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Cytogentic studies of tissues derived from cattle exhibiting the weak calf syndrome

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CYTOGENETIC STUDIES OF TISSUES DERIVED FROM CATTLE EXHIBITING THE WEAK CALF SYNDROME

by

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Several cytogenetic staining techniques were used to examine and characterize the chromosome complements of cells derived from various tissues of cattle. Comparisons of chromosome complements were made between cells of normal animals and cells of animals tentatively diagnosed as afflicted with Weak Calf Syndrome (WCS).

In addition, chromosome analyses were done on a transformed cell line of purported bovine origin (Calf B Sg Tr) which was thought to have acquired transformed properties as a consequence of the pathobiology associated with WCS.

Chromosome complements of non-transformed WC-derived tissues were cytogenetically normal for all parameters tested. Transformation was not observed in any of the WC-derived tissues. The chromosome complement of the Calf B cell line was not interpretable by comparison to normal bovine karyotypes. Examinations of the Calf B cell line as well as other transformed cell lines of purported weak calf and lamb origin by isozyme analysis and virus susceptibility studies revealed that all of these cell lines were inaccurately specified.

These studies offer a limited data base for the suggestion that transformation may not be a reproducible sequelae of long-term *in vitro* culture of WC-derived tissues.

CYTOGENETIC STUDIES OF TISSUES DERIVED

FROM CATTLE EXHIBITING THE WEAK CALF SYNDROME

If a man wishes to be sure of the road he treads
on, he must close his eyes and walk in the dark.

St. John of the Cross
The Dark Night of the Soul

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ABBREVIATIONS

ADP	adenosine diphosphate
A-T	adenine-thymine
ATP	adenosine triphosphate
BSS	Balanced Salt Solution
BT	bovine turbinate cell line
C	temperature Celsius
Calf B Sg Tr	Calf B salivary gland transformed
cyclic AMP	adenosine 3´: 5´-cyclic monophosphoric acid
cm	centimeter
CPE	cytopathic effect
DMBA	7,12-dimethyl-benz(a)anthracene
DNA	deoxyribonucleic acid
E	effector chromosome
EST	esterase
et al.	et alii (L, and others)
FCS	fetal calf serum
g	grams
G-C	guanine-cytosine
G6PDH	glucose-6-phosphate dehydrogenase
h	hours
HBT-3	human breast tumor cell line
HBT-39	" " " " "
HEK	human embryonic kidney line
HeLa	human cervical carcinoma cell line
Hep-2	human epithelial cell line

H7BSG	cell line of unknown origin
IDH	isocitrate dehydrogenase
JT	Jack (Ward) tissue sample
LAH	lactalbumin hydrolysate
LDH	lactate dehydrogenase
m	median
MDH	malate dehydrogenase
μ	micro-
MEM	Minimal Essential Medium
min	minutes
ml	milliliters
mm	millimeters
mM	millimolar
MNNG	N-methyl-N´-nitro-N-nitrosoguanidine
n	haploid number; also population size of sample
N	normality
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBT	p-nitro blue tetrazolium
NCTC	tissue culture medium
OSG-17	ovine transformed cell line
OV(H)Tr	ovine transformed passaged in hamsters
p	short arm of a chromosome
PBS	phosphate buffered saline
Pd	phosphate buffered saline without calcium and magnesium
PGM	phosphoglucomutase

PMS	phenazine methosulfate
PPLO	pleuropneumonia-like organisms
q	long arm of a chromosome
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
S	suppressor chromosome; also Svedburg unit; also cell cycle phase of synthesis
SC	single cell isolation
SCE	sister chromatid exchanges
SG	salivary gland
sm	submedian
SSC	standard saline citrate
st	subterminal
t	terminal
TDW	triple distilled water
390K	bovine kidney transformed cell line
Tris	2-Amino-2(hydroxymethyl)-1,3-propandiol
v	volume
Vero	African Green Monkey kidney cell line
w	weight
WCS	Weak Calf Syndrome

CHAPTER 1

INTRODUCTION

HISTORY

Signs and Gross Pathology

Weak Calf Syndrome (WCS) was first reported in the Bitterroot Valley of Southwestern Montana in 1964 (97). Briefly, signs characteristic of the syndrome are a reluctance on the part of the neonate to stand or move, a crusted and reddened muzzle, diarrhea and failure to nurse. Postmortem lesions include subcutaneous hemorrhages and edema, both of which are especially notable in the joint tissues. Gastrointestinal tract ulcerations, petechial hemorrhages of internal organs and muscular hemorrhages frequently accompany the appearance of the syndrome. Perhaps most noteworthy among the signs and lesions is the absence or involution of the thymus. Similar or related syndromes have subsequently been described in diverse locations throughout Northwestern and Midwestern United States (15, 97).

Etiology

The epizoology, pathology and etiology (ies) of WCS have not been clearly delimited (10, 15, 97). What has emerged from previous investigations, however, is the recognition of a constellation of circumstances which seem to be consistently associated with appearance of the disease. Factors contributing to the syndrome are, in broad terms; stress, nutritional parameters, presence of various

microbial agents, hormone anomalies and suppressed cell-mediated immunological response (15, 97). Lack of a clearly defined monocausal etiology makes the notion of synergistic effects operating between these factors very attractive on an intuitive level.

Studies done at the University of Montana showed that primary tissue cell cultures obtained from calves exhibiting the syndrome were transformed after prolonged in vitro culture (46). As such the cells exhibit the required characteristics of transformed cells. Among these requirements are immortality of transformed cells in culture, change in morphology, change in social behavior and synthesis of new antigens at the cell surface (76). These cultured tissues produced a transmissable filterable agent capable of inducing intranuclear eosinophilic inclusion bodies. Also, the agent was implicated in the induction of chromosome changes in cell cultures derived from tissues of embryonic and newborn bovine kidneys and salivary glands and from an ovine salivary gland.

CHROMOSOMES

Definition

The term chromosome has taken on increasingly broad meanings as our concepts of cytogenetics expand. The term will be used in this thesis to denote those bodies evident in the metaphase nucleus of eukaryotes when stained with certain dyes. At the metaphase stage of mitosis the nucleus has disappeared, and the chromosomes in their most compact form are lining up across the center of the cell.

Chromosomes of a given eukaryotic cell can be visualized by various staining techniques and can be arranged into morphologically identical pairs. The arrangement of cut-out photomicrographs of the somatic metaphase set into morphologically identical pairs is called a karyotype.

Chromosome number and structure are characteristic for all higher species. The normal somatic chromosome complement of cattle is 60, i.e., 2n = 60 (34, 50, 61). Different modal chromosome numbers may be observed, but the number within the individual will be constant. The cattle karyotype consists of 58 t autosomes and 2 sm sex chromosomes. The t and sm are symbols reflecting the variation in centromeric position. These various locations are relative positions on a continuum between two extreme geometric points (from median to terminal) according to the nomenclature of Levan et al. (51). The use of small case letters denote regions whereas large case denote exact points. Submedian (sm) and subterminal (st) always denote regions.

All autosomal pairs constitute a continuous series arranged in descending order of lengths and numbered in that order. This protocol is not strictly adhered to with respect to cattle karyotypes according to the convention established by Evans et al. (31).

The development of techniques for the longitudinal differentiation of chromosomes has made it possible to distinguish among individual pairs within a complement. Prior to 1971, only gross morphological features such as length, position of centromere (hence arm ratio), and to a lesser extent, the presence or absence of structures as satellites or secondary constrictions could be used to identify chromosomes.

Of course, observation of characteristic breaks or addition/deletions have been useful in making certain predictions about phenotypic irregularities, but data of this nature are not universally regarded as unequivocal. In fact karyotypic variation in many disease states suggested that chromosome changes were merely an epiphenomenon and not associated with initiation of disease.

Neoplasms are often accompanied by cytogenetic alterations. The assessment of whether specific observable chromosome changes are absolutely required for neoplastic transformation is made difficult by conflicting data even with known carcinogens (28). Reported instances of neoplasia without concomitant observable chromosome anomalies lends further credibility to the view that such changes are not consistent or specific (28, 63). On the other hand, several investigators have proposed that chromosome changes are non-random (4, 83). Chromosome variability for a given disease is explained by considering that the condition may be produced by different etiologic agents. For a genetically heterogenous population also, it may not be valid to think that a single etiologic agent can cause the same chromosome anomaly in cells from each individual (83). The logical conclusion holding that chromosome changes are consistent and specific is: chromosomal changes precede morphological transformations. Sachs and his colleagues (39, 40) have postulated the existence of chromosomes that cause malignancy (effector, E) and chromosomes that suppress (S) malignancy. They propose that the determining factor in whether a cell is or is not malignant is the balance in the number of E and S chromosomes. They

have presented evidence that there are constant chromosomal changes reflecting the malignancy of the clones.

Chromosomal Rearrangements

Chromosome breakage may result in reunion of fragments and novel arrangements of different segments. Rearrangements may be classified as either stable or unstable dependent on whether the resulting chromosomes can be perpetuated. If the new structures are unstable they will be lost in subsequent generations. Genetic information may be gained or lost or the old genetic complement may be maintained but simply repackaged. In the latter event, new linkage groups and position effects on genes provide opportunity for chromosomal evolution (93).

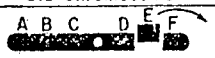
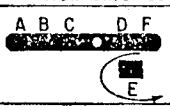
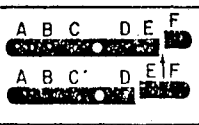
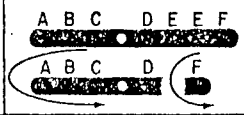
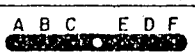
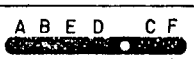
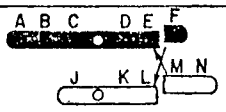
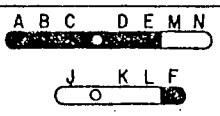
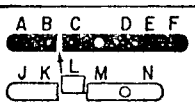
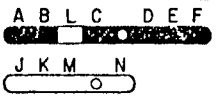
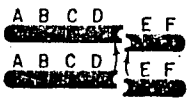
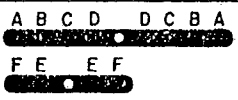
Figures 1 and 2 illustrate some common chromosomal rearrangements and how they are produced. The unstable rearrangements are most easily identified cytogenetically. Banding techniques have made the stable rearrangements easier to detect.

One important type of translocation not illustrated is the Robertsonian translocation. Robertsonian translocations are chromosomal rearrangements in which two telocentric or acrocentric chromosomes fuse at the centromere to form a metacentric chromosome. These rearrangements may result in the loss of a centromere and some surrounding heterochromatin (nucleolar organizers?), but because no essential genes are lost, the translocation is viable with no phenotypic effects (109).

Effects of Diverse Agents

A variety of physical, chemical and biological agents can break chromosomes (26, 38, 93).

