retroviruses on electron microscopic examination. These particles are biochemically defined only by their RNA and reverse transcriptase content. The purified enzyme obtained from such particles was strongly inhibited by hyperimmune sera raised against reverse transcriptase of the gibbon ape leukaemia virus and the woolly monkey type C virus but to a much lesser extent by antisera directed to the enzyme of Rauscher murine leukaemia virus (R-MuLV). No inhibition was found with antisera to avian sarcoma virus, RD114 virus and Mason-Pfizer monkey virus reverse transcriptase (Todaro and Gallo, 1973; Gallagher et al., 1974; Mondal et al., 1975). Normal human cellular DNA polymerases are not inhibited by these antisera. Cross neutralization is also observed in inhibition experiments using the gibbon ape or the woolly monkey virus enzyme and antibodies to R-MuLV reverse transcriptase (Scolnick et al., 1972). These immunological data suggest that a putative human type C virus is related to the primate viruses SiSV and GALV.

Supporting evidence for this relationship is obtained in experiments showing that the ^{3}H-DNA transcription product synthesized in the presence of actinomycin D in the endogenous leukaemic cell reverse transcriptase reaction hybridizes best to SiSV RNA and much less to MuLV RNA (Gallo et al., 1973; Miller et al., 1974). This study was extended by Reitz et al. (1976) who found that the cDNA synthesized by cytoplasmic viral-like particles from a leukaemic patient hybridized to some extent to RNA from both SiSV and BaEV.

1.3.3. Simultaneous detection test

The simultaneous detection of high molecular weight RNA and reverse transcriptase activity, standardized by Schlom and Spiegelman (1971) has been applied to tissues obtained from a great variety of human malignancies. This test is based on the observation that, in retroviruses, the initial transcription product catalysed by the viral enzyme is found complexed to its 70S RNA template (Spiegelman et al., 1970). The simultaneous detection test involves isolation of cytoplasmic particles with a density of 1.16 g per ml, detergent treatment of the particles resulting in a density shift to 1.24 g per ml [which is also found by the conversion of type C virions to cores (Stromberg, 1972)] and finally demonstration of endogenous DNA synthesis and determination of the sedimentation coefficient of the DNA-RNA hybrid and the DNA fragment. Positive simultaneous detection tests have been obtained with almost every kind of human tumour tested: leukaemias (Baxt et al., 1972), leukaemias in remission (Viola et al., 1976), lymphomas, including Hodgkin's and Burkitt's disease, reticulum cell sarcomas (Spiegelman et al., 1973; Kufe et al., 1973a, 1973b), plasma of leukaemic patients (Yaniv et al., 1973), malignant melanomas

(Birkmayer et al., 1974; Balda et al., 1975), brain tumours (Cuatico et al., 1973), idiopathic neurological disease (Viola et al., 1975) and lung and gastrointestinal carcinomas (Cuatico et al., 1974). Only in the case of leukaemias and lymphomas wasthe DNA product shown to share sequence homology with R-MuLV or RD114 RNA (Baxt et al., 1972; Kufe et al., 1973a; 1973b; Spiegelman et al., 1973). In all of these experiments, control normal cell extracts were always negative. Remarkable, however, is still the high frequency of positive results, whereas presumptive viral markers are only rarely detected with other techniques.

The relevance of the data obtained with the simultaneous detection test is discussed by Gallo and Reitz (1976). The results obtained with this test must be considered with caution with regard to human type C viruses. The above-mentioned relatively high frequency of detection also holds for reverse transcriptase. While the viral enzyme seems to be relatively easily detected, other viral structural proteins are hardly ever found.

1.3.4. Viral antigens

The detection of viral specific RNA and polypeptides cross-reacting with the major viral structural protein (p30) of baboon virus in tissues of baboons (Sherr and Todaro, 1974a) and other Old World monkeys (Sherr et al., 1974b) indicated that the endogenous virogenes can be partially expressed. Apparently, the major antigenic determinants of the p30 polypeptides found in different Old World monkey species have not undergone extensive evolutionary divergence (Sherr et al., 1974b). This has led to the search for such interspecies specific, cross-reacting antigens in human tissues. With the aid of a competition radioimmunoassay, antigens which competed with the p30 protein of M7-baboon virus were detected in two human tumours (one lymphocytic lymphoma and one ovarian carcinoma) (Sherr and Todaro, 1974b). In another study, Sherr and Todaro (1975) obtained positive results for p30 related to SiSV in partially purified extracts of peripheral blood leukocytes of 5 patients with acute leukaemia. Although control experiments (among others, competition assays with radiolabelled p30 of a murine and feline leukaemia virus and SiSV) were included in these studies, no data are available on the proteolytic activity of the tissue extracts. Proteolytic enzymes present in the tissue extracts can break down the labelled p30 polypeptides. Thus, in a competition radioimmunoassay, displacement of the labelled viral protein can be the result of proteolytic cleavage and this may lead to false positive results (Charman and Gilden, 1978). The same argument holds for the study of Strand and August (1974b), who obtained evidence for a p30 related to those of known animal type C viruses in both normal and malignant human tissues. In interspecies specific assays for antigenic determinants, they found p30 proteins competing with radiolabelled p30 of SiSV or RD114. In an immuno-

diffusion assay, immunoprecipitates were found in two of the positive tissue extracts with anti-RD114 and anti-SiSV sera.
These positive results are in contrast with those obtained by other groups also using interspecies specific competition radioimmunoassays, indicating the lack of detectable p30 antigens in human tissues (Charman et al., 1976; Stephenson and Aaronson, 1976; Charman and Gilden, 1978). Stephenson and Aaronson (1976) especially showed convincing evidence for the lack of viral p30, at least in the 130 human normal and neoplastic tissue specimens they tested. They found that p30 antigens detectable at very low levels in mammalian cells could be purified and that they retained their antigenicity. Using the same approach in some human tissues [including cells of the positive patient HL-8 of Sherr and Todaro (1975)], competition was found with SiSV p30. However, this was shown to be nonspecific. Their specificity testing included DEAE chromatography; in all suspected samples, the reactivity was dispersed throughout the column without evidence of a discrete peak. The suspected extracts were further purified by agarose-gel filtration. Following this, all positive human samples lost their reactivity under conditions where trace amounts of control labelled viral p30 could be purified.

Another technical approach for the detection of viral footprints is the use of fluorescent antibody assays with which antigens in or on single cells can be visualized. With the aid of the indirect cytoplasmic immunofluorescence test, SiSV-p30 related antigens could be detected in acetone-fixed cell cultures of human mesenchymal tumours, e.g., osteosarcoma, giant cell tumour, chondrosarcoma and chondroblastoma (Zurcher et al., 1975). Different animal and human control cell lines and animal virus-positive and negative lines were included for specificity testing of the antisera. This antiserum characterization is essential for this kind of immunological test. In another immunofluorescence study (Smith et al., 1977), human tumour cells were screened for RD114- and SiSV-p30 related antigens. In this study, a fibrosarcoma cell line was positive in the immunofluorescence assay after incubation with antiserum directed to RD114-p30 but negative with SiSV-p30 antiserum. All other lines used, e.g., chondrosarcoma, giant cell tumour and osteosarcoma cell lines, were negative with both antisera. In both studies (Zurcher et al., 1975; Smith et al., 1977), the observed fluctuation of the presumptive viral antigens with cell passage number is remarkable. In the positive chondrosarcoma line included in the first study of Zurcher et al. (1975), De Man et al. (1977) using electron microscopy and the simultaneous detection test, found evidence for replicating type C particles.

In the NZB mouse strain, a spontaneous autoimmune disease syndrome has been linked with the expression of high titres of xenotropic type C virus-virus (Levy, 1975). Although recent genetic studies (Datta et al., 1978) have raised doubts about this relationship, investigators have still sought for the presence of viral markers in tissues of human patients with systemic lupus erythematosus (SLE), a disease comparable to the murine immune complex glomerulonephritis. Lymphocytes of patients with SLE were reported to possess an antigen on their membrane that reacted with an antiserum prepared against a murine type C virus (SP104) (Lewis et al., 1974b). In spleen cells of SLE patients, on the contrary, no mRNA's with homology to cDNA of SP104 virus RNA were found (Quimby et al., 1978). Indirect immunofluorescence studies on cryostat sections of unfixed kidney and spleen of an SLE patient revealed antigens reacting with antisera to p30 of MuLV, RD114, SiSV and feline leukaemia virus (FeLV) (Mellors and Mellors, 1976). The staining observed with anti-MuLV and anti-FeLV sera can be due to interspecies specific antigenic determinants shared by the putative viral proteins and the p30 polypeptides of MuLV and FeLV. However, the endpoint titres of anti-RD114 and anti-SiSV sera were similar when measured on positive control cells and on SLE tissue, indicating the involvement of group or type specific determinants. A comparable phenomenon was observed by Strand and August (1974b), who detected relatively high concentrations of competitor proteins resembling especially RD114 and SiSV p30 in extracts of a human lupus kidney. Besides the detection of viral antigens in SLE patients, antibodies could be eluted from the glomerular immune deposit in two SLE patients (Mellors and Mellors, 1978). These antibodies reacted mainly with the p30 of RD114. Human SLE is apparently an interesting disease for further virological studies.

1.3.5. Antiviral antibodies

Evidence for antibodies to type C viruses in human sera is sparse, as would be expected from the low level, if any, of detectable antigens in human biopsies. The most commonly used method for detection of antiviral antibodies in sera is immunoprecipitation of radioactively labelled viral proteins with immunoglobulins. With such a test, Charman et al. (1974; 1975), Charman and Gilden (1978), Stephenson and Aaronson (1976) and other groups (personal communication) found no proof for immunoglobulins in hundreds of human sera reacting with murine and primate type C viral structural proteins. On the other hand, Snyder et al. (1976) and Kurth et al. (1977) independently reported the presence of naturally occurring antibodies in human sera that reacted with BaEV and SiSV in the radioimmunoprecipitation assay. Snyder et al. (1976) found in human sera of healthy individuals and patients with neoplastic diseases relatively high titres of precipitating antibodies to labelled intact SiSV. This reactivity could be partly absorbed with fetal calf serum and absorption with the major glycoprotein (gp70) of SiSV completely abolished the precipitating capacity of the human sera. On further study, Snyder and Fox (1978) detected a minor component (gp55) in fetal calf serum which binds to virus in culture fluids. Most likely, human sera possess natural antibodies to this calf

serum component. Thus, the precipitation activity in human sera may be defined in terms of two specificities: one present in a 70,000 dalton fraction of SiSV, the other in a minor component of fetal calf serum. There is, however, an inconsistency in the study of Snyder et al. (1976): incubation of human high titre sera with iodinated gp70 of SiSV did not result in precipitation above background levels. It has been argued that antigenicity of the viral proteins is lost during the purification and labelling process. This is not very likely, since antiviral antibodies can readily be detected in natural or experimental animal leukaemia models using the same technique (Charman et al., 1975). In fact, any reasonable explanation for the above mentioned discrepancy is lacking at the moment.
Kurth et al. (1977) adapted the radioimmunoprecipitation assay for the detection of antibodies of "low avidity". In this test, sera of normal individuals exhibited precipitating activity for viral proteins of both SiSV and BaEV. During pregnancy an increase in titre of antibodies reacting with BaEV was observed (Hirsch et al., 1978). In the same women, a cell-mediated immunity against BaEV-infected cells was also found to be selectively expressed during pregnancy. Thiry et al. (1978) also observed a cell-mediated immunity to BaEV and Mason-Pfizer monkey virus during pregnancy.

Another approach is to look for antibodies directed against viral reverse transcriptase. Most investigators have not found anti-reverse-transcriptase activity in human sera. However, one group reported that immunoglobulins purified from plasma of 2 AML patients inhibited the *in vitro* reverse transcriptase reactions catalysed by the enzymes of both SiSV and BaEV (Prochownik and Kirsten, 1976). It has also been reported that the surface of some fresh human leukaemic blood cells contains IgG's reactive with certain viral polymerases (Jacquemin et al., 1978).

1.3.6. Viral RNA and DNA

Viral-related nucleotide sequences in RNA and DNA from human tumour cells have been found by the use of ^{3}H-cDNA and ^{125}I-RNA probes from mammalian type C viruses (for critical reviews on this subject, see Gallo and Reitz, 1976; Gallagher, 1977). Complementary DNA from R-MuLV hybridized with RNA extracted from polysomal fractions obtained from human leukaemias and lymphomas (Hehlmann et al., 1972a; 1972b) and sarcomas (Kufe et al., 1972). Cellular RNA from normal control cells did not show significant annealing to the R-MuLV probe. Employing more stringent hybridization conditions and a better characterized probe Larsen et al. (1975) detected viral-related sequences in a few leukaemic patients. Cellular RNA was enriched for polyadenylated messengers and showed a significant amount of hybridization with a cDNA probe from Moloney murine leukaemia virus.

Although the first experiments detecting RNA related to R-MuLV are disputable (low hybridization counts, lack of hybridization kinetics, poorly characterized and unrepresentative probes), Aulakh and Gallo (1977) using "recycled" cDNA from R-MuLV, found proviral sequences distantly related to the murine virus in DNA from spleens of patients with lymphoma and leukaemia. Such sequences were absent in DNA from apparently uninvolved tissues of the same patients. Proviral sequences related or identical to SiSV have never been found in DNA obtained from normal individuals or leukaemic patients (Benveniste et al., 1974b; Scolnick et al., 1974; Reitz et al., 1976; Wong-Staal et al., 1976). Nevertheless, RNA extracted from previously mentioned cytoplasmic particulate 1.16 fractions of two leukaemic patients was shown to be hybridizable to an SiSV probe (Gallo et al., 1973). However, these results and those of other investigators (Hehlmann et al., 1972a; 1972b; Kufe et al., 1972; Larsen et al., 1975) detecting cellular RNA related to viral cDNA are most likely not specific for leukaemic cells, as discussed elsewhere (Gallo and Reitz, 1976; Gallagher, 1977). When high concentrations of cellular RNA are used in these kinds of studies, an SiSV cDNA probe, for instance, can also hybridize significantly to RNA from normal phytohaemagglutinin-stimulated lymphocytes. However, most investigators used fresh unstimulated leukocytes as the normal control.

Proviral sequences related to BaEV can be found in tumorous tissues from a number of leukaemic patients (Reitz et al., 1976; Wong-Staal et al., 1976). Normal human cell DNA does not contain such genetic information. In these experiments, representative iodinated viral RNA was used as a probe. The results show that an extensive amount (maximally 70 %) of BaEV genome is present in the leukaemic cell DNA and that the hybrids have a high thermal stability, indicating a low degree of base pair mismatching.

1.3.7. Isolation of putative human type C viruses

An alternative to looking for viral markers in fresh or frozen human biopsies is the use of cultures of human tumour material. Indeed, many reports have described the detection of viral-like particles in cultured cells of patients with malignant haemopoietic disorders (Kotler et al., 1973; 1975; Shubladze et al., 1974; Vosika et al., 1975; Weimann et al., 1975a; 1975b; Klucis et al., 1976; Sawada et al., 1977). Criteria for the definition "viral-like" have been high-molecular weight RNA, reverse transcriptase, buoyant density and electron microscopic morphology, which, in many instances, is disputable. In most studies, these viral-like particles have not been shown to be infectious for secondary cells and characterization by means of immunological and nucleic acid hybridization techniques is lacking. It is obvious that the validity of these results remains to be established. Nevertheless, a number of more detailed studies reporting the detection and/or isolation of type C viruses from human biopsy cultures have been presented. Mak et al. (1974a) used short-term suspension cultures of bone marrow of patients with leukaemia in relapse and in remission. The bone marrow was stimulated for proliferation in vitro by the addition of human leukocyte conditioned medium (Aye et al., 1972). An increase in both intracellular and extracellular reverse transcriptase was found following culture. The reverse transcriptase activity was associated with viral-like particles with characteristic retrovirus buoyant densities (Mak et al., 1974b). The enzyme, however, could not use the synthetic template-primer complex polyriboadenylic acid.oligodeoxythymidylic acid, a combination which can be readily used by all known retrovirus reverse transcriptases (for reviews on the utilization of synthetic template-primer combinations by cellular and viral polymerases, see Bollum, 1975; Gallo et al., 1975). In a further study, Mak et al. (1975) detected high-molecular weight RNA associated with reverse transcriptase in particles released in the medium of leukaemic bone marrow cultures. The base sequences of the RNA in the particles was characterized by synthesizing cDNA in an endogenous reaction. The cDNA showed about 50 % homology with SiSV RNA and much less with MuLV RNA. The same has been found by others (Gallo et al., 1973). It is not very likely that these particles represent replicating biologically active virions.

Priori et al. (1971) reported on the establishment of a human lymphoma cell line (ESP-1) which produced type C virus. This virus, originally thought to be of human origin, was shown to be closely related to murine leukaemia viruses. Murine type C virus group-specific antigens were detected in ESP-1 cells (Gilden et al., 1971) and reverse transcriptase found in the culture could be inhibited by antiserum raised against the enzyme of murine type C virus (Scolnick et al., 1972).

Another presumptive human type C virus (RD114 virus) (McAllister et al., 1972) appeared to be an endogenous domestic cat virus, as has been discussed previously (Chapter I, 1.2).

A permanently established human malignant lymphoma cell line has been reported to produce type C virus (Kaplan et al., 1977). Attempts to infect secondary cells with this isolate have failed so far. The main characterization of the virions was done by reverse transcriptase inhibition tests. The isolated viral enzyme could be partially neutralized by antibodies to the reverse transcriptases of both RD114 virus and SiSV. However, the results presented raise some doubt about the specificity of the anti-reverse transcriptase antisera used in this study. The RD114 reverse transcriptase antiserum completely inhibited the homologous RD114 virus enzyme as well as polymerases of SiSV and GALV. Such a cross-neutralization of polymerases of the RD114-BaEV virus group and the SiSV-GALV group has never been observed before (Sherr et al., 1974a).

Typical budding and mature type C viruses were found in cocultures of rat cells with lung tissue of a patient with both pulmonary adenocarcinoma and chronic lymphocytic leukaemia (Gabelman et al., 1975). The particles isolated from tissue culture fluids had the biochemical and biophysical characteristics of mammalian type C viruses; the major internal polypeptide (p30) was serologically related to the homologous polypeptide of SiSV and GALV and, according to nucleic acid hybridization studies, the virus was highly related to SiSV. It was infectious only for rat cells and induced plaques in the so-called KC test (for a review on the KC test, see Chapter III, 3.1.). This test is specific for viruses of the RD114-BaEV group, while the isolated virus was serologically related to SiSV.

A human embryonic lung fibroblast strain (HEL-12) spontaneously released type C virus after about 6 months in culture (Panem et al., 1975). The virus was immunologically related to both SiSV (for p30 polypeptide and viral polymerase) and RD114 virus (only tested for p30) (Panem et al., 1977). It was infectious for cells of homologous and heterologous species. Early passages of HEL-12 cells contained HEL-12 proviral DNA sequences and RNA from virus-producing HEL-12 cells hybridized substantially to SiSV cDNA but not to BaEV cDNA (Prochownik and Kirsten, 1977). These data suggest the existence of two different primate viruses produced by HEL-12 cells: one related to the SiSV-GALV group and the other to the RD114-BaEV group, with the former virus in great excess. Other experiments of this group suggest that complementary DNA of the HEL-12 viruses, although related to SiSV and BaEV, is a better probe than these primate viruses for detecting viral footprints in humans. HEL-12 virus related sequences were found in the DNA of two cancer patients: one with osteogenic sarcoma and one with acute myeloid leukaemia (Prochownik and Kirsten, 1977). Some patients with myeloid leukaemia have circulating immunoglobulins which specifically inhibit the reverse transcriptases of HEL-12 virus (Prochownik and Kirsten, 1976). In addition, type C virus specific glomerular immune complexes were detected with antiserum directed against HEL-12 virus in patients with SLE (Panem et al., 1976). By use of the immunofluorescence assay on cryostat sections, HEL-12 virus related antigens were found in 24 of 24 human placentas (Sawyer et al., 1978). The immuno fluorescence reaction could be absorbed out with purified HEL-12 virus but not completely with SiSV or BaEV.

The best characterized and most convincing type C viral isolate obtained from human tumour material is the so-called HL-23 virus. Leukaemic blood leukocytes from a patient (HL-23) with acute myeloid leukaemia were initiated and sustained for proliferation and differentiation _in vitro_ by the addition of medium conditioned by a particular whole human embryo cell strain (Gallagher and Gallo, 1975). The expression of a complete type C virus is apparently not related to the initiated proliferation and differentiation, since other leukaemic cell cultures did not release virus under identical growth conditions (Gallagher et al., 1975). This virus was infectious for secondary cells and showed the _in vitro_ host range of SiSV (Teich et al., 1975).

In further studies, the existence of two different viruses in the isolate was revealed. By immunological (type-specific radioimmunoassay of p12 and p15 polypeptides) and molecular hybridization (thermal stability) criteria, the viruses were found to be completely identical to, respectively, SiSV and M7-BaEV (Okabe et al., 1976; Chan et al., 1976).

It was originally thought (Gallagher and Gallo, 1975) that the induced differentiation of the HL-23 acute myeloid leukaemic cells in suspension culture led to virus production. However, many other leukaemic suspension cultures, also stimulated to proliferation and differentiation with medium conditioned by human embryonic fibroblasts, showed no evidence of virus activation (Gallagher et al., 1975).

The picture that emerges from this review on primate and human type C viruses is complex (Table 1). At least in normal cell cultures from different primate species of Old and New World origin, endogenous type C viruses of unknown oncogenicity can be activated. The naturally occurring exogenous primate, woolly monkey and gibbon ape viruses are oncogenic for gibbons and other experimental animals. As far as the higher apes and man are concerned there is no direct evidence for the existence of endogenous viruses. In man, in particular, primate viruses have served as probes for the detection of human viruses. Viral footprints are inconsistently found in man. Nevertheless, the isolation of putative human viruses related to the woolly monkey virus or the baboon virus has been reported several times by different groups. Both the woolly monkey and the baboon virus are exogenous for man, suggesting that these viruses are spread horizontally in the human population. Certainly, much further study is needed to elucidate the possible role of type C RNA tumour viruses in the etiology of human leukaemia.

1.4. OBJECTIVE OF THE PRESENT STUDY

The discoveries of Vilhelm Ellerman and Oluf Bang (1909) and Peyton Rous (1911) of the viral etiology of chicken leukaemias and sarcomas in the beginning of this century gave new directions to cancer research in general. But it was not until the nineteen-fifties through the work of John Bittner (1942) and Ludwik Gross (1951) that the virus theory of cancer became more popular. A viral etiology of human cancer has been subsequently postulated. Some recent findings suggest the existence of type C retroviruses in man.

The objective of the present work was to search for an etiological role of retroviruses in human leukaemia. If such a role of type C viruses in the development of human leukaemias can indeed be found, this will certainly have prophylactic and therapeutic consequences. Immune prevention of viral induced neoplastic diseases has been extensively described for both RNA and DNA tumour viruses. For example, passive immunization can lead to prevention of herpes virus induced malignant lymphoma in monkeys (Laufs and Steinke, 1975). A herpes virus in chickens causes a lymphoproliferative disease which can be controlled by vaccination with an antigenically related herpes virus of turkeys (Biggs, 1975). A therapeutic effect has been achieved by passive immunization of Friend murine leukaemia virus (F-MuLV) infected mice (Hunsmann et al., 1975). Vaccination of mice with isolated viral proteins can lead to resistance to a subsequent challenge with infectious F-MuLV (Schäfer et al., 1976).

Different approaches can be used in studies on the detection of human leukaemia viruses, e.g., searching for complete virus and viral footprints in fresh human biopsies or employing different culture techniques with human tumour material and subsequent screening for virus production. We chose the technique of cocultivation as used by the group of Todaro for the detection of primate viruses. Our cocultivation studies finally led to the isolation of a presumed human type C virus which appeared to be oncogenic for experimental animals.

CHAPTER II

EXPERIMENTAL PROCEDURES

2.1. CELL CULTURES

All cells were grown in Dulbecco's modified Eagle's minimal essential medium (Flow Laboratories, Irving, Scotland) supplemented with 10 % (vol/vol) heat-inactivated (40 min at 56° C) fetal calf serum (Flow) and with-without antibiotics. When cells had grown to monolayers at 37° C in 75 cm^2 plastic tissue culture flasks (Flow), they were removed from the flasks by trypsinization [0.25 % trypsin (wt/wt) in Eagle's balanced salt solution]. The cell suspensions were spun for 10 min at 200 x g. The pellets were resuspended in medium and seeded into new flasks.

The cell lines used in our studies are listed in Table 2. Continuous lines of bat, dog, human, marsupial, mink, mouse, and rabbit origin were used. Primary cultures of whole BALB/c mouse embryos, whole WAG/Rij rat embryos, whole human embryos, human embryonic kidney and adult human kidney were prepared in our own laboratory according to the techniques described by Paul (1970). Cell cultures of human, mouse and rat origin productively infected with the woolly monkey (simian) sarcoma-leukaemia virus (SiSV) or the Rauscher murine leukaemia virus (R-MuLV) were used. Two viral-transformed nonproducer rat cell lines were also included: the XC and K-NRK lines.

Karyotypic and isoenzyme analyses of the cultures (kindly performed by Dr. P. Meera-Khan, Anthropogenetic Institute, University of Leiden, Leiden, The Netherlands) were repeatedly performed in order to ascertain the species origin of the lines used. Tests for mycoplasma (cultures and electron microscopy) were always negative.

In the cocultivation experiments described in Chapters III and IV, human nucleated bone marrow cells (10^6 - 10^7) were cultured on top of an exponentially growing layer of indicator cells in 75 cm^2 tissue culture flasks. When the monolayer had grown to confluency the bone marrow cells growing in suspension were removed, spun down gently (100 x g) and plated together with the trypsinized indicator cells. The replating procedure for the bone marrow suspension was carried out with the first 3 cocultivation passages. After that time, bone marrow cells were no longer detected in the cultures by light microscopy and karyotypic analysis.

To avoid contamination with viruses and cells, the lines were handled in 4 different tissue culture cabinets in 3 separate buildings. All uninfected cell lines and the rat nonproducer lines were handled in a laminar flow tissue culture cabinet located in the Radiobiological Institute TNO. In the same building the cocultivation experiments were carried out in a biohazard cabinet. The Rauscher virus infected cells were handled in a

Table 2

ANIMAL CELL LINES

Name of cell line	Species	ATCC No.[a]	Reference
TblLu (NBL-12), adult lung	BAT	CCL 88	-
A7573, fetal thymus	DOG	-	Nelson-Rees et al., 1976
A204, rhabdomyosarcoma	HUMAN	-	Giard et al., 1973.
FB289, whole embryo	HUMAN	-	-
HEK, embryonic kidney	HUMAN	-	-
HK, adult kidney	HUMAN	-	-
NC37, lymphoid	HUMAN	-	Reedman and Klein, 1973
PtK 1 (NBL-3), adult kidney	MARSUPIAL	CCL 35	-
Mvl Lu (NBL-7), fetal lung	MINK	CCL 64	Henderson et al., 1974
BALB/3T3, embryo	MOUSE	-	Aaronson and Todaro, 1968
MEF, whole embryo	MOUSE	-	-
SIRC, cornea	RABBIT	CCL 60	Leerhoy, 1965
REF, whole embryo	RAT	-	-
Virus-infected			
NC37 + SiSV, NC37 infected with simian sarcoma-leukaemia virus	HUMAN	-	-
BALB/3T3 + R-MuLV, 3T3 infected with Rauscher murine leukaemia virus	MOUSE	-	-
REF + SiSV, REF infected with simian sarcoma-leukaemia virus	RAT	-	-
XC, sarcoma induced by Rous sarcoma virus	RAT	CCL 165	Svoboda, 1960
K-NRK, normal kidney cells transformed by Kirsten strain of mouse sarcoma virus	RAT	-	Klement et al., 1971

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[a] American Type Culture Collection (ATCC), Catalogue of Strain II, 1975, Rockville, Maryland, USA.

biohazard cabinet in a separate building of the Radiobiological Institute. The woolly monkey virus producing lines were cultured in a biohazard tissue culture cabinet in the herpesvirus laboratory of the Primate Center TNO.

2.2. VIRUS TRANSMISSION IN VITRO

Type C retroviruses can be transmitted in vitro by simple incubation of cell monolayers with virus-containing preparations. When supernatants of virus-producing cultures were used in our experiments, the fluids had to be clarified by centrifugation (15 min at 10,000 x g) and then filtered through a millipore filter (0.45 µm pore size) (Millipore, Benelux, Brussels, Belgium). Virus production and subsequent spread within the culture is dependent on cell proliferation (Humphries and Temin, 1972; Fischinger et al., 1975; Paskind et al., 1975; Varmus et al., 1977); hence, it is important to use exponentially growing cells for infection. To increase the efficiency of virus transmission, the cells were treated with the polycation diethylamino ethyldextran (DEAE-dextran) and virus dilutions were made with polybrene (1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide) (Sigma Chemical Company, St. Louis, Missouri, USA). These chemicals most likely cause an increased surface affinity for virus and an increased fluidity of the cell membrane (Toyoshima and Vogt, 1969). For example, focus formation by mouse sarcoma virus and the plating efficiency of mouse leukaemia virus can be enhanced by pretreating the cells with DEAE-dextran (Somers and Kirsten, 1968; Rowe et al., 1970).

In our experiments, the following procedure was employed for virus transmission. Noninfected cells were plated (10^5 per ml) and, after 24 h, the culture fluid was removed and the cells were treated with 25 µg of DEAE-dextran (Pharmacia, Uppsala, Sweden) per ml for 30 min at 37° C. The dextran was then pipetted off and the culture was infected with a virus-containing preparation (0.5 ml per 75 cm^2 culture flask and 0.2 ml per 60 mm petri dish). Serial dilutions of virus preparations were made in ice-cold medium without serum and with 4 µg of polybrene added per ml. After adsorption for 2 h at 37° C, unattached virus was removed and fresh medium was added. The medium was changed every 2 days.

2.3. BIOLOGICAL ASSAYS FOR TYPE C RETROVIRUSES

Two biological test systems were used in the experiments for the calculation of virus titres of tissue culture fluids and the quantification of virus production by virus-infected cells: 1) the XC cell plaque assay, useful in titrating certain kinds of type C "leukaemia" viruses; and 2) the focus formation technique used in determining sarcoma virus titres in tissue culture fluids.