DIAGRAMMATIC REPRESENTATION OF STABLE CHROMOSOMAL REARRANGEMENTS

Old Chromosome	New Chromosome	No. of Breaks	Cytologic Effect	Genetic Effect	Rearrangement
		2	Chromosome shortened	"E" segment lost	Deletion (Deficiency)
		2	Chromosome lengthened	"E" segment added	Duplication
		2	None	Change in gene order	Paracentric inversion
		2	Centromere position shifted	Change in gene order	Pericentric inversion
		2	Two morphologically altered chromosomes	Genes shuffled into new linkage groups	Reciprocal translocation
		3	Chromosome lengthened	"L" segment added	Insertion
		1*	Chromosomes are exactly metacentric	Entire chromosome arm is duplicated and other arm lost	Isochromosome

*Misdivision of centromere after chromosome replication.

FIG. 1

DIAGRAMMATIC REPRESENTATION OF UNSTABLE CHROMOSOMAL CONFIGURATIONS

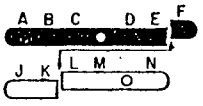
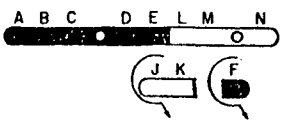
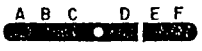
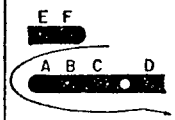
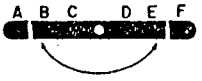
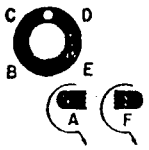
Old Chromosome	New Chromosome	No. of Breaks	Cytologic Effect	Rearrangement
		2	Chromosome with two centromeres	Dicentric chromosome
		1	Chromosome segment lacking a centromere	Acentric fragment
		2	Two proximal ends of chromosome are joined	Ring chromosome

FIG. 2

Figs. 1 & 2 used by permission of author: M. W. Shaw (93).

X-rays, ultraviolet light, cold shock, magnetic fields and sound waves are numbered among the physical clastogens. The term clastogen is a counterfeit label of convenience first suggested by Shaw (93). The Greek word root "clast" means to break, fragment or fracture.

X-rays have been shown to induce several types of structural rearrangements in human chromosomes such as dicentric and tricentric chromosomes, reciprocal translocations, inversions, ring chromosomes and acentric fragments. However, no Robertsonian translocations have been observed in such irradiated cells (14, 41, 92).

Shaw (93) and Heidelberger (38) have reviewed the effects of chemical agents on chromosomes. Chemicals may overlap in their effects. Some chemicals which are clastogenic may be mutagenic, teratogenic and/or carcinogenic as well.

Parenthetically, it should be noted that most research documenting chromosome changes resulting in transformation of cells has been done with fibroblasts. Fibroblast cells produce sarcomas on inoculation into suitable hosts. Studies on the malignant transformation of epithelial cells, which generate carcinomas and are thus regarded as more clinically important, are still in preliminary stages. Conclusions drawn from studies of fibroblasts may not be applicable to epithelial cells. These studies also may not reflect the <u>in vivo</u> state.

Metabolic products such as aflatoxins provide an interesting example of a chemical carcinogen which must be enzymatically activated to a chemically reactive form before it can react with cellular macromolecules to exert its effect. Studies are in progress to evaluate the effects of procarcinogens on liver cultures which offer special advantages beyond

the sensitivity of these cells to procarcinogens. One advantage is that these cells are epithelial-like in nature and thus qualifying them as a model for carcinoma production. The liver is also the organ with the broadest capability of metabolic activation, and use of liver cells provides considerable potential for direct comparison with the extensive research on the action of carcinogens on liver (108).

Bacteria, mycoplasmata, protozoa, rickettsiae and viruses are examples of biological clastogens (4, 36, 82, 96).

Agrobacterium tumefaciens induces crown gall tumors in many higher plant species. Studies of chromosomes in cultures derived from tumors of Crepis capilaris showed a tendency to reduction in numbers of chromosomes and new chromosomes resulting from translocations in later transfers of the cell lines (85). These findings with plant chromosomes are relevant since there are no known plant clastogens to which animal chromosomes have been found to be immune (93).

With particular reference to virus-induced changes, varying types of chromosomal modifications have been observed. Among these modifications are an inhibition of cellular division, breaks, chromosomal pulverizations and alterations in the numbers of chromosomes (5).

In contrast to ionizing radiation, virus-induced chromosome changes do not tend toward structural chromosome rearrangements. Their clastogenic effect is similar to that of ribosides and deoxyriboside analogues (4).

McDougall (58) has demonstrated random chromatid and isochromatid gaps and breaks in diploid human embryo kidney cells following infection by several types of adenovirus except types 12 and 31. Adenoviruses

types 12 and 31 (both oncogenic in hamsters) tended to produce breaks localized on the long arm of chromosome 17. When it is considered that types 12 and 31 increase thymidine kinase activity two- or three-fold in human cells in vitro and that a locus for this enzyme is found on chromosome 17, a possible explanation for the gaps may lie in the suggestion that virus-induced transcription resulted in a region of uncondensed host DNA and produced the virus-induced enzyme as a product.

The demonstration of such a "marker chromosome" has been invaluable in the mapping of human chromosomes. Cell fusion experiments using inactivated Sendai virus or polyethylene glycol have made it possible to assign many gene functions to human chromosomes.

The adenoviruses, herpesviruses, papovaviruses and poxviruses are DNA virus groups which have oncogenic members. The oncornaviruses contribute the RNA viruses known to cause tumors in certain animals. Their role in the causation of human cancer is uncertain, but many of these viruses can cause transformation of primary tissue culture cells. In fact, a remarkable feature of cell transformation is the diversity of viruses that can effect it. As stated earlier, the transforming event is generally accompanied by cytogenetic alterations.

However, the clastogenic effect of physical, chemical and biological agents is not restricted solely to malignant transformation. DiPaolo and Popescu (28) have studied immediate effects of chemical carcinogens with various chromosome banding techniques. They have found the majority of damaged cells not viable and have shown that cultured Syrian hamster fetal cells maintain their chromosome abnormalities or deviations from the normal modal number for up to 20 subcultures. Experiments

treating Syrian hamster cells with N-methyl-N´-nitro-N-nitrosoguanidine (MNNG), an alkylating agent, showed MNNG produced mainly chromatid aberrations (as contrasted with chromosome aberrations). The frequency of aberrations was shown to be independent of the length of exposure. Further, G-band analysis showed the breaks and gaps to be randomly distributed but involving primarily the negative bands. They concluded that with human and hamster material, chemical carcinogens effected nonspecific chromatin lesions probably as a result of toxicity since transformation was not obtained. In fetal rat cells, however, treatment with 7,12-dimethyl-benz(a)anthracene (DMBA) may have induced a specific lesion.

Studies are being pursued now to evaluate effects of chemicals on frequency of sister chromatid exchanges (SCE) (28, 75). The SCE frequencies are regarded as more sensitive indicies of chromosome damage than the frequency of chromosome aberrations. Early evidence indicates that chemicals which cause a high frequency of transformation also induce a high frequency of SCE.

Chromosome damage caused by physical, chemical and biological agents may have a common contributory mechanism. Structural chromosome changes may be mediated by a cytoplasmic event whereby damage to lysosomes results in liberation of deoxyribonuclease which attacks and breaks the interphase chromosomes (2).

Numerical errors in chromosome distribution among progeny cells has also been postulated to result from disorganization of a cyclic-AMP-dependent network of microtubules and microfilaments (78).

TECHNIQUES USED IN THE CHARACTERIZATION OF CHROMOSOMES

C-banding

C-banding is a staining technique which demonstrates certain types of constitutive heterochromatin.

Two broad classes of heterochromatin have been distinguished (6, 7, 52). One type known as facultative heterochromatin is heteropycnotic (precocious condensation) only in certain cells or at certain stages and does not affect all homologues. Facultative heterochromatin consists of structural genes which may be cytologically condensed and genetically inactivated depending upon physiological and developmental processes. The heteropycnotic character of the X chromosomes in female mammals exemplifies facultative heterochromatin. This genetically inactivated heterochromatin is commonly referred to as a Barr body, but the term X-chromatin body is preferred. This phenomenon may represent a "dosage compensation" mechanism. The Lyon hypothesis or single active X theory of dosage compensation in mammals has been named after the author (54, 55, 56). The other type, constitutive heterochromatin, shows variable degrees of heteropycnosis in all cells and is integrated into the structures of all chromosomes (both homologues behave the same way). Parenthetically, it should be noted that some heterochromatin exhibits a behavioral pattern which is intermediate between the two types.

Both types share some properties. They both can be seen as heterochromatic masses in interphase cells (86). Autoradiographic studies show that DNA of heterochromatin replicates later in interphase than

DNA of euchromatin (33, 52, 100). Late replication does not differentiate the two types of heterochromatin. Both display the characteristic of close chromatid apposition at metaphase (86). As judged by their failure to incorporate tritiated uridine, both appear to be genetically inert (22). However, this apparent inactivation cannot be considered absolute since the Drosophila Y chromosome, which is entirely heterochromatic, is known to carry genes which control the development of spermatozoa (106).

Constitutive heterochromatin contains most of the non-transcribed highly repetitive DNA sequences of the genome (104, 109). These redundant DNA sequences are referred to as satellite DNA (not to be confused with the chromosome structures called "satellites"). Calf satellite DNA I has been shown to consist of a repeating unit of 1460 nucleotide pairs which when hydrolyzed by various restriction endonucleases yield defined subsatellite fractions (81). In the case of guinea pig α-satellite, two of the six possible reading frames yield nonsense codons (95). RNA-DNA hybridization experiments have failed to reveal the presence of RNA complementary to mouse satellite DNA (32).

Constitutive heterochromatin sequences are preferentially located in regions near the centromeres in all mammals so far examined. The discovery of a procedure for staining constitutive heterochromatin came as a by-product of an experiment designed to determine the cytological location(s) of mouse satellite DNA by in situ nucleic acid hybridization. Pardue and Gall (71) noticed that in areas where satellite DNA is located (centromeric regions of all autosomes and the X chromosome), deeply staining regions were obtained with Giemsa whereas the remaining arms

were lightly stained. Arrighi and Hsu (3) showed similar deeply staining regions involving human repetitious DNA. They concluded that these regions represent constitutive heterochromatin of the human complement, that each chromosome has a characteristic amount and location of this substance and that facultative heterochromatin is not revealed by this technique since the two X chromosomes behave similarly in staining property.

The discovery of a staining procedure was important for the following reasons: (1) it provided a simple method for revealing constitutive heterochromatin at metaphase as well as other stages, (2) it correlated heterochromatin and repeated DNA sequences, (3) it contributed to a greater understanding of karyologic and molecular evolution and (4) it led to the discovery of Giemsa banding (G-bands) of chromosomes. This was important (as noted at the outset) in the longitudinal differentiation of chromosomes (45).

The existence of constitutive heterochromatin as a defined substance renders the view of heterochromatin as simply a state of the chromatin untenable. In other words, the concept that all types of heterochromatin could be active and decondensed in some cells at some stage of development just as the mammalian X could be active and euchromatic in one cell and inactive and heterochromatic in another has largely been discarded. Evidence is accumulating which lends support to the view that constitutive heterochromatin is not transcribable and thus is not simply inactivated as is the case with facultative heterochromatin. Its condensed nature may be a consequence of a repetitive constitution rather than histone binding (16).

Repeated DNA sequences may definitely be correlated with constitutive heterochromatin. The temptation exists to interpret that highly repetitive DNA is responsible for C-band staining. The preparations are treated with acid, alkali and/or elevated temperatures and presumably the cellular DNA is denatured. The incubation at 65°C in saline-citrate solution presumably renatures the DNA. Thus, the differential staining may be a result of highly repetitious DNA renaturing, while low repetitious and unique DNA do not.

The denaturation-renaturation scheme is regarded skeptically by many because the DNA in situ may behave differently from the DNA in solution in response to various treatments.

Attempts have been made to elucidate the strandedness of the DNA molecules in the chromosome with acridine orange fluorescence in combination with the treatments. If the color changes associated with acridine orange fluorescence in relation to strandedness are reliable, then the denaturation-renaturation mechanism may be operative (21). In addition, Mace et al. (57) have provided evidence favoring this mechanism using fluorescein-labeled antibody against single-stranded DNA which was obtained from a patient with systemic lupus erythematosis. The antibody produced no fluorescence in control preparations but gave fluorescence when the preparations were "denatured". If the preparations were allowed to "denature" and to "renature", the C-band regions failed to show fluorescence. The actual molecule(s) that are stained by the Giemsa stain have not been characterized. The possibility exists that the constitutive heterochromatin presents itself because it renatures quickly and is not extracted by the procedure (21).

As stated previously, heterochromatin appears to be centromerically located in the majority of chromosomes of most mammalian species. The amount of heterochromatin on each particular chromosome appears to be characteristic, but at least in man, polymorphism does occur among individuals (24, 25). One or more chromosomes (e.g. 1, 9, 16) may contain heterochromatic regions twice as long as that of its homologue, but these individuals show no phenotypic abnormality. This observation also lends support to the concept that heterochromatin is genetically inert. Variability of the C-bands may be accounted for by an increased frequency of unequal crossing-over, since it is conceivable that any sequence can synapse with any other sequence of the homologue during meiosis due to the many repeated DNA sequences.

Interstitial and terminal C-bands are observed in some species. These could have originated from centromeric regions but were displaced by inversions or translocations. In some animals many chromosome arms are totally heterochromatic (e.g. the hamster genus Mesocricetus) (44).

Heterochromatin can also be found in the sex chromosomes. Species with large X chromosomes invariably contain a large amount of constitutive heterochromatin in the X chromosomes. When the Y chromosome is a tiny element, heterochromatin is not present, but when the arm areas are slightly larger (greater than 2μ in length), it is invariably heterochromatic in its entirety. Presumably, the functional portion is included but is masked by the heterochromatic part. In man the C-band technique is excellent for identifying the Y chromosome when other methods give equivocal results.

The location of constitutive heterochromatin in regions near the centromeres, telomeres, nucleolar organizers and its insertion into other regions of chromosomes suggests a structural role in chromosome organization (7, 109). Another postulated function is providing structural support for the centromere, thereby ensuring correct separation of chromosomes during cell division (7). Alternatively, constitutive heterochromatin may serve to isolate and protect vital groups of genes from crossing-over and thus preserve them from change. An example used is the separation of the genes coding for 18S and 28S rRNA in the nucleolar organizer. Such protection is essential when it is considered that these cistrons which are linked and repeated in tandem have been amazingly conserved throughout evolution (109). There is evidence that crossing-over in plants and animals is less frequent in heterochromatin that in euchromatin. Additionally, constitutive heterochromatin may have a role in the attraction of homologous chromosomes prior to meiotic synapsis (109). Evidence from the observations of centromeric heterochromatin during male meiosis in the mouse supports this possibility. Conspicuous heterochromatic "knobs" are seen associating in pairs toward the end of interphase or at the beginning of meiotic prophase. Condensed regions may attract homologous chromosomes at the onset of meiosis, but they probably prevent the intimate pairing that is necessary for crossing-over.

Heterochromatin may also control the association of chromosomes, which although not homologous carry genes with a functional relationship. In man, the acrocentric chromosomes 13, 14, 15, 21 and 22 carry

the nucleolar organizers and are found in close association in both meiosis and mitosis (109).

Certain consequences are predicted if constitutive heterochromatin, through its repetitive DNA content, did effect pre-meiotic alignment. First, there would be limitations on the number of mutations a region of heterochromatin could sustain without loss of ability for alignment. Second, the species specific base composition of repetitive DNA could erect fertility barriers by preventing pairing of heterochromatin from closely related species. Third, constitutive heterochromatin would play a role in speciation if barriers could be erected between strains of the same species that have become geographically separated because of the apparent high susceptibility of the DNA of heterochromatin to mutation. Finally, constitutive heterochromatin in the centromeric regions could play another role in speciation by facilitating Robertsonian type translocations (109).

Evidence is accumulating that constitutive heterochromatin may be involved with the development of neoplasia following viral infections or the actions of mutagenic agents. For example, satellite DNA replicates at the beginning of S phase instead of at the end following infection of mouse fibroblasts with polyoma virus (94). Responses of various cell lines from the African Green Monkey to infection by SV40 depend on the presence or absence of heavy satellite DNA (60, 80). The possibility exists that insertion of these oncogenic viruses into the satellite DNA of constitutive heterochromatin disturbs chromosome organization and function (109). Because of its high susceptibility to breakage by mutagenic agents, its repetitive nature and its tendency

to form aggregates during the cell cycle, constitutive heterochromatin may be involved in the structural aberrations found in other types of neoplasia (non-viral) (65, 66).

G-banding

The development of the C-band technique led to the hypothesis that chromosomal dyes may bind differentially to the various species of DNA. This in turn led to the demonstration of chromosome crossbands of varying width and shades when stained with Giemsa, Leishman's, Wright's, etc. (29, 49, 74, 87, 88, 91, 98, 102, 105). These crossbands were termed G-bands.

The mechanism by which G-bands are induced has not been elucidated. Hsu's group (8) has presented evidence that removal of histone fractions f1 and f2a are at least partially responsible for the induction of G-bands. The f2b and f3 fractions appear not to be involved. They also suggested that fixation in methanol and acetic acid (3:1) or 0.2 N HCl removed the histone fractions f1 and f2a and thus allowed induction of the G-bands. These data may explain why a banding pattern could be obtained without need of any pretreatment by trypsin since fixative (methanol:acetic acid, 3:1) is used routinely in the harvest of cells (59, 110).

Yunis (111) has reviewed the evidence that chromosome bands represent a conformational feature of mammalian chromosomes. Salient features of this model include a chromosome composed of long chromatin fibers, the bulk of which is arranged in condensed and predominantly horizontal loops. These loops are visualized as stained bands in the

light or fluorescent microscope and are rich in repetitive and nongenic DNA. This DNA is considered of intermediate repetitiveness and corresponds to the facultative heterochromatin observed in interphase nuclei. The unstained bands excepting the centromeric regions are composed of genic and intergenic DNA arranged in a looser and more vertical manner. Pericentromeric and perinucleolar bands are the highly repetitive constitutive heterochromatin described in the previous section. The prediction of this model, in view of the presence of large amounts of nongenic DNA, is that chromosome band defects would occur with moderate to no phenotypic effects.

Additionally, it appears that the G-band patterns observed at metaphase result from the progressive condensation of numerous smaller bands shown in the more elongated late prophase and prometaphase. This phenomenon occurs as a result of chromosome contraction. The higher resolution afforded by prophase chromosomes may make it possible to construct a map such as that known for the giant chromosomes of Drosophila (112).

Q-banding

Quinacrine mustard and quinacrine dihydrochloride, both acridine derivatives, have been shown to effect differential fluorescence in UV light in a variety of chromosomes of plants and animals including man (11, 12, 13). The combination of position, width and brightness of the "Q-bands" proved to be so unique that virtually every chromosome pair of the human karyotype can be recognized with relative ease.

In general, Q-bands may be correlated with G-bands. A good preparation by G-banding technique may in some cases make the recognition of individual chromosomes easier and more accurate than a quinacrine fluorescent pattern, but the Giemsa banding does not offer consistent results. The quinacrine fluorescent methods are valued for their reliability in this respect. Also, other stains may be applied after quinacrine. The technique suffers from the liability that fluorescence of chromosomes bound to quinacrine fades quickly.

Hsu (45) has reviewed the evidence that uninterrupted stretches of A-T (adenine-thymine) regions effect an enhancement of fluorescence, while a progressive quenching of quinacrine fluorescence is observed with increasing guanine content of DNA. The dyes are thought to bind to chromosomal DNA by intercalation. Other mechanisms such as non-histone protein-DNA interaction may also contribute to differential chromosome fluorescence (23).

R-banding

Chromosomes stained by acridine orange and certain antibiotics produce a reverse fluorescent banding pattern (R-bands) to that given by the quinacrines (30, 35, 103). Reverse binding is thought to occur as a result of specific binding to G-C (guanosine-cytosine) rich regions of DNA.

In summary, each staining procedure offers distinct advantages and disadvantages. When used to their best effect, different areas within a single chromosome can be localized and studies of structural abnormalities can be made easier.

KARYOTYPIC STUDIES OF BOVINE TISSUES

Normal Tissue

The cattle karyotype consists of 58 t autosomes and 2 sm sex chromosomes. "Short arms" are sometimes observed on the autosomes of cattle. For this reason, the autosomes of cattle have often been described as acrocentric. However, Hansen (37) has shown that the "short arms" do not show fluorescence and that the centromeric heterochromatin can appear irregularly angular. Therefore, the autosomes of cattle may be telocentric, i.e., the centromere is terminally located in all autosomes.

The sex chromosomes are identified by their submetacentric centromeres. The X chromosome is about the same size as the largest autosomal pair. The Y is among the smallest of the complement.

Interest in bovine cytogenetics has created a need for international agreement standardizing banded bovine karyotypes. For this reason the "Reading Numbering System" will be used in this thesis (53).

Cancer Eye and Other Malignant Tissues

Bovine ocular squamous cell carcinoma, commonly known as Cancer Eye, is cosmopolitan in its incidence among cattle (84). Ushijima (101) has not been able to observe an abnormal karyotype in cells associated with this condition. This is surprising in light of the fact that while chromosomal abnormalities may be undetectable in early tumors, they almost invariably appear at later stages or after metastasis (9).