2.3.1. XC cell plaque assay

Leukaemia viruses can replicate in tissue culture but do not produce cytopathologic lesions *in vitro*. There are, however, exceptions on this rule due to the peculiarity of certain cell lines, e.g., the XC cell line. Klement et al. (1969) found that the XC cell line, which stems from rat tumour cells originally induced by the Prague strain of Rous sarcoma virus, forms syncytia when in contact with cells producing murine leukaemia viruses. The viruses produce focal areas of infection consisting of multinucleated giant cell syncytia. This observation led to the development of a plaque assay (Rowe et al., 1970) for type C retroviruses, called the XC cell cytopathogenicity assay (for detailed information on the XC cell test and its application, see Chapter III, 3.1). The experimental procedure for this assay as described by Rowe is as follows (Fig. 2A): Cells permissive for replication of the leukaemia virus are seeded (2×10^5 cells per petri dish) and the following day virus samples are inoculated onto the subconfluent cultures. Before inoculation, the cultures are exposed to 25 µg per ml DEAE-dextran for 30 min. The cell layers are irradiated (2000 rad) four days after inoculation to prevent further growth and 2×10^5 XC cells are added per dish. Four days later, the cultures are fixed in methanol and stained with May-Grünwald. The syncytia are visible chiefly because of the failure of the XC cells to infiltrate the cell sheet in the infected areas. In permissive cells, a linear dose-response relationship is usually found between virus concentration and syncytium count (one-hit kinetics). All our experiments were done in quadruplicate. Petri dishes were coded and syncytia counted independently by two persons using an inverted microscope at a magnification of 75x. Results were expressed as plaque forming units per ml tissue culture fluid. The infectivity of the virus stock (endpoint titre) was calculated from the number of plaques (syncytia) found at different dilutions.

Niwa et al. (1973) developed the so-called reverse XC test, in which the plating sequence of the cells is reversed (Fig. 2B). First, XC cells are plated and, one day later, virus-infected cells are seeded on top of the XC cells. Syncytia are counted 4 days later. It is claimed that the reversed version of the XC test is more sensitive than the classical one for assaying leukaemia viruses. In the experiments described in Chapter III, a modification of the technique of Niwa is employed when assays are done with bone marrow of virus-infected mice (Fig. 2C).

2.3.2. Sarcoma virus genome rescue technique and focus formation

Murine sarcoma viruses (MSV) transform cells of various species. Stocks of transforming MSV always contain helper transformation-defective viruses which compensate for the incapacity of the transforming viruses to replicate by phenotypic mixing. Quantitative assays based on the defectiveness of the MSV genome have been developed for leukaemia viruses

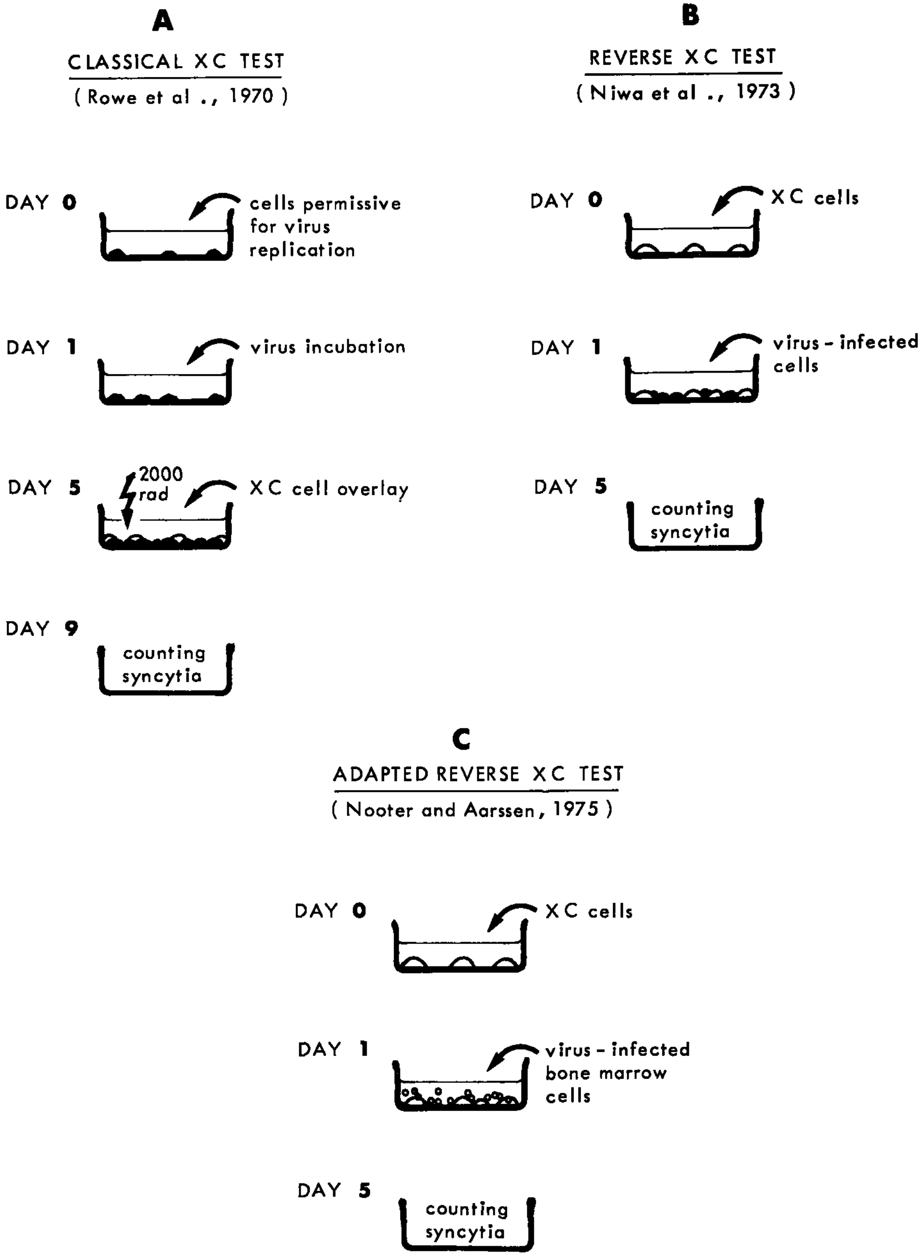

Figure 2: Schematic representation of the various XC tests.

(Hartley and Rowe, 1966; Bassin et al., 1971a; Fischinger et al., 1974) (for detailed information on the MSV genome, see Chapter V, 5.1.). In our studies, MSV-transformed rat nonproducer (NP) cells were applied in order to detect human type C "helper" viruses. The Kirsten strain of MSV transformed rat NP cell line (designated as K-NRK) used in Chapter V is described elsewhere (Aaronson and Weaver, 1971; Klement et al., 1971).

MSV genome rescue experiments were carried out by cocultivation of equal numbers of rat NP (K-NRK) cells with presumed helper virus producing cell lines. The amount of rescued MSV was titrated by the focus assay of Hartley and Rowe (1966). Supernatants of the cultures taken from the 8th day were clarified by centrifuging for 15 min at 10,000 x g and then filtering through a millipore filter (0.45 µm pore size). These were then assayed for transformation (0.2 ml virus medium per dish) on mouse, rat, rabbit and human cells. Cultures for focus formation were prepared by inoculation of 2 x 10^5 target cells per 60 mm petri dish. On the next day, these cultures were treated with DEAE-dextran (25 µg per ml for 30 min) and inoculated with dilutions of the supernatant to be tested. Eight days after infection, foci of transformed cells were counted unstained with the aid of an inverted microscope (75 x). Results were expressed as focus forming units per ml tissue culture fluid. The infectivity of the virus stock (endpoint titre) was calculated from the number of foci found at different dilutions. Tissue culture fluids that could not be assayed immediately were stored at -70° C.

2.4. SEROLOGICAL DETECTION AND CHARACTERIZATION OF TYPE C RETROVIRUSES

The antigens of type C viruses can be divided into type, group and interspecies specific ones (Schäfer et al., 1975). Due to this phenomenon, it is possible to assess, besides the detection of virus, also the serological relationship between different viruses. Two fluorescent antibody techniques were used in our studies: 1) the indirect immunofluorescence assay on acetone-fixed cells for the detection of cytoplasmic viral antigens; and 2) the Sepharose bead immunofluorescence assay, which is comparable with a solid phase radioimmunoassay. This technique is useful for the detection of soluble antigens in e.g., tissue culture fluids or animal sera but can also be applied to the detection of antibodies (for detailed information on the above mentioned techniques, see Haaijman, 1977).

2.4.1. Cytoplasmic indirect immunofluorescence assay

In this assay, the preparation is first incubated with an unlabelled antiserum directed against the antigen of interest (Fig. 3). The binding of the unlabelled antibody is demonstrated after a second incubation with a fluorescent antiserum directed against the immunoglobulin (Ig) molecules

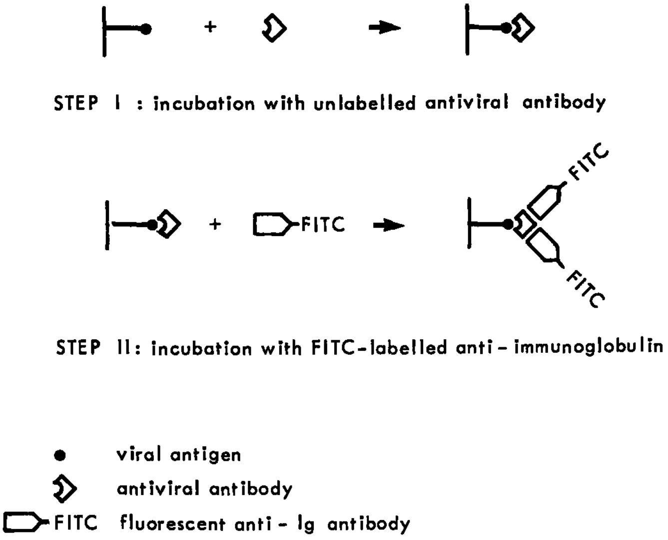

Figure 3: Schematic representation of the indirect method in immunofluorescence microscopy. The indirect technique requires two incubation steps.

of the animal species in which the unlabelled antibody was raised. For the cytoplasmic indirect immunofluorescence assay (IFA), 50 µl of a suspension of freshly trypsinized cells (10^5 per ml) were transferred into wells of Teflon coated microscope slides. The cells were allowed to grow at 37° C for 20 h in humidified CO_2 incubator. They were then fixed in cold acetone and stored at -20° C until use. Ten spots on these slides were used for incubation. Seven of the wells were filled with 2-fold dilutions (starting with 1 : 10) of the antiviral serum in phosphate-buffered saline (PBS), one of the wells with the appropriate normal serum and 2 wells with PBS only. After the first incubation (30 min at 37° C), the slides were washed four times with PBS and subjected to a second incubation period (30 min at 37° C) with a fluorescein isothiocyanate (FITC) conjugated goat antiserum to Ig of the species used for the production of the antiviral antisera (Nordic Immunological Laboratories, Tilburg, The Netherlands) (dilution 1 : 20). One of the PBS filled wells was again filled with PBS. After being washed, the cells were covered with Elvanol and a cover glass. The slides were examined for cytoplasmic granular fluorescence with a Leitz Orthoplan microscope using an FL 40x/1.30 objective and GW 4x oculars. Epiilumination was accomplished with a mercury arc HBO 100W/2. The filters used were 2x KP490 + 1 mm GG455 and K515.

Results are expressed as endpoint titres, representing the reciprocal of the highest antiserum dilution in which virus-specific cytoplasmic fluorescence was still observed. Positive and negative control cells were included in each IFA and double blind scoring of the slides was carried out by two independent investigators.
Microphotographs were taken with an Orthomat camera on Ilford HP 4 film.

2.4.2. Sepharose bead immunofluorescence assay

The method as developed for the quantification of immunoglobulins (Haaijman and Brinkhof, 1977) was adapted for the detection of viral antigens and antiviral antibodies (Koch et al., 1977).

2.4.2.1. Detection of viral antigens and antiviral antibodies

Fifty microliters of a 1% suspension of Sepharose 4B beads (Pharmacia, Uppsala, Sweden) (1 ml bead pack to 100 ml of PBS) coupled with IgG fractions of antisera (for coupling technique, see below) were incubated for 1 h at 21° C with 50 µl of a test sample diluted in PBS and containing 2 % bovine hemoglobin in flat bottom microtitration plates under continuous agitation (Fig. 4). After repeated washing with PBS, the beads were incubated with antisera conjugated with FITC. After again washing, the individual bead fluorescence was measured with a microfluorometer. At least 5 beads were measured per sample dilution. For the experiments described in Chapter V, a calibration curve was prepared by incubation of the beads with different concentrations of purified SiSV or R-MuLV, which were disrupted by incubation with 0.3 % Nonidet P-40 for 1 h. The amount of viral protein was determined by the method of Lowry et al. (1951). Linear regression analysis was applied to fluorescence values falling in the concentration-dependent range.

For the detection of antiviral antibodies in hyperimmune sera or sera of expermental animals beads coupled with disrupted virus were incubated with IgG fractions of the test sera diluted in PBS (Fig. 4). After washing the beads were incubated with goat antiserum to rabbit or rat Ig conjugated with FITC. After again washing, the individual bead fluorescence was measured.

2.4.2.2. Coupling of antibodies or antigens to Sepharose beads.

IgG fractions of antiviral antisera, disrupted (0.3 % Nonidet P-40) purified virus preparations or fetal calf serum were coupled covalently to Sepharose 4B beads by the cyanogen bromide method of March et al. (1974), using 2-3 mg protein per ml of activated beads. Residual active groups were deactivated by treatment of the beads with 0.5 M ethanolamine in 2 % sodium hydrogen carbonate, pH 9.5, for 6 h. After coupling and deactivation, the beads were washed and stored in PBS containing 0.01 % merthiolate at 4° C.

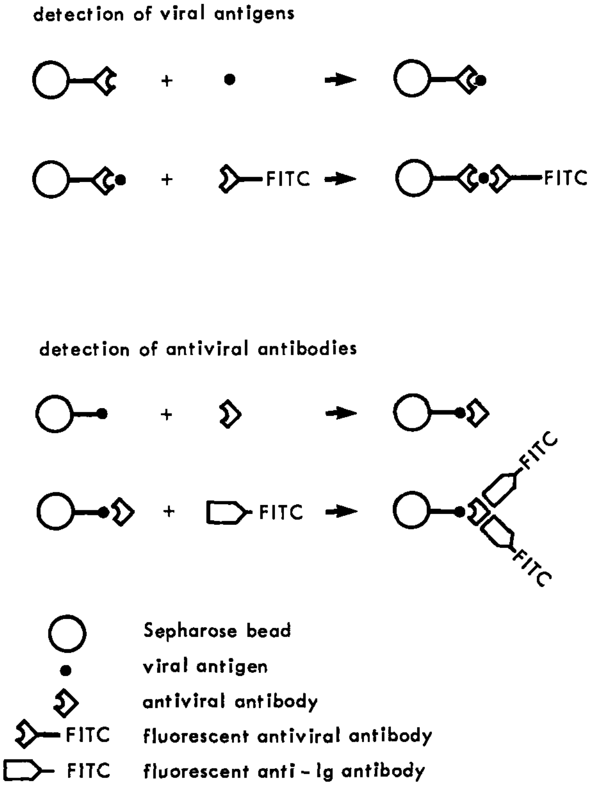

Figure 4: Schematic representation of the Sepharose bead immunofluorescence assay for the detection of viral antigens and antiviral antibodies.

2.4.3. Antisera

The antiviral antisera used are listed in Table 3. Antisera were subjected to chromatography on QAE-Sephadex A50 (Pharmacia) in ethylenediamineacetate (pH 8.0; I: 0.1) (Joustra and Lundgren, 1970). The isolated IgG fractions were dialyzed against PBS and stored at -20° C in 1 ml aliquots at a final protein concentration of 10 mg per ml.

2.4.3.1. Absorption of antisera

Anti-fetal calf serum activity was removed from the antisera by affinity chromatography. IgG fractions of antisera were placed on a column consisting of Sepharose 4B beads coupled with fetal calf serum according to the technique of March et al. (1974) and recollected. Thereafter, residual activity against fetal calf serum and retroviruses was measured in the Sepharose bead assay.

Table 3

ANTIVIRAL ANTISERA

Name of antiserum	Prepared in	Against	Endpoint titre[a]	Assayed on	Reference
GA-EMuLV	Goat	Purified endogenous ecotropic mouse type C virus	2560	mouse BALB/3T3 cells producing Rauscher virus	Nooter et al., 1975
Ra-R-MuLV-45	Rat	Syngeneic lymphoma cells induced by Rauscher murine leukaemia virus	2560		Zurcher et al., 1975
Ra-R-MuLV-76	Rat		5120		Ibid.
RA-R-MuLV	Rabbit	Purified Rauscher murine leukaemia virus	320		Zurcher et al., 1978
RA-R-MuLV-p30	Rabbit	p30 polypeptides of Rauscher murine leukaemia virus[b]	320		-
GA-SiSV	Goat	Autologous skin fibroblasts transformed by simian sarcoma virus[c]	160	rat REF cells producing simian sarcoma-leukaemia virus	-
RA-SiSV	Rabbit	Purified simian sarcoma-leukaemia virus[d]	320		-
GA1-SiSV-p30	Goat	p30 polypeptides of simian sarcoma-leukaemia virus[e]	320[f]		Strand and August, 1973
GA2-SiSV-p30	Goat	p30 polypeptides of simian sarcoma-leukaemia virus[g]	-		-
RA-SiSV-p30	Rabbit	p30 polypeptides of simian sarcoma-leukaemia virus[b]	320		Zurcher et al., 1978

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[a] Immunofluorescence endpoint titres were determined as described under 2.4.1. The titres given here are mean titres; often titres one log lower or higher are observed. Specificity testing of the antisera was included in each experiment and the results are shown in the respective Tables in Chapters III, IV, V and VI.

[b] The purified Rauscher murine leukaemia virus and simian sarcoma-leukaemia virus preparations used for p30 isolation were obtained through the Office of Program Resources and Logistics, Viral Oncology, NIH, Bethesda, Maryland, USA

[c] The antiserum was kindly provided by Dr. C. Bergholz, Rush-Presbyterian-St. Lukes Medical Centre, Chicago, Illinois, USA, and was reported to have a neutralizing activity for simian sarcoma-leukaemia virus (50 % inhibition of focus formation) at 1 : 512.

[d] The antiserum was kindly provided by Dr. H. Daams, The Netherlands Cancer Institute, Amsterdam, The Netherlands and was reported to have an endpoint titre of 320.

[e] The antiserum was kindly provided by Dr. M. Strand, formerly of Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York, USA.

[f] Endpoint titre was assayed on marmoset cells producing simian sarcoma-leukaemia virus.

[g] The antiserum was obtained through the Office of Program Resources and Logistics, Viral Oncology, NIH, Bethesda, Maryland, USA.

In the experiments in Chapter IV, the viral origin of the antigens detected in the immunofluorescence assay was demonstrated by specific absorption. An 0.4 ml aliquot of an IgG fraction of the RA-SiSV-p30 antiserum was absorbed (60 min at 37° C followed by 60 min at 4° C and thereafter ultracentrifugation) with either purified SiSV (1.5 ml SiSV, grown in rat cells; 0.4 mg protein per ml) or purified tissue culture derived mouse mammary tumour virus (MuMTV) (2 ml MuMTV; 0.28 mg protein per ml). The absorbed sera were tested for residual anti-SiSV activity by use of the IFA.

2.5. ENZYMATIC ASSAY FOR TYPE C RETROVIRUSES

In 1964, Temin postulated the once controversial provirus hypothesis which states that RNA tumour viruses replicate through a DNA intermediate. Essential for this theory appeared to be the existence of an RNA directed DNA polymerase. It is now known that all RNA tumour viruses contain an enzyme that preferentially transcribes RNA into DNA and which is therefore called reverse transcriptase. This enzyme is located within the viral core, which is surrounded by an envelope containing lipids and proteins. For full DNA polymerase activity in vitro, the envelope must be disrupted. The enzyme can then be assayed for by using either a synthetic template-primer complex or the virion RNA strand as a natural template.

2.5.1. Synthetic template-primer assay

Two synthetic polynucleotides were used in each experiment: oligo dT(12-18)-poly rA and oligo dT(12-18)-poly dA (Temin and Baltimore, 1972). Reverse transcriptase preferentially uses the RNA template (poly rA) as compared to the synthetic DNA template (poly dA) (Goodman and Spiegelman, 1971). Reverse transcriptase determination in tissue culture medium involves the following steps. Virus particles are spun down at high-speed centrifugation and are thereafter disrupted by Nonidet P-40 at the appropriate pH and in the presence of a "reducing agent" (dithiotreitol) as enzyme stabilizer. The enzyme reaction is carried out in a salt mixture, supplemented with labelled triphosphate and a primer-template.

Twenty-five ml of tissue culture medium were clarified by centrifuging for 15 min at 10,000 x g. The supernatant was layered on 10 ml of 20 % (wt/wt) sucrose in 10 mM Tris, pH 7.4, in a Beckman SW27 centrifuge tube and centrifuged at 100,000 x g for 2 h at 4° C. After resuspending the pellets in 50 µl of 50 mM Tris, pH 7.4, supplemented with 1 mM dithiotreitol (Calbiochem, San Diego, California, USA) and 0.1 % (vol/vol) Nonidet P-40 and incubating at 0° C for 15 min, 20 µl aliquots were incubated at 37° C for 30 min in a final volume of 100 µl containing 50 mM Tris, pH 7.4, 1 mM dithiotreitol, 5 mM $MnCl_2$, 1.2 mM $MgCl_2$, 70 mM KCl, 0.02 % Nonidet P-40, 12 µCi ^{3}H-thymidine triphosphate (TTP) (spec. act. 15 Ci/mMol)

(RadioChemical Centre, Amersham, England), 32 µM dTTP and 4 µg oligo dT (12-18)-poly rA or oligo dT(12-18)-poly dA (Sigma). Poly dT synthesis was terminated by the addition of 1 ml 25 % (wt/wt) trichloroacetic acid containing 0.05 M sodium pyrophosphate and 50 µg yeast RNA as carrier. Acid insoluble radioactivity was collected on millipore filters and washed several times with 5 % trichloroacetic acid. The filters were dried and counted in a liquid scintillation device in counting solution.

The results are expressed as counts per minute (cpm) ^{3}H-thymidine monophosphate incorporated per ml tissue culture fluid. In all Tables, corrections for background cpm have been made.

2.6. ELECTRONMICROSCOPIC DETECTION AND CHARACTERIZATION OF TYPE C-RETRO VIRUSES

Three electron microscopic techniques were used: 1) a standard technique for the examination of cell packs and tissues, which allows the localization of particles within the cells; 2) a millipore filter technique for the detection of viruses in tissue culture fluids; and 3) immunoelectron-microscopy with ferritin-labelled antibodies. This technique is comparable with an indirect immunofluorescent antibody technique but, instead of a fluorescent substance, ferritin conjugated to antibodies is used.

2.6.1. Electron microscopy of cells and tissues

Cell packs and tissue pieces were fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, postfixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, dehydrated through graded ethanol series and propylene oxide and embedded in epon. Ultrathin sections were mounted on uncoated 200 mesh copper grids and double stained with uranyl acetate and lead citrate.

The sections were examined for the presence of viral particles in a Philips 300 electron microscope at instrument magnifications of 15,960x and 30,000x.

2.6.2. Millipore filter technique

This technique allows the detection of very small numbers of virus particles in tissue culture supernatants. The method described here is a modification of that of Miller et al. (1973). Millipore membrane filter discs (type 5 VSWP) of 16 mm diameter (pore size 25 nm) were punched from larger filters supported on a rubber mat using a sharpened stainless steel tube of 16 mm inside diameter. Discs were positioned on top of supports made of Epon embedding medium in cellulose nitrate tubes (Beckmann 302235). Cell-free culture fluids were mixed with sodium cacodylate-buffered 2 % stabilized glutaraldehyde and were put in cellulose nitrate tubes on top of the millipore membrane discs and centrifuged at 150,000 x g for 45 min in a Beckman centrifuge (50 Ti rotor at 4° C).

The supernatant fluid was discarded and the tubes were split open with a razor blade to remove the discs with the sediments. They were cut into thin oblong strips and immersed in phosphate buffered 1 % osmium tetroxide solution for 60 min at 4° C. Strips were dehydrated in a graded series of isopropanol, treated with toluene and flat embedded in Epon. After polymerization, strips were orientated in the ultramicrotome so that the section could be cut (with a diamond knife) perpendicular to the plane of the millipore membrane.

Ultrathin sections were collected on uncoated grids (200 mesh) and their contrast were enhanced with an aqueous solution of uranyl acetate and lead citrate.

2.6.3. Immunoelectronmicroscopy

The method used was described by Calafat et al. (1974). Virus producing and control cells were grown in small petri dishes to near confluency and washed twice with Hanks' solution and then incubated for 3 h at room temperature with 0.5 ml rabbit anti-SiSV serum (RA-SiSV) (dilution 1 : 5). The cells were washed twice with Hanks' solution and then incubated for 90 minutes at room temperature with 0.5 ml ferritin-labelled goat antiserum to rabbit IgG prepared as described by Calafat et al. (dilution 1 : 50). After being washed three times, the cells were fixed in 3.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1, postfixed in 1 % osmium tetroxide in the same buffer and scraped off the dishes with a rubber policeman and centrifuged.

The pellets were dehydrated and embedded in a mixture of Epon and Araldite. Thin sections were stained with uranyl acetate and lead hydroxide. The sections were examined for the presence of virus-specific staining of budding and mature virus particles and staining of the cell membrane.

2.7. HUMAN BONE MARROW

In the experiments described in Chapters III and IV, bone marrow from normal individuals and patients with a variety of malignant and nonmalignant disorders was cocultivated with animal indicator cells for the detection of primate type C retroviruses.

Bone marrow was collected in heparinized glass bottles containing 4 ml Dulbecco's minimal essential medium. To remove the majority of erythrocytes, the cells were allowed to sediment by gravity in 1 % methylcellulose in culture medium for 20 min. All bone marrow samples were taken before chemotherapy was initiated, except when stated otherwise in the text. The specific diagnosis, age and sex are mentioned in the text (Chapter III) or in Tables 8, 9 and 10 (Chapter IV).

2.8. ANIMAL EXPERIMENTS

The following animal species were used in the experiments described in Chapters III, V and VI: 1) congenital athymic nude mice, obtained in 1975 from the University of Leuven (Leuven, Belgium); 2) BALB/c mice from an inbred breeding couple obtained from the Charles River Breeding Laboratories (North Wilmington, Massachusetts, USA); and 3) WAG/Rij rats, a small Wistar strain obtained from Glaxo Laboratories (Uxbridge, Middle Essex, England). All animals used in our work were bred SPF (Specific Pathogen Free) in the Radiobiological Institute, but were kept "clean conventional" after weaning (Solleveld, 1978).

In Chapter III, a quantitative assay was developed for virus-infected haemopoietic cells obtained from BALB/c mice inoculated with leukaemia viruses. Two virus-induced leukaemia models were used: one induced by the Rauscher murine leukaemia virus (Rauscher, 1962) and the other by the Soule murine leukaemia virus (S-MuLV) (Soule and Arnold, 1970). Both viruses were inoculated into experimental animals as crude cell-free extracts which were prepared as follows. Leukaemic spleens induced by either R-MuLV or S-MuLV were homogenized in a Sorvall Omni-Mixer for 1 min at 19,000 rpm in 5 volumes of PBS. The homogenate was centrifuged at 25,000 x g for 30 min and the sediment was homogenized again in the same amount of fresh PBS before being recentrifuged. All operations were carried out at 4^{o} C. The two supernatants were combined and stored in cold-resistant plastic bags (Cryofoil II, Cryoson, Midden Beemster, The Netherlands) in liquid nitrogen. The Rauscher virus was obtained in 1967 from Dr. F. Rauscher Jr. (formerly of the National Cancer Institute, Bethesda, Maryland, USA). Fifty eight-week-old female BALB/c mice were given intraperitoneal injections (0.1 ml) of an R-MuLV preparation and at the indicated intervals 4 animals were killed, the spleens were weighed and bone marrow samples were pooled (Chapter III). Bone marrow was obtained from the femurs as described by Bradley and Metcalf (1966).

The Soule virus was obtained from Dr. C.M. McGrath (Michigan Cancer Foundation, Detroit, Michigan, USA) and was propagated *in vitro* in the MCF-6 cell line (Soule and Arnold, 1970). The first animals were made leukaemic by a cell-free extract of an MCF-6 cell pack; thereafter, 50 newborn BALB/c mice were inoculated intraperitoneally (0.1 ml) with a crude cell-free extract of a leukaemic spleen. At the indicated intervals, the mean of the spleen weights of 4 animals was expressed as a percentage of control, nontreated, animals of the same age (Chapter III).

CHAPTER III

DETECTION OF TYPE C VIRUSES IN HUMAN LEUKAEMIC BONE MARROW WITH THE XC CELL PLAQUE ASSAY

INTRODUCTION

Sufficient evidence is now available that viruses are involved in the induction of various tumours in animals of many species, among which are birds, rodents and primates. It is, therefore, presumed that at least some neoplastic diseases of man are caused by type C retroviruses. During the past years, this hypothesis has been substantiated by publications dealing with the "biochemical" presence of type C viruses. Even the isolation of such particles from human cells in culture is reported (see review in Chapter I).

The technique of cocultivation of leukaemic bone marrow with suitable substrates for virus replication in combination with biological and biochemical methods for the detection of retroviruses is essential in our studies on the detection of human viruses. This cocultivation method has been profitable for the isolation of type C retroviruses from primate species from which no viruses could be retrieved by other means (Todaro et al., 1978a; 1978b). In such cocultivation experiments, is it assumed that, if a few particles are released by the test cells, they infect the indicator cells and this subsequently results in increased viral replication.

In contrast to the sarcoma viruses, most leukaemia viruses cannot be assayed satisfactorily *in vitro* for their oncogenic potential (for a review on *in vitro* transformation by RNA tumour viruses, see Nooter and Bentvelzen, 1977). Several other methods are available for the assessment of infectious entities. *In vitro* assays for the replicative capacity of leukaemia viruses are, for instance, the reverse transcriptase assay (Temin and Mizutani, 1970; Baltimore, 1970), assays for viral specific antigens by radioimmunoassay (Parks and Scolnick, 1972), complement fixation (Sarma et al., 1964; Hartley et al., 1965) or immunofluorescence (Pinkel et al., 1966), induction of resistance to transformation with a sarcoma virus (Sarma et al., 1967), rescue of a sarcoma virus from a "non-producer" cell line (Huebner et al., 1966; Fischinger and O'Connor, 1968; Rispens and Long, 1970) and plaque assays (Rowe et al., 1970; Bassin et al., 1971a; Kawai and Hanafusa, 1972; Fischinger et al., 1974).

The technique used in the work described here is the XC cell cytopathogenicity assay. The classical XC plaque assay of Rowe et al. (1970) had to be adapted to the use of haemopoietic cells and this was evaluated in an animal model system. With this modified XC test, we detected the presence of type C particles in bone marrow of a leukaemic child. Later, peripheral buffy coat cells from the same patient were cultured with human embryonic fibroblasts and virus was detected in this coculture. The rate of in vitro replication of the virus was very low; nevertheless, it could be shown that the viral antigens were related to the woolly monkey (simian) sarcoma-leukaemia virus (SiSV).