Especially noteworthy with respect to the characteristics of WC-derived transformed cell lines is the cytogenetics of canine venereal tumor. The tumor (also known as the Sticker venereal sarcoma), has been known for about a century and is apparently transmitted from dog to dog by transfer of cellular material. Makino (62) has reviewed the subject and presented evidence for a universal karyotype of this disease with modes at or near 59 chromosomes (dogs 2n = 78). Other remarkable features of the karyotype are: (1) reduction in chromosome number is accompanied by the formation of biarmed chromosomes (all the autosomes are normally acrocentric), (2) the mechanism may be one of centric fusion, (3) the presence of inclusion bodies have been reported in the cells of this tumor, but a virus has not been demonstrated and (4) estimation of nuclear DNA content has not shown any loss of chromosomal material, in fact, the DNA content is higher in tumor than in normal cells.

The data on modal DNA values is important because these values are obtained at interphase and therefore not subject to bias. Distributions of modal chromosome numbers in tumors tend to be biased by the exclusion of those that are not technically favorable. Difficulties encountered include breakage of metaphases, loss of chromosome material during preparation, poor chromosome morphology and differential chromosome contraction. There is a general tendency of tumor cells to band less well than normal cells.

Most pertinently, a bovine fibrosarcoma from a 7-year-old Holstein cow has been reported which presents two unusual karyotypes consisting of 24-26 atelocentrics (m and sm chromosomes) and 12 or 13 acrocentrics

(27). The rearranged chromosomes were postulated to have arisen by a process of centric fusion.

WC-derived Transformed Cell Lines

As mentioned previously, primary tissue cell cultures obtained from calves exhibiting WCS were transformed after prolonged *in vitro* culture. Jannke (46) has described in detail the properties of some of these cell lines. Briefly, the cells' change in social behavior included increased cloning efficiency in soft agar, loss of contact inhibition, immortality in culture and tumor production upon inoculation into immunosuppressed hamsters. Eosinophilic nuclear inclusion bodies were also evident in a kidney cell line (47). Of particular interest to the discussion at hand are the reported chromosome alterations. Instances of chromosomal fragmentation, polyploidy, aneuploidy and subdiploidy were observed and an extensive translocation of chromosomes was present in all transformed cell chromosome spreads. That polyploidization and aneuploidy were observed is not unusual. There is evidence for a sequence of events during transformation, at least in murine cell strains (42, 43). The sequence of events was diploidy → tetraploidy → hypotetraploidy or, less often, diploidy → hypodiploidy → hypotetraploidy.

What is remarkable about the transformed cells' karyotypes involves two points: (1) the fact that cells of diverse origin all exhibited similar karyotypes, i.e., a predominance of biarmed elements and (2) that the modes stayed very near those of the normal bovine complement.

Patterns of chromosome change during in vitro transformation is thought to parallel those occurring during carcinogenesis. Initial damage to cells results in a population exhibiting diverse chromosome aberrations. Subsequently, it is thought that the dominance of one stem line represents the clonal development of a population of cells having similar karyotypes (4). While these processes may indeed be operative, the existence of similar karyotypes from a "stable" cell line of embryonic bovine kidney origin, a transformed bovine kidney and salivary gland and a transformed ovine salivary gland is striking.

Without banding techniques the nature of the observed changes would be impossible to determine.

STATEMENT OF THESIS

This study was designed to investigate the cytogenetic nature of the chromosomes in tissues derived from cattle exhibiting the Weak Calf Syndrome.

Specific aspects of the chromosome studies involving the WC-derived tissues included: (1) conventional staining analysis in an effort to determine if these tissues presented an aneuploid chromosome number or an easily detectable altered morphology, (2) C-banding analysis in an attempt to determine (as noted for the cytogenetics of canine venereal tumor and the bovine fibrosarcoma) if centric fusion resulting in multiple biarmed chromosomes may be the general tendency of chromosome rearrangements in the cellular transformation of WC-derived tissues (and possibly all acrocentric and telocentric chromosomes?), (3) G-banding analysis in an attempt to elucidate the origin of the chromosomal fragments

involved in the rearrangements involving the transformed lines and (4) given the large literature on the progression of tumor development, it became pertinent to ascertain whether a clone established from a single cell of the already transformed line would produce identical karyotypes. If identical karyotypes were obtained, this may be taken as presumptive evidence that the observed changes are fixed characteristics that occurred sometime during the sequences leading to transformation and further, the lesions produced in this way were stable. Alternatively, karyotypic variation among the population of cells from a single clone would denote a continual chromosomal interchange.

CHAPTER 2

MATERIALS AND METHODS

SOURCE OF TISSUES

Bovine peripheral blood leukocytes were obtained at Rasmussen's Processing Plant, Missoula, MT, through the courtesy of Bob Younger and Dennis Merritt.

Organs for primary tissue cultures were obtained from calves diagnosed as presumptive WCS-positive by Jack Ward, D.V.M. The calves were born at various ranches in the Bitterroot Valley of Montana. Organs utilized were kidneys, salivary glands, testicles and thymus.

Bovine turbinate (BT) cells were obtained from the State Veterinary Research Laboratory in Bozeman, MT.

PROCESSING ANIMAL ORGANS

Samples were collected at necropsy and placed in a Whirl Pak sterile plastic bag (NASCO, VWR Scientific) containing 20 to 50 ml Minimal Essential Medium (MEM) (GIBCO) supplemented with 10% fetal calf serum (FCS) (GIBCO). Samples were processed immediately upon arrival at the laboratory. Only tissues less than 24 h old were processed. Tissues were handled aseptically in a sterile petri dish and were bathed in a solution of phosphate buffered saline without calcium and magnesium ions (Pd). The Pd solution also contained 1.0% glucose (w:v) and penicillin/streptomycin to concentrations of 100 units/ml and 100 µg/ml respectively. Outer protective capsules,

adherent adipose tissue and connective tissues were removed with the aid of sterile scissors and forceps. In the case of kidney tissue the cortex was retained, and the white medulla was discarded. Tissues were minced using scissors to sizes approaching 1 mm^3 and transferred to a trypsinization flask. Tissues were washed with one or two rinses of Pd (pH 7.2). Trypsin (Difco) solution (0.25% in Pd) was added in sufficient volumes to immerse tissues completely. A magnetic stirring bar was introduced, and the flask was placed on a magnetic stirrer for 10 min and set at a speed which did not cause the trypsin to foam. The cell-trypsin suspensions which were saved for cell recovery were decanted through a guaze-covered funnel into a 500 ml centrifuge bottle (Bellco Glass) containing 50 ml culture medium. Medium was poured through the funnel after each decantation to promote greater yields of cells and to inhibit further action of trypsin. The medium used routinely was NCTC 135 (GIBCO) supplemented with 20% FCS (GIBCO) and L-glutamine (J.T. Baker) to a concentration of 20 mM. Gentamycin (Schering Corp.) and mycostatin (Squibb) were also included (along with the penicillin/ streptomycin added routinely during formulation) to control possible contamination. Gentamycin was added to a concentration of 50 µg/ml, and mycostatin was added to a concentration of 50 units/ml. The trypsinization procedure was repeated three times. Products of the first trypsinization were discarded. Centrifuge bottles containing digest mixture were kept on ice.

The filtrate was centrifuged at 700-1000 rpm for 10 min. The supernatant fluid was discarded, and the resultant cell pellet was dispersed in 5 ml medium. Any volume above 5 ml was assumed to

constitute packed cell volume, and the cells were resuspended in medium at a relative concentration of 1 ml packed cells to approximately 30 ml medium. Suspended cells were dispensed into either 25 cm^2, 75 cm^2 or 150 cm^2 plastic tissue culture flasks (Corning) depending on resultant cell volumes. The medium was gassed with 5% CO_2, and the flasks were placed at 37°C. After 72 h of incubation the cultures were washed to remove debris, and the attached cells were refed. When confluency was attained, the monolayers were subpassaged at high cell densities to enhance monolayer formation. Copies of the various cell lines were frozen at -70°C (Revco) and in liquid nitrogen at -195.8°C.

CELL STORAGE

Actively growing monolayer cells were washed twice with Pd, trypsinized and washed in 5-10 ml medium. Transformed and stable cell lines were diluted to concentrations from 5 X 10^5 to 5 X 10^6 cells/ml of medium supplemented with 20% FCS. Primary cell lines were suspended at a cell density of 1 X 10^7 cells/ml. Dimethyl sulfoxide (Mallinckrodt) was added to the medium as a cryoprotective agent to a concentration of 10% (v:v). One ml of the suspension to be frozen was placed in sterile 2.0 ml ampoules or 0.5 dram borosilicate glass vials (VWR Scientific). After sealing, the ampoules or vials were wrapped in a folded sheet of paper toweling and then in one layer of aluminum foil. The package was labeled and placed at -70°C (Revco). Freezing cells in liquid nitrogen was accomplished by placing sealed vials or ampoules on aluminum canes (Minnesota Valley Engineering) and submerging the canes in a 0.05% methylene blue solution for

approximately 30 min at $4^{o}C$. This enabled detection of improperly sealed ampoules/vials and also allowed time for the cryoprotective agent to equilibrate with the cells. Canes were rinsed in cold tap water at the end of this time and placed in a cylinder containing 95% alcohol. The cylinder was placed at $-70^{o}C$ for 2-3 h after which the canes were transferred immediately to the liquid nitrogen storage container ($-196^{o}C$).

Frozen cells were recovered by thawing immediately at $37^{o}C$ with moderate agitation, washing the cells once with medium and seeding into a 25 cm^2 culture flask. After gassing, cells were incubated at $37^{o}C$ for 24 h at which time the medium was changed.

GROWTH AND MAINTENANCE OF CELL CULTURES

NCTC 135 medium was used to maintain cells in culture. Media formulations have been reproduced in the Appendix. Fetal calf serum was added to concentrations of 5, 10, 15 and 20% by volume depending on the growth characteristics of cells.