3.1. XC CELL PLAQUE ASSAY

Rat XC tumour cells carrying the Rous sarcoma virus (RSV) genome (Svoboda, 1960) have been used for the development of a mixed cytopathogenicity test for tissue culture cells infected with certain types of viruses: murine leukaemia viruses (MuLV) (Klement et al., 1969; Rowe et al., 1970), murine myeloma virus (Volkman and Krueger, 1973), murine sarcoma virus (Trowbridge et al., 1973), feline leukaemia virus (Rangan et al., 1972a) and viruses of the simian sarcoma-gibbon ape leukaemia virus group (Rangan et al., 1972b). The XC test is based upon the observation of Klement et al. (1969) that XC cells when cocultivated with mouse embryo cells releasing MuLV form syncytia consisting of multinucleated giant cells. This led to the development of a plaque assay (Rowe et al., 1970): mouse embryo cells are infected with MuLV and, after six days, irradiated with ultraviolet light, thus preventing further outgrowth of the cells. The irradiated monolayer is then overlaid with XC cells. A few days later, the plates are fixed and stained and plaques can be counted. The number of syncytial plaques is linearly related to the dilution of MuLV. XC cell syncytium formation is thought to be due to cellular fusion (Klement et al., 1969). It appeared that virus-specific components of the cellular membrane and/or the type C virus itself activated XC cells to form syncytia (Johnson et al., 1971).

The baboon endogenous virus (BaEV), endogenous RD114 cat virus and Mason-Pfizer monkey virus (M-PMV) fail to induce syncytia in XC cells. However, human malignant glioma KC cells carrying the RSV genome (Pontén and MacIntyre, 1968) rapidly develop syncytia when overlaid with cells infected with BaEV (Ahmed et al., 1975), RD114 (Rand and Long, 1972) and M-PMV (Ahmed et al., 1974; Rand et al., 1974). Other type C viruses, e.g., of feline, mouse, rat, hamster, woolly monkey and gibbon ape origin, do not induce syncytia in the KC cell line.

The precise mechanism of syncytium induction in either XC cells or KC cells is not clear. It is also not known precisely why a given virus induces syncytia in one cell type and not in another. In the case of BaEV, the high degree of responsiveness of KC cells to BaEV, as compared with XC cells, can be due to the fact that human cells are generally more easily infected with BaEV than are rat cells (Ahmed et al., 1975). However, the woolly monkey virus can propagate in human cells (Rhim et al., 1975a) but does not induce syncytia in KC cells. Obviously, the failure of SiSV to induce syncytia in KC cells cannot be explained by the viral host range.

Rand et al. (1976) used the KC and XC retrovirus indicator lines for the detection of virus producing human cells. Most human cell lines were negative; however, some human choriocarcinoma cells fused with both lines, but no evidence of overt virus production was found in these cells.

EXPERIMENTAL PART

3.2. MODIFICATION OF THE XC TEST FOR BONE MARROW

The XC cell assay used here is a modification of the so-called reversed assay of Niwa et al. (1973). Bone marrow cells of mice with virus-induced leukaemia instead of virus-infected mouse embryo fibroblasts are plated over an XC underlayer in an attempt to quantify the number of virus-infected haemopoietic cells. The change in plating sequence in the reversed assay is in favour of bone marrow cells, of which only a selected portion adheres to the culture surface (Nooter and Bentvelzen, 1975). Besides this, the XC cell underlayer can provide an appropriate environment for bone marrow cells (Metcalf, 1973).

It is demonstrated in this Chapter that XC cells cocultivated with bone marrow cells of MuLV infected BALB/c mice form syncytia and that syncytium formation increases during the progression of the disease. Quantification of the syncytium forming capacity of type C viruses in XC monolayers was originally expressed as plaque forming units (Rowe et al., 1970) and, on that basis, we retain this nomenclature, although the syncytia described in this study do not always look like real plaques.

Eight-week-old female BALB/c mice were injected with a crude Rauscher murine leukaemia virus (R-MuLV) (Rauscher, 1962) preparation. Newborn BALB/c mice were injected with a crude Soule murine leukaemia virus (S-MuLV) (Soule and Arnold, 1970) preparation.

Susceptible mice injected with a large inoculum of R-MuLV develop an erythroblastosis with accumulation of immature erythroid cells in liver, spleen, bone marrow and blood. From day 7 onwards, the spleen weight increases and can reach a weight of 2.5 g in the terminal stage of the disease after 4-5 weeks.

The MCF-6 cell line (Soule and Arnold, 1970) produces a virulent murine leukaemia virus designated by us as S-MuLV. Injection of this virus into neonatal BALB/c mice leads to splenomegaly due to the development of lymphosarcomas of T-cell origin (Harris and Zipori, 1977) . The lymphosarcomas are mainly found in spleen and lymph nodes and occasionally infiltrate into the liver and kidneys. The thymus is mostly not involved. The animals die within 3 to 5 months.

3.2.1. Results

Cocultivation of XC cells and bone marrow cells of leukaemic BALB/c mice, either induced by Rauscher or Soule virus, results in syncytium formation as shown in Table 4 and Fig. 5. With our procedure, the best time to count syncytia is day 5; on day 4, the cell layer is not confluent and, on day 6, the cell layer is rather closely built up and here and there piled up, which may influence counting. Normal mouse bone marrow

Table 4

XC CELL PLAQUE ASSAY OF MuLV-INFECTED MOUSE BONE MARROW

Cocultivation of XC cells with:	No. of plaques[a]
None[b]	18[c]
Normal BALB/c bone marrow	
5.0×10^4 [d]	14[c]
1.0×10^5	12[c]
2.0×10^5	19[c]
Bone marrow of R-MuLV-infected BALB/c mice[e]	
5.0×10^4	1430
1.0×10^5	2510
2.0×10^5	3870
Bone marrow of S-MuLV-infected BALB/c mice[f]	
5.0×10^4	1702
1.0×10^5	3358
2.0×10^5	5175
Bone marrow of mammary tumour-bearing BALB/c mice[g]	
1.0×10^5	22[c]

[a] Mean of 4 dishes

[b] XC cells (2×10^5 per dish) without cocultivation of bone marrow cells.

[c] Small plaques consisting of syncytia with less than 4 nuclei.

[d] Number of cells cocultivated with 2×10^5 XC cells per dish.

[e] Injected 3 weeks earlier with Rauscher murine leukaemia virus (R-MuLV).

[f] Injected 2 months earlier with Soule murine leukaemia virus (S-MuLV).

[g] Injected with the mouse mammary tumour virus and killed in the terminal stage of the disease.

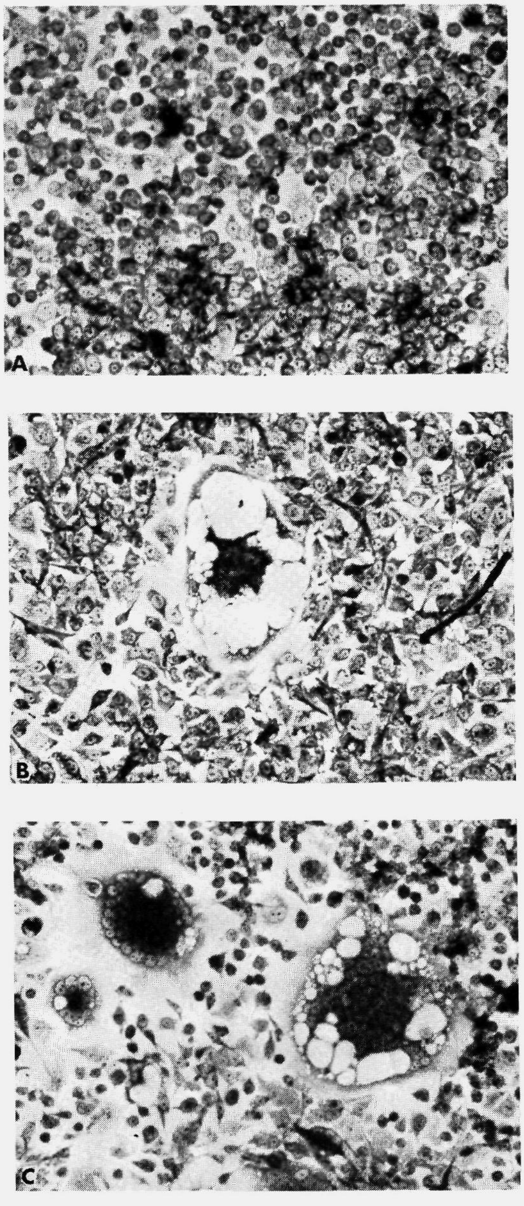

Figure 5: Syncytia induced by cocultivation of XC cells and bone marrow of mice with virus-induced leukaemia. Magnification x 210.
A. Control XC culture; B. Bone marrow of Rauscher murine leukaemia virus-infected mice; C. Bone marrow of Soule murine leukaemia virus-infected mice.

cells and bone marrow cells of mammary tumour bearing animals do not induce plaque counts above control values (XC plaque counts without overlayer).

With leukaemic bone marrow, doubling the number of bone marrow cells leads to approximately the same increase in XC plaques. At a very high cell concentration, a deviation from linearity which is probably due to confluency of plaques is observed. Figure 5 shows plaques induced by R-MuLV and S-MuLV. Bizarre, strongly vacuolized giant cells with numerous nuclei or nuclear fusion are formed. Large plaques are never found in the control groups (normal bone marrow and bone marrow of mammary tumour bearing mice); only a few cells grouped together with the beginning stages of cell and nuclear fusion are seen.

Figure 6 shows the plaque forming capacity of bone marrow during the progression of the erythroblastosis induced by R-MuLV as measured by spleen weight. As early as day 2, there is an increase in this capacity in R-MuLV-infected mice as compared with normals. This increase is consistent during the further development of the malignancy. Comparable results are obtained with S-MuLV-infected mice (Fig. 7). Because of the fact that the Soule virus was inoculated into newborn mice, the spleen weights are now expressed as percentages of those of control nontreated animals. The maximum number of plaques per 10^5 bone marrow cells is about two times higher than in Rauscher virus-injected mice.

3.2.2. Discussion

In the conventional XC cell assay, MuLV-infected mouse embryo fibroblasts are cocultivated with XC cells in order to quantify the original input of virus.

In our experiments, the number of syncytia formed is regarded as a quantification of the number of virus-infected and virus-producing cells. During the progression of the diseases, as measured by spleen weights, this number increases. A remarkable observation was that there is an early increase in the syncytium number in bone marrow of infected mice, even before there are conspicuous clinical signs of the malignancies. Noteworthy about the modified XC cell assay are the following facts: 1) it is a reliable test; repetition of the experiments led to the same results (data not shown); 2) it is a sensitive method; plaque counts range from zero to a few thousand; and 3) relatively small numbers of test cells are needed.

In the case of virus-induced leukaemia, bone marrow seems to offer a suitable tissue for cocultivation with XC cells and the subsequent quantification of virus-infected cells.

The XC test was adapted to the use of bone marrow in the belief that such a system could be an aid in the study of candidate viruses of possipossible etiological significance to human leukaemia.

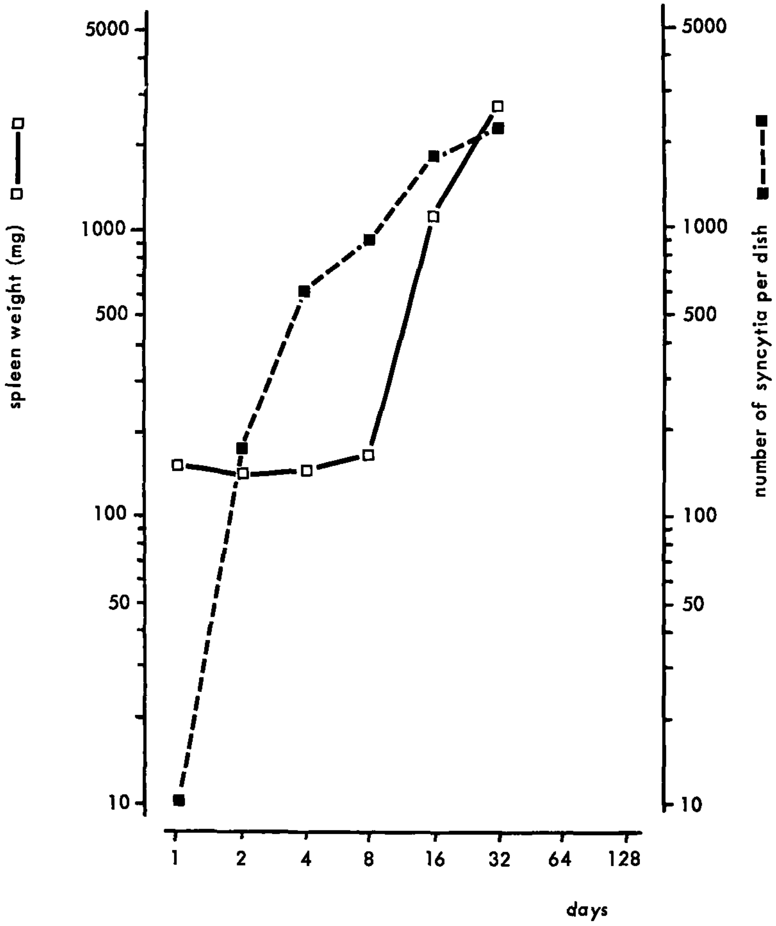

Figure 6: Spleen weight and plaque forming capacity of bone marrow at different intervals after Rauscher murine leukaemia virus infection. Spleen weights are the mean of 4 animals. Plaque numbers are the mean of 4 dishes.

3.3. DETECTION OF TYPE C VIRUSES IN HUMAN BONE MARROW

In the next experiments aimed at the detection of putative human leukaemia viruses, bone marrow of normal individuals and patients with a variety of haemopoietic disorders were assayed in the modified XC test. The bone marrow samples were collected from 4 normal adult donors, 2 adult patients with acute myeloid leukaemia, 1 child with acute lymphoblastic leukaemia, 2 adult patients with chronic myeloid leukaemia, 3 with chronic lymphatic leukaemia, 1 with aplasia and 1 with secondary polycythaemia.

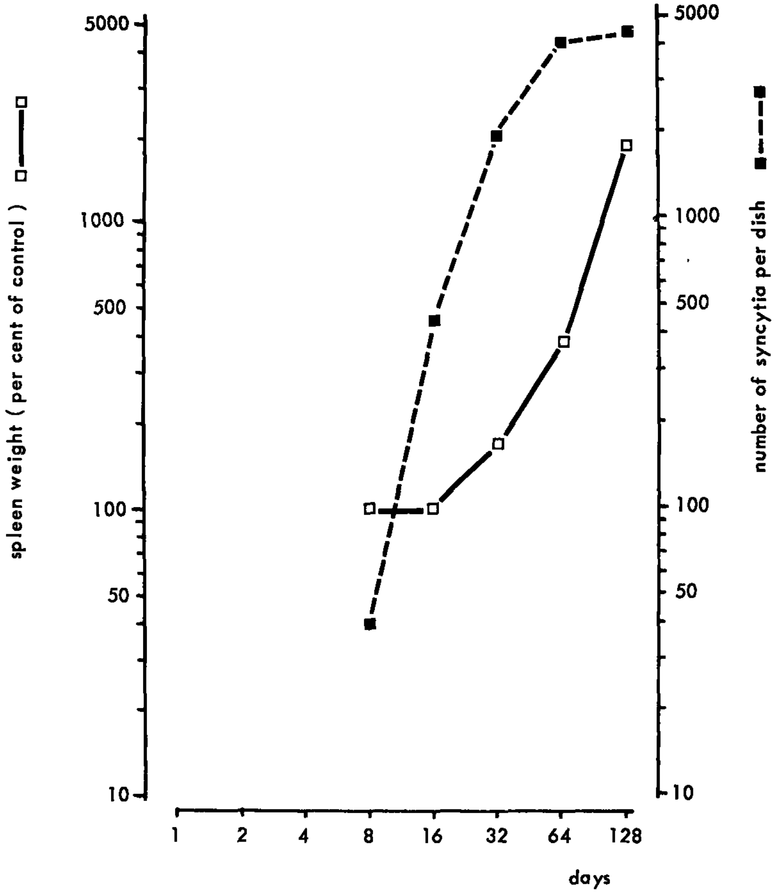

Figure 7: Spleen weight and plaque forming capacity of bone marrow at different intervals after Soule murine leukaemia virus infection. Spleen weights are the mean of 4 animals and are expressed as percentages of control values. Plaque numbers are the mean of 4 dishes.

Induction of syncytia was found in one case. Only the bone marrow of the 4-year-old patient with a lymphosarcoma that had progressed to a state of acute lymphoblastic leukaemia was positive in our assay. The detection and the partial characterization of a type C retrovirus obtained from the bone marrow of this leukaemic child is reported here.

3.3.1. Detection with the XC cell plaque assay

Bone marrow cells were cultured in liquid suspension (2×10^6 cells per 2 ml per tube) and phytohaemagglutinin (PHA) (10 µl per ml, Burroughs, Wellcome, Lot. No. 9268) was added as the growth initiator (Nowell, 1960). After 3 days of culture, the supernatant was examined for the presence of type C particles using the Millipore filter technique and was found to be positive (Fig. 8A). The Millipore filter technique is described by Miller et al. (1973). In short, virus particles in the culture fluid are spun down on a Millipore membrane filter in an ultracentrifuge tube. The filter is processed with the standard electron microscopic preparation technique and only the membrane surface has to be examined under the electron microscope for particles.

The *PHA* stimulated bone marrow cells were seeded on top of a 24-h culture of XC cells. Four days later, conspicuous syncytial plaques which were similar to those reported to occur after cocultivation of XC cells and cells infected with a murine leukaemia virus were found (Table 5).

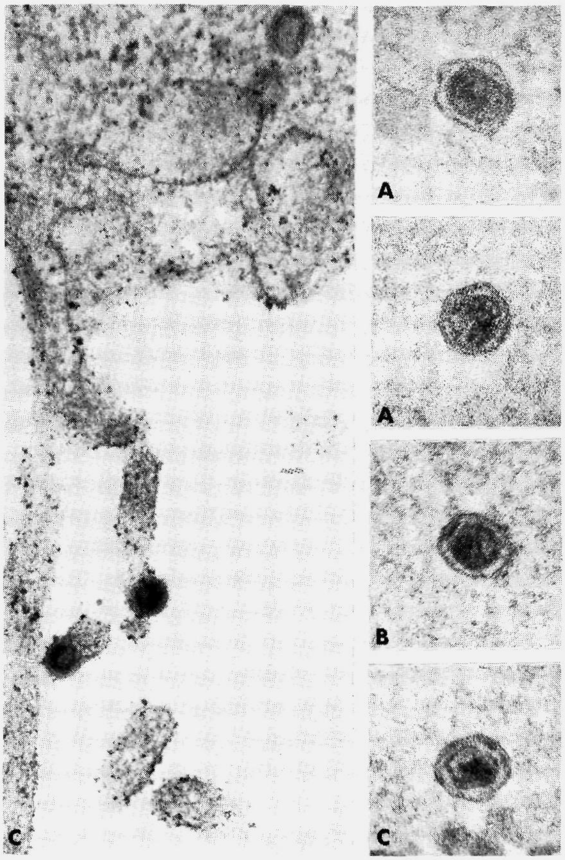

Figure 8: Type C particles detected in cells and in supernatants of culture fluids.
A. Particles from mitogen-stimulated human bone marrow cells; ø 110-115 nm.
B. Particle from cocultivated human embryonic kidney (HEK) cells;
C. Mature and budding particles from cocultivated human embryonic fibroblasts (FB 289); budding particles 95-100 nm.

Table 5

DETECTION OF TYPE C VIRUS BY THE XC CELL ASSAY AND ELECTRON MICROSCOPIC EXAMINATION OF CULTURE FLUIDS

Cocultivation of XC cells	No. of cells plated	No. of plaques[a]	E.M.[b]
None	-	17[c]	-
Normal human BM[d]	1.0×10^5	5-10[c]	N.T.[e]
	1.0×10^6	12-16[c]	-
Leukaemic BM, stimulated by PHA[f]	1.0×10^5	471	+
Leukaemic BM, unstimulated	1.0×10^5	393	+
	5.0×10^5	901	N.T.
Coculture XC + leuk.BM, unstimulated			
2nd passage	1.0×10^5	520	+
4th passage	1.0×10^5	606	+
8th passage	1.0×10^5	35	-
Coculture XC + normal BM			
3th passage	1.0×10^5	15[c]	-

[a] Mean of 4 dishes

[b] Electron microscopic observation of type C particles with the Millipore filter technique

[c] Small plaques consisting of syncytia with less than 4 nuclei

[d] Four normal bone marrows (BM) were tested

[e] Not tested (N.T.)

[f] Phytohaemagglutinin (PHA)

Some small plaques which contained only a few nuclei were found in the control XC cultures (XC cells overlayered with normal bone marrow). The same cytopathogenic effect was found when fresh leukaemic bone marrow cells were cocultivated directly with XC cells. After trypsinization of the XC cells which had been in contact with the leukaemic cells (mentioned in Table 5 as coculture XC + leukaemic BM), the production of syncytia as tested on an XC cell monolayer persisted on further passaging. However, syncytium formation ceased after about 6 weeks in culture. The 8th passage of coculture XC + leukaemic BM was negative in the XC plaque assay. Cocultures of XC + normal BM cells were negative for syncytium induction in an XC monolayer.

The XC cultures which showed a positive cytopathogenic effect also released type C particles, as revealed by electron microscopy (Table 5). Control XC cultures or mixed cultures of XC with normal bone marrow cells were repeatedly negative for virus particles.

3.3.2. Transmission and characterization of the virus

Because of the cytopathogenic effect of the virus in XC cells, the putative human virus was transmitted to another cell line by cocultivation. A subconfluent monolayer of a secondary culture of human embryonic kidney (HEK) cells was cocultivated with irradiated (2000 rad) cells of a coculture of XC cells + leukaemic bone marrow (4th passage). The advantage of radiation in the cocultivation procedure is that the irradiated cells die slowly in the course of a week, during which they can continue virus production. The second passage of the cocultivated HEK cells induced plaques in the XC test (310 plaques per dish per 10^5 cells plated) and type C particles were found in the supernatant with the aid of the electron microscope (Fig. 8B). However, the 6th passage no longer had plaque forming capacity, indicating that virus production had stopped; this was similar to what was found for the 8th and subsequent passages of coculture XC + leukaemic bone marrow.

Indirect cytoplasmic immunofluorescence tests were carried out on acetone-fixed cultured cells to assess the serological relationship of the putative human virus to other mammalian type C retroviruses as well as to exclude the possibility of laboratory contamination. At the time when the putative human virus was detected, the only type C viruses used in our laboratories were murine leukaemia viruses. For that reason, several antisera to murine type C viruses were tested on the cultures (Table 6). Polyvalent rat antisera to Rauscher murine leukaemia virus numbers 45 and 76 have been used in studies on the presence of type C retroviral antigens in human bone tumour cultures (Zurcher et al., 1975). Their specificity has been extensively investigated. They show a broad group-specific reactivity with various ecotropic and xenotropic mouse viruses (data not shown). They also possess a broad interspecies reactivity when tested on woolly monkey fibrosarcoma cells producing SiSV.

Antiserum 45 reacts with the virus isolated from the cocultures of the leukaemic child after passaging in HEK cells. A goat antiserum to an endogenous type C retrovirus isolated from a mouse tumour cell line EMT6 (Rockwell et al., 1972) and designated as GA-EMuLV gives similar reactions. The strongest antiserum to R-MuLV (no. 76), however, does not react with the putative human virus. This finding strongly indicates that the weak but positive reaction with the two antisera is not due to contamination of the cultures with a murine virus but to an interspecies antigenic determinant not detected by antiserum 76.

Table 6

IMMUNOFLUORESCENCE ENDPOINT TITRATION OF CELL CULTURES WITH ANTISERA TO ANIMAL TYPE C VIRUSES

Cells	Antisera[a]			
	Ra-R-MuLV		GA-EMuLV	GA1-SiSV-p30
	no. 45	no. 76		
BALB/3T3	-	-	-	-
BALB/3T3 + R-MuLV[b]	2,560	5,120	2,560	20
XC	-	-	-	-
XC + leuk. BM, 2nd passage	40	-	20	40
XC + 5-bromodeoxyuridine + dimethylsulphoxide[c]	160	80	-	40
HEK	-	-	-	-
HEK + irradiated XC + leuk. BM[d], 2nd passage	40	-	20	40
HEK + irradiated XC	-	-	-	-
Marmoset cells infected[e] with SiSV	320	80	80	320

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[a] Fluorescence staining was scored as positive when the reciprocal of the endpoint titer of the antiviral antiserum was 20 or higher.

[b] BALB/3T3 cells infected with Rauscher murine leukaemia virus (R-MuLV)

[c] Subconfluent monolayer of XC cells treated for one day with 5-bromodeoxyuridine (20 µg per ml) and 2 % dimethylsulphoxide

[d] Human embryonic kidney (HEK) cells cocultivated with irradiated (2000 rad) cells of the coculture XC + leukaemic bone marrow.

[e] Marmoset cells producing woolly monkey sarcoma-leukaemia virus.

A goat antiserum (GA1-SiSV-p30) directed against the purified major structural polypeptide of SiSV, with a molecular weight of 30,000 dalton (p30), gives a good reaction with marmoset cell cultures harbouring the primate virus, although the acetone-fixed slides have been kept for 18 months at -20°C. The weak reaction (end point titre, 1 : 40) with the putative human virus grown in HEK cells is probably due to poor replication of the virus and in turn to low production of p30 in the cytoplasm. However, an antigenic dissimilarity between the simian and the human virus cannot be excluded.

Another source of contamination may be the XC cells. Control XC cultures proved to be consistently negative with all antisera. The cocultivation procedure may have activated an endogenous rat virus. Rat antiserum no. 76, however, reacts with endogenous viruses activated in XC cells by 5-bromodeoxyuridine (20 µg per ml) and 2 % dimethylsulphoxide (Klement et al., 1972; Grollé et al., 1973) but not with XC cells cocultivated with the leukaemic cells. Goat antiserum EMuLV showed quite the opposite pattern, suggesting that contamination with endogenous rat virus can be excluded.

A second detection was made one month later from the same patient, while in remission, by cocultivating the buffy coat of the peripheral blood with human embryonic fibroblasts, FB 289. Within two weeks, virus was demonstrable by reverse transcriptase assay (up to 20 x 10^3 cpm per ml), electron microscopy (Fig. 8C) and the immunofluorescence test. In the latter assay, antiserum no. 76 gave no reaction, whereas anti-SiSV-p30 was positive with a titre of 1 : 40 (Fig. 9). But again the virus did not propagate very well in tissue culture. The positive immunofluorescence staining disappeared on further passages of the cocultivated FB289 cells.

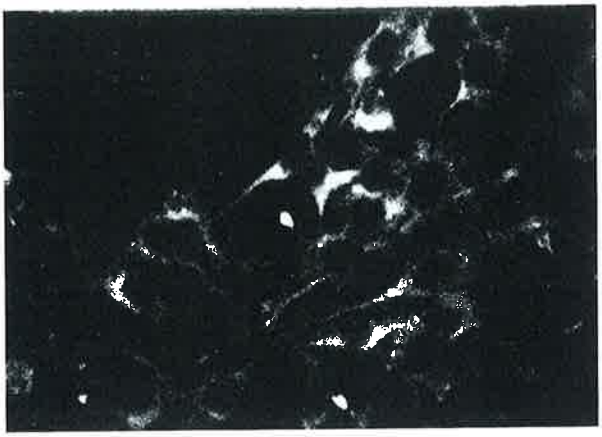

Figure 9: Human embryonic fibroblasts (FB 289) cocultivated with white blood cells of the child with lymphosarcoma. The cells were incubated with a goat antiserum to the major structural polypeptide (p30) of SiSV (dilution 1 : 20). Magnification x 575.

3.3.3. Discussion

In our experiments, we added PHA to a leukaemic bone marrow suspension in order to stimulate cell proliferation. This resulted in the electron microscopic detection of type C virions. It is not very likely that this virus release was due to a specific activation by the T-cell mitogen, as is described for activation of endogenous mouse viruses from B lymphocytes by the B-cell mitogen lipopolysaccharide (Schumann and Moroni, 1976). Unstimulated and PHA-stimulated leukaemic bone marrow both had a high syncytium inducing capacity when seeded on top of an XC monolayer. Most likely due to the cytopathogenic effect, the putative human viruses could no longer be propagated in the XC cells.

We have to consider the possibility that other than type C retroviruses induced plaques in the XC monolayers. For instance, herpesviruses (cytomegalo, varicella, simplex) adenoviruses, paramyxoviruses (human respiratory syncytical virus, measles virus, mumps virus) and foamy viruses can be isolated from human tissues and they can have cytopathogenic effects in cell cultures which sometimes resemble XC syncytia. However, morphologically and immunologically, these viruses have nothing in common with type C RNA tumour viruses. Exceptions are the foamy or syncytium-forming viruses. They have a 60-70 S single stranded RNA and are similar to type C viruses in morphology and in physical-chemical structure, they possess the enzyme reverse transcriptase and can persist in cell cultures (for a review on foamy viruses, see Hooks and Gibbs, 1975). However, group and interspecies specific antigens present in type C viruses are not found in the foamy viruses and our immunofluorescence data clearly show that the human bone marrow derived viruses are related to mammalian type C retroviruses.

Another source of contamination capable of inducing a cytopathogenic effect in XC cells can be introduced by bovine sera used in the cultures. These sera are frequently contaminated with bovine viruses, among which can be the bovine syncytial virus (BSV) (Van der Maaten et al., 1973) and the bovine leukaemia virus (BLV) (Calafat et al., 1976). Both of these viruses induce multinucleated giant cells in mixed cell cultures of mammalian origin (Cornefert-Jensen et al., 1969; Ferrer and Diglio, 1976). However, BLV has no cytopathogenic effect in rat (XC) and mouse (3T3) cells and does not share antigenic determinants with other mammalian or avian leukaemia viruses (Gilden et al., 1975; McDonald and Ferrer, 1976; Devare and Stephenson, 1977). BSV can be ruled out on the basis of morphology: the envelope structure is quite distinct from that of type C viruses (Dermott et al., 1971).

Rous sarcoma virus transformed mammalian cells generally do not release infectious virus, with the exception of a marsupial cell line (Svoboda et al., 1974). Hamster and rat tumours induced by RSV, e.g., are mostly virus-free but contain the complete viral genome, since cells yield infectious RSV when grown in mixed cultures with chick cells (Svoboda et al., 1968). Apparently, RSV can be rescued from the nonproducing lines but mammalian type C viruses have never been shown to activate this nonreplicative RSV genome in XC cells. Besides this, our immunofluorescence data cannot be influenced by the eventual presence of avian viruses, because the avian and mammalian retroviruses do not share antigenic determinants (Hilgers et al., 1972).

The detection of viral antigens related to SiSV in the cytoplasm of XC cells cocultivated with leukaemic bone marrow and of virus producing human embryonic cells strongly suggests that we are dealing with human derived virions. The positive reactions with two MuLV-directed antisera appear to be due to viral interspecies specific antigenic determinants shared by mouse and primate type C viruses. A remarkable observation in our putative human virus producing lines is the poor virus release, also encountered by others working with human tissue derived retroviruses. We maintained our putative human virus-infected lines in culture for months and virus production (extracellular reverse transcriptase) was screened for every two weeks; however, it was never high enough for transmitting the virus to other more permissive mammalian cell lines.

Biochemical studies are required to assess in more detail the relationship of these viruses to other animal viruses and to determine whether they are exogenous or endogenous to human cells. Virus production in culture, however, is nil and that is a limiting factor for these kinds of studies.

CHAPTER IV

TYPE C VIRAL ANTIGEN DETECTION IN COCULTURES OF HUMAN LEUKAEMIC BONE MARROW AND DOG CELLS

It was shown in the previous Chapter that cocultivation of human tumour cells with animal indicator cells is a promising method for the detection and isolation of putative human viruses. Due to the cytopathogenic effect of type C viruses in the rat XC cell line, these indicator cells are suitable only for virus detection. We here describe cocultivation experiments with the fetal dog thymus cell line A7573 which has been shown to be suitable for the propagation of woolly monkey (simian) sarcoma-leukaemia virus (SiSV)-related viruses (Teich et al., 1975).

Dog thymus cells were cocultured with human bone marrow samples from normal individuals, leukaemic patients and nonleukaemic patients (see Tables 8, 9 and 10). SiSV cross-reacting antigens were detected in some of these cocultures by means of the indirect cytoplasmic immunofluorescence assay (IFA). For childhood leukaemia, 5 out of 9 cocultures showed virus-specific IFA staining. Detailed characterization of the antiviral antisera was also partly a subject of this study and was carried out with the classical IFA on a variety of virus-negative and virus-positive cells and by using a solid phase immunofluorescence test: the Sepharose bead immunofluorescence assay (Haaijman and Brinkhof, 1977).

4.1. CHARACTERIZATION OF THE ANTISERA

The following antiviral antisera were used: 1) an antiserum (RA-SiSV-p30) directed against the major internal structural polypeptide of SiSV with a molecular weight of 30,000 dalton (p30); and 2) an antiserum (RA-R-MuLV) directed against purified Rauscher murine leukaemia virus (R-MuLV).

4.1.1. Sepharose bead IFA

The method as developed for the quantitative assay of immunoglobulins (Haaijman and Brinkhof, 1977) has been adapted to the detection of viral antigens (Koch et al., 1977). Briefly, Sepharose beads coupled with fetal calf serum (FCS) or disrupted virus were incubated with antiviral antisera diluted in phosphate-buffered saline in microtitration plates. After washing, the beads were incubated with goat antirabbit immunoglobulin (Ig) antiserum conjugated with fluorescein isothiocyanate. Finally, after removing the unbound conjugate, the individual bead immunofluorescence was measured with a microfluorometer.

The unabsorbed rabbit antiserum to the p30 major structural polypeptides of SiSV when tested with the Sepharose bead IFA showed high reactivity with SiSV and R-MuLV as well as with FCS (Fig. 10A).

Anti-FCS activity present in the antiviral antiserum was removed by affinity chromatography. IgG fractions of antisera were placed on a column consisting of Sepharose 4B beads coupled with FCS and recollected. Thereafter, residual activity to FCS, SiSV and R-MuLV was measured in the

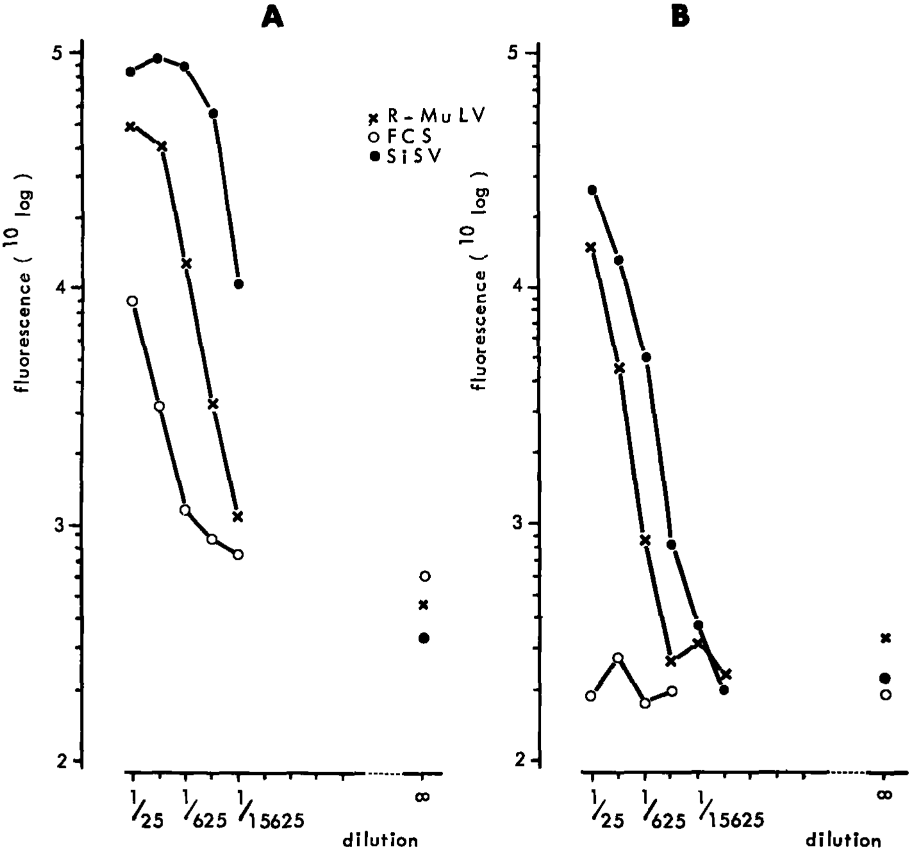

Figure 10: Characterization of RA-SiSV-p30 antiserum with the Sepharose bead immunofluorescence assay.
A. Before absorption with FCS. Sepharose beads coated with ● SiSV, x R-MuLV or o FCS were incubated with fivefold dilutions of an IgG fraction of RA-SiSV-p30 serum. Fluorescence staining of the Sepharose beads was achieved by subsequent incubation with fluorescein isothiocyanate-conjugated goat antiserum directed against rabbit Ig.
B. After absorption with FCS. An IgG fraction of RA-SiSV-p30 serum was absorbed with Sepharose beads coated with FCS. After absorption, residual activity against ● SiSV, x R-MuLV and o FCS was measured as described in A.

Sepharose bead IFA. After absorption, the unwanted reactivity with FCS was removed without drastically affecting the reaction to the viruses (Fig. 10B). The antiserum to SiSV-p30 has a broad interspecies activity. It strongly reacts with SiSV and to a somewhat lesser extent with R-MuLV, clearly demonstrating the interspecies specific antigenic determinants of the p30 molecules.

The rabbit antiserum directed against R-MuLV was also absorbed for FCS activity and tested in the same way as was the RA-SiSV-p30 serum. It showed an interspecies activity with SiSV and before absorption a reaction with FCS (Fig. 11A). The reaction with FCS was specifically removed by absorption (Fig. 11B).

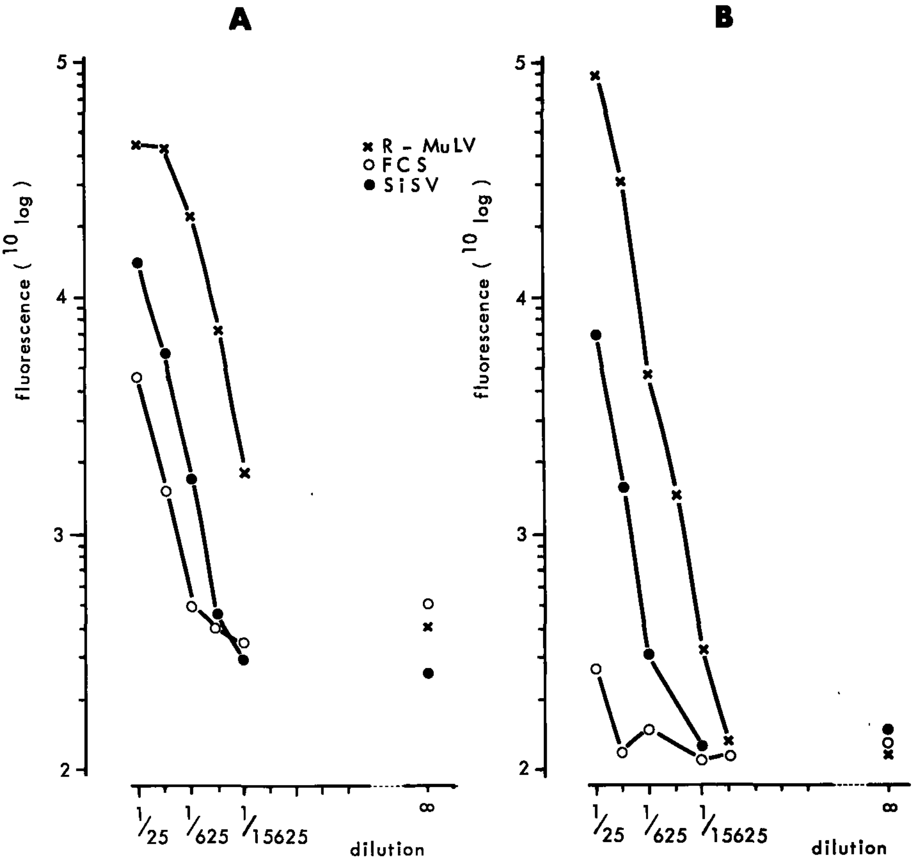

Figure 11: Characterization of RA-R-MuLV antiserum with the Sepharose bead immunofluorescence assay.
A. Before absorption with FCS. Sepharose beads coupled with ● SiSV, x R-MuLV or o FCS, were incubated with five-fold dilutions of the IgG fraction of antiserum.
B. After absorption with FCS. Residual activity against ● SiSV, x R-MuLV and o FCS was measured as described in A.

4.1.2. Indirect cytoplasmic IFA

In the cytoplasmic IFA, the antiviral antisera which have not been absorbed for anti-FCS activity react with every cell line tested. After absorption with FCS, the antiserum to SiSV-p30 gives a strong reaction (endpoint titre, 1 : 320-640) with cultures productively infected with SiSV and a weakly positive reaction with cultures releasing R-MuLV (endpoint titre, 1 : 40-80) (Table 7). No reaction was observed with cultures producing bovine leukaemia virus. The RA-R-MuLV antiserum gave a considerably stronger reaction with cells producing R-MuLV (endpoint titre, 1 : 160-320) than with SiSV-positive cells (endpoint titre, 20-40). These IFA data are in agreement with those obtained with the Sepharose bead assay. Neither antiserum reacted with a variety of animal (n = 7) and human (n = 12) cell lines which do not release a retrovirus. The virus negative lines included the dog line and the human lymphoid NC37 cell line. The SiSV preparation used for isolation of the p30 polypeptides and subsequent preparation of antiserum was grown in this NC37 line.

Table 7

IMMUNOFLUORESCENCE ENDPOINT TITRES OF CONTROL CELL CULTURES

Control cell cultures	Antisera[a] RA-SiSV-p30	 RA-R-MuLV
Virus positive:		
REF + SiSV[b]	320-640	20- 40
BALB/3T3 + R-MuLV[c]	40- 80	160-320
BoEF + BoLV[d] (n= 3)	<10	<10
Virus negative:		
Animal BALB/3T3, REF, R1-69[e] SIRC[f], A7573, FRhL[g], BoEF (n= 7)	<10	<10
Human skin fibrobl. (7), lung fibrobl. (1), R970[h], WHE[i], A204[j] NC37[k] (n=12)	<10	<10

[a] Results are expressed as endpoint titres, representing the reciprocal of the highest antiserum dilution with which virus-specific cytoplasmic fluorescence was still observed. The antisera were absorbed with fetal calf serum.

[b] Primary culture of rat embryonic fibroblasts (REF) productively infected with SiSV.

[c] Mouse BALB/3T3 cells productively infected with R-MuLV.

[d] Bovine embryonic fibroblasts (BoEF) productively infected with bovine leukaemia virus (BoLV) (kindly provided by Dr. A. Ressang, Centraal Diergeneeskundig Instituut, Rotterdam, The Netherlands).

[e] Rat osteosarcoma cell line (R1-69) (Barendsen et al., 1977).

[f] Rabbit cornea cell line (SIRC)

[g] Rhesus monkey fetal lung cell line (FRhL) (Wallace et al., 1973).

[h] Human osteosarcoma cells nonproductively infected with the Kirsten strain of the mouse sarcoma virus (R970) (Rhim et al., 1975c).

[i] Primary culture of whole human embryo cells (WHE).

[j] Human rhabdomyosarcoma cell line (A204).

[k] Human lymphoid cell line (NC37).

4.2. DETECTION OF SiSV-p30 RELATED ANTIGENS

Human bone marrow cells were cultured on top of an exponential phase growing layer of fetal dog thymus (A7573) cells. The cells were transferred every 5 days. Prior to trypsinization, the supernatant with floating bone marrow cells was removed, centrifuged and replated together with the trypsinized A7573 cells. After three passages of cocultivation, human bone marrow cells were no longer detected by light microscopy and karyotypic analysis. The nomenclature for the cocultures is as follows: the dog cell line used is listed first (A) followed by the number of the bone marrow donor. Bone marrow was derived from 17 leukaemic patients, 12 patients with nonleukaemic bone marrow and 9 normal individuals. The specific diagnoses, age and sex are mentioned in the respective tables. All samples were taken before chemotherapy was initiated.

After one to two months of culture, reactions appear with the RA-SiSV-p30 antiserum but not with the RA-R-MuLV antiserum in some cocultures (Tables 8, 9, and 10).

The cytoplasmic fluorescence was of a granular nature (Figs. 12, 13 and 14). Thirty to 60 % of the cells stained positive. The endpoint titre for the positive cocultures never exceeded 1 : 80. The earliest positive

Table 8

IMMUNOFLUORESCENCE ENDPOINT TITRES OF ANTISERA TO TYPE-C RETROVIRUSES IN COCULTURES OF HUMAN LEUKAEMIC BONE MARROW AND DOG CELLS

					Antisera[a]	
Coculture	Passage no.	Diagnosis	Sex	Age in years	RA-SiSV-p30	RA-R-MuLV
A1	9	ALL[b]	♂	3	+ (40)	-
A2	8	ALL	♂	5	-	-
A3	13	ALL	♂	6	+ (80)	-
A4	10	AML[c]	♂	8	+ (40)	-
A5	6	ALL	♀	5	+ (80)	-
A8	9	AML	♂	66	-	-
A24	9	CML[d]	♂	31	-	-
A25	8	AML	♂	70	-	-
A26	8	CML	♂	76	-	-
A28	8	ALL	♀	5	+ (40)	-
A30	8	AML	♂	74	-	-
A31	8	AML	♂	52	+ (40)	-
A32	8	AML	♀	73	-	-
A35	8	AML	♀	46	-	-
A36	8	AML	♀	9	-	-
A37	8	AMML[e]	♀	cong.[f]	-	-
A38	9	ALL	♂	1	-	-

[a] Fluorescence staining was scored as positive when the reciprocal of the endpoint titre of the antiviral antiserum was 40 or higher. The numbers in parentheses indicate endpoint titres which are expressed as the reciprocal of the highest dilution showing virus-specific staining. Antisera were absorbed with fetal calf serum.

[b] Acute lymphatic leukaemia (ALL).

[c] Acute myeloid leukaemia (AML).

[d] Chronic myeloid leukaemia (CML).

[e] Acute myelo-monocytic leukaemia (AMML).

[f] Congenital (cong.).

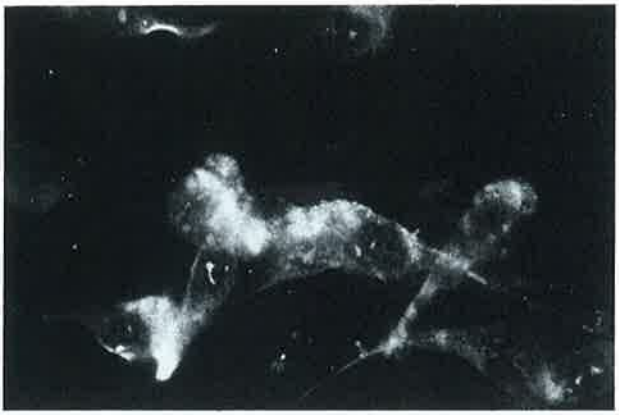

Figure 12: Cytoplasmic fluorescence of coculture A1 after incubation with rabbit antiserum to the major structural polypeptides (p30) of SiSV (RA-SiSV-p30) (dilution 1 : 20). Magnification x 900.

Figure 13: Cytoplasmic fluorescence of coculture A3 after incubation with RA-SiSV-p30 antiserum (dilution 1 : 20). Magnification x 900.

Figure 14: Control A7573 cells after incubation with RA-SiSV-p30 antiserum (dilution 1 : 20). Magnification x 900.

Table 9

IMMUNOFLUORESCENCE ENDPOINT TITRES OF ANTISERA TO TYPE C RETROVIRUSES IN COCULTURES OF NONLEUKAEMIC BONE MARROW FROM VARIOUS PATIENTS

Coculture	Passage no.	Diagnosis	Sex	Age in years	Antisera[a] RA-SiSV-p30	 RA-R-MuLV
A6	6	lymphosarcoma	♀	8	-	-
A7	11	renal transplantation	♀	44	-	-
A9	9	aplastic anemia	♂	8	-	-
A15	9	pyruvate kinase deficiency	♂	39	-	N.T.[b]
A16	10	pure red cell aplasia	♂	18	-	N.T.
A18	8	myelofibrosis	♂	68	-	-
A19	10	aplastic anemia	♀	12	-	N.T.
A20	10	non-Hodgkin lymphoma	♂	8	+ (40)	-
A23	10	non-Hodgkin lymphoma	♂	30	-	N.T.
A27	8	bronchus carcinoma	♂	56	-	-
A29	8	myelofibrosis	♂	75	-	-
A33	8	myelofibrosis	♂	72	-	-

[a] See legend Table 8.
[b] Not tested (N.T.).

Table 10

IMMUNOFLUORESCENCE ENDPOINT TITRES OF ANTISERA TO TYPE C RETROVIRUSES IN COCULTURES OF NORMAL HUMAN BONE MARROW AND DOG CELLS

Coculture	Passage no.	Sex	Age in years	Antisera[a] RA-SiSV-p30	 RA-R-MuLV
A10	8	♀	14	-	-
A11	11	♀	11	-	N.T.[b]
A12	9	♂	9	-	-
A13	8	♀	62	-	-
A14	8	♂	24	-	N.T.
A17	9	♀	51	-	N.T.
A21	11	♂	32	-	-
A22	11	♂	8	+ (40)	N.T.
A34	8	♀	14	-	-

[a] See legend Table 8.
[b] Not tested (N.T.).

cocultures were found at passage 6. Four positive cocultures were studied for a longer period of time at two-week intervals. The positive IFA staining persisted for about 2 months (up to passage 18 to 20). A decrease was observed during the following passages and the staining finally disappeared completely (maximally at passage 23).

SiSV-p30-related antigens were found in a total of 8 out of 38 cocultures. The majority of positive cases were detected in the group of leukaemic donors (6 out of 17) (Table 8). Of these 6 positive cocultures, 5 were derived from leukaemic children. In the group of nonleukaemic patients, only 1 out of 12 cocultures was scored as positive (Table 9). The positive sample came from a child with a non-Hodgkin lymphoma. Only 1 out of 9 cocultures derived from normal bone marrow donors contained SiSV-p30 related antigens (Table 10). This sample was derived from an 8-year-old child.

Reproducibility of detection of virus-related antigens in such cocultures was demonstrated in two separate instances. A portion of the bone marrow specimen of 2 leukaemic patients (numbers 1 and 5) was stored in liquid nitrogen. When the IFA appeared to be positive on cocultures of these bone marrow samples, the stored specimen were tested at a later time and found to be positive as well.

4.3. Absorption tests

To demonstrate the viral nature of the antigens detected in the immunofluorescence assay, IgG fractions of the RA-SiSV-p30 antiserum which wereabsorbed with fetal calf serum were further absorbed with either purified SiSV (grown in rat cells) or purified mouse mammary tumour virus (MuMTV) (for absorption procedure, see Chapter II, 2.4.3.1.). The absorbed sera were tested for residual anti-SiSV activity by use of the cytoplasmic IFA.

Specificity of the detection of SiSV-p30 related antigens in the cocultures was demonstrated by the absorption of the reactivity with purified SiSV and not with purified MuMTV (Table 11). The positive staining of both SiSV-producing cells and a positive coculture (A1) was completely abolished after absorption of the RA-SiSV-p30 antiserum with SiSV (a 6 log and at least a 3 log decrease in endpoint titre, respectively), while absorption with MuMTV resulted in only a slight decrease in the titre of the antiserum (in both instances, a one log decrease).

Table 11

ABSORPTION OF RA-SiSV-p30 ANTISERUM

Absorption with[a]	IFA endpoint titres[b] on REF + SiSV[c]	IFA endpoint titres[b] on coculture A1
None	320	40
purified SiSV[d]	<10	<10
purified MuMTV[e]	160	20

[a] IgG fractions of the antiserum were used. Before absorption with specific retroviruses the IgG fractions were first absorbed with fetal calf serum. SiSV used for p30 isolation was grown in human NC37 cells.
[b] Endpoint titres are expressed as the reciprocal of the highest dilution showing virus specific staining.
[c] Rat embryonic fibroblasts (REF) productively infected with SiSV.
[d] SiSV used in the absorption test was grown in rat cells.
[e] Mouse mammary tumour virus (MuMTV).

4.4. EXTRACELLULAR REVERSE TRANSCRIPTASE DETERMINATIONS

In addition to screening the cocultures for SiSV-p30 related antigens, extracellular reverse transcriptase was assayed. Five of the 8 SiSV-p30 positive cocultures were tested for the presence of viral polymerase in the supernatants for a maximum of 8 months (Table 12). Coculture A1 showed a somewhat increased level of reverse transcriptase after 1 month in culture. However, all other determinations were negative.

4.5. DISCUSSION

In a previous immunofluorescence study (Zurcher et al., 1975), SiSV-related antigens were detected in human bone tumour cultures; however, antigen expression was always low and varied with passage number. The objective of the current experiments was to obtain evidence for SiSV-related information in human leukaemia.

Experiments with animal cells *in vitro* have shown the existence of endogenous xenotropic type C viruses, which mostly cannot propagate in high

Table 12

REVERSE TRANSCRIPTASE ACTIVITY IN SUPERNATANTS OF COCULTURES POSITIVE FOR SiSV-p30 RELATED ANTIGENS

	Reverse transcriptase activity[a] after months in culture					
Cocultures	1	2	3	4	6	8
A1	14.7	0.4	0.9	1.6	1.1	1.7
A3	0.9	1.8	2.3	0.4	1.7	N.T.[b]
A4	1.7	0.4	0.8	1.9	2.3	N.T.
A5	2.5	0.5	1.3	0.5	0.6	N.T.
A22	0.7	0.9	0.1	3.0	N.T.	N.T.

[a] Reverse transcriptase activity per ml culture fluid, expressed as cpm x 10^{-3} ^{3}H-TMP incorporated on an oligo dT(12-18)-poly rA template-primer. The underlined number is regarded as positive.

[b] Not tested (N.T.).

titre in cells of the species of origin (Weiss, 1969; Levy, 1973; Livingston and Todaro, 1973; Todaro et al., 1974). However, cocultivation with permissive cell lines from heterologous species frequently led to the isolation of such viruses. Using the cocultivation method, we detected SiSV-p30 related antigens in 8 out of 38 cultures of human bone marrow and the canine cell line, A7573. Despite the fact that some cocultures harboured SiSV-related polypeptides, all extracellular polymerase determinations were negative except for coculture A1. This discrepancy can be due to a slow rate of release of mature virions. Our results can be explained by postulating that the intimate contact between the bone marrow cells and the indicator cells allowed the transfer and subsequent propagation of the few virus particles present in the original bone marrow samples. However, an explanation for the absence of overt virus production and the disappearance of the viral antigens from the positive cocultures is lacking at the moment.

The specificity of the antisera and the immunofluorescence technique is a crucial requirement for the demonstration of viral footprints in these cocultures. Theoretically, sera of bovine origin may be a source of unwanted contamination with bovine leukaemia viruses when employed in cell cultures. However, no reactions were found between the p30-antisera and bovine leukaemia virus producing fibroblasts.

As the SiSV used for antiserum preparation was grown in a human lymphoid cell line (NC37), it is possible that the purified SiSV-p30 polypeptides were contaminated with human lymphoid cell antigens. Two arguments against the involvement of such cellular antigens in our IFA are: 1) the control, uninfected NC37 cells do not react in the cytoplasmic IFA; and 2) absorption of the RA-SiSV-p30 antiserum with SiSV grown in rat cells fully blocked the reaction with the primate virus producing cells and the cells of coculture A1 specificially.

Finally, the possibility has to be considered that the cocultivation procedure activated endogenous retroviruses which immunologically cross-react with SiSV in the A7573 cells. However, no retroviruses are known from the species <u>Canis familiaris</u> up to now, which makes this possibility very unlikely.

An overall view of the results is presented in Table 13. Seven of the 8 positive cocultures were derived from bone marrow of children. Five of the 6 positive donors in the leukaemic group were children. The two positive cases in the nonleukaemic and normal groups were also derived from children.

These results suggest an association between childhood leukaemia and this virus-related antigen.

The antigens detected in the cocultures are most likely produced by a transmissible agent derived from the human cells, which proved to be poorly replicative in various tissue culture systems in our investigations.

These results and our earlier studies (Chapter III) suggest childhood leukaemia to be a highly suitable disease entity for further virological studies.

Table 13

DETECTION OF ANTIGENS RELATED TO SiSV IN COCULTURES OF HUMAN BONE MARROW AND DOG CELLS

Bone marrow donors		No. of positive cocultures[a]/ total no. of cocultures	
<u>Patients</u>	Leukaemic	total	6/17
		adults[b]	1/8
		children	5/9
	Nonleukaemic	total	1/12
		adults	0/8
		children	1/4
<u>Normal individuals</u>		total	1/9
		adults	0/4
		children	1/5

[a] Cultures were scored as positive when the reciprocal of the endpoint titre of the anti-SiSV-p30 antiserum was 40 or higher.

[b] Adult individuals are those of the age of 18 or older.

CHAPTER V

DETECTION OF HUMAN TYPE C "HELPER" VIRUSES

INTRODUCTION

A type C retrovirus has been isolated from a spontaneous woolly monkey fibrosarcoma in tissue culture (Theilen et al., 1971). The virus appeared to consist of a replication defective sarcoma virus associated with a helper virus [woolly monkey (simian) sarcoma-leukaemia virus (SiSV)] which allows replication of the sarcoma component (Wolfe et al., 1972a; Scolnick and Parks, 1973). The defective sarcoma genome can be rescued from transformed cells by helper viruses of different mammalian origin. Since a putative human type C virus could be replication defective, it seemed worthwhile to cocultivate human tumour cells with cells producing a helper leukaemia virus. Theoretically, the rescued human sarcoma virus genome can then be assayed in vitro on susceptible target cells. Attempts in our laboratory to rescue a sarcoma genome from a human osteosarcoma cell line which showed viral antigens in the immunofluorescence assay (Zurcher et al., 1975) have failed so far.

On the other hand, the replication defective mouse sarcoma virus (MSV) can be rescued by leukaemia viruses of other mammalian species, including different primate type C viruses (Rhim et al., 1975b) and the human derived HL-23 retrovirus (Teich et al., 1975). Hence, cocultivation of mammalian cells nonproductively transformed by MSV and human tumour cells is a possible method for the detection of human helper viruses. We performed a few cocultivation experiments in which MSV transformed human cells were cultured in the presence of human leukaemic cells. However, no release of transforming viruses was ever found. In the experiments described in this Chapter, instead of tumour cells, we used a coculture of dog cells and human leukaemic cells, which was positive in the immunofluorescence assay for SiSV-p30 antigens (see Chapter IV). In the first part of this Chapter, we give a theoretical view of the sarcoma viruses (for a recent review on the genome of sarcoma viruses, see Van Zaane and Bloemers, 1978) and, in the Experimental Part, we describe the isolation of a helper virus of putative human origin.

5.1. SARCOMA VIRUSES

Harvey (1964), Moloney (1966) and Kirsten and Mayer (1967) independently described the isolation of viruses which induce sarcomas in mice from stocks of the Moloney and Kirsten leukaemia virus. These virus strains were called, respectively, the Harvey, Moloney and Kirsten strains of MSV. Besides oncogenic activity in vivo, MSV can induce foci of morphologically altered cells on infection of mouse cells in vitro (Hartley and Rowe, 1966; Ting, 1966). These foci can be readily counted and permit a quantitative in vitro assay of the MSV in cell cultures. The capacity to transform cells in vitro appeared to be a general characteristic of all avian and mammalian sarcoma viruses.

Most avian and mammalian sarcoma viruses are defective in replication. The defective nature of MSV was revealed when the titrations of some virus stocks did not show a linear dose-response curve: addition of an excess of leukaemia "helper" virus restored the linearity (Hartley and Rowe, 1966). It appeared that MSV transformation could also follow a one-hit kinetic curve when spread of progeny virus from cell to cell was prevented by suspending the cells in soft agar (Bassin et al., 1970). Here, the colonies were derived from a single cell transformed by a single MSV particle. The helper virus appears to supply viral polymerase, glycoproteins and perhaps other viral structural components to the MSV genome (Peebles et al., 1976a). An MSV genome enveloped in structural proteins provided by the helper virus (phenotypic mixing) is called a pseudotype and such a virus particle is neutralized by antiserum raised against the helper virus (Huebner et al., 1966). In addition to antigenicity, the host range of the pseudotype, both in vivo and in vitro, is also determined by the helper virus (Harvey and East, 1971). Not only murine leukaemia viruses (MuLV) but also type C viruses of other species are able to rescue a defective MSV genome (Aaronson, 1971; Rhim et al., 1975a; 1975b).

The sarcoma viruses have been obtained by inoculation of murine leukaemia viruses in mice and rats. Most likely the MSV genomes are derived from recombination between leukaemia viruses and the genetic information in cells from which MSV isolates were originally derived. The genomes of these sarcoma viruses contain sequences homologous to both the leukaemia viruses and the species of origin (Benveniste and Scolnick, 1973; Scolnick et al., 1973; Scolnick and Parks, 1974; Anderson and Robbins, 1976; Fraenkel and Fischinger, 1976; Hu et al., 1977).