Medium was changed weekly or when the pH of the medium became too acid as evidenced by the yellow color of the indicator dye. Cells were passed when confluency was attained or when cell density was high enough to produce acid conditions in 24 h. Cells were passaged by the standard trypsinization procedure. Medium was removed by aspiration and the cell layer was washed twice with Pd. Trypsin (Difco) solution (0.25% in Pd without versene for primary cells) was added in 1.0 ml amounts for 25 cm^2 and 75 cm^2 flasks and 1.5 ml for 150 cm^2 flasks. Trypsin versene solution (0.15%) was used for stable and

transformed lines (Appendix). Cells overlayed with trypsin solution were incubated at 37°C for a period not exceeding 15 min. Following detachment of cells, 5-10 ml of medium were added to the flask in order to aid further detachment by means of forced pipetting action and also to inactivate the trypsin. Suspended cells were removed to a 15 ml conical centrifuge tube and centrifuged at 700-1000 rpm for 5-10 min. The supernatant fluid was aspirated, and the cell pellet was resuspended in the desired volume of medium by forced pipetting. Cells were dispensed into new flasks in appropriate concentrations. Transformed and stable cell lines could be seeded in concentrations as low as 1×10^4 cells/25 cm^2 flask and still not result in an overlong period to attain confluency. Primary cells were seeded into Leighton tubes and 25 cm^2 flasks at about $1 - 2 \times 10^5$ cells/ml and into 75 cm^2 flasks at about $2 - 3 \times 10^5$ cells/ml. Fresh culture medium was added in volumes appropriate to flask size (5-10% of flask volume). The pH of the medium was adjusted by gassing the flasks with a 5% CO_2 mixture in air, and the flasks were incubated at 37°C.

The Calf B salivary gland transformed cell line (Calf B Sg Tr) exhibited overt manifestations of contamination with pleuropneumonia-like organisms (PPLO). As such the cells were granular in appearance with lacy edges and produced acid pH conditions within 24 h which could be somewhat abrogated by treatment with 50-100 µg/ml of tetracycline (Sigma). Several protocols were used in an attempt to eliminate these organisms. The most successful method was that of Johnson and Orlando (48). Nearly monolayer cultures of Calf B cells were grown in NCTC 135 plus 10% FCS supplemented with 50 µg/ml Novobiocin (Sigma) for 7 days

at 37°C followed by an 18 h exposure to heat (41°C). After treatment, cultures were fed fresh medium without Novobiocin but supplemented instead with an anti-PPLO agent (GIBCO) (Tylocine, 100X, 6,000 µg/ml). Tylocine was used routinely thereafter as an additive to the medium used on Calf B Sg Tr cells.

SINGLE CELL ISOLATIONS

Single cell isolations were attempted by three methods: (1) soft agar isolation techniques, (2) dilution techniques with subsequent planting in flat bottomed wells of microtiter plates (Falcon Plastics, MicroTest II, 3040) and (3) dilution techniques with subsequent planting in 25 cm^2 tissue culture flasks.

Soft agar isolation was accomplished using the method of Ushijima (101). In this method, cells suspended in a 0.3% agar solution are grown on a 0.7% agar pad. Ionagar (Oxoid #2, Consolidated Labs) was prepared at concentrations of 0.6 and 1.4% in triple distilled water (TDW) and autoclaved to insure sterility. Agar of the respective concentrations was dispensed in appropriate aliquots into test tubes. NCTC (2X) with 40% FCS was filter sterilized (0.22 µm filter, Millipore Filter Corporation) and mixed in equal volumes with the agar solutions at 45°C. Approximately 5 ml of the resulting 0.7% agar pad was added per Corning flask and allowed to solidify.

Cells suspended in NCTC + 20% FCS were serially diluted in tubes containing the 0.3% agar mixture to obtain a final concentration of 10 cells/2-3 ml. Approximately 2-3 ml of this mixture was overlayed on the previously poured 0.7% agar pad. Flasks were gassed as for

freshly passaged cell cultures after a brief period to allow for solidification of the overlay. Cultures were inspected for growth periodically. Cells were recovered with a Pasteur pipette; released from the agar matrix by forced pipetting; placed in 25 cm^2 flasks with a reduced volume of medium (2-3 ml) and gassed.

The dilution techniques differed only in the concentration of cells desired at the endpoint. The microtiter plate technique (2) required a final concentration of 1 cell/ml. This suspension was placed into each of 3 microtiter wells in 33.0 µl/well volumes. Each well then received 0.27 ml medium additionally. The wells were gassed, covered and incubated at 37°C in a water jacketed CO_2 incubator (Bellco Glass, Inc.). The flask technique (3) aimed for 5-10 cells/ml. Cells were simply planted in a reduced volume of medium (1-3 ml), gassed and allowed to attach for 24-48 h. A rubber policeman was used to disrupt cells in flasks containing more than one clone.

CHROMOSOME PREPARATIONS OF LEUKOCYTES CULTURED FROM PERIPHERAL BLOOD

Chromosome preparations were made from whole blood by modification of a conventional air-drying procedure (64). Fresh blood was collected in a test tube containing preservative-free heparin. Leukocytes were consolidated by centrifugation at 1700 rpm for 30 min. The buffy coat was collected with a Pasteur pipette, and 2 drops were added to 5 ml chromosome medium (Appendix). Alternatively, 2 to 5 drops of whole blood were usually added to some tubes of chromosome medium. The mixture was incubated 3 days at 37°C and was resuspended once or twice daily. The pH was maintained near neutrality. After 3 days spindle fiber

formation was arrested by adding colcemid (Ciba) stock solution (Appendix) to cultures at a volume of 20 µl/ml of culture medium and left for 1.5-2 h. Cultures were harvested by centrifugation for 10 min at 900 rpm, and all but approximately 0.5 ml of the supernatant fluid was removed. Cells were resuspended by a gentle swirling of the tube and transferred to a siliconized 12 ml round-bottomed centrifuge tube with a siliconized Pasteur pipette. The cells were swollen by adding 8.0 ml of prewarmed (37-41°C) hypotonic potassium chloride (KCl) (0.075 M) and allowing the mixture to incubate 5-7 min at 37°C. Cells were centrifuged at 600 rpm for 5 min, and all but approximately 0.5 ml of the supernatant fluid was removed. The cells were fixed in 5 ml of 3:1 methanol:glacial acetic acid solution for 20 min at room temperature. After being washed twice more with fixative, the cells were resuspended in 0.5-1.0 ml of fixative depending on their number. Air-dried spreads were prepared on tissue culture clean slides.

CHROMOSOME PREPARATION OF TISSUE CULTURES

Colcemid was added to actively growing 18-24 h old cultures at concentrations and durations described above.

For monolayer cultures, hypotonic treatment with KCl was found to be unsuitable. Metaphase chromosome spreads were disintegrated or dispersed too widely to be useful. A prewarmed (37-41°C) solution of 1 part MEM:3 parts TDW was used instead. The cultures were allowed to stand 20 min at 37°C after the addition of a 1-3 mm layer of this solution to the culture flask. An equal volume of fixative (freshly prepared and kept at 4°C) was added to the hypotonic solution with

care being taken to avoid turbulence. The flask was first allowed to stand 5 min at room temperature before being violently shaken to aid detachment of cells. The fluid was transferred to a siliconized centrifuge tube with a siliconized Pasteur pipette and allowed to stand for 20 additional min at room temperature. Cells were fixed and processed as for leukocytes.

STAINING TECHNIQUES

Giemsa (conventional chromosome staining)

Giemsa staining was used routinely to check for cell densities and for suitability of the spreads on freshly prepared air-dried slides. The stain was formulated with 24 ml distilled water plus 0.5 ml pH 6.8 citrate-phosphate buffer (0.1 M citric acid-0.2 M dibasic sodium phosphate) plus 0.5 ml stock Giemsa (Allied Chemical) (Appendix). The staining was done with freshly prepared stain for 5 min. The slides were then flooded with distilled water and allowed to air dry.

C-banding

The C-banding was carried out by a modified method of Sumner (99). Air-dried spreads were treated with 0.2 N HCl for 30 min at room temperature and rinsed with deionized water for 1 min. Slides were placed in freshly prepared 5% aqueous $Ba(OH)_2 \cdot 8\ H_2O$ at 37-50°C for 5-15 min and were rinsed thoroughly with several changes of deionized water. Slides were incubated in standard saline citrate (SSC) (0.3 M NaCl containing 0.03 M tri-sodium citrate) for 2 h at 65°C and rinsed briefly

with deionized water. Staining was effected using a solution of 5 ml Giemsa in 50 ml of Gurr's buffer prepared from proprietary tablets (E. Gurr Ltd.). Stain was applied for 5 min and the slides were then rinsed briefly with deionized water and allowed to air dry.

G-banding

G-bands were induced using Seabright's (91) modified method. Air-dried preparations were aged at least 1 week in a dessicator prior to use. Aged slides were dipped in freshly prepared 0.1% trypsin (Difco) dissolved in Gurr's buffer prepared from a tablet (pH 6.8). A 15 sec exposure was found to produce well-defined bands. Exposures for longer periods resulted in overtreatment. After trypsin treatment the slide was flooded with phosphate buffered saline (PBS, Appendix). Staining was done with a solution of Leishman's stain prepared by diluting 1 part Leishman stock (Appendix) with 4 parts Gurr's buffer (pH 6.8) prepared from a tablet. Staining times approaching 4 min were found to give best results. Slides were rinsed in distilled water and allowed to air dry.

PHOTOMICROSCOPY

Chromosome spreads were studied with an American Optical (AO) Series 20 Microscope (2071M) with a 50 watt Mercury Vapor Lamp (AO 2055, L1) providing excitation energy.

A neutral density filter (#310-601) allowing 50% transmission and a green filter (#2061) were used routinely in scanning and black & white photography.

Routinely, spreads were examined at 1000X and photographed at 5000X.

Representative spreads were photographed with AO #1053 35 mm camera back equipped with a special adapter for fitting AO #1191 Lens and Shutter Assembly. Exposures were controlled with Expo Star Shutter Control (AO #1190). High contrast copy film 5069 (Kodak), ASA 64, was used routinely.

Prints were made on Luminos single weight universal F paper. Cut-out photomicrographs were arranged into karyotypes, rephotographed with a Polaroid MP-3 using Kodak 4X5 Professional Copy Film and printed on Kodabromide F-5 paper.

IDENTIFICATION AND NOMENCLATURE

As stated previously, increased interest in bovine cytogenetics has created a need for an international agreement standardizing arrangements of G-banded cattle chromosomes. For this reason the "Reading Numbering System" was used in this thesis (53).

Chromosomal rearrangements were described by the nomenclature established for the human karyotype (18, 72, 73), with modification for the bovine chromosomes.

The system used in numbering the bands was also based on the conventions established for the human karyotype (72), with modifications made for bovine chromosomes as suggested by Lin (53) when agreement was possible. This system will be described in detail later when it is illustrated with accompanying figures.

STARCH GEL ELECTROPHORESIS

Tissue culture cell extracts were prepared by growing monolayer cells in serum-free medium (MEM + 0.25% lactalbumin hydrolysate (LAH) + L-glutamine + gentamycin (50 µg/ml)) for 48 h prior to harvest. Cells were washed with Hanks' Balanced Salt Solution (BSS) (Appendix). The cell pellet was resuspended in TDW, frozen at -70°C and thawed prior to use.

Cell extracts from whole tissues were prepared by first freezing the tissue at -70°C, thawing and homogenizing the tissues with an equal volume of TDW. Samples were centrifuged at 1000 rpm for 10 min after which the supernatant fluid was removed and saved. Supernatant fluids were also frozen at -70°C and thawed prior to use.