A great part of the MSV genome is (endogenous) sarcoma-virus specific and at the 5'-end of the genome is a part of the leukaemia virus genome (Shih et al., 1978; Tronick et al., 1978). This leukaemia virus specific information is part of the gag-gene region and the portion of it can vary a great deal among the different classes of defective sarcoma virus genomes. With the aid of mammalian sarcoma-virus transformed cells the subgene order within the leukaemia virus gag-gene has been elucidated (Barbacid et al., 1976) (see also Chapter I, 1.1.2).Each sarcoma virus genome variant represents a distinct genotype which is stable even when the sarcoma virus is carried by a different helper or passed through cells of different lines (Aaronson et al., 1972; Peebles et al., 1973).

When tissue culture cells are infected with sarcoma viruses, different classes of transformed cells can be produced, e.g.: 1) sarcoma virus transformed (sarcoma virus genome plus, leukaemia virus genome minus) (S^+L^-) cells which do not release infectious virus except upon superinfection with helper virus (Peebles et al., 1971). The S^+L^- genome can code for the mouse helper virus 30,000 molecular weight major structural polypeptide (p30) (Bassin et al., 1971b) the 12,000 molecular weight polypeptide (p12) and the 15,000 molecular weight polypeptide (p15) (Barbacid et al., 1976); 2) cells transformed by sarcoma virus in the absence of infectious virus production expressing only p12 and p15 (Barbacid et al., 1976); 3) sarcoma virus nonproducer (NP) cells which are transformed by sarcoma virus in the absence of helper virus, and which lack any evidence for antigen expression (Aaronson and Rowe, 1970; Aaronson and Weaver, 1971; Klement et al., 1971; Rhim et al., 1973a; Barbacid et al., 1976). All these sarcoma genomes can be rescued by superinfection with helper virus.

EXPERIMENTAL PART

5.2. DETECTION OF TYPE C HELPER VIRUSES

In the previous Chapter, it was shown that cocultivation of human bone marrow cells with animal indicator cells leads to the transient expression of viral antigens. In the experiments described here we applied Kirsten strain of MSV-transformed nonproducer rat kidney cells (designated as K-NRK) in order to detect helper virus activity in coculture A1 (described in Chapter IV). The rat (K-NRK) NP cells do not produce infectious virus or leukaemia virus antigens (Aaronson and Weaver, 1971; Klement et al., 1971). We chose culture A1 for testing with K-NRK cells because A1 was the only coculture of dog thymus cells with human bone marrow which showed a small burst of extracellular reverse transcriptase activity (Chapter IV, Table 12).

Cells of the 9th passage of coculture A1 (negative for reverse transcriptase) were cocultivated with equal numbers of rat NP (K-NRK) cells. This coculture is indicated by KA1. Reverse transcriptase activity was detected in the supernatant after 8 days (Table 14). With the electron microscopic technique of Miller et al. (1973), mature and immature type C particles could be detected in the supernatant of the first passage of KA1 and not after repeated trials in supernatants of the A1 and K-NRK cultures alone. Successful rescue of the MSV genome by a helper virus is determined

Table 14

RESCUE OF MSV GENOME FROM RAT NP (K-NRK) CELLS

		Focus formation of rescued virus[a] assayed on			
Cultures	R.T. activity[b]	mouse[c] BALb/c-MEF cells	rat[c] WAG/Rij-REF cells	rabbit SIRC cells	human[c] kidney cells
A7573	0.4	0	0	0	0
K-NRK	0.9	0	0	0	0
Al-5th pas.	14.7	0	0	0	0
KA 1-1st pas.[d]	16.9	0	56	1250	956
KA 1-3rd "	7.2	N.T.	18	846	740
KA 1-5th "	3.0	N.T.	0	14	N.T.
KA 1-7th "	N.T.	N.T.	0	0	N.T.
KA 1st " [e]	0.3	0	0	0	N.T.

[a] Average number of foci per plate; 0 indicates that the foci were not detected in 0.2 ml of undiluted filtered virus preparation and N.T. indicates not tested.

[b] Extracellular reverse transcriptase (R.T.) activity expressed as cpm x 10^{-3} ^{3}H-TMP incorporated in a synthetic template-primer complex oligo dT(12-18)-poly rA per ml culture fluid.

[c] Primary cultures of BALB/c mouse embryo (MEF), WAG/Rij rat embryo (REF) and human kidney (HK) cells.

[d] Rat (K-NRK) NP cells were cocultured with equal numbers of presumed helper virus releasing cells. Supernatants from these cultures were examined for the presence of reverse transcriptase activity and focus formation.

[e] Normal A7573 cells were cocultured with K-NRK cells.

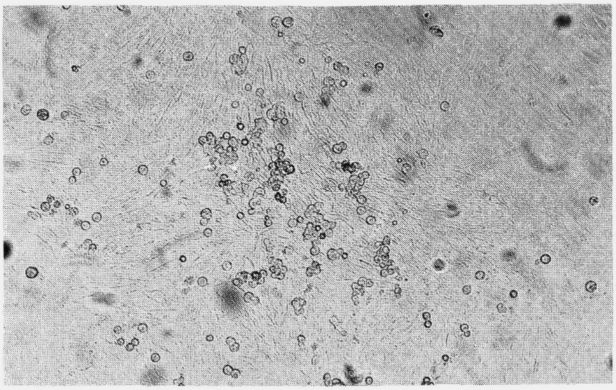

Figure 15: Focus of rabbit SIRC cells induced by MSV rescued from rat NP cells. Magnification x100.

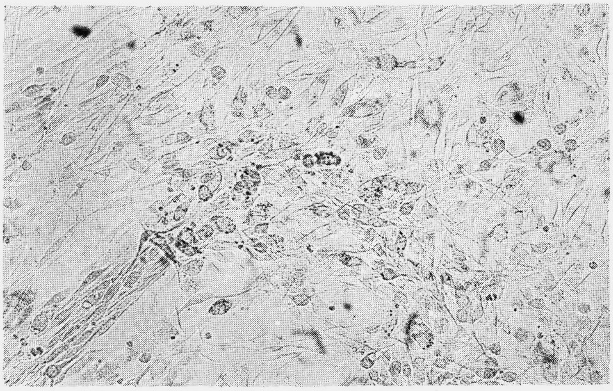

Figure 16: Focus of rat embryo cells induced by MSV rescued from rat NP cells. Magnification x 100.

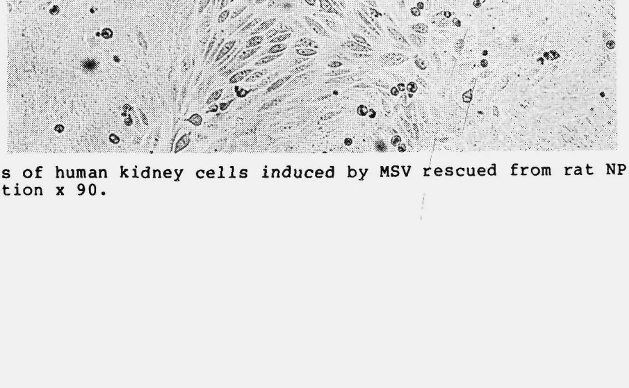

Figure 17: Focus of human kidney cells *induced* by MSV rescued from rat NP cells. Magnification x 90.

by focus assay. Supernatants of various passages of coculture KA1 were assayed for transformation on mouse, rat, rabbit and human cells (Table 14). A high focus forming capacity was found during 3 subsequent passages on rat, rabbit and human cells but not on mouse cells. A1 culture fluid like those from parental A7573 cultures did not induce transformation in any of the four target cells, neither did the supernatant of K-NRK cells and of a coculture of normal dog cells with K-NRK cells (coculture KA).

Four days after addition of the KA1 supernatants to the SIRC cultures, rounded refractile cells appeared and distinct groups of such cells were found after 6 days. These groups or foci could be easily distinguished from the background of SIRC cells (Fig. 15). Foci induced in rat embryo cells had a somewhat different appearance due to the detachment of several transformed cells and multinucleated giant cells were also formed (Fig. 16). Foci induced in human kidney cultures were mainly composed of fusiform altered cells (Fig. 17). Supporting evidence for the viral nature of the observed transformation is focus formation inhibition by specific antiviral antisera.

Complete neutralization of focus formation of the 2nd passage of the KA1 culture fluid on SIRC cells was achieved by incubation of about 80 focus forming units with goat anti-SiSV serum (GA-SiSV) (dilution 1 : 40) (Table 15). Incubation with rat anti-MuLV, rat-preimmune and normal goat serum had no appreciable effect on focus formation. Remarkable is the fact that virus production by the KA1 culture ceased after a few passages of cocultivation, as indicated by reverse transcriptase and focus formation activity.

Table 15

NEUTRALIZATION OF FOCUS FORMATION BY SPECIFIC ANTISERUM

KA 1-2nd pas. supernatant incubated[a] with	Focus formation assayed on rabbit SIRC cells[b]
DMEM[c]	84
Rat anti-MuLV serum (EMuLV)[d]	68
Rat preimmune serum	72
Goat anti-SiSV serum (GA-SiSV)[d]	0
Goat normal serum	60

---

[a] One ml of 1 : 1 dilution of supernatant was incubated with 1 ml of an an 1 : 40 dilution of serum for 45 min at room temperature, passed through a 0.45 µm filter and inoculated into rabbit SIRC cells.

[b] 0 indicates that foci were not detected in 0.2 ml of undiluted virus preparation.

[c] Dulbecco's Minimal Essential Medium.

[d] For antisera, see "Experimental Procedures".

5.3. VIRUS PRODUCTION AND HOST RANGE

SIRC cells incubated with supernatant of coculture KA1 (2nd passage) showed elevated extracellular viral polymerase activity in the supernatant during early passages, but this again decreased upon further passage. These infected SIRC cultures had a "wild" appearance with many foci consisting of cells which tended to pile up, but which were loosely attached to the monolayer. Several foci of SIRC cells (indicated by SKA) transformed by KA1 supernatant were transferred to small petri dishes and checked for virus production (Table 16). One of the lines (indicated by SKA21-3) derived from clone 3 started to produce large amounts of virus as estimated by reverse transcriptase assay.

Table 16

VIRUS PRODUCTION BY VARIOUS SKA21 CELL CLONES

Clone number	Reverse transcriptase activity[a]
1	8.7
2	10.0
3	14.4
4	5.1

[a] Measured 15 days after establishing of the clones. Enzyme activity is expressed as cpm x 10^{-3} ^{3}H-TMP incorporated on a synthetic template-primer complex oligo dT(12-18) rA per ml culture fluid.

Although SKA 21-3 cells are derived from a focus of transformed cells, the morphology of the virus-producing rabbit cells gradually became indistinguishable from the uninfected SIRC cells. However, when transplanted subcutaneously into adult athymic nude mice (2 x 10^6 cells per animal), 5 out of 5 mice developed subcutaneous tumours within two weeks. Inoculation of control SIRC cells into nude mice never resulted into tumour formation. The subcutaneous tumours were histologically undifferentiated sarcomas. After trypsinization, the cells were reestablished in culture and were confirmed as rabbit cells by iso-enzyme analysis (personal communication, Dr. P. Meera-Khan, Anthropogenetic Institute, University of Leiden, Leiden, The Netherlands).

Another characteristic of transformed cells which resembles the tumour formation _in vivo_ is the growth in soft agar. Purified agar (Difco Laboratories, Inc.) was dissolved in culture medium to 0.5 % concentration. One ml of 0.5 % agar was then added per 35 mm petri dish and allowed to solidify as a bottom layer. The SKA21-3 cell suspension (10^4 cells per ml) was mixed with one ml 1 % agar medium supplemented with 10 % fetal calf serum to a final concentration of 0.5 % agar. Dishes were incubated in a humidified atmosphere (5 % CO_2) at 37° C and, within a week, the SKA21-3 cells formed colonies of closely packed cells, with a plating efficiency of about 1 %. Control SIRC cells had no colony forming capacity in agar.

The high rate of virus release by the SKA21-3 cells persisted in subsequent passages. Figure 18 shows the electron microscopy of budding and mature type C particles found in SKA21-3 cells. The nucleoid of the budding particle is almost completed and there is already discontinuity between the viral envelope and the plasma membrane. The mature particles have an irregular shape and the inner components have a disorganized structure.

Table 17 shows the different viral parameters in subsequent passages of SKA21-3 cultures. Extracellular reverse transcriptase activity was high from the 6th passage and remained high. The SKA21-3 cells were originally transformed by MSV pseudotype with human helper virus and supernatants of the SKA21-3 cultures still contain cell transforming activity. The focus assay of Hartley and Rowe (1966) was carried out on secondary rat embryo cells. A linear dose-response curve with titres of up to 8×10^3 focus forming units (FFU) per ml was found (Table 17). Focus formation could be abolished by previous incubation of the virus containing supernatant (about 80 FFU; 45 min. at room temp.) with a goat anti-SiSV serum (GA-SiSV), but not with an antiserum directed against Rauscher murine leukaemia virus (R-MuLV) (no. 76). These neutralization data are in agreement

Table 17

BIOLOGICAL AND BIOCHEMICAL PARAMETERS OF SKA21-3 VIRUSES

Passage number of SKA21-3 cells[a]	Reverse transcriptase[b]	Focus formation[c]	XC-plaque formation[d]
3	14	N.T.	N.T.
6	480	8.1×10^3	9.6×10^5
12	510	2.6×10^3	2.4×10^5
20	337	3.2×10^3	N.T.

---

[a] Rabbit cornea cells (SIRC) producing human helper virus pseudotype of MSV

[b] Extracellular reverse transcriptase activity per ml culture fluid expressed as cpm $x10^{-3}$ ^3H-TMP incorporated on a synthetic template-primer complex oligo dT(12-18)-poly rA.

[c] Focus assay was carried out on secondary rat embryo cultures and expressed as focus forming units per ml; N.T. = not tested.

[d] The XC plaque assay was carried out on dog thymus cells and expressed as plaque forming units per ml.

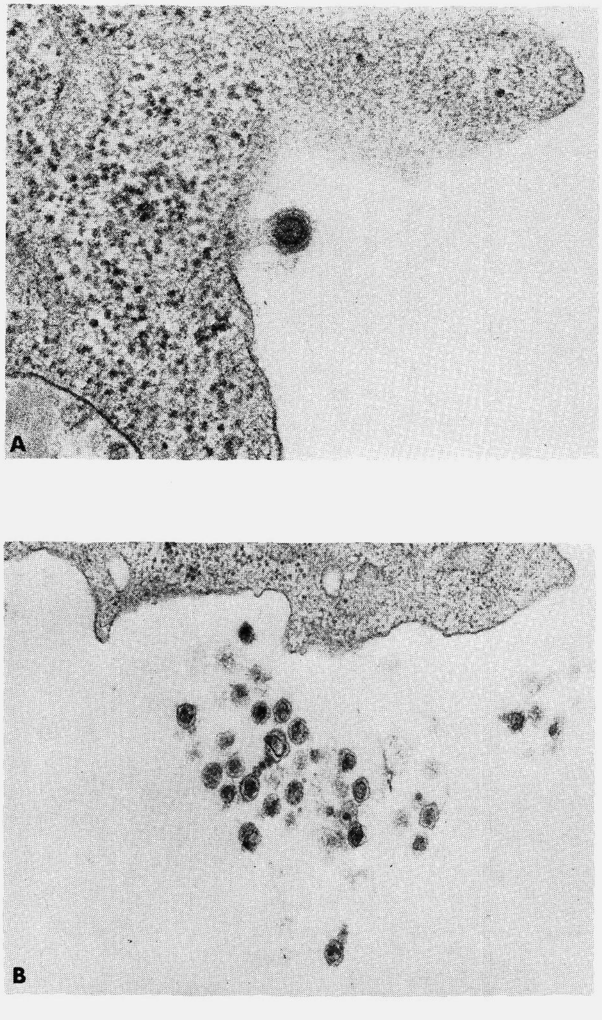

Figure 18: Type C particles in SIRC cells infected with the human helper virus pseudo-*type of MSV. A. Budding particle; magnification x 50,000.* B. Extracellular mature particles; magnification x 23,000.

with the previously reported SiSV-antiserum-neutralization of the focus forming activity of the KA1 supernatant.

The SKA21-3 virus induced syncytia in the XC cell line (Table 17, Fig. 19). The classical XC test (Rowe et al., 1970) was carried out with dog thymus cells and plaques were counted at day 9. A linear relation was found between plaques and dilution.

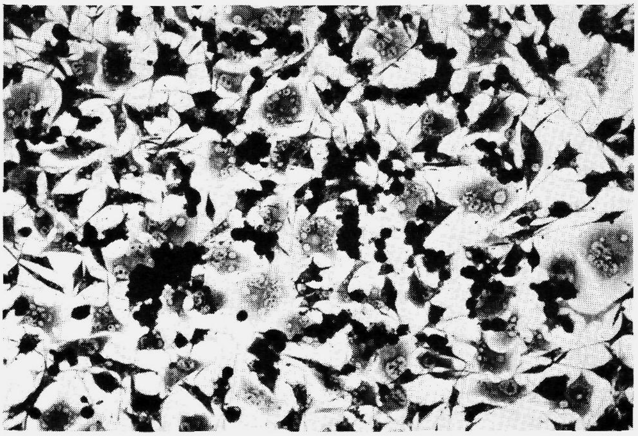

Figure 19: Syncytia induced by SKA21-3 virus in the XC cell line. Magnification x 140.

Virus released in SKA21-3 culture fluid appeared to have a buoyant density of 1.16 g per ml, which is characteristic for type C retroviruses. Ultrapellets obtained from tissue culture fluid were layered on a linear 20 to 50 % (wt/wt) sucrose gradient and centrifuged at 100,000 x g for 16 h at 4° C. The gradient was fractionated and monitored for density. Each fraction was diluted with Tris buffer (10 mM, pH 8.0) and centrifuged at 100,000 x g for 60 min at 4° C. The pelleted virus was resuspended and 20 µl aliquots were used in the synthetic template-primer assay (Fig. 20).

Host range studies on the SKA21-3 virus were carried out by transmission on various mammalian cell lines. Table 18 shows that the virus can replicate in bat, dog, human, marsupial, mink, rabbit and rat cells, but not in mouse cells. This host range restriction for mouse cells had already been found in the focus formation assay (Table 14).

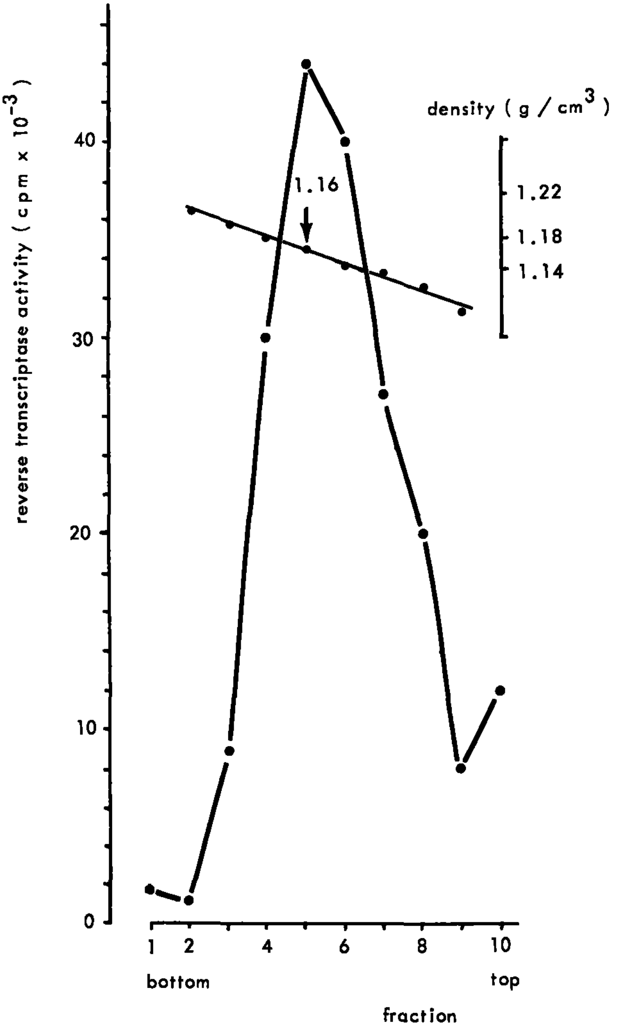

Figure 20: Sucrose gradient banding profile of particle-bound reverse transcriptase activity in SKA 21-3 culture fluid. Polymerase activity was assayed with the oligo dT(12-18)-poly rA template and expressed as cpm x 10^{-3} ^{3}H-TMP incorporated per 20 µl test sample.

Table 18

TRANSMISSION OF SKA21-3 VIRUS

Species	Host cell line	Reverse transcriptase activity[a] after months in culture		
		1	2	3
Bat	Tb 1 Lu	452	361	552
Dog	A7573	N.T.[b]	367	N.T.
Human	A204	17	129	122
Marsupial	PtK1	112	432	317
Mink	Mv 1 Lu	N.T.	257	101
Mouse	BALB/3T3	0.4	0.9	0.2
Rabbit	SIRC	40	895	46
Rat	K-NRK	101	580	930

[a] Reverse transcriptase activity expressed as cpm x 10^{-3} ^{3}H-TMP incorporated on a synthetic template-primer.

[b] Not tested (N.T.).

5.4. IMMUNOLOGICAL CHARACTERIZATION OF THE SKA21-3 VIRUS

5.4.1. Immunofluorescence test on fixed cells

Antisera directed against the major internal structural protein (p30) of both woolly monkey virus (RA-SiSV-p30) and Rauscher murine leukaemia virus (RA-R-MuLV-p30) were used in indirect immunofluorescence studies on acetone-fixed cells to assess the serological relationship of the putative human virus to other mammalian type C retroviruses. As can be seen in Table 19, the virus-producing SKA21-3 cells have SiSV-p30 related antigens in their cytoplasm. Weak positive reactions were seen with the antiserum directed against the p30 of murine type C virus. The murine and simian virus antisera had titres on the respective positive cells of 160-320 and 320-640. The weak reactions of the simian virus antiserum with MuLV infected cells and of the MuLV antiserum with SiSV producing cells are probably due to cross-reacting interspecies specific antigens. The SiSV-p30 antiserum had the same endpoint titres when assayed on rat cells producing SiSV and on SKA21-3 cells. This suggests that the SKA21-3 virus is closely related antigenically to the simian sarcoma-leukaemia virus, based on the p30 data and on the neutralization data (envelope proteins).

Table 19

IMMUNOFLUORESCENCE ENDPOINT TITRES OF SKA21-3 CELLS

Cell lines	Antisera[a] RA-SiSV-p30	 RA-R-MuLV-p30
SKA21-3	320-640	20- 40
SIRC	<10	<10
REF + SiSV[b]	320-640	20- 40
REF	<10	10
BALB/3T3 + R-MuLV[c]	40- 80	160-320
BALB/3T3	<10	<10

[a] Results are expressed as endpoint titres, presenting the reciprocal of the highest antiserum dilution with which virus-specific cytoplasmic fluorescence was still observed.

[b] Rat embryonic fibroblasts (REF) producing simian sarcoma-leukaemia virus (SiSV).

[c] Mouse BALB/3T3 cells producing Rauscher murine leukaemia virus (R-MuLV).

5.4.2. Sepharose bead assay on tissue culture fluids

Further serological characterization and quantification of the putative human type C retrovirus produced in tissue culture was carried out with the Sepharose bead immunofluorescence assay (for a detailed description of the technique, see Chapter II). The results shown in Figure 21 clearly demonstrates that SKA21-3 cells release antigens closely related to those of the simian sarcoma virus into the medium. Sepharose beads loaded with a goat antiserum (GA2-SiSV-p30) against the p30 molecule of SiSV (Seph-GA2-SiSV-p30) were incubated with either dilutions of purified SiSV (obtained from the National Cancer Institute, Bethesda, Maryland, USA; prepared by Electro-Nucleonics Lab., Inc.) or with dilutions of SKA21-3 fourth and 20th passage cell-free culture supernatant. Supernatants of uninfected SIRC and R-MuLV-producing BALB/3T3 cells served as controls. Staining of bound viral proteins was obtained by a subsequent incubation with a conjugated goat antiserum directed against SiSV-p30 (GA2-SiSV-p30-FITC). The protein concentration was adjusted to 10 mg per ml for each culture fluid. The antiserum directed against SiSV-p30 did not cross react with purified R-MuLV protein. The initial concentration of the SiSV preparation was 0.6 mg per ml. From Figure 21, it can be roughly estimated that the SKA21-3 supernatant (20th pass.) contained approximately 1 μg SiSV related proteins per ml, since a 25-fold dilution of the culture fluid roughly corresponds with a 15,000 dilution of the purified virus.

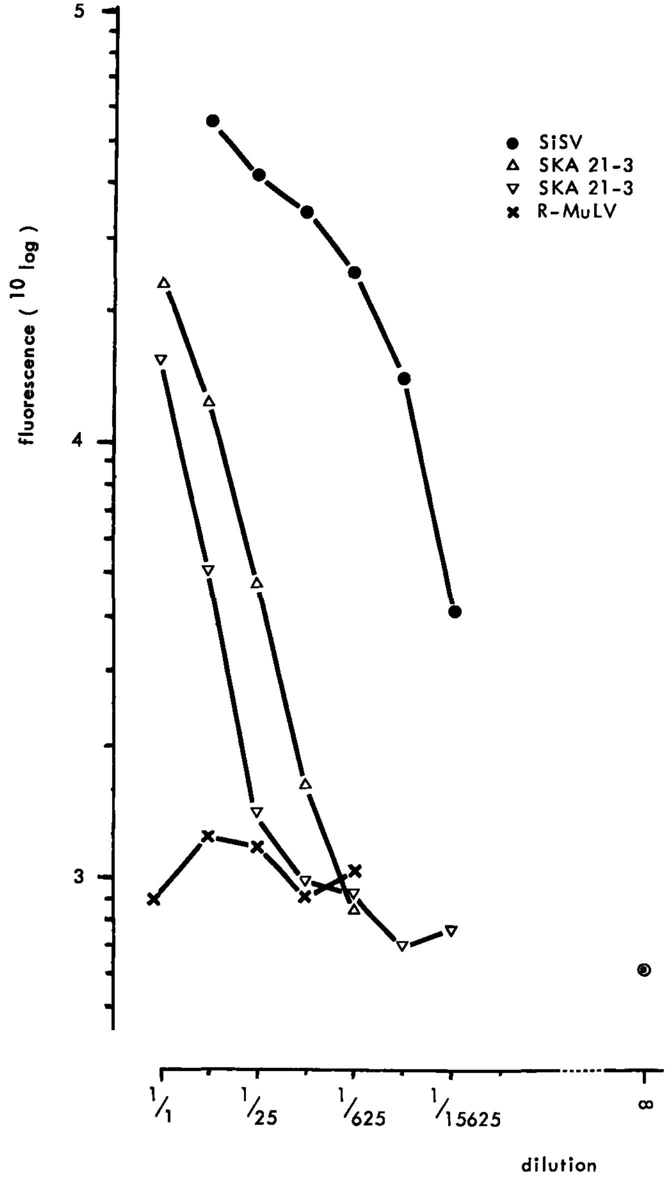

Figure 21: Characterization of viral proteins in the culture fluid of SKA21-3. Sepharose beads coated with an immunoglobulin G fraction of a goat serum directed against the p30 of SiSV (Seph-GA2-SiSV-p30) were incubated with fivefold dilutions of samples of:

● SiSV (purified); ▽ SKA21-3 (4th passage) (culture fluid); △ SKA21-3 (20th passage) (culture fluid); × BALB/3T3 + R-MuLV (culture fluid) and subsequently with GA2-SiSV-p30-FITC.

The protein concentration of all undiluted samples was 10 mg per ml.

A more detailed calibration curve was prepared to quantitatively measure the amount of SiSV related viral proteins. This calibration curve (shown in Fig. 22) has a sigmoid shape with a background plateau at 30 and a saturation plateau at 1200 ng per ml of SiSV. The fluorescence is linearly dependent on the virus concentration between these two values. No reactivity of this antiserum was found with fetal calf serum, even at concentrations of 10 mg protein per ml.

The concentration of SiSV-related proteins in different cultures was determined on the basis of the calibration curve (Table 20). The amount of viral antigens in the supernatants of two different passages of SKA21-3 culture was respectively 635 and 3332 ng per ml. The concentration in supernatants of cultures infected with SiSV was considerably lower (73 and 151 ng per ml), indicating that the SKA culture is a good virus producer.

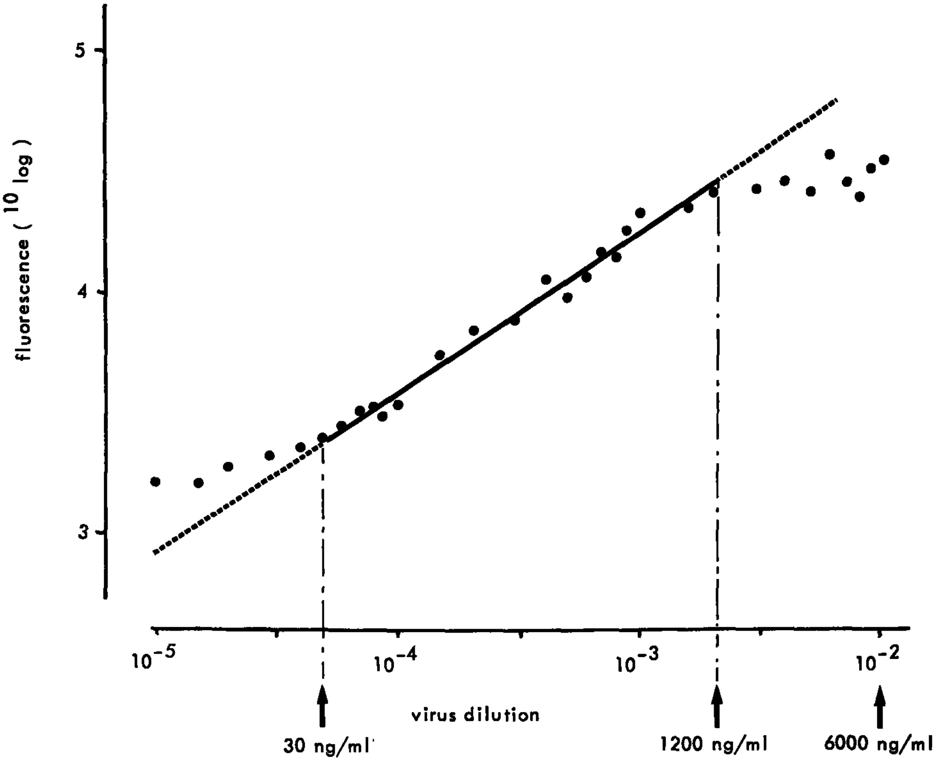

Figure 22: Calibration curve for SiSV antigens with Sepharose beads coupled with an immunoglobulin G fraction of a goat serum directed against the p30 protein of SiSV (Seph-GA2-SiSV-p30). The undiluted preparation contained 0.6 mg protein per ml. Seph-GA2-SiSV-p30 was incubated with different dilutions of the SiSV preparation and subsequently with GA2-SiSV-p30-FITC. The equation for the solid line was calculated by the least squares method ($y = 6.23 + 0.66\ x$; regression coefficient = 0.9902). The concentration of the undiluted sample was taken as 1 on the abscissa.

Table 20

AMOUNT OF SiSV EQUIVALENTS IN SUPERNATANTS OF VIRUS PRODUCING CULTURES

Cultures	ng SiSV equivalents ± SE[a] per ml culture fluid
SKA21-3 - 4th passage	635 ± 84
SKA21-3 - 20th passage	3332 ± 373
NC37 + SiSV[b]	73 ± 6
REF + SiSV[c]	151 ± 8
R-MuLV[d] (purified) - 1.7 mg/ml	<30
fetal bovine serum - 10 mg/ml	<30

[a] Mean ± SE of quadriplos.
[b] NC37 cells producing simian sarcoma-leukaemia virus (SiSV).
[c] Rat embryonic fibroblasts (REF) productively infected with SiSV.
[d] Rauscher murine leukaemia virus (R-MuLV).

5.4.3. Immunoelectronmicroscopy on SKA21-3 cells

Further study on the antigenic properties of the SKA21-3 virions was performed by immunoelectronmicroscopy with ferritin-conjugated antibodies. SKA21-3 cells and control SIRC cells were incubated with a rabbit antiserum (RA-SiSV) directed against purified SiSV. After washing, an additional incubation followed with ferritin-labelled goat anti-rabbit immunoglobulin G. The cells were then processed for electron microscopic examination. Anti-SiSV serum reacted solely with SKA21-3 cells and the ferritin label was found in discrete regions of the cell membrane (Fig. 23). The outer surfaces of budding and mature virions were specifically labelled. Staining of the cell surface was never observed in control SIRC cells.

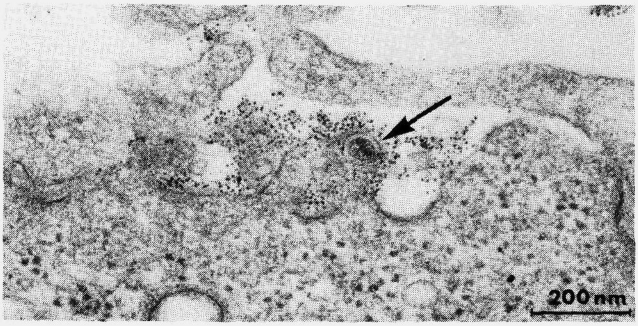

Figure 23: Immunoelectron microscopy on SKA21-3 cells. SKA21-3 cells incubated with a rabbit anti-SiSV serum were washed and incubated again with a ferritin-labelled goat antirabbit IgG. After being washed the cells were scraped of the surface, pelleted and further processed for electron microscopy. Labelling of the outer surface of a virus particle and of the cell membrane is seen.

5.5. DISCUSSION

Cocultivation of canine thymus cells with human leukaemic bone marrow (culture A1) resulted in a temporary release of type C virions as measured by extracellular reverse transcriptase activity. Cocultivation of the infected canine cells with the nonproducing cell line K-NRK transformed by MSV yielded a new pseudotype of MSV that could transform rat embryo, rabbit SIRC and human kidney cells but not mouse embryo cells.

The supernatants from the parental coculture A1 failed to transform any of the target cells. This suggests that the presumed human helper virus is not transforming. Comparison of the foci induced by the human helper virus MSV pseudotypes shows that the morphological appearance of a focus of MSV-transformed cells can differ substantially. It has been shown that these pronounced differences depend, among other things, on the type of target cell and the MSV strain used (Levy et al., 1973; Rhim et al., 1973b). The focus forming activity of the MSV-pseudotypes produced in this investigation was neutralised by an antiserum to the woolly monkey virus but not by an antiserum directed to murine leukaemia virus, indicating a close antigenic relationship of envelope proteins of SiSV and the presumed human helper virus. An irregular pattern of virus release at several passages of coculture KA1 was observed. Nevertheless, the cocultivation and subsequent rescue method seems to be highly sensitive for the detection of virus, as MSV-pseudotypes were produced in the cocultivation of K-NRK and A1 cells at a time that no viral polymerase could be detected in supernatant of the latter culture.

A cell line (SKA21-3) derived from a focus of transformed rabbit SIRC cells became a high producer of typical type C retroviruses as determined by electron microscopy, polymerase activity, buoyant density and plaque and focus formation. MSV-transformed lines often revert to a normal "flat-growing" phenotype while retaining the MSV genome (Fischinger et al., 1972). We observed that the MSV-transformed SKA21-3 line became flat growing and morphologically untransformed. However, upon seeding in soft agar, they formed colonies and were tumorigenic in nude mice. These results indicate by both criteria that the SKA21-3 cells have the properties of transformed cells.

By different serological methods, it was shown that the putative human type C virus is antigenically related to the woolly monkey virus in both internal and external structural proteins. The tests used included immunofluorescence on fixed cells (p30 proteins), neutralization of focus formation (envelope proteins) immunoelectronmicroscopy (envelope proteins) and the Sepharose bead assay on tissue culture fluids (p30 proteins). With the latter assay, the lowest concentration of viral antigens which can be detected is 1.5 ng per sample of 50 µl. Such a sensitivity is comparable to that of the radioimmunoassay which is widely used in tumour virology. The test, using antisera to purified SiSV-p30 polypeptides (GA2-SiSV-p30), proved to be specific, in that no reactions were found with culture fluids from uninfected cell lines or with large amounts of fetal calf serum. Only a very weak reaction of the antiserum to SiSV-p30 was found with high concentrations of Rauscher murine leukaemia virus, indicating that this antiserum (GA2-SiSV-p30) was mainly directed against the type specific determinants of the viral p30 molecule.

The A1 coculture which preceeded the SKA21-3 line was negative in the Sepharose bead assay (data not shown), while cytoplasmic IFA was positive for SiSV-p30. There was probably synthesis of internal viral proteins, but hardly any release of complete virions.

The presumed human type C virus shows a wide host range _in vitro_ as determined on a variety of mammalian cell lines. Such a wide infectious capacity is also reported for another human tissue derived type C virus HL-23V) (Teich et al., 1975). The SKA21-3 isolate, just like SiSV (Rhim et al., 1975c), shows a host range restriction for mouse cells.

The final SKA21-3 isolate was obtained by a number of successive cultivation procedures using 3 mammalian cell lines. It is possible that endogenous retroviral information of one of the cell lines used is picked up during this procedure. However, preliminary data on RNase T1 oligonucleotide fingerprints of the genomes of SiSV, GALV, HL-23V and SKA21-3 virus show that both the woolly monkey virus-related HL-23 component and the SKA21-3 isolate are "identical" to SiSV but distinguishable from the gibbon isolate (personal communication, Dr. B.G. Sahagan and Dr. W.A. Haseltine, Sidney Farber Cancer Institute, Boston, Mass., USA). Other arguments against recombination of the putative human SKA virus with endogenous viral genes are: 1) for the first line used for cocultivation (line A7573), no retroviruses are known at present. Even activation of possible endogenous canine retroviruses with halogenated pyrimidines (Lowy et al., 1971) was not successful (data not shown); and 2) the second cocultivation line (K-NRK) harbours, besides the Kirsten-MSV genome, also endogenous rat viruses (Klement et al., 1971; 1972). It is not very likely that the culture procedure triggered the release of an endogenous rat virus. Arguments against this are based on: a) serological data which show that the SKA virus is indistinguishable from the woolly monkey virus; and b) cocultivation experiments of K-NRK cells plus normal A7573 cells, which never led to the rescue of the replication-defective MSV genome.

Primate related type C viruses can be divided into 2 main groups on the basis of nucleic acid homology and antigenic relationship: the woolly monkey (SiSV)-gibbon ape (GALV) virus group and the endogenous cat (RD114)-baboon (BaEV) virus group. Experiments performed in the laboratories of Dr. R.C. Gallo suggest that the SKA21-3 isolate harbours 2 distinct primate viruses: one related to the RD114-BaEV group and the other to the SiSV-GALV group with the latter virus in far excess. This preliminary conclusion is based on reverse transcriptase inhibition, complement fixation, competition radioimmunoassay for p30, p15 and p12 and nucleic acid homology.

This remarkable finding is in agreement with experiments on two other human derived type C isolates. The HL-23 virus isolated from cultured human leukaemic cells contains 2 viruses: one indistinguishable from the prototype SiSV, the second indistinguishable from the M7-BaEV isolate (Chan et al., 1976: Okabe et al., 1976). The other human isolate (HEL-12), spontaneously released by human embryonic lung fibroblasts, was shown by the immunofluorescent antibody technique to contain one virus related to SiSV and another to RD114 virus (Panem et al., 1977). Whether this association of SiSV with BaEV in human material represents a functional relationship is completely unknown at the moment.

CHAPTER VI

ONCOGENICITY OF SKA21-3 VIRUSES IN RATS

INTRODUCTION

Final proof that viruses are oncogenic in man will always be difficult to obtain. Besides seroepidemiological evidence, transformation *in vitro* by the agent and tumour induction in experimental animals are the appropriate experiments to "prove" the carcinogenic action of viruses in man.

In this Chapter, we demonstrate the oncogenicity of a human tissue derived type C viral isolate in rats. The virus has been described in detail in the previous Chapter. Briefly a putative human helper virus was able to rescue the mouse sarcoma virus (MSV) genome from nonproducer rat cells. The presumed human helper virus pseudotype of MSV induced foci of transformed cells in mammalian cell monolayers. One clone of such transformed rabbit (SIRC) cells (referred to as SKA21-3) became a high producer of type C virions. The SKA21-3 viruses appeared to be antigenically related to the woolly monkey (simian) sarcoma-leukaemia virus (SiSV). In the next part of the Introduction to this Chapter, the pathogenic lesions induced by mammalian type C retroviruses are reviewed and, in the Experimental Part, the oncogenic potential of the presumed human isolate is tested in two types of experiments: (1) inoculation of SKA21-3 cells producing the MSV pseudotypes plus the human helper virus; and 2) inoculation of cells producing the helper virus alone.

6.1. PATHOLOGIC LESIONS INDUCED BY MAMMALIAN TYPE C RETROVIRUSES

The mammalian RNA tumour viruses can be divided into 3 groups on the basis of the pathologic lesions they are associated with: the mammary tumour, leukaemia and sarcoma viruses. The mouse mammary tumour virus (MuMTV) induces carcinomas of the mammary gland in mice. The leukaemia and sarcoma viruses, which are referred to as type C viruses, are associated with neoplasms of mesenchymal origin in a variety of animal species. We will discuss here the lesions induced by the mammalian type C leukaemia and sarcoma viruses.

6.1.1. Leukaemia viruses

After the discovery of Gross (1951) that the leukaemia of the inbred AK mouse strain could be cell-free transmitted to other mouse strains, many murine leukaemia virus isolates have been described (for a detailed account of individual viruses, see Gross, 1970). Most of them have been recovered in the laboratory after repeated transplantation and/or cell-free transmission of spontaneous or induced tumours in various mouse strains and may have arisen as a result of recombination of different endogenous viruses (Troxler et al., 1977). These "laboratory" viruses, which may be genotypically altered by the isolation procedures, provide a highly valuable tool for studying the mechanism of carcinogenesis. However, the naturally occurring virus-associated neoplasms are important as model systems for the etiology of human cancer. Such naturally occurring neoplasms with a presumed viral etiology are found in, for instance, wild mice (Gardner et al., 1973), inbred laboratory mice (Peters et al., 1973; Hartley et al., 1977), domestic cats (Jarrett et al., 1964), cattle (Miller et al., 1969) and primates in captivity (Theilen et al., 1971; Kawakami et al., 1972).

The pathogenic spectrum of the various murine leukaemia viruses can vary a great deal. They may induce polycythemia as well as different forms of leukaemia and lymphoma (for a review on the pathobiology of virus-induced murine leukaemias, see Siegler, 1968; Fey, 1974). However, the most common virus-induced lesions in mice are the thymic lymphosarcomas with or without leukaemia. Myeloid leukaemia, erythremic myelosis and reticulum cell sarcoma can also be induced by injection of murine type C viruses. From the wild mouse (Mus musculus), type C retroviruses with a neurotropic and lymphomagenic activity have been isolated. Inoculation of newborn wild mice leads to the development of lymphosarcomas and/or a neurogenic type of lower limb paralysis. The latter is due to vacuolar neuronal degeneration and gliosis, especially in the anterior horns and adjacent white matter of the spinal cord, resulting in neurogenic atrophy of the hind leg musculature (Gardner et al., 1973; Officer et al., 1973).

Feline type C leukaemia virus isolates obtained from spontaneous lymphosarcomas upon injection into newborn cats induce: lymphosarcomas, lymphocytic leukaemias and reticulum cell sarcomas (Jarrett et al., 1964; Rickard et al., 1969; Theilen et al., 1970), together referred to as lymphoreticular neoplasms (Jarrett et al., 1966). Other disorders of the haemopoietic tissues (e.g., granulocytic leukaemia, erythremic myelosis, aplastic anaemia, myelofibrosis) form a quantitatively minority of diseases experimentally induced by or naturally associated with feline leukaemia virus infections (Herz et al., 1970; Jarrett, 1971).

Evidence has accumulated for the viral etiology of enzootic leukaemia in adult cattle and sheep. The bovine and ovine leukaemia viruses are most likely closely related (Rohde et al., 1978). These retroviruses are consistently associated with bovine and ovine lymphosarcoma (Miller et al., 1969; Paulsen et al., 1972; Van der Maaten et al., 1974; Piper et al., 1975). Inoculation of the bovine leukaemia virus induced lymphoid tumours in sheep (Olsen et al., 1972).

Type C retroviruses are found to be the major etiological factors in the development of lymphocytic and myeloid leukaemias and lymphosarcomas in gibbons in captivity in 3 independent colonies (Kawakami et al., 1972; Snijder et al., 1973; DePaoli et al., 1973; Gallo et al., 1978). Young gibbons develop myeloproliferative disorders upon inoculation with the gibbon ape leukaemia virus (personal communication, Dr. T. Kawakami, Comparative Oncology Laboratory, University of California, Davis, California, USA). A closely related infectious type C virus has been isolated from a woolly monkey with a spontaneous fibrosarcoma (Theilen et al., 1971; Wolfe et al., 1972a). This viral isolate consists of a replication-defective woolly monkey (simian) sarcoma virus, with an excess of helper (leukaemia) virus (Scolnick and Parks, 1973). Attempts to induce leukaemias or lymphosarcomas in marmosets with the natural helper virus (the sarcoma-associated virus) of the simian sarcoma virus have failed so far (Bergholz et al., 1977).

6.1.2. Sarcoma viruses

Most sarcoma viruses are replication-defective and cannot produce infectious progeny unless the cells infected by the sarcoma virus are also infected with a leukaemia virus which acts as a helper (see review, Chapter V, 5.1.). The Harvey, Moloney and Kirsten mouse sarcoma viruses are probably formed by recombination of cellular "oncogenes" with endogenous viral genes coding for viral replication functions (Martin and Weiss, 1974; Shih et al., 1978; see also Chapter V, 5.1). These transforming viruses are actually "made" in the laboratory (for a historical review, see Gross, 1970). Most likely, the nontransforming leukaemia viruses served as a transducing agent capable of picking up cellular oncogenic information. Apparently this phenomenon, if it indeed exists, can also happen spontaneously in nature, since naturally occurring sarcoma viruses have been isolated from "spontaneous" sarcomas in inbred mice (Finkel and Biskis, 1968; Gazdar et al., 1972), cats (Snyder and Theilen, 1969; Gardner et al., 1970; Snyder, 1971) and a lower primate species (Theilen et al., 1971; Wolfe et al., 1971).

The pathology of MSV has been extensively reviewed by Harvey and East (1971). In general, these viruses induce solid mesenchymal tumours (sarcomas) at the site of inoculation in mice, rats, hamsters and Mastomys, with a short latency period. The lesions induced by the various sarcoma virus isolates can differ; we here will review the pathologic lesions induced by the Kirsten strain of MSV (MSV-K). In rodents, MSV-K produces sarcomas accompanied by erythroblastic splenomegaly and concomittant anaemia and cystic haemorrhagic and osteolytic lesions. This complex of lesions is referred to as "early" changes induced by MSV (Harvey and East, 1971). The "late" effects of MSV in susceptible species often consist of generalized lymphosarcoma. MSV-K evokes a distinct late response in rats which is defined as "malignant haemopoietic tumour of undifferentiated type, not involving the thymus" (Harvey and East, 1971).

Infectious stocks of MSV are always composed of a mixture of leukaemia and sarcoma viruses. Thus, animals injected with infectious MSV can develop two types of diseases: sarcomas and leukaemias. Presumably, the above-mentioned early effects are caused by the sarcoma virus genome and the late effects by the helper virus genome (Scher et al., 1975). The MSV-K-induced sarcomas have an angiomatous component and consist of pleomorphic undifferentiated mesenchymal cells with areas of fibroblastic or histiocytic differentiation. Sarcomatous areas are often present in the spleen, lymph nodes, liver, lungs and bone marrow. A general feature of the MSV-sarcomas and the late differentiated or undifferentiated haemopoietic tumours is their transplantability in newborn syngeneic recipients. The erythroblastic spleen cells induced in the early phase of the disease cannot be transplanted (Kirsten and Mayer, 1969).

The feline sarcoma virus can experimentally give rise to fibrosarcomas in cats and many other species as well, including the dog, rabbits, sheep, pigs and monkeys (Gardner et al., 1971; Wolfe et al., 1972b; Essex, 1974).

The simian sarcoma virus isolated from a naturally occurring fibrosarcoma of a woolly monkey induces fibrosarcomas in marmosets (Wolfe et al., 1971; Deinhardt et al., 1972).

EXPERIMENTAL PART

6.2. INDUCTION OF SARCOMAS

Ten newborn BALB/c mice were injected subcutaneously with SKA21-3 cells (2-5 x 10^6 cells per animal). (For a schematic representation of the isolation procedure and subsequent _in vitro_ and _in vivo_ passage of SKA21-3 viruses, see Figure 24). None of the inoculated mice developed signs of disease up to one year post injection. This is in agreement with the previously reported _in vitro_ host range of the putative human tissue derived helper virus (Chapter V). However, 20 out of 20 newborn WAG/Rij rats inoculated with SKA21-3 cells (2 - 5 x 10^6 cells per animal) developed typical MSV lesions with a latency period of 1 to 4 months. In addition, these rats also showed various proliferative lesions of the haemopoietic tissues. Many animals were anemic with haematocrit values sometimes as low as 10 %. Also posterior paralysis was common. Grossly firm, sometimes haemorrhagic, tumour nodules were found at the site of inoculation. Spleen and lymph nodes were often enlarged and haemorrhagic.

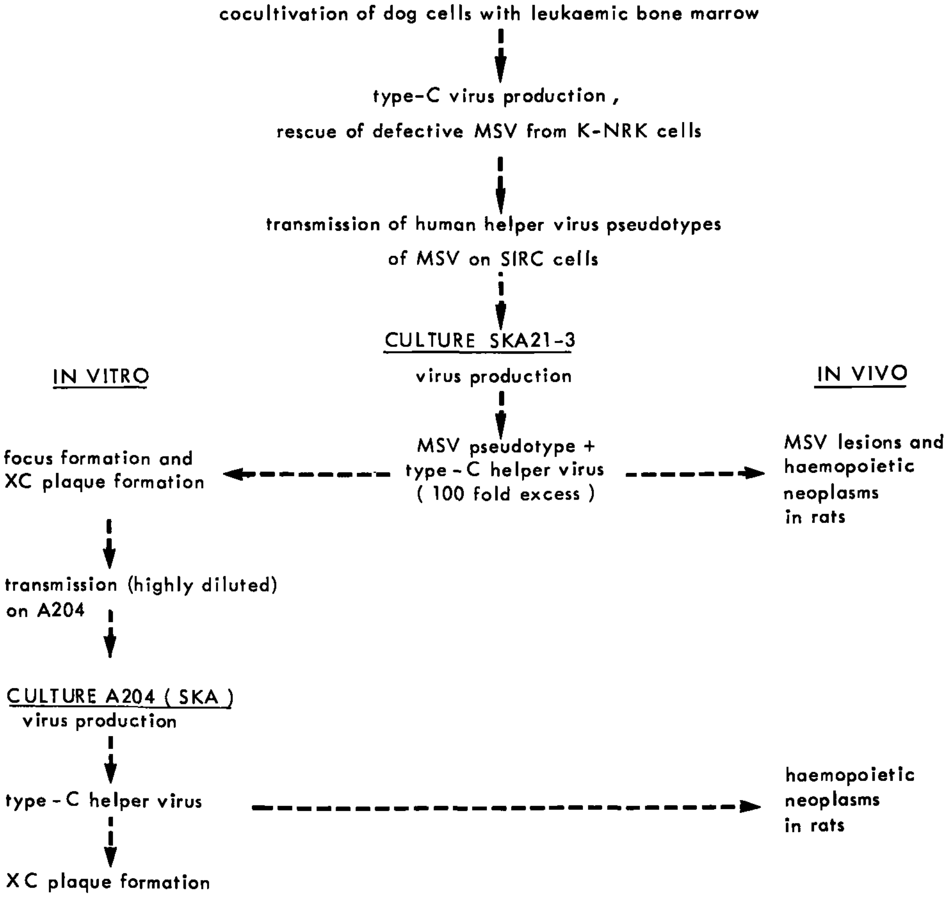

Figure 24: Schematic representation of the isolation procedure and subsequent passages of the SKA21-3 viruses.

The subcutaneous tumours were characterized histologically by a proliferation of fibroblasts, histiocytes and small vessels with extensive invasion of the surrounding tissue (Fig. 25). Cellularity varied appreciably between different areas of the tumour. Foci of erythropoiesis and haemorrhages were common. The tumour cells showed extreme nuclear polymorphism and mitoses were easily seen. There was considerable variation in the extent of the lesions among the different animals. Sarcomatous tissue was regularly present at the inoculation site and in lymph nodes and spleen. Less frequently, sarcomatous tissue was found in other organs such as brain, liver, lung, kidney, bone marrow and uterus. While the presence of MSV lesions was a constant finding in these rats, a variety of proliferative lesions of the haemopoietic tissues was observed as well (Table 21).

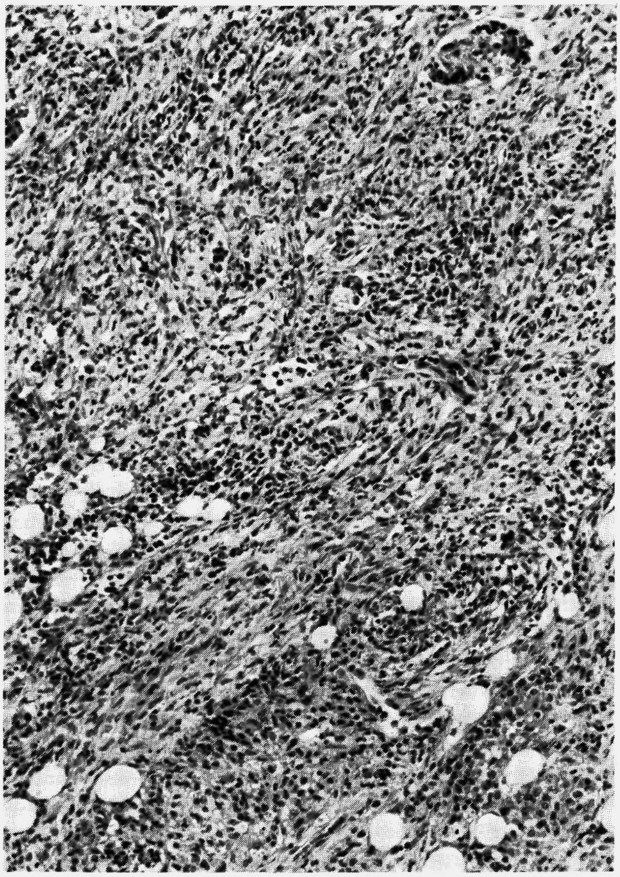

Figure 25: Subcutaneous sarcoma tissue in a rat neonatally inoculated with SKA21-3 cells. Fibroblastic and histiocytic differentiation (lower right corner) can be seen. HPS. Magnification x 210.

Proliferation of erythroid elements in spleen and liver was especially frequent (Fig. 26). In 4 cases, the erythroblastic proliferation was so extensive with such destructive growth in many tissues that a diagnosis of erythremic myelosis was made.

In 4 animals, a generalized lymphoblastic lymphoma developed (Fig. 27). The lymphoid origin was apparent from the cellular characteristics and from the fact that the tumour primarily affected lymphoid tissue (cortex of lymph nodes, white pulp of spleen, peribronchial lymphoid tissue and thymus).

In 3 cases, the tumour cells were undifferentiated. Combinations of proliferative lesions of erythropoietic elements and malignant undifferentiated haemopoietic tumours or lymphoblastic lymphomas were common. Thus, in 9 of the 20 rats inoculated, a malignant proliferation of haemo-

Table 21

HISTOLOGICAL DIAGNOSES OF 20 RATS AFTER NEONATAL INOCULATION WITH SKA21-3 CELLS

	MSV lesions[a]	Myeloproliferative diseases					Number of rats	
		benign		malignant				
		erythroid	myeloid	erythremic myelosis	lymphoblastic lymphoma	undifferentiated		
	+	-	-	-	-	-	4	11
	+	+	-	-	-	-	6	
	+	+	+	-	-	-	1	
	+	-	-	+	-	-	3	9
	+	+	-	-	+	-	1	
	+	+	-	-	+	+	1	
	+	-	+	+	+	-	1	
	+	+	-	-	+	-	1	
	+	+	-	-	-	+	1	
	+	-	-	-	-	+	1	
total	20	11	2	4	4	3	20	

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[a] As described under 6.2. in text.

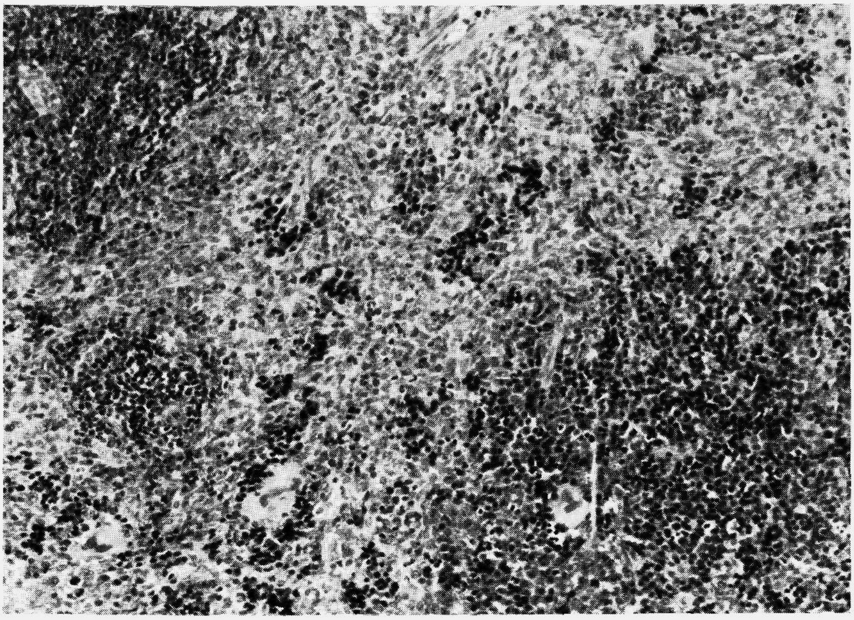

Figure 26: Abundant erythropoiesis in the spleen of a rat neonatally inoculated with SKA21-3 cells. Note normal white pulp (left upper corner). HPS. Magnification x 240.

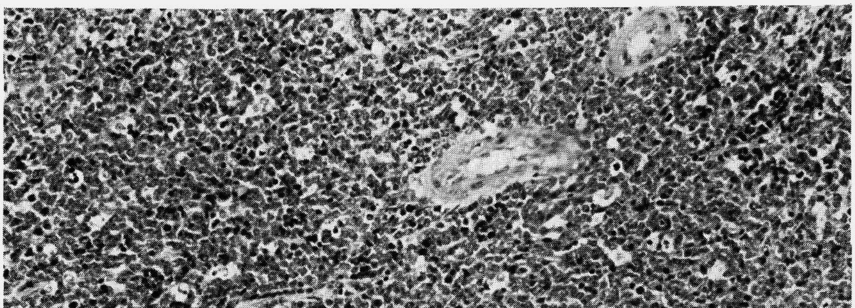

Figure 27: Lymphoblastic lymphoma in white pulp of the spleen in a rat neonatally injected with SKA21-3 cells. HPS. Magnification x 240.

poietic tissue elements could be demonstrated histologically.
Extensive epidural growth of malignant cells of haemopoietic tissue origin with invasions of the meninges and spinal cord often led to posterior paralysis (Fig. 28).

Subcutaneous inoculation of uninfected SIRC cells into newborn rats never led to local outgrowth of these cells nor did the animals show any other signs of disease during a 12 month observation period.

Some of the subcutaneous tumours were analysed for isoenzyme patterns (kindly performed by Dr. P. Meera-Khan, Anthropogenetic Institute, University of Leiden, Leiden, The Netherlands) and were found to be of rat origin. This suggests that the induced lesions were the result of virus produced by the inoculated SIRC cells.

6.3. *INDUCTION OF TUMOURS OF THE HAEMOPOIETIC TISSUES*

Evidence for the oncogenic potential of the presumed human type C virus was obtained by inoculation of cells producing the helper virus without MSV pseudotypes. Transmission of the helper virus freed of sarcoma virus was performed by endpoint dilution.