Horizontal starch gel electrophoresis was accomplished following the method of Allendorf et al. (1).

Two different buffer systems were used:

(1) Described by Ridgway et al. (79). Gel: 0.03 M Tris -- 0.005 M citric acid, pH 8.5. Electrode: 0.06 M lithium hydroxide - 0.3 M boric acid, pH 8.1. Gels were made using 99% gel buffer and 1% electrode buffer.

(2) Described by Clayton and Tretiak (20). Gel: 0.002 M citric acid, pH 6.0. Electrode: 0.04 M citric acid, pH 6.1. Each buffer was pH adjusted with N-(3-aminopropyl)-morpholine.

Gels were made using 14% starch (Electrostarch Co., Madison, WI) in the appropriate buffer. Samples were applied to the gels on a saturated wick of filter paper (grade 470) sandwiched between a cut which ran the length of the gel. Wicks were removed when a tracking dye had penetrated the gels approximately 0.25 cm (10-15 min).

A potential of 200 volts (or 75 milliamperes) (Heathkit) was applied across the gel until the dye marker had migrated 4-6 cm from the origin; i.e., after about 3-4 h. Gels were cooled during the run by placing frozen ice packs on their tops.

Enzymes stained for, abbreviations used and specific staining procedures are reproduced in Table I. Table I represents a shortened version of a more extensive published table of enzymes (1). The table is reproduced in part with the author's permission.

Three different stain buffers were used. These are referred to by Roman numerals in Table I.

I. 0.2 M Tris-HCl, pH 8.0

II. A solution of 1:4 of stain buffer I and water.

III. The Ridgway buffer system gel mixture; i.e., 99% gel buffer and 1% electrode buffer.

Gels were incubated at 37°C to develop the stains. Destaining was accomplished using a 1:4:5 acetic acid-methanol-water mixture.

Table I. Enzymes stained for, abbreviations and specific stain recipes used for starch gel electrophoresis of various tissue cell extracts.

Enzyme	Abbreviation	NBT/PMS	Cofactor	Stain buffer	Other Components
Esterase	EST			III	5 ml 1% α-naphtyl-acetate in 1:1 of acetone and water 150 mg Fast Blue BB salt
Glucose-6-phosphate dehydrogenase	G6PDH	X	NADP	II	200 mg Glucose-6-phosphate
Isocitrate dehydrogenase	IDH	X	NADP	II	200 mg DL-isocitric acid
Lactate dehydrogenase	LDH	X	NAD	III	25 ml 0.5 M DL-Na-lactic acid
Malate dehydrogenase	MDH	X	NAD	III	25 ml 0.5 M DL-Na-malate pH 7.0
Phosphoglucomutase	PGM	X	NADP	II	300 mg K-glucose-1-phosphate 50 u G6PDH 5 drops α-D-glucose-1,6 diphosphate

The stain buffers I-III are given in the text. If not stated otherwise, 50 ml of stain buffer was used. X in the NBT/PMS column indicates the presence of both NBT and PMS. For 50 ml of the stain buffer, 10 mg NBT, 5 mg PMS and 5 mg of the cofactor was used. All stains using NADP also included 10 mg $MgCl_2$. ADP = adenosine diphosphate; ATP = adenosine triphosphate; NAD = β-nicotinamide adenine dinucleotide; NADP = nicotinamide adenine dinucleotide phosphate; NBT = p-nitro blue tetrazolium; PMS = phenazine methosulfate

CHAPTER 3

RESULTS

CYTOLOGICAL AND CYTOGENETIC CHARACTERISTICS OF PRIMARY ORGAN CULTURES OF WC-DERIVED TISSUES

Eighteen cell cultures derived from tissues of 7 calves (Bos taurus) tentatively diagnosed at necropsy as being afflicted with WCS were studied at various passage levels and compared cytologically and cytogenetically with bovine tissues obtained from a local abattoir. All 7 calves were born in the Bitterroot Valley of Southwestern Montana during the 1977 calving season.

Terminology Used For Non-transformed WC-derived Tissues

Primary cell cultures derived from animals thought to be afflicted with WCS were distinguished by assigning cell line-like designations even though none of these tissue cultures could be said to have become established as cell lines. For example, the kidney from the fifth animal processed was designated as JT-5-77 K. The "J" stands for Jack Ward, the collector of the specimen. The "T" designates a tissue sample as opposed to a serum sample. The year the sample was collected is indicated by the 77 following the animal number, and the organ represented is designated by K, Thy, SG or TE in the case of the kidney, thymus, salivary gland or testicular tissues respectively. Passage numbers were added after the organ designation, and a culture retrieved from storage carried the suffix "A" after the passage number. Thus a thymus cell culture from the second animal processed which was in the

5th level of passage and which had been recovered from storage would be labeled JT-2-77 Thy 5A.

Cytological Analysis of Non-transformed WC-derived Tissues

Cytologically, none of the primary cell cultures exhibited any of the characteristics of transformed cell lines. These primary cell cultures grew in lines of orientation, were contact inhibited and could not be passaged indefinitely. Cells derived from tissues thought to represent weak calves had a tendency to palisade (grow in three dimensions), but stacks of cells growing in this fashion would subsequently detach and die. Growth rates could be arranged on a continuum from slow growing kidney cells through faster growing thymus, salivary gland and testicular cells. Some of the kidney cell cultures (JT-2-77 K3, JT-4,-5,-6 and -7) had cells which when stained with hematoxylin and eosin stains contained eosinophilic intranuclear inclusion bodies. Inclusions were small and not pronounced. Some cell cultures exhibited cytopathology at various passage levels. The usual cytopathology observed was vacuolation, followed by degeneration imparting a stringy appearance to the cells and subsequent death. Results of attempts to isolate agents responsible for these changes were equivocal.

Cytogenetic Analysis of Normal and Non-transformed WC-derived Tissues

Chromosome complements of tissues thought to be from calves afflicted with WCS were cytogenetically normal for all parameters tested.

Conventional Staining Analysis

Conventional staining with Giemsa revealed the normal diploid number (2n = 60) with 58 telocentric autosomes and 2 submetacentric sex chromosomes. A representative karyotype derived from one of these Weak Calf cell cultures (JT-4-77 SG) is depicted in Figure 3. The karyotype illustrates the normal chromosome complement. The sex chromosomes are easily identified by their submetacentric centromeres. The X chromosome is about the same size as the largest autosomal pair. The Y is among the smallest chromosomes of the complement. Two large sm X chromosomes were apparent in female karyotypes.

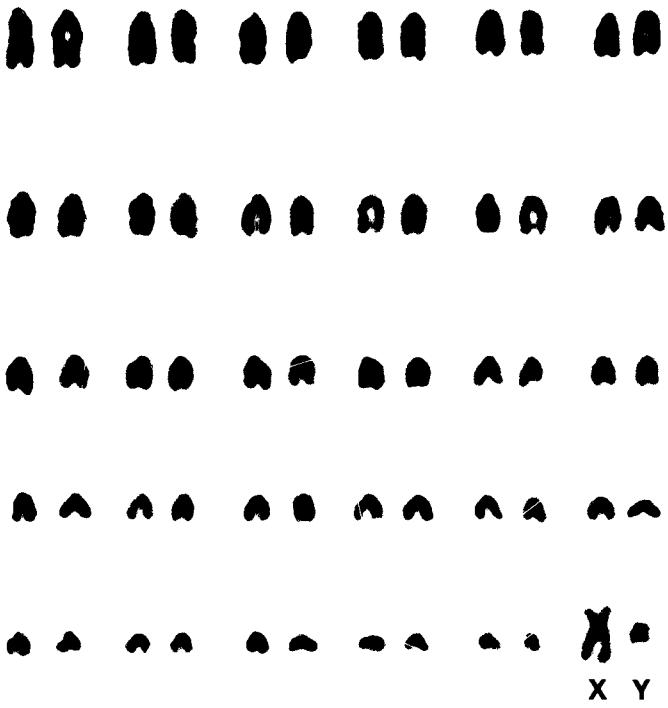
C-band Analysis of Normal and Non-transformed WC-derived Tissues

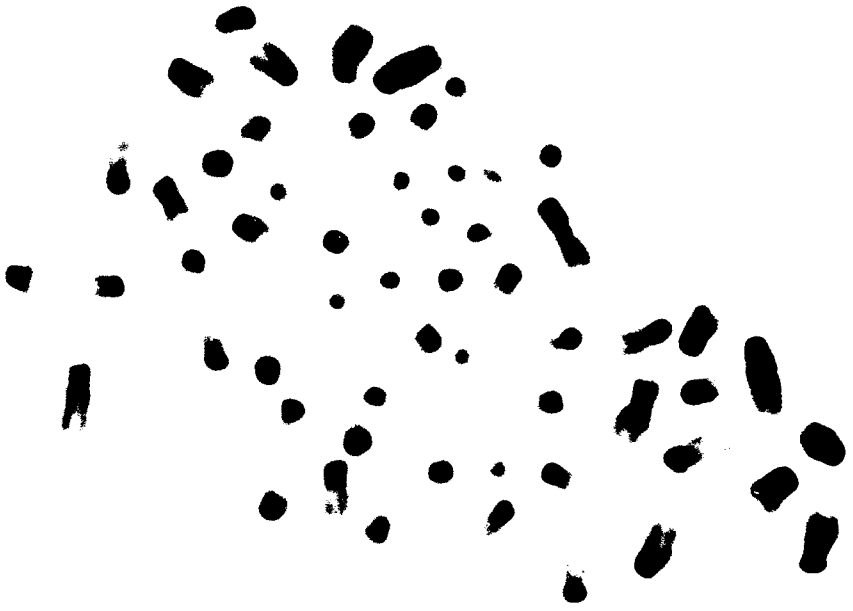
Application of C-banding techniques to representative chromosomes revealed the existence of what may be identified as constitutive heterochromatin localized at the centromeric regions of all autosomes. Regions of euchromatin stain light gray. Figure 4 is representative of a partial metaphase plate of a WC-derived cell culture, JT-6-77 SG2A.

Figure 5 depicts the C-banded karyotype of a so-called "stable" bovine turbinate (BT) cell line. Briefly, stable cells are altered primary cells which have survived beyond the limited passage time of normal primary cells. Stable lines are thought to have undergone a "transforming event" even though they continue to exhibit contact inhibition. This BT line was in its 31st level of passage, and the line was derived from a female as evidenced by the presence of 2 X chromosomes. BT-31 appears to be an example of a euploid stable cell

Fig. 3. Representative conventionally stained karyotype of a cell taken from primary tissue cell culture of WC-derived tissues, JT-4-77 SG. The karyotype illustrates the normal bovine chromosome complement.

Fig. 4. Partial metaphase plate of a cell taken from primary tissue cell culture of a WC-derived tissue, JT-6-77 SG2A. The chromosomes are C-banded.