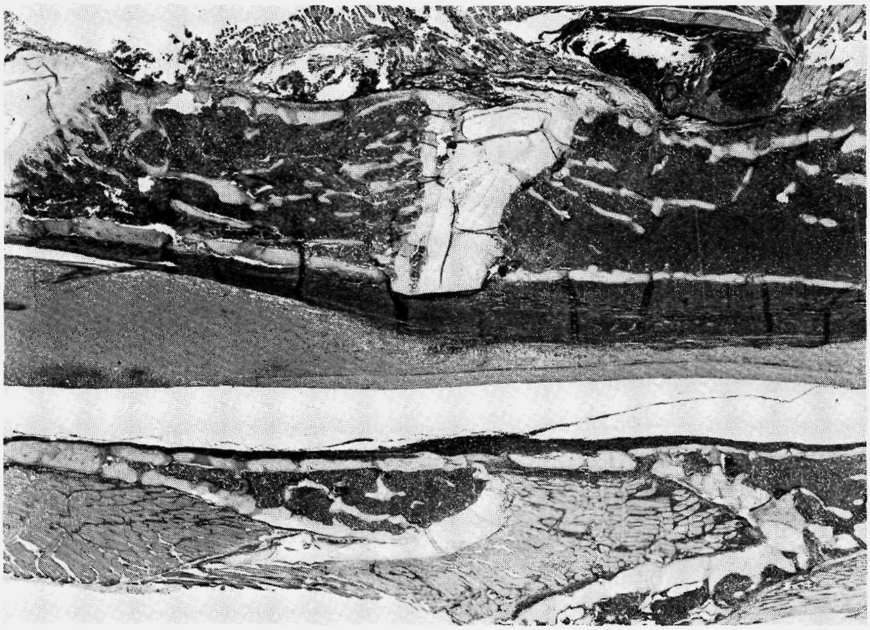

Figure 28: Involvement of vertebral bone marrow by an undifferentiated malignant neoplasm of haemopoietic tissue origin with extensive invasion of adjacent skeletal muscle and compression and invasion of spinal cord in a rat neonatally injected with SKA21-3 cells. HPS. Magnification x 18.

6.3.1. Endpoint dilution of SKA21-3 viruses on A204 cells

Both focus formation and XC plaque induction are biological techniques with which the ratio sarcoma virus-helper virus in the virus stock can be assayed. The SKA21-3 virus stock had an MSV: helper virus ratio of 1 : 100 (Chapter V, Table 17). Serial dilutions, up to 10^{-5}, were made of the virus stock produced by SKA21-3 and incubated on human rhabdomyosarcoma (A204) cells. After a few weeks, the cultures started to release virus as could be demonstrated by electron microscopy, reverse transcriptase activity, focus formation and XC plaque formation (Table 22). SKA21-3 virus dilution 10^{-5} on A204 cells, referred to as culture A204(SKA), had high polymerase activity, XC plaque formation (10^4 plaque forming units per ml), but no foci of transformed cells were induced upon inoculation onto rabbit SIRC and rat embryo cells.
Obviously, the MSV genome was titrated out of the SKA21-3 isolate and the virions propagated in A204(SKA) cells were most likely the presumed human type C helper. A204(SKA) cells had high immunofluorescence titres of SiSV-p30 related antigens in their cytoplasm and, in the culture fluids, about 800 ng SiSV-equivalents per ml were detected by the Sepharose bead immunofluorescence assay. With the nucleic acid hybridization technique, SiSV-related provirus could be detected in A204(SKA) cells (personal communication, Dr. R.G. Smith, National Cancer Institute, Bethesda, Maryland, USA). However, no indications for the presence of MSV proviral DNA were found.

6.3.2. Inoculation of A204(SKA) cells

Ten newborn BALB/c mice were injected subcutaneously with A204(SKA) cells (2-5 x 10^6 cells per animal). None of these mice developed signs of disease up to one year postinjection; the same was found for the inoculation of SKA21-3 cells. After inoculation of 20 newborn WAG/Rij rats with 2-5x10^6 A204(SKA) cells, no subcutaneous sarcomas were observed, but 11 of these rats died between 5 and 9 months after injection with a variety of proliferative lesions of the haemopoietic tissues (Table 23). Seven animals were still alive after 12 months and 2 died of sepsis at the age of 9 months.

Proliferation of erythropoietic elements (whether or not accompanied by a proliferation of other haemopoietic tissue elements) was a common finding in these rats. It varied from erythropoiesis of variable severity in spleen and liver (2 cases) to a generalized invasive neoplastic growth of erythroblasts (erythremic myelosis) (4 cases).

In 7 cases, a diagnosis of lymphoblastic lymphoma was made (Fig. 29). In two of these cases, a myeloid reaction was present as well. In one animal, areas of erythremic myelosis and areas with proliferation of undifferentiated blast-like cells were found in addition to the malignant tumour of lymphoblasts. The malignant character of the afore-mentioned

Table 22

DILUTIONS OF SKA21-3 VIRUS ON A204 CELLS

	Dilutions							
	10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	Reverse[a] trans-criptase	Focus[b] formation	Reverse trans-criptase	Focus formation	Reverse trans-criptase	Focus formation	Reverse trans-criptase	Focus formation
A204 -5th passage	70×10^3	-[c]	221×10^3	84	129×10^3	3	106×10^3	0

---

[a] Extracellular reverse transcriptase activity expressed as counts per minute ^{3}H-TMP incorporated on a synthetic template-primer complex oligo dT(12-18)-poly rA per ml culture fluid.

[b] Focus assay was carried out on secondary rat embryo cultures and expressed as focus-forming units per ml; 0 indicates that foci were not detected in 0.2 ml undiluted tissue culture fluid.

[c] Too numerous to count.

Table 23

HISTOLOGICAL DIAGNOSIS OF 11 RATS AFTER NEONATAL INOCULATION WITH A204(SKA) CELLS

	MSV lesions[a]	Myeloproliferative disease					Number of rats	
		benign		malignant				
		erythroid	myeloid	erythremic myelosis	lymphoblastic lymphoma	undifferentiated		
	-	+	-	-	-	-	1	
	-	-	-	+	+	+	1	10
	-	-	-	+	-	-	3	
	-	-	+	-	+	-	2	
	-	-	-	-	+	-	3	
	-	+	-	-	+	-	1	
Total	0	2	2	4	7	1	11	

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[a] As described under 6.2 in text.

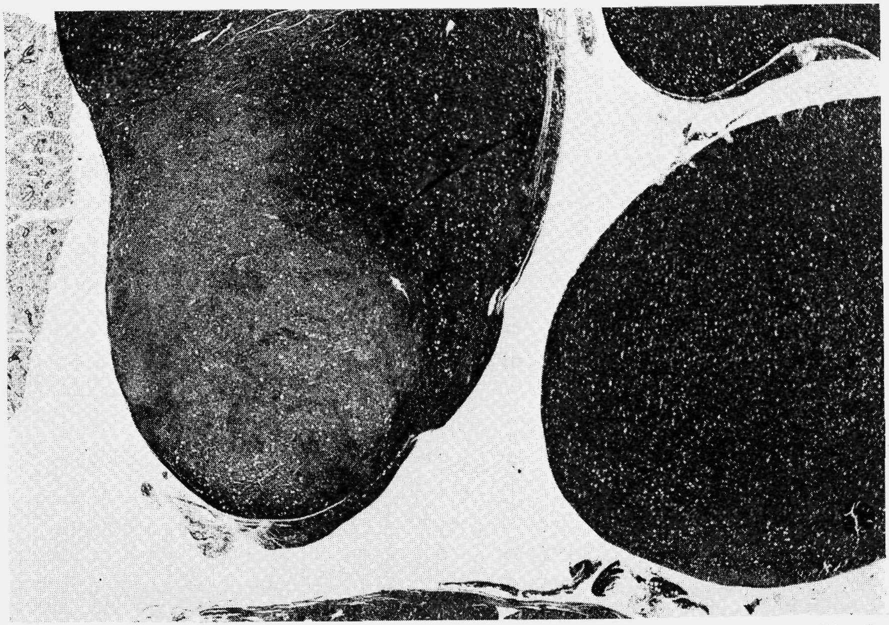

Figure 29: Enlarged lymph node with lymphoblastic lymphoma in a rat neonatally inoculated with A204(SKA) cells. Note extensive areas of necrosis (left), starry sky appearance and extracapsular invasion. HPS. Magnification x 18.

erythremic myelosis was reflected by the appearance of malignant subcutaneous tumours consisting of undifferentiated blast cells two weeks after inoculation of 5 x 10^6 tumour cells into newborn syngeneic rats. These animals died within 1 month after inoculation. At necropsy it appeared that the spleen, lymph nodes, lungs and kidneys contained tumour cells.

In the majority of the 10 rats with malignant haemopoietic tumours, the disease had progressed to the leukaemic form as could be concluded from the presence of many neoplastic cells inside blood vessels in the histological sections. None of the 11 necropsied animals showed signs of fibrohistiocytic sarcomas such as are found on inoculation of the MSV-helper virus combination. The hind limb paralysis which was present in several of these rats was due to tumour growth in the meninges and compression or invasion of the spinal cord. Subcutaneous inoculation of uninfected A204 cells into newborn rats never led to local outgrowth of these cells nor to the development of haemopoietic tumours during the 12 month observation period.

Some of the tumorous spleens were analysed for isoenzyme patterns (kindly performed by Dr. P. Meera-Khan) and the cells proved to be of rat origin. This suggests that the induced haemopoietic tumours were the result of virus production by the inoculated A204(SKA) cells.

From these studies, it appears that the helper virus alone may cause a proliferation of various progenitor cells of the haemopoietic tissues comparable to the spectrum of myeloproliferative lesions caused by FeLV (see Theilen, 1975) and that this proliferation can vary from a benign possibly reactive process to a generalized malignant neoplastic growth.

6.4. VIRAEMIA AND ABSENCE OF CIRCULATING ANTIBODIES

Sera of the experimental rats were examined for the presence of virus particles, viral antigens and antiviral antibodies.

Blood samples of 2 rats, one with a generalized lymphoblastic lymphoma and one with an erythremic myelosis, were made cell-free and processed for electron microscopic examination by the Millipore filter technique. The cell-packs from the same blood samples were processed according to the standard electron microscopic technique. Mature virus particles were found in sera and budding virions in peripheral blast cells of both animals.

Sera of rats which developed sarcomas or haemopoietic neoplasms contained SiSV-p30 related antigens, as estimated by the Sepharose bead immunoassay technique (Table 24). Beads coated with hyperimmune goat serum directed against SiSV-p30 (GA-SiSV-p30) were incubated with rat sera and stained with goat antiserum to rat IgG conjugated with fluorescein isothiocyanate. Of the 12 animals tested, 5 had high levels of SiSV-p30 equivalents in their serum, as estimated with the aid of a reference curve; 6 had intermediate levels and only one was negative.

Table 24

ANTIGENS RELATED TO SiSV-p30 IN SERA OF INOCULATED RATS

Rats inoculated with	No. animals tested	SiSV-p30 related antigens[a]
SKA21-3 cells	3	+
A204(SKA) cells	1	-
	3	+
	5	++

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[a] Sera are regarded as (+) if the amount of SiSV-p30 equivalent, as determined with the Sepharose bead assay, is between 50 and 200 ng per ml. Less than 50 ng is regarded as (-) and more than 200 ng as (++).

No circulating antiviral antibodies were found with either the Sepharose bead test or a radioimmunoprecipitation assay (kindly performed by Dr. P. Herbrink, Department of Pathology, University of Leiden, Leiden, The Netherlands).

6.5. RECOVERY AND CHARACTERIZATION OF VIRUS

Mature, immature and budding virions were found in thin sections of a tumorous spleen by direct examination with the electron microscope.

Virus was recovered from tumorous spleens and lymph nodes by the cocultivation method (Table 25). When lymph node cells of a rat with SKA21-3 induced generalized sarcomas were cocultivated with dog thymus (A7573) cells, the culture (A3894) became a producer of type C retrovirus after a few weeks, as assayed by reverse transcriptase. The supernatants contained focus forming activity when assayed on rat embryonic fibroblasts. Cells of this coculture were positive for SiSV-related antigens as shown by immunofluorescence tests on acetone-fixed cells. Weak cross-reactions were found with antiserum against Rauscher murine leukaemia virus. Spleen tissue of 2 rats with haemopoietic tumours were assayed for infectious virus by cocultivation with A7573 cells (Table 25). A few weeks after the initiation of the coculture, extracellular viral polymerase was found. The cocultures (A1117 and A3109) were positive for SiSV-related antigens. The reverse transcriptase positive culture supernatants were repeatedly negative for focus formation.

The antisera used for the serological identification of the viruses retrieved from the tumorous animals were examined for cross-reactivity with endogenous rat type C retrovirus (Table 25). Cell cultures producing endogenous type C rat viruses were made as follows: exponentially growing rat embryo cells were treated with 5-bromodeoxyuridine (20 µg per ml) for 24 h in order to activate endogenous rat viruses (Grollé et al., 1973). Three days after treatment, culture supernatant assayed for reverse transcriptase was positive (10 x 10^3 cpm per ml) and the cells were fixed with acetone. Only hyperimmune serum directed against Rauscher virus reacted with the halogenated pyrimidine-treated rat cells (titre 1 : 80); the antiserum to SiSV gave negative results on cells producing endogenous rat virus. However, the immunofluorescence endpoint titres of the cocultures A3894, A1117 and A3109 were high with the SiSV antiserum. Apparently, the virus recovered by cocultivation from tumorous organs is serologically related to SiSV and probably not to the endogenous rat virus.

Table 25

VIRUS DETECTION IN COCULTIVATIONS OF TUMOROUS RAT TISSUES

Cell lines	Reverse transcriptase activity[a]	Focus formation[b]	Immunofluorescence endpoint titres[c] Antisera RA-SiSV-p30	Immunofluorescence endpoint titres[c] Antisera RA-R-MuLV
Control lines				
REF+SiSV[d]	60	5	320	80
REF+BUdR[e]	10		10	40,80[f]
REF	0.6	0	10	20
BALB/3T3+R-MuLV[g]	910	0	40	320
BALB/3T3	0.3	0	10	20
A7573	1.1	0	<10	N.T.[h]
Coculture of MSV tumour				
A3894[i]	113	10	≥ 160	80
Cocultures of leukaemic spleens				
A1117[i]	35	0	≥ 160	>80
A3109[i]	10	0	≥ 320	40

[a] Expressed as cpm x 10^{-3} ^{3}H-TMP incorporated on the oligo dT(12-18)-poly rA template.

[b] Expressed as focus forming units per ml culture fluid.

[c] Expressed as the reciprocal of the highest dilution showing virus-specific fluorescence.

[d] Rat embryo (REF) cells productively infected with simian sarcoma-leukaemia virus (SiSV).

[e] REF cells treated with 5-bromodeoxyuridine (BUdR) (20 µg per ml) for 24 h.

[f] Results of two separate experiments.

[g] BALB/3T3 cells infected with Rauscher murine leukaemia virus (R-MuLV).

[h] Not tested with RA-R-MuLV antiserum. With other anti-R-MuLV antisera the endpoint titres on A7573 cells were ≤ 20.

[i] Tumorous tissue was cocultivated with A7573 cells.

6.6. DISCUSSION

The association between a transmissible virus related to the woolly monkey and gibbon ape leukaemia virus and a few cases of human leukaemia (Gallagher and Gallo, 1975; Gabelman et al., 1975; Chapter V) suggests an etiological role particularly because of the proven oncogenicity of this group of viruses in subhuman primates (Wolfe et al., 1971; Kawakami, personal communication). The SiSV-component of the well-described HL-23V isolate retrieved from blood cells of a leukaemic patient (Teich et al., 1975) could transform cells in vitro (Markham et al., 1978), and could induce fibrosarcomas in marmosets (Bergholz et al., 1977). However, development of haemopoietic neoplasms induced by this presumed human virus or the prototype of the woolly monkey type C helper virus has never been reported. In this Chapter, we have demonstrated that inoculation of cells producing MSV pseudotypes and presumed human helper virus induce sarcomas and haemopoietic tumours in rats. Tumour induction by inoculation of cell-free virus preparations was not attempted because of the previously reported (Deinhardt et al., 1972) poor response to this type of administration of SiSV as compared to inoculation of virus producing cells in susceptible animals. However, serum from an A204(SKA) inoculated rat with a lymphoblastic lymphoma again caused haemopoietic neoplasms upon inoculation into newborn WAG/Rij rats (data not shown).
Viruses serologically identical to the inoculated human type C virus were isolated by cocultivation of canine A7573 cells and tumorous lymph nodes and spleens of animals injected with virus-producing SKA or A204(SKA) cells. This finding coupled with the viraemia found in the inoculated animals shows that the human-derived virus can replicate in rats. Obviously, the lesions induced in the animals described in this Chapter are due to spread of viruses released by the inoculated SKA21-3 and A204(SKA) cells.

Several strains of murine leukaemia virus originated from the inoculation of human tumour material (see Gross, 1970). It became obvious that this procedure led to the activation of endogenous ecotropic murine type C retroviruses. There is no indication for the involvement of an endogenous rat virus in the induction of haemopoietic neoplasms by A204(SKA) cells. The virus isolated from tumorous material in our studies is serologically closely related to SiSV. The SiSV antisera used showed no cross-reactions with the rat endogenous type C virus when tested in either the cytoplasmic immunofluorescence test or in the Sepharose bead assay (data not shown). Because of the lack of antiserum directed against the endogenous rat virus, an antiserum to a murine leukaemia virus was used. This was justified by the observations of shared antigenic determinants by endogenous rat virus and murine virus (Table 24; Grollé et al., 1973; Sherr et al., 1975).

Another complication is the passage of the virus through various cell lines before its "leukaemogenicity" was demonstrated. Recombination between an "innocent" human-derived helper virus and the MSV genome and/or cellular genes of one of the cell lines used might have produced a leukaemogenic virus lacking the capacity to induce sarcomas. Mammalian type C viruses grown in animals or cultured cells of heterologous species can recombine with host cell genes to form virions with altered biological properties (Stephenson et al., 1974a; Sherr et al., 1976). We have no data for our own work supporting this recombination hypothesis at this time.

In our experiments, inoculation of virus-producing cells into adult rats did not result in signs of disease (data not shown). One hundred per cent of the MSV pseudotype-injected newborn rats and about 50 % of the helper-virus injected newborn animals developed a malignancy. In experimental tumour induction with primate Herpes viruses, the age of the recipient is not a restricting factor for tumour development (Deinhardt, 1975). However, except for the murine erythroblastosis viruses, the majority of oncogenic type C retroviruses are highly age-depent in inducing neoplastic growth (see Gross, 1970). The exact mechanism underlying this phenomenon is not yet understood and a discussion on it is beyond our scope.

CHAPTER VII

GENERAL DISCUSSION

The isolation of type C RNA tumour viruses from a few cases of human leukaemia and the occasional finding of viral footprints in human biopsies is in favour of a theory of the viral etiology of human leukaemia. However, the evidence published so far, on the presence of viral markers (nucleic acids, proteins and antibodies) in human leukaemia are highly controversial (for a review, see Chapter I). In this Chapter it will be attempted to explain the various inconsistencies with reference to specific animal models.

7.1. ENDOGENOUS VIRUSES

Both DNA and RNA viruses have been found in various animal species to be associated with leukaemias and lymphomas. There are, however, presently no good reasons to speculate that human leukaemias and lymphomas, except for Burkitt's disease, are caused by DNA viruses (Green and Wold, 1976). In contrast, the best candidates are to be found in the group of type C RNA tumour viruses. A role of exogenous RNA tumour viruses in the etiology of naturally occurring cancer has now been established in a wide range of animal species (Tooze, 1973). However, as far as the endogenous type C viruses are concerned, their role in the development of naturally occurring neoplasms is still a matter of debate (Niman et al., 1977) and is in fact only convincingly established in mice.

Endogenous type C viral genes representing part of the normal cellular genetic constitution are found in many animal species, among which are birds, mice, rats, cats, pigs and New and Old World primates (Benveniste et al., 1974a; Todaro et al., 1978a; 1978b) (for a review on endogenous viruses, see Aaronson and Stephenson, 1976). It is in fact assumed that each species harbours such viruses. Their function is not yet known (Todaro, 1975) but some of them have been shown to be oncogenic (Stephenson et al., 1974b; Greenberger et al., 1975). A prototype of naturally occurring leukaemia associated with viruses, is found in the AKR mouse strain. This strain has a high incidence of leukaemia due to the spontaneous activation of endogenous viruses (Hartley et al., 1977; Elder et al., 1977) which is followed later by lymphatic leukaemia, while the endogenous viruses remain generally repressed in other mice strains with a low leukaemia incidence (Gross, 1970). Endogenous type C viruses are released in cell cultures of normal tissues either spontaneously or after activation by different stimuli, e.g., treatment of the cultures with halogenated pyrimidines (Lowy et al., 1971) or inhibitors of protein synthesis (Aaronson and Dunn, 1974), stimulation of lymphocyte cultures with lipopolysaccharides (Moroni and Schumann, 1975) or Graft-versus-Host reactions in vitro (Hirsch et al., 1972).

Attempts to activate endogenous viruses in human cell cultures have failed so far, at least in the 200 individual cultures of human origin examined by Stephenson and Aaronson (1976). However, man is not unique in that respect: in his closest relatives, the apes (gibbon, orangutan, gorilla and chimpanzee), endogenous type C viruses have also not been identified. In fact, the evidence obtained so far for the human situation suggests the existence of horizontally spreading human viruses.

7.2. PUTATIVE HUMAN EXOGENOUS VIRUSES

In our work, the technique of cocultivation was paramount and appeared to be sensitive for the detection of presumed human type C viral footprints, most likely of exogenous viruses. Particularly in cocultures of dog cells (permissive for replication of primate viruses) with bone marrow of leukaemic children, woolly monkey (simian) sarcoma-leukaemia virus (SiSV)-related markers were detected. In this respect, the successful repetition of the experiments with stored bone marrow samples of certain positive patient biopsies is particularly convincing. This suggests that intimate contact between leukaemic cells and permissive target cells can lead to a transient expression of putative human viruses. This transient virus expression could not be influenced by treating the cocultivated dog cells with halogenated pyrimidines and/or glucocorticoids (data not shown) (Dunn et al., 1975; Niwa et al., 1975; Lowy and Scolnick, 1978). To my knowledge, most studies directed at the detection of human viruses have been done on biopsies from adult patients. Although the results in human leukaemia are generally negative, our work presented in the previous Chapters suggests that a viral association is more overt in childhood leukaemia. This could mean that in contrast to adult leukaemia viral antigens or antiviral antibodies can easily be detected in fresh tumour material and sera of leukaemic children.

Although occasional virus isolates have been obtained from human biopsies, there is no present evidence that such viruses are consistently present in human leukaemias. The experimental approaches employed have been electron microscopy, tissue culture, immunological and biochemical methods. As most available data are controversial, many investigators have seriously questioned the role of viruses in human leukaemia. In fact, the criticism at the moment of a model of viral leukaemogenesis in man is that there are no satisfactorily consistent reports on serology, provirus and virus isolation.

7.2.1. SiSV and BaEV in human tissues

In man, most convincing evidence for the existence of type C retroviruses concerns two primate viruses, SiSV and the endogenous baboon virus (BaEV). These type C viruses, however, are exogenous for man and therefore must have been acquired sometime during life. But then, according to the "provirus" theory (Temin, 1964) (which has been proven in the meantime), proviral DNA sequences must be detectable in the tumour cells (e.g. Shoyab et al., 1974). Indeed, BaEV proviral sequences in leukaemic cell DNA from several patients have been reported by one group (Reitz et al., 1976; Wong-Staal et al., 1976). It is remarkable, that in the original leukaemic spleen cells of patient HL-23 from which finally BaEV and SiSV-related viruses could be retrieved (Chan et al., 1976; Okabe et al., 1976), only BaEV provirus was found. Although the HL-23 leukaemic cell cultures readily released SiSV (Gallagher and Gallo, 1975), woolly monkey virus related proviruses could not be detected in the patient's tumour tissue (Reitz et al., 1976). These observations led to several assumptions. The most simple one assumes that the HL-23 viruses are laboratory contaminants. For the following reasons, however, this does not seem to be the case: 1) the viruses have been isolated from the patient on two separate occasions. The first time was from the patients peripheral blood; the other (6 months later) was from bone marrow when the patient was in remission (Gallagher et al., 1975); and 2) the simultaneous presence of both SiSV and BaEV in the isolate. This argument also holds true for other presumptive human isolates (Panem et al., 1977; Chapter V, this thesis). The coexistence of BaEV and SiSV in a number of isolates cannot be due to mere coincidence; it may suggest a functional relationship.

Another way of explaining the lack of SiSV provirus in human leukaemic cell DNA is the hypothesis of partial provirus (onc-gene?) integration and the assumption that only a selected part of the leukaemic cell population harbours complete infectious SiSV. Evidence for incomplete provirus integration has been described for host cell integrated prophages, representing about one half of the complete phage genome (Fischer-Fantuzzi and Calef, 1964). In the "higher" animal model, some recent evidence for the partial integration of provirus into host cell DNA was presented by Shevlyaghin et al. (1977). Inoculation of adult rats with the avian Rous sarcoma virus (RSV) results in the induction of different kinds of tumours. Interesting tumours were those which did not contain infectious sarcoma virus. Fusion of these tumour cells with chicken embryo cells resulted in rescue of the sarcoma genome. However, rescue of the RSV genome was not successful in one group of tumours. These tumours were negative for viral antigens and there were no detectable antiviral antibodies in rats bearing these tumours. These animals with viral induced tumours are extremely interesting because of their possible relevance to the human situation.
Using the technique of nucleic acid hybridization, Svoboda et al. (1977)

detected less than 50 % of the genome sequences in an RSV-induced mouse tumour cell line [unfortunately no data were available on whether the transformation specific (src) sequences were present in these cells]. Apparently partial integration of an retroviral genome and the establishment and/or maintenance of a transformed cell state can occur.

7.2.2. Viral antigens and antiviral antibodies

In general, viral induced tumours contain viral structural proteins. Exceptions to this have been described, e.g., tumours induced by the mouse sarcoma virus (MSV) in hamsters and guinea pigs do not release infectious virus nor do they contain viral antigens (Huebner et al., 1966; Rhim et al., 1976). However, the sarcoma cells do contain a rescuable, replication-defective sarcoma virus genome. An explanation for this phenomenon could be that the MSV helper virus can enter the cells, but that there is a block for integration of the helper virus genome. This implies that the tumours arose by outgrowth of single MSV-transformed cells.

Cells transformed in vitro can also show low or nonexistent levels of viral RNA expression (Deng et al., 1974; Peebles et al., 1976b). Thus, it is theoretically possible that some human tumours are composed of viral-transformed "nonproducer" cells. This is not very likely, however because, in that case, proviral sequences should still be detectable in the tumour cells (provided of course that the nucleic acid hybridization probe employed is representative for the unknown virus).

Another way to explain the absence of detectable viral markers is by assuming that only a small population of cells harbours DNA provirus and viral proteins. In that case, viral footprints cannot be detected in, for instance, leukaemic spleen tissue. This hypothesis implies that either the majority of leukaemic cells are intrinsically normal (they are "reactive" cells) or that most proviral sequences are lost from the chromosomes of the leukaemic cells during the process of oncogenesis (for a discussion on this subject, see also Gallo et al., 1977). At the moment, there are no additional arguments in favour of one or the other theory.

The absence of detectable viral proteins in human tumour cells is reflected in the absence of antiviral antibodies in human sera. Sera of animals exogenously infected with a retrovirus are either viraemic (antigenaemic) or contain antiviral antibodies (Kawakami et al., 1978). Even endogenous viruses can provoke a humoral immunity in their natural hosts, e.g., ecotropic mouse viruses (Nowinski and Kaehler, 1974; Ihle and Lazar, 1977) and xenotropic baboon viruses (Weislow et al., 1976).

The fact that human sera in general are negative for antiviral antibodies (when rigid criteria are applied) suggests that either retroviruses are not ubiquitous in the human population or that yet unknown mechanisms play a role in the apparent absence of detectable antibodies. Mechanisms which can theoretically explain the lack of detectable immune responses are rare. At least man can mount an immune response to type C viruses. Inoculation of formalin inactivated Rauscher murine leukaemia virus (R-MuLV) in melanoma patients led to the formation of high titres of antibodies directed against the major internal structural polypeptide (p30) and the envelope glycoprotein (gp70) (Hersh et al., 1974; Charman et al., 1975).

A natural defence mechanism for horizontal virus transmission has been considered to explain the absence of virus and antibodies in human sera. Sera from normal primates, including man, have the capacity to inactivate by lysis a number of retroviruses, among which are avian myeloblastosis virus, FeLV, MuLV, SiSV and gibbon ape leukaemia virus (GALV) (Welsh et al., 1975). The mechanism of viral lysis is complement-dependent (Cooper et al., 1976) and a role for antibody in this process could not be found. This inactivation was evidenced by the release of reverse transcriptase and high-molecular-weight RNA from the internal virus cores and by the loss of biological activity (focus formation and XC cell plaque formation). Sera from lower animals such as chickens and rodents do not contain this lytic activity.

However, Gallagher et al. (1978) found that the retrovirus lytic activity present in gibbon sera does not necessarily protect against infectious transmission of GaLV. Despite the presence of lytic activity in gibbon sera, these animals can be viraemic or can have antibodies. It is reasonable to assume that, if retroviruses do infect man, the findings in the gibbon ape also apply to man.

It should be emphasized that the absence of virus expression in human leukaemia cannot be taken to argue against a viral etiology of this disease in man. Although in a few species among which cattle, cats and gibbons overtly expressed viruses are associated with leukaemia, these animals are exceptions rather than the rule. In many other species a viral etiology of leukaemia has not yet been found and species with known virus-associated leukaemias also have other forms of leukaemia in which viruses are apparently not involved. For example, the spontaneous lymphomas from younger cats frequently show significant levels of feline leukaemia virus (FeLV) gene expression (viral RNA and major structural protein, p30), whereas no FeLV expression is detectable in the vast majority of the lymphomas in older cats (Niman et al., 1977). This could mean that viruses are not at all involved in the etiology of the "virus-free" neoplasms. On the other hand, it is quite possible that expression of the viral genome is a rare event in many naturally occurring virally induced neoplasms. For obvious reasons, in tumour virology, only those models in which excessive virus production can be observed have been studied *in extenso*. Thus, it would be important to investigate viral systems in which virus expression occurs less overtly.

7.3. IRRADIATION INDUCED LYMPHOMAS

A probably more suitable animal model is provided by the X-irradiation induced lymphomas in mice. Fractionated doses of irradiation result in a high incidence of thymic lymphomas in susceptible mice after about 6 to 12 months (Kaplan and Brown, 1952). From such irradiation induced lymphomas, virus isolates which upon injection into newborn mice again caused thymic lymphomas have been obtained (Gross, 1959; Lieberman and Kaplan, 1959). Thus, it was hypothesized that irradiation activated latent oncogenic endogenous viruses, which caused the primary lymphomas. However, with the technique of nucleic acid hybridization, viral RNA could not be detected in the tumour cells nor were viral proteins found in tumours on radioimmunoassay (Ihle et al., 1976b). These results are inconsistent: although infectious viruses can be isolated from the induced tumours, virus expression is nill. Therefore, it seems that viruses can be isolated occasionally from the X-irradiation induced lymphomas and, although these viruses are oncogenic, they are probably not involved in the induction of the primary tumours. On the other hand, there are still arguments in favour of a role of viruses in X-ray-induced lymphomas, e.g.: 1) a transient virus expression is observed after irradiation during the early stages of the disease (Haas, 1977); and 2) vaccination of young mice with a nonleukaemogenic virus can protect against a subsequent challenge with X-rays (Lieberman and Kaplan, 1977). Similar findings were reported by Peters et al. (1977). Inoculation of inactivated MuLV provoked immunity to endogenous type C virus and led to prevention of radiation lymphomas. Nevertheless, there is, for yet unknown reasons, no influence of the natural humoral immune response on the development of radiation lymphomas; regardless of the presence of antiviral antibodies, the lymphoma incidences are high (Ihle et al., 1976a). The fact that antiviral antibodies are not protective against virus-induced tumour development in a natural situation is also encountered in another system. Antibodies to the mouse mammary tumour virus in mouse sera are correlated with the incidence of mammary tumours and mouse strains with the highest tumour incidence are the strains with the highest antibody levels (Ihle et al., 1976c).