X Y



line since the normal bovine chromosome complement is present. However, other metaphase spreads of this line (one example obtained from BT-34) have presented with an aneuploid number, i.e., 59. The mechanism(s) causing this hypodiploid complement may be nondisjunction or anaphase lagging.

C-band analysis of a normal male leukocyte produced essentially similar results as illustrated in Figure 6.

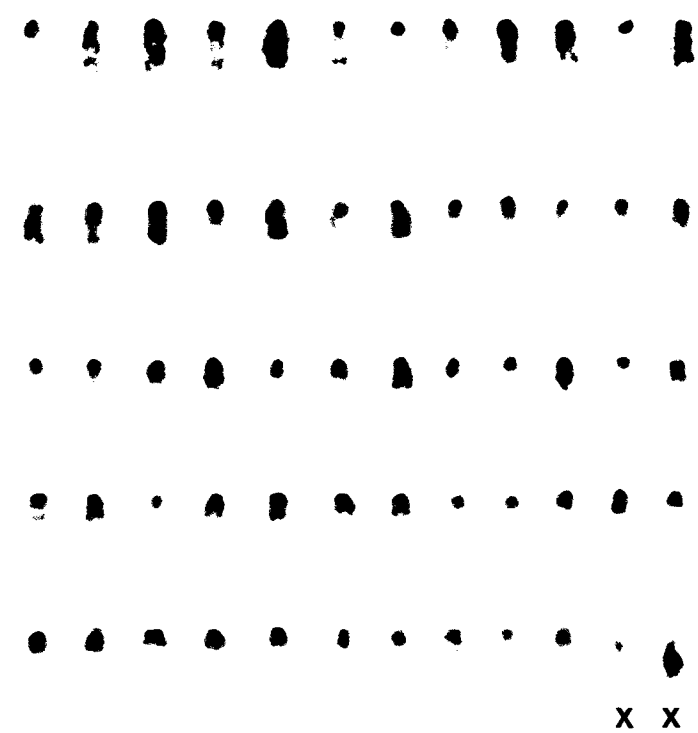
These C-banded karyotypes share several noteworthy features. The constitutive heterochromatin is confined to the regions coincident with the centromeres of all the autosomes. There is a lack of interstitial (within the arms) and telomeric (at the distal ends of the chromatids) constitutive heterochromatin.

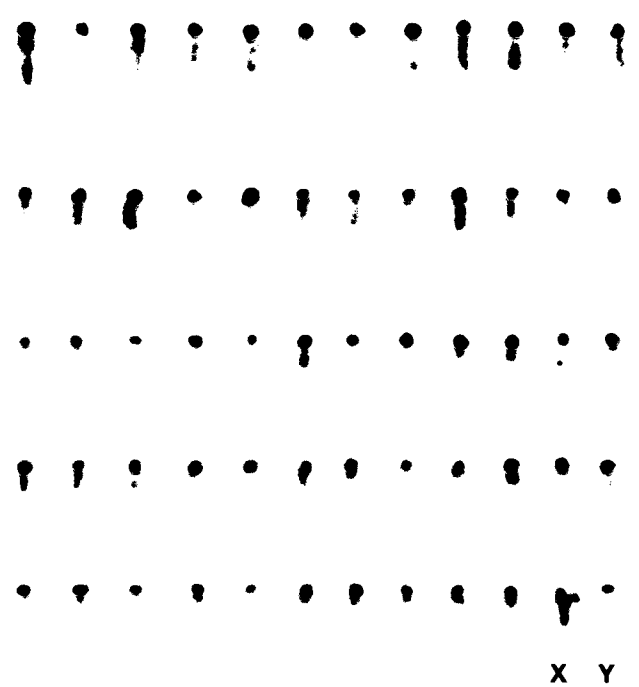
The X chromosomes appear to lack constitutive heterochromatin (Figs. 4 & 5). The dark region at the centromere of the X chromosome of Figure 6 is merely artifact, and the darkly staining appendage was left extending from the chromosome to assure that this interpretation would be made. The short arms of some of the X chromosomes illustrated do appear to stain with a density which is intermediate between centromeric heterochromatin and euchromatin. If this phenomenon is not merely a result of darkroom manipulations, then it still must be stated that the species of DNA being stained is not the same as the species found at the centromeres of the autosomes.

The Y chromosome may exhibit centromeric heterochromatin. The short arm and centromere are dark but again of intermediate intensity between euchromatin and the species of heterochromatin found at the centromeres of the autosomes. Lack of density may result from a

Fig. 5. C-banded karyotype of a cell derived from tissue cell culture of BT-31, a stable cell line.

Fig. 6. Representative C-banded karyotype of the bovine male. The cell was taken from a leukocyte culture.


X X


X Y

different species of heterochromatin being present or from diminished amounts of constitutive heterochromatin.

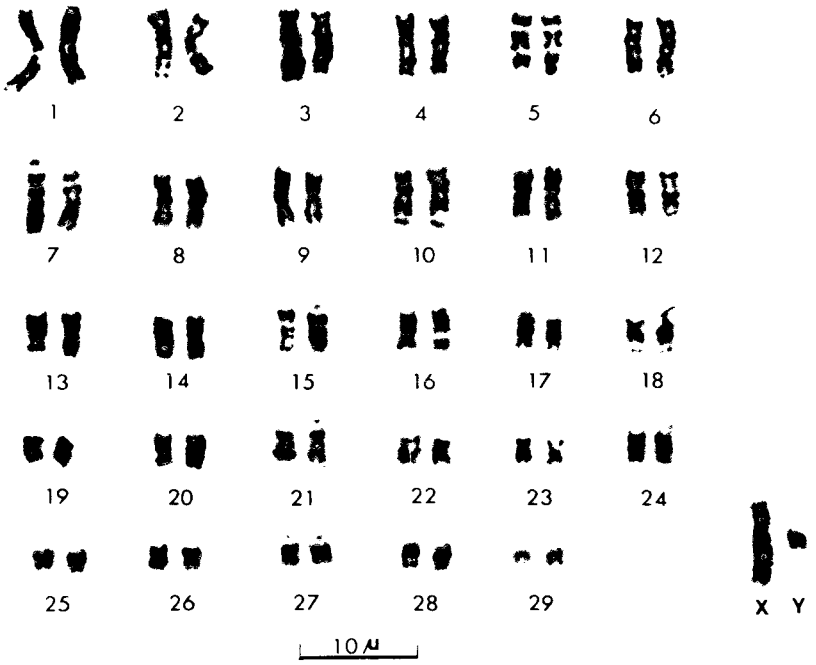
G-band Analysis of Normal and Non-transformed WC-derived Tissues

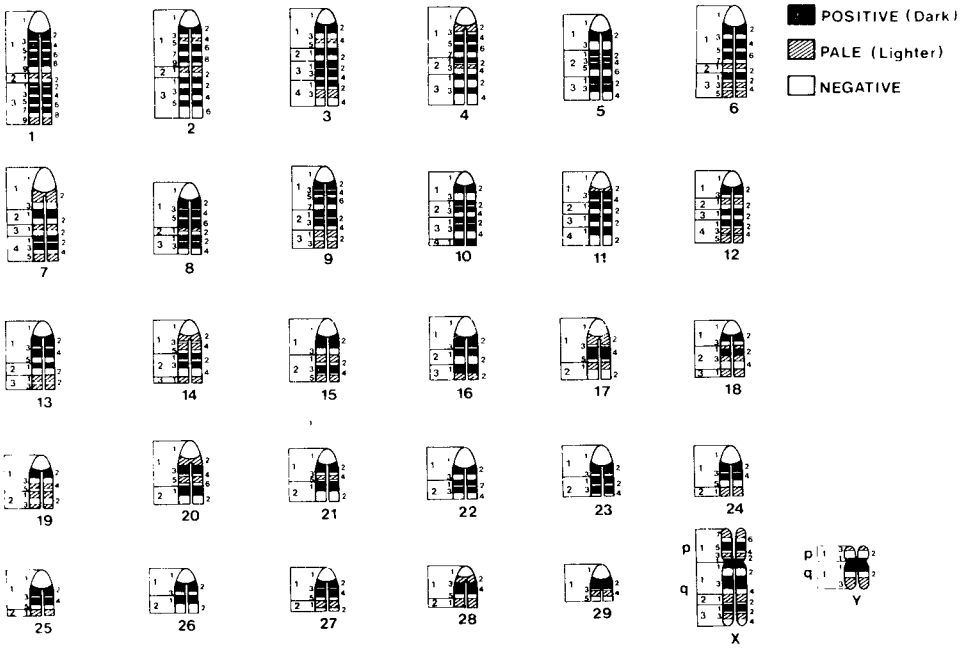
Homologous chromosomes have been paired by their similarities in banding patterns and arranged according to the convention established by the Reading Numbering System (53) (Fig. 7). Figure 7 is used with permission of C. C. Lin and represents the state of the art of bovine G-band analysis. Most other workers have difficulty attaining this level of quality. A diagrammatic representation of the same karyotype is shown (Fig. 8) and is also used with permission (53).

As stated previously, the system used in numbering the bands was based on conventions established for the human karyotype (72, 73) with modifications made for bovine chromosomes as suggested by Lin (53): "Regions" are defined by morphologically distinct landmark bands. Landmark bands can either be dark staining or pale staining bands. In the autosomes, the first region is described by the negatively staining centromeric-paracentromeric area and is designated as band 1. Band 2 is designated as the pale or positive band next to the proximal negative band in this region. With the exception of region 1, all other regions start with a positive or pale band. Negative or pale bands may end a region. To facilitate ready visual recognition of a region within a chromosome, a pale band is taken to represent a slightly lighter band when compared to the darker bands. To indicate the pale band in the middle of chromosome 1 then, the designation is 1,21.

Fig. 7. Representative G-banded karyotype of the bovine male arranged according to the Reading Numbering System (53). Used with permission of C. C. Lin.

Fig. 8. Diagrammatic representation of the normal male bovine karyotype arranged according to the Reading Numbering System. Used with permission of C. C. Lin (53).


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
X Y
10 μ


POSITIVE (Dark)
PALE (Lighter)
NEGATIVE
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
p
q
X
p
q
Y

The centromeric-paracentromeric band in chromosome 5 is designated as 5,11, and the positive band next to the proximal negative band in this region is called 5,12. The symbols p and q are used to indicate the short and long arms of a chromosome respectively and are of course applicable only in the case of the sex chromosomes in the bovine karyotype. The centromeric-paracentromeric regions of all the autosomes are G-band negative while these regions in the sex chromosomes are G-banded positive. With the sex chromosomes, numbering the bands of each arm also starts from the paracentric area. Therefore, the first band of the first region is called p11 in the short arm or q11 in the long arm. Using this numbering system Lin (53) has assigned 310 bands in the bovine karyotype.