The parallels between irradiation induced lymphomas in mice and leukaemias in man are obvious: 1) lack of overt virus expression; 2) lack of sero-epidemiological evidence; and 3) infrequent isolation of infectious type C viruses.

What can be learned from this model is that tumour induction can follow unpredictable pathways and that viruses may play a role in a manner quite distinct from that found in, for instance, the AKR mouse. Besides this, it is shown by the radiation thymic lymphoma model (Haran-Ghera, 1977; Kaplan, 1977) and other models of virus induced neoplasia (Nandi and McGrath, 1973) that tumour induction is not a single-hit event, even when viruses are involved. Tumour formation is rather the result of a complex interaction of, e.g.: 1) viruses; 2) environmental influences (irradiation); 3) genetic constitution (virus host range; target cell susceptibility to transformation); 4) immunologic competence; 5) hormonal constitution; and 6) age and sex of the host.

7.4. ONCOGENICITY OF HUMAN VIRUSES

The assessment of a viral etiology of human leukaemia involves, among other things, the detection and isolation of the virus and demonstration of oncogenicity in its natural host. For obvious reasons, the latter criterion cannot be fulfilled in humans. Tumour induction in animals and *in vitro* transformation of human target cells with putative human oncogenic agents are the appropriate experiments. However, a few examples may show that final proof for oncogenicity of a virus in its natural host is often difficult to obtain. This problem is especially encountered in the case of Epstein-Barr virus (EBV) which is thought to induce African Burkitt's lymphomas in man. EBV can transform human cells *in vitro* (Yata et al., 1975) and is oncogenic for primates (Falk et al., 1976). Probably, under natural conditions, this virus is only oncogenic for man when special environmental conditions are provided (de-Thé et al., 1978). However the most convincing evidence for the supposed etiological role of EBV in the development of Burkitt's lymphomas would be obtained by prevention of the disease with specific vaccines (Epstein, 1978).

The monkey herpes viruses, H. saimiri and H. ateles are not oncogenic for their natural hosts, respectively, squirrel and spider monkeys, but cause lymphomas in other monkey species (Laufs and Meléndez, 1973; Ablashi et al., 1971). Human adenovirus type 12 can produce solid tumours upon inoculation into newborn hamsters (Trentin et al., 1962) but is not found to be associated with cancer in man. As far as our own work is concerned, the cocultivation procedures finally resulted in the isolation of a putative human type C virus which did not transform cells *in vitro* but which caused haemopoietic neoplasms in rats. The lack of capacity to transform cells *in vitro* is, however, a normal characteristic of "leukaemia" viruses. Recent results in our laboratory show that the woolly monkey sarcoma-leukaemia virus complex can induce histiocytic sarcomas in rats and that the woolly monkey leukaemia (helper) virus alone induces haemopoietic tumours in rats (Nooter and Zurcher, to be published). The demonstration of leukaemogenic activity of the SKA21-3 virus and the woolly monkey leukaemia virus suggests that these viruses can play a role in the development of human leukaemia.

7.5. ORIGIN OF PUTATIVE HUMAN VIRUSES

Animal species with which man is in close contact, e.g., cats and cattle, suffer from horizontally spread oncogenic type C viruses. Virus infected cats and cows thus provide a substantial reservoir of type C viruses in man's environment and, as interspecies transfer of retroviruses can occur, bovine and cat lymphosarcomas have been considered as possible human health hazards (McClure et al., 1974). Seroepidemiologic studies were initiated to identify antibodies against the bovine leukaemia virus (BoLV) in sera of farm families, farm employees and veterinarians. However, all sera tested in one study, including those of the 200 controls, were negative for antibodies to BoLV (Donham et al., 1977). In another study more than 2000 human sera, including sera from laboratory workers and patients with malignancies, were screened for antibodies and FeLV proteins (Krakower and Aaronson, 1978b). Again, there was no evidence of FeLV infection in man.

Several (mostly unpublished) claims have been made of the detection of presumed human viruses. After more thorough studies, these viruses appeared to be mouse type C viruses. Xenotropic mouse viruses can propagate in human cells. Thus, there is still the remote possibility that these presumed human viruses did not arise by laboratory contamination but were indeed associated with the human tumours under investigation (Weiss, 1975).

Until now, the two primate viruses, SiSV and BaEV, repeatedly appear in studies on viral involvement in human neoplasia. These viruses are exogenous for man, which would imply that, for instance, the endogenous baboon virus must have left its host of origin and spread horizontally in the human population. Indeed, interspecies transmission of type C genes has been postulated (Todaro, 1975). A few species of the genus Felis contain the endogenous RD114 virus. However, homologous sequences are present in cellular DNA of all Old World monkeys and apes, suggesting that an endogenous primate virus became a horizontally spread infectious virus that has accidentally been integrated into the germ line of an ancestor of modern cats. Actually, it is thought that all exogenous viruses have been developed from endogenous virogenes through an intermediate form, with or without interspecies transfer (Gillespie et al., 1975).

For SiSV, which is also exogenous for woolly monkeys, the situation is quite different from that of the baboon virus. At least, there is no reason to believe that SiSV is a woolly monkey virus: 1) only one SiSV isolate is known; and 2) no viral footprints are found in other woolly monkeys (Wong-Staal et al., 1975; Kawakami et al., 1978). Besides this SiSV proviral sequences are not present in normal or malignant cellular DNA in other New and Old World monkeys and apes, nor are other viral markers found. An exception is the gibbon ape, with its exogenous gibbon ape leukaemia virus (GALV) which is closely related to SiSV. This suggests that, in fact, the gibbon ape is the natural host for SiSV. However, antibodies directed to GALV have been found predominantly in gibbons which are exposed to intensive contact with man (Kawakami et al., 1978). Thirty per cent of the zoo animals examined, 60 per cent of the pet gibbons and zero per cent of the gibbons housed at institutions had specific antiviral antibodies. Thus, it is more likely that SiSV-GALV related viruses are horizontally spreading human type C viruses.

Finally, mice are considered as the original species in which the SiSV-GALV related viruses once were endogenous. Nucleotide sequence homology has been found for SiSV and laboratory mouse (_Mus musculus_) DNA (Wong-Staal et al., 1975). As most gibbon viruses have been obtained from animals living under natural conditions in South-East Asia, a search was made for related viruses in Asian murine species. Indeed, an endogenous xenotropic type C virus has been isolated from the South-East Asian murine species _M. caroli_ (Lieber et al., 1975). This virus is immunologically (reverse transcriptase, and p30) closely related to SiSV and GALV. Apparently, viruses of the SiSV-GALV group originated in Asian mice and subsequently entered primates. Paradoxically, the xenotropic and ecotropic _M. musculus_ viruses show a greater extent of homology (maximally 21 and 17 per cent) to the genomes of woolly monkey and gibbon ape viruses than does the _M. caroli_ virus (maximally, 6 and 13 per cent) (Benveniste et al., 1977).

7.6. VIRAL ETIOLOGY OF CHILDHOOD LEUKAEMIA

From the work presented in the previous Chapters, it seems that viral footprints are more easily detected in children than in adults. To arrive at a working hypothesis for the involvement of an exogenous virus in childhood leukaemia, one has to consider the following items: 1) contagiousness of a viral disease; 2) virus reservoir and route of transmission of a putative human type C virus; and 3) viral markers in the leukaemic cells and antiviral antibodies in the sera. A discussion of these subjects followed by a working hypothesis is given below.

At the moment, there is no good evidence for a contagious character of childhood leukaemia. This is not in contradiction with a possible viral etiology of childhood leukaemia: a) when the viruses are widespread among the human populations; and b) when the development of leukaemia is a complex, multifactorial phenomenon. These facts are encountered in the African Burkitt's lymphomas. These tumours are endemic in certain areas of Africa and are caused by the ubiquitous Epstein-Barr virus, in association with, among other things, environmental cofactors (de-Thé et al., 1978).

Virus transmission from parent to offspring (vertical) and from individual to individual (horizontal) require abundant virus production. Sources of virus with regard to transmission are rather restricted: saliva, milk, urine, faeces, sperm and placenta. In the cat, saliva proves to be the sole source of virus (Francis et al., 1977). In gibbon apes, however, urine and faeces are the main sources of virus excretion (Kawakami et al., 1977). Exogenous murine leukaemia viruses are transmitted mainly via milk (Law and Moloney, 1961). Obviously, the route of transmission in man is unpredictable. Besides indirect evidence for congenital transmission, there is at present absolutely no direct proof for any horizontal or vertical spread of putative human viruses along the aforementioned possible routes of transmission.

In mammals, most work on transmission of exogenous viruses is done in the mouse mammary tumour virus (MuMTV) system. The common route of extrachromosomal vertical transmission of MuMTV in the female mouse is via the mother's milk. Although biologically active MuMTV can be found in placentas of high-cancer strain (Mühlbock et al., 1952), only a few reports suggest intrauterine MuMTV transfer (Fekete and Little, 1942). In mice, extrachromosomal male transmission of MuMTV has also been described (Bittner and Frantz, 1954; Bentvelzen, 1968). Vasectomized male mice were shown to transmit the virus to females, demonstrating that is the seminal fluid which contains the virus and not the spermatozoa. Nevertheless, reports on direct in utero infection of the offspring by the male mouse are rare (Andervont, 1963). Thus, in analogy to the MuMTV model, direct in utero infection of human embryos with viruses is not very likely. Nevertheless, there are indications for vertical extrachromosomal transmission in man. Human embryo cells (Panem et al., 1975) and placental tissues (Sawyer et al., 1978) were found to be positive for proteins cross-reacting with exogenous type C viruses in immunofluorescence assays.

Another aspect of congenital virus transmission is germline integration of exogenous viruses, a mechanism by which exogenous viruses can become endogenous. Studies by Jaenisch (1976) have shown that mouse preimplantation embryos are rather resistant to germline integration by exogenous murine leukaemia virus. Nevertheless, newborn gibbons from viraemic mothers show GALV proviral DNA sequences in nontarget tissue (muscle) (Kawakami et al., 1978). It is not known whether congenital transmission in this case resulted in germline integration of GALV. Remarkable is the fact that SiSV-related proviral DNA sequences have been found in a patient with an osteogenic sarcoma. However, these proviral sequences were not found in the tumour, but in the liver (Prochownik and Kirsten, 1976).

The absence of viral markers in human leukaemic cells has already been discussed in this Chapter. In fact, the lack of detectable proviral sequences in leukaemic cell DNA is the most puzzling and can be explained (see also Gallo et al., 1977), for instance,by partial provirus integration, by the loss of provirus due to the chromosomal rearrangement often found in leukaemic cells or by a reactive nature of the leukaemic cell population. The latter assumption implies that the normal haemopoietic cell regulating mechanism is somehow affected by viruses. The possibility of the use of inappropriate "probes" (that is, probes of the known animal viruses), which results in missing the unknown human viruses, can apply, but cannot explain the absence of detectable SiSV provirus in patient HL-23 (Reitz et al., 1976). It is not possible at the moment to chose for one or the other above-mentioned theories.

The majority of human leukaemic cells does not express viral antigens. The same is found for irradiation induced lymphomas in mice. Since the products of the putative viral onc-gene have not been fully identified, their participation in physical carcinogenesis is quite possible. Another interesting model in this respect is the cat leukaemia model. A high percentage (50-70 per cent) of free roaming adult cats in urban areas has antibodies to an antigen (FOCMA-Feline Oncornavirus Associated Cell Membrane Antigen) associated with the feline leukaemia virus (Jarrett, 1976). This antigen is distinct from the structural proteins of this virus, but is probably virus-coded (Stephenson et al., 1977). It is associated with oncogenic transformation and may represent the onc-gene product of the feline sarcoma-leukaemia virus group (Sliski et al., 1977), comparable to the recently described onc-gene product of Rous sarcoma virus (Brugge and Erikson, 1977). This would suggest: a) that many cats are exposed to viruses of this group; and b) that immunosurveillance plays a significant role in the control of the disease, since only a very few of such cats develop leukaemias or sarcomas (Essex et al., 1975). Antibodies to FOCMA occur separately from those directed against the major envelope glycoprotein gp70 (Essex and Lamon, 1976). In analogy with this model, it is possible that human leukaemic cells contain such a nonvirion antigen which is coded for by putative human type C viruses.

At present, there is no reasonable explanation for the lack of antiviral antibodies in human sera. This represents a weakness in any theory of a viral etiology of human leukaemia. Nevertheless, an (incomplete) working hypothesis on a viral etiology of childhood leukaemia could be as follows:

Type C retroviruses, exogenous for man, are transmitted from parents to offspring. This extrachromosomal, congenital transmission takes place <u>in utero</u>, either transplacentally or via seminal fluid. Somewhere during embryogenesis haemopoietic cells can become transformed and are then subject to immunological control mechanisms. Escapes from control can lead to leukaemia.

Optimistic supporters of the idea of a viral etiology of human leukaemia often argue that in fact a comparable situation was once envisaged for EBV, which is accepted nowadays to be a human cancer virus. However, the two situations are not comparable. In the case of EBV, tumour formation finally appeared to develop along known pathways. For almost all African Burkitt's lymphomas, a close association with EBV has been found. The tumour cells carry the viral genome and contain virus-coded antigens and sera of Burkitt's lymphoma patients have high titre antiviral antibodies (for a review on the relation EBV-Burkitt's lymphomas, see Gunven, 1975). In contrast, in the case of a viral etiology of adult leukaemia in man, the putative human RNA tumour viruses are apparently not associated with the tumour cells in a manner described for the available animal models. Therefore, some animal models, for instance the irradiation induced thymic lymphomas in mice, deserve new experimental approaches.

Further progress with regard to the question as to whether SiSV is associated with childhood leukaemia would be achieved with answers to the following questions: 1) is the virus widespread in the human population?; 2) do the leukaemic cells contain any viral footprints?; 3) what is the mode of transmission of the virus?; and 4) is there any immunological response to the virus?, if not, why.

Such answers are needed not only for theoretical reasons, but may be important for prophylaxis and therapy of the disease. Besides this, subsequent steps could be the unraveling of a possible interplay of human viruses with environmental factors, such as radiation, in the origin of human leukaemia.

SUMMARY

Type C RNA tumour viruses are associated with neoplasms of the haemopoietic organs in several vertebrate species. Such viruses have also been isolated from two primate species: woolly monkey (simian sarcoma - leukaemia virus, SiSV) and gibbon ape (gibbon ape leukaemia virus, GALV). In addition, infectious type C viruses of unknown oncogenic potential have been recovered from baboons (baboon endogenous virus, BaEV). In contrast to the baboon type C viruses, SiSV and GALV are exogenous. That is, their genetic information can be acquired by the host cell genome in the form of integrated proviral DNA as a result of horizontal transmission. It is assumed that at least some neoplastic diseases of man are caused by type C retroviruses. During the past years, this hypothesis has been substantiated by publications dealing with the "biochemical" presence of type C viruses in man. Even the isolation of type C viruses from normal and neoplastic human biopsies in tissue culture has been reported. Chapter I extensively reviews type C RNA tumour viruses in general, the primate type C viruses and the search for human viruses.

The technique of cocultivation of human leukaemic bone marrow with substrates suitable for virus replication in combination with biological and biochemical methods for the detection of retroviruses is essential in our studies on the detection of human viruses. The cocultivation procedure was utilized because it provides an increased contact between target cells and presumed virus-containing cells over an extended period of time.

Chapter II is concerned with the experimental procedures used in our studies on the detection and characterization of presumed human viruses. These include cell culture techniques, biological and biochemical assays for type C leukaemia and sarcoma viruses and serological techniques for the characterization of leukaemia viruses.

The XC cell cytopathogenicity assay for the detection of putative human viruses is discussed in Chapter III. Leukaemia viruses can be titrated in the classical XC test. Virus transmission on susceptible target cells induces giant cell syncytia in a subsequent rat XC cell overlay. This classical XC test had to be adapted to the use of haemopoietic cells and was evaluated in an animal model. In the adapted XC test, virus-infected bone marrow cells are plated over an XC cell monolayer. From the results obtained with murine leukaemia virus-infected mice, it became apparent that: 1) the adapted XC test is reliable and sensitive; 2) relatively small numbers of test cells are needed; and 3) in the case of virus-induced leukaemia, bone marrow seems to offer a suitable tissue for cocultivation with XC cells and the subsequent quantification of virus-infected cells.

In the other experiments described in Chapter III, aimed at the detection of putative human leukaemia viruses, bone marrow of normal individuals and patients with a variety of haemopoietic disorders was assayed in the adapted XC test. Induction of syncytia was found in one case of childhood leukaemia. The XC cultures which showed a positive cytopathogenic effect also released type C particles, as revealed by electron microscopy. The establishment of good virus producer lines, even after transmission to secondary cells, was not successful. Nevertheless, it could be shown that the putative human virus was at least antigenically related to SiSV.

In Chapter IV, cocultivation experiments which were performed with the fetal dog thymus cell line A7573 instead of rat XC cells are described. Human bone marrow of leukaemic patients, nonleukaemic patients and normal individuals was cocultivated with A7573 cells. These cocultures were screened for retrovirus antigens by means of the indirect cytoplasmic immunofluorescence assay (IFA). Rabbit antisera against the major structural protein (p30) of SiSV and against purified Rauscher murine leukaemia virus were used. After two to three months in culture, 6 of 17 cocultures derived from leukaemic patients showed positive staining in the IFA with the anti-simian virus serum. Five of these 6 positive cultures were derived from leukaemic children. One of 12 cocultures of the nonleukaemic groups and one of 9 normal bone marrow cocultures were positive with the simian virus antiserum. None of the 38 cocultures stained positive in the IFA with Rauscher virus antiserum. It was shown by absorption experiments that the positive IFA results were virus-specific. These results provide evidence that SiSV related information was present in the original bone marrow samples and that cocultivation with permissive mammalian cells enabled the detection of viral footprints.

Quantitative assays based on the defect in the murine sarcoma virus (MSV) genome have been developed for leukaemia viruses. In Chapter V, we applied MSV transformed rat "nonproducer" (K-NRK) cells in order to detect human "helper" viruses in a coculture of dog thymus cells with bone marrow of a leukaemic child. The replication defective MSV was rescued from the K-NRK cells and the resulting MSV pseudotype with the presumed human helper virus induced foci of transformed cells in various mammalian cell lines. One clone (SKA21-3) of rabbit cornea (SIRC) cells transformed by such a pseudotype rescued from nonproducing K-NRK cells proved to be a good producer of type C virions. Electron microscopy of thin sections of SKA21-3 cells showed typical budding and mature type C particles. Viruses released in the SKA21-3 culture fluids had a buoyant density of approximately 1.16 g per ml, which is typical for type C viruses.
The supernatant of SKA21-3 cultures contained 10^3 to 10^4 focus forming units and approximately 10^5 XC plaque forming units per ml. There was an excess of helper virus, the ratio being 1 : 100. The serological relation-

ship of the SKA21-3 viruses to other mammalian type C viruses was assessed by focus formation neutralization tests, indirect cytoplasmic immunofluorescence tests, the Sepharose bead immunoassay and immunoelectron microscopy. With all four techniques, the SKA21-3 viruses appeared to be closely related to SiSV in both internal and/or envelope proteins.

The oncogenicity of the SKA21-3 viral isolate is reported in Chapter VI. Two types of experiments were performed: 1) inoculation of SKA21-3 cells producing the MSV pseudotypes plus helper virus; and 2) inoculation of cells producing the presumed human helper virus alone.

The MSV genome was eliminated from the SKA21-3 virus stock by incubation of human rhabdomyosarcoma (A204) cells with a high virus dilution (10^{-5}). After a few generations, this culture, referred to as A204(SKA), began to produce type C virions as detected by electron microscopy, reverse transcriptase activity and XC-plaque formation. No foci of transformed cells were induced upon inoculation of A204(SKA) supernatant onto rabbit SIRC and rat embryo cells.

SKA21-3 and A204(SKA) cells were inoculated subcutaneously into newborn BALB/c mice and newborn WAG/Rij rats. None of the BALB/c mice developed signs of disease upon injection with either SKA21-3 or A204(SKA) cells. This is in agreement with our findings on the in vitro host range of the SKA21-3 viruses. However, newborn rats inoculated with SKA21-3 cells developed typical MSV lesions after 1 to 4 months (angiomatous fibrohistiocytic sarcomas at the inoculation site and elsewhere in the body) and enlarged erythroblastic spleens accompanied by severe anemia. Also a variety of malignant neoplasms of haemopoietic tissue origin was observed.

Five to 9 months after inoculation of A204(SKA) cells, rats developed haemopoietic neoplasms. The majority of these were diagnosed as lymphoblastic lymphomas with a leukaemic involvement of the peripheral blood. These lymphomas caused severe enlargement of the lymph nodes. No fibrohistiocytic tumours, as seen in rats injected with SKA21-3 cells, were observed in these rats injected with the helper virus-producing A204(SKA) cells. SiSV-related type C viruses could be recovered from sera and tissue specimens of diseased animals from both groups, indicating that the observed lesions were most likely due to virus released by the inoculated SKA21-3 and A204(SKA) cells.

In Chapter VII, our experimental results are discussed in a broad context. The following items are discussed: putative human endogenous and exogenous viruses, the origin of the putative human exogenous viruses (SiSV and BaEV) and a comparison with the available data on human leukaemia with the mouse irradiation induced thymic lymphomas. It is attempted to give explanations for inconsistent results: the absence of proviral sequences in human leukaemic cell DNA, antiviral antibodies and viral antigens and, on the other hand, the isolation of SiSV and BaEV related type C viruses from human biopsies. Finally, a model for viral etiology of childhood leukaemia is postulated.

SAMENVATTING

C-type RNA tumorvirussen zijn geassocieerd met neoplastische aandoeningen van de bloedvormende organen in verschillende gewervelde diersoorten. Dergelijke virussen zijn ook geïsoleerd uit twee verschillende apesoorten: het wolaapje, met het wolaap sarcoom-leukemievirus (SiSV) en de gibbon met het gibbonleukemievirus (GALV). Bovendien zijn C-type virussen geïsoleerd uit bavianen (het endogene bavianenvirus, BaEV). Enige oncogene werking van dit virus is niet bekend. In tegenstelling tot het bavianenvirus, zijn het wolaap-en gibbonvirus exogeen, d.w.z. hun genetische informatie wordt verkregen door het gastheercel-genoom in de vorm van een geintegreerd proviraal DNA als gevolg van horizontale overdracht.

Het is logisch te veronderstellen dat tenminste enige vormen van kanker bij de mens veroorzaakt worden door C-type RNA tumorvirussen. Gedurende de laatste jaren wordt deze hypothese gesteund door publikaties over de biochemische aanwezigheid van C-type virussen in de mens. Zelfs is de isolatie beschreven van dit type virus uit in kweekgebrachte biopsieën van normale individuen en neoplastische patiënten.

In hoofdstuk I worden de C-type RNA tumorvirussen uitvoerig besproken alsmede de C-type virussen geïsoleerd uit apen en het onderzoek naar mogelijke C-type virussen bij de mens.

In hoofdstuk II worden de experimentele procedures beschreven die in ons werk gebruikt werden voor de opsporing en karakterisatie van menselijke virussen (b.v. celkweektechnieken, biologische en biochemische bepalingsmethoden voor C-type leukemie- en sarcoomvirussen en serologische technieken).

Het gebruik van de z.g. XC cel test voor de opsporing van mogelijk menselijke C-type virussen wordt beschreven in hoofdstuk III. Met behulp van de klassieke XC test kunnen C-type leukemievirussen getitreerd worden. Deze test moest worden aangepast voor het gebruik van beenmerg en dit werd geëvalueerd in een diermodel. In onze aangepaste XC test worden virus-geinfecteerde beenmergcellen uitgezaaid over een XC cel onderlaag in plaats van virus-geïnfecteerde fibroblasten. De resultaten verkregen met C-type leukemievirus geinfecteerde muizen tonen duidelijk aan dat: 1) de test betrouwbaar en gevoelig is; 2) betrekkelijk kleine hoeveelheid testcellen nodig zijn in de test; en 3) in het geval van virus-geinduceerde leukemie beenmergcellen een geschikt substraat lijken te zijn voor gebruik in de XC test.

De andere experimenten beschreven in hoofdstuk III zijn er op gericht virussen te ontdekken in menselijk beenmerg. Slechts één beenmergspecimen van een leukemisch kind, vertoonde een positieve XC test. Tevens werd met het electronenmicroskoop C-type virussen gevonden in het supernatant van deze positieve XC celkweken. Het bleek niet mogelijk om goede virus producerende lijnen te maken. Desalniettemin kon worden aangetoond dat het verondersteld menselijke virus verwant was aan het wolaapvirus.

In hoofdstuk IV worden kweekexperimenten beschreven met een hondecellijn (A7573) als doelwitcel in plaats van de ratten XC lijn. Beenmerg van leukemische patiënten, niet-leukemische patiënten en gezonde individuen werden samengekweekt met de A7573 cellen. Deze cocultures werden onderzocht op antigenen met behulp van de indirekte cytoplasmatische immunofluorescentietechniek (IFA). Konijnenantisera gericht tegen het belangrijkste interne structurele eiwit (p30) van SiSV en tegen een muizeleukemievirus werden gebruikt. Na twee tot drie maanden in kweek vertoonden 6 van de 17 cocultures van leukemische patiënten cytoplasmatische fluorescentie met het antiserum gericht tegen het wolaapvirus. In 5 van deze 6 positieve kweken was het beenmerg afkomstig van leukemische kinderen. Eén van de 12 cocultures van de niet-leukemische groep en één van de 9 normale beenmerg cocultures waren positief met het SiSV antiserum. Geen van de 38 cocultures kleurde in de IFA-test met het antiserum gericht tegen het muizevirus. Door middel van absorptietesten kon worden aangetoond dat de IFA resultaten virus-specifiek waren. Deze resultaten suggereren dat SiSV-verwante informatie in het oorspronkelijke beenmergspecimen aanwezig was en dat het samenkweken mét virus-ontvankelijke cellen de opsporing van virale voetsporen mogelijk maakt.

Kwantitatieve bepalingen gebaseerd op het incomplete karakter van muizesarcoomvirussen (MSV) zijn voor C-type leukemievirussen ontwikkeld. In hoofdstuk V is het gebruik beschreven van MSV-getransformeerde rattecellen (K-NRK) voor de opsporing van menselijke "helper" virussen in een cocultivatie van hondecellen met beenmergcellen van een leukemisch kind. Inderdaad kon het MSV genoom "gered" worden uit de K-NRK cellen. Het resulterende pseudotype-deeltje gevormd door MSV en het veronderstelde menselijke helper virus induceerde foci van getransformeerde cellen in verschillende zoogdiercellijnen. Een kloon (SKA21-3) van konijnencornea (SIRC) cellen getransformeerd door een bovengenoemd MSV-pseudotype bleek een goede producent te zijn van pseudotype deeltjes en helper virus partikels. Dit is gebaseerd op electronenmicroscopie, bepaling van het virale enzym en bepaling van de hoeveelheid helper virus en transformerend virus. De verhouding pseudotype partikels en helper partikels in het SKA21-3 weefselkweek supernatant was ongeveer 1 : 100. De serologische verwantschap van de SKA21-3 virussen met andere zoogdier type C virussen werd bepaald met cytoplasmatische immunofluorescentie, de Sepharose bollen immunofluorescentietest, immunoëlectronenmicroscopie en inhibitie van transformatie. Met behulp van deze technieken werd aangetoond dat de SKA21-3 virussen immunologisch sterk verwant zijn aan het wolaapvirus voor zowel interne als enveloppe eiwitten.

In hoofdstuk VI wordt de oncogeniciteit van de SKA21-3 virussen gerapporteerd. Twee verschillende experimenten werden uitgevoerd: 1) inspuiting van SKA21-3 cellen, die de MSV-pseudotype deeltjes en het helper virus produceren; en 2) inspuiting van cellen die alleen het vermeende menselijke helper virus produceren. Voor dat laatste experiment moest het MSV-genoom geëlimineerd worden uit het SKA21-3 virus bevattende supernatant. Dit gebeurde door eindpunt-verdunning (10^{-5}) op menselijke (A204) cellen. Deze kweek [A204(SKA)] werd een goede virusproducent. Het supernatant had geen celtransformerende aktiviteit meer, hetgeen suggereert dat het MSV inderdaad was geëlimineerd. Daarna werden SKA21-3- of A204(SKA)-cellen subcutaan ingespoten in pasgeboren muizen en ratten. Geen van de ingespoten muizen kreeg enige ziekte, hetgeen overeenkomt met in vitro gegevens van de SKA21-3-virussen. Echter één tot vier maanden na inspuiting van SKA21-3-cellen ontwikkelden ratten subcutane tumoren. Uit histologisch onderzoek bleek dat op de plaats van inspuiting en in lymfklieren en milt angiomateuse fibrohistiocytaire sarcomen aanwezig waren. Vijf tot 9 maanden na inspuiting van A204(SKA)-cellen stierven de ratten aan neoplastische groei van cellen van de bloedvormende organen, die merendeel werden gediagnostiseerd als lymfoblastische lymfomen. De lymfomen werden voornamelijk in de lymfklieren gevonden en vaak was er een leukemische uitbreiding in het perifere bloed. Op de plaats van inspuiting werd geen fibrohistiocytaire groei gevonden zoals na inspuiting van SKA21-3-cellen. C-type virussen verwant aan het wolaapvirus konden worden geïsoleerd uit sera en weefsels van de zieke dieren. Dit wijst er op dat de geïnduceerde lesies het gevolg waren van virus, geproduceerd door de ingespoten SKA- en A204(SKA)-cellen.

In hoofdstuk VII worden de resultaten in breder verband besproken. De volgende onderwerpen komen aan de orde: menselijke endogene en exogene C-type virussen, de herkomst van de mogelijk menselijke exogene virussen (SiSV en BaEV) en een vergelijking van leukemie bij de mens met stralingsleukemie bij de muis. Verklaringen worden gegeven voor tegenstrijdigheden in de resultaten, n.l. de afwezigheid van provirus in DNA van menselijke leukemiecellen en de afwezigheid van virale antigenen en daartegen gerichte antilichamen enerzijds en de succesvolle isolatie van C-type virussen verwant aan SiSV en BaEV uit menselijk materiaal anderzijds.
Tot slot is een mechanisme voor een mogelijke virale etiologie van kinderleukemie gepostuleerd.

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