A slightly more contracted G-banded karyotype is also illustrated (Fig. 9). The number of bands depends on the degree of contraction of the chromosome. This karyotype also represents a different effect of trypsin. Very small changes in detail of technique also induce variability in chromosome appearance. Variation in ambient temperatures, water temperatures, slide temperatures as well as numerous other factors can effect change in chromosome appearance.

RESTAINING WITH OTHER DYES

Chromosome preparations stained by one of the standard methods (Giemsa, Orcein) could be destained with 70% alcohol or acetic acid or both and subsequently treated with other dyes including the fluorochromes. Slides were left in the alcohol 30 min (90).

Fig. 9. Representative G-banded karyotype of the bovine female arranged according to the Reading Numbering System (53).


1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 23 24
25 26 27 28 29 X X

In this regard, conventionally stained slides could yield adequately stained C-banded preparations, but G-banded preparations would disintegrate when subjected to the C-banding technique. C-banding was an irreclaimable method and should therefore be the last stain applied.

RESULTS OF ANALYSIS OF THE CALF B SG TR CELL LINE

The Calf B salivary gland transformed (Calf B Sg Tr) cell line is an established cell line which was thought to have acquired transformed properties as a consequence of the pathobiology associated with WCS.

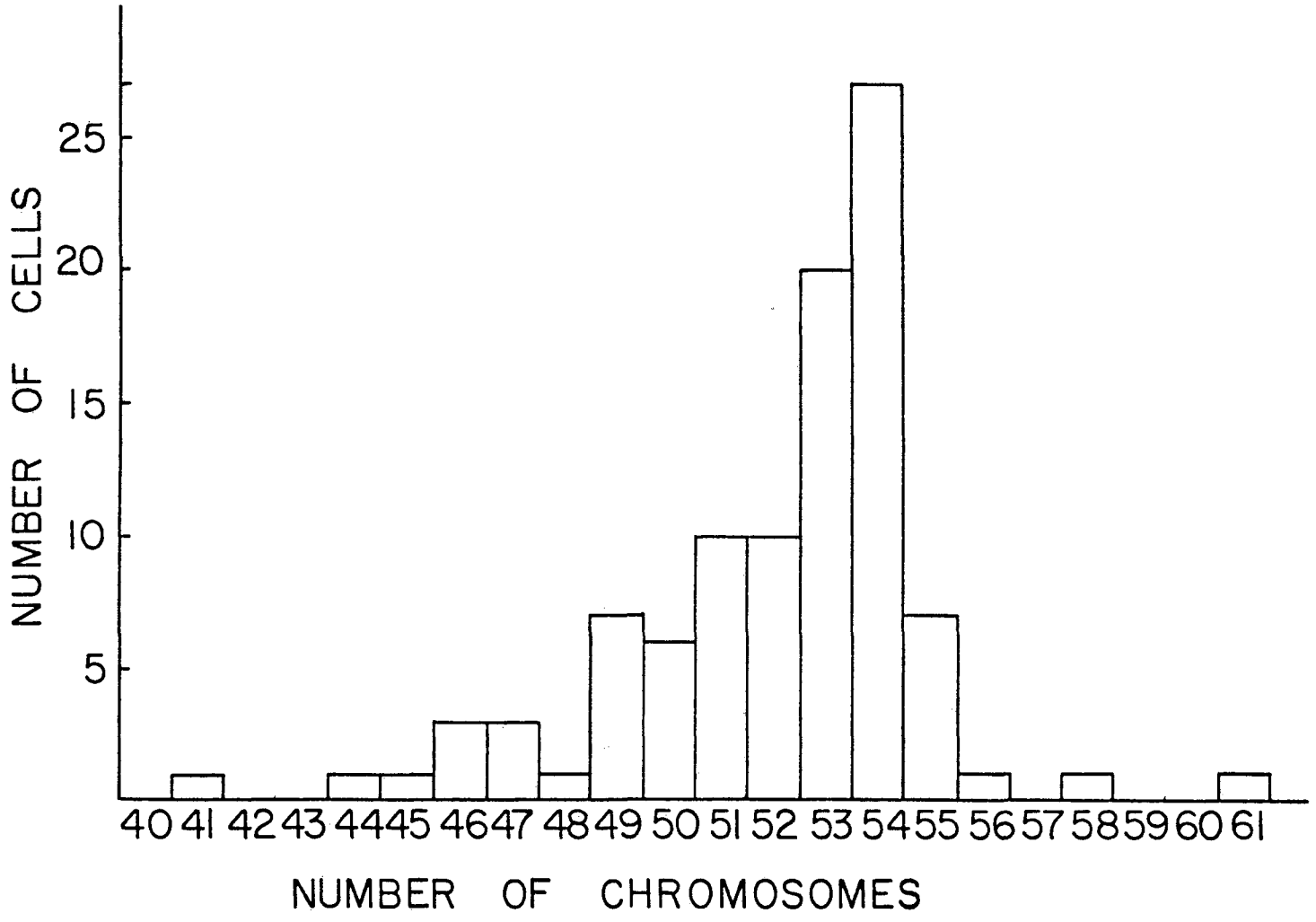
Modal Number

The Calf B Sg Tr cell line exhibits a modal number of 54. Figure 10 is a histogram representing the frequency distribution of 100 Calf B cells (n = 100) in regard to chromosome number. These data indicate that elements are more easily lost than gained and may reflect some dysfunction of the spindle fiber attachment sites at the nucleoplasm "matrix" that is thought to compose the centromere. This study is biased by the criteria used for inclusion of a metaphase plate. An acceptable metaphase spread was one which was confined to a roughly circular area (not drawn out across the field) and which included both marker chromosomes.

Conventional Staining Analysis

Karyotypes of various single cell clones are illustrated and are roughly comparable (Figs. 11 - 14). There has been an increase in the number of arms from 62 found in the normal bovine karyotype to a number

Fig. 10. Histogram representing frequency distribution of 100 Calf B Sg Tr cells in regard to chromosome number.


NUMBER OF CELLS
25
20
15
10
5
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61
NUMBER OF CHROMOSOMES

near 108. Two acrocentric marker chromosomes which are achromatic over the proximal parts of their q arms are readily apparent and consistently observed. The karyotype appears to be composed of approximately 43 atelocentrics and about 11 acrocentrics. It is difficult to determine if a true telocentric element exists. The last clear element of Figure 13 may be a minute or just an artifact.

C-band Analysis

Every centromere is stained by this method and therefore the X chromosome has probably been lost (Figs. 15 & 16). There appear to be no acentrics as revealed by this technique. Once again, the markers do not stain in the proximal regions of their q arms. In contrast to the normal C-banded bovine karyotype, marked regions of interstitial and telomeric heterochromatin are revealed in the p arms of some of the elements. These are especially evident in the first row of Figure 16.

G-band Analysis

Two G-banded karyotypes are illustrated (Figs. 17 & 18). One represents a cell derived from a single cell clone (SC-3) while the other (Figure 17) represents the parent cell line.

As usual the individual elements have been arranged by length. Almost every element is biarmed. Because of the difficulties involved in obtaining synchronized cells between two different preparations (thus the chromosomes have contracted at differing rates between the two metaphases), the individual elements are not strictly comparable by position. Nevertheless, the G-banded chromosomes have specific

Fig. 11. Karyotype of a conventionally stained cell derived from a single cell clone of the Calf B Sg Tr cell line. Calf B Sg Tr SC-2 is illustrated.

Fig. 12. Karyotype of a conventionally stained cell derived from a single cell clone of the Calf B Sg Tr cell line. Calf B Sg Tr SC-3 is illustrated.

banding characteristics and individual chromosomes can be identified. With other words, landmark bands for individual chromosomes appear to be consistent and reproducible.

In the more extended karyotype (Fig. 18), the markers appear in the 4th row, 5th element from the left, and 5th row, 2nd element from the left. This karyotype has 56 elements, but the last two may be minutes or artifacts. The more contracted karyotype (Fig. 17), displays the markers in the 3rd and 5th rows. This karyotype has 51 or 52 elements depending on whether the last is a true chromosome.

If a postulate is advanced that endopolyploidy must have occurred to effect these changes, then it should be possible to demonstrate 4 duplications of each arm. Few elements can be found that conform to this prediction.

Given that landmark bands appear to be consistent and reproducible, identification of structural changes should be possible. However, close analysis has resulted in the conclusion that the karyotype of the Calf B Sg Tr cell line is uninterpretable by comparison to normal G-banded bovine chromosomes. One tentative hypothesis entertained to account for these results was that a centric fusion resulting in multiple bi-armed chromosomes had probably occurred but lack of similarity in band patterns between the transformed karyotype and the normal karyotype suggested that structural rearrangements had occurred at sometime during the sequences leading to transformation. Alternatively, inability to interpret the banding patterns of the transformed karyotype imposed the necessity of proving (by ways other than banding) that the cells were, in fact, of bovine origin.

Fig. 13. Karyotype of a conventionally stained cell derived from a single cell clone of the Calf B Sg Tr cell line. Calf B Sg Tr SC-3 is illustrated. Last clear element may be a minute or just an artifact.

Fig. 14. Karyotype of a conventionally stained cell derived from a single cell clone of the Calf B Sg Tr cell line. Calf B Sg Tr SC-4 is illustrated.

Fig. 15. Karyotype of a C-banded cell derived from tissue culture of the Calf B Sg Tr cell line.

Fig. 16. Karyotype of a C-banded cell derived from the Calf B Sg Tr cell line. Note the marked regions of interstitial and telomeric heterochromatin in the p arms of some of the elements in the first row.

[illegible]

[illegible]

[illegible]

Fig. 17. Karyotype of a G-banded cell derived from the Calf B Sg Tr cell line.

Fig. 18. Karyotype of a G-banded cell derived from a single cell clone of the Calf B Sg Tr cell line. Calf B Sg Tr SC-3 is illustrated. Note the more extended nature of this karyotype as compared with Fig. 17.

[illegible]

[illegible]

[illegible]

ISOZYME MOBILITY PATTERNS

All cell lines previously reported as transformed sheep (ovine) or transformed WC-derived cell lines were examined to determine their adherence to purported biological origin.

Cells were examined for the presence of six enzymes: esterase (EST), glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and phosphoglucomutase (PGM). Enzymes from Calf B Sg Tr cells comigrated with the comparable enzymes from cells of African Green Monkey origin for all enzymes tested. In addition, enzymes from the cell line designated variously as OSG-17, OVTr, or OV(H)Tr (passaged in hamsters), of purported ovine origin, comigrated with enzymes from cells of human origin. Isozymes from the cell line designated as 390K of purported bovine origin, also comigrated with isozymes from cells of human origin. Fetal calf serum was shown to have faint activity when stained for LDH and protein, but LAH was shown to have no detectable activities. Some of these results are depicted in Figure 19.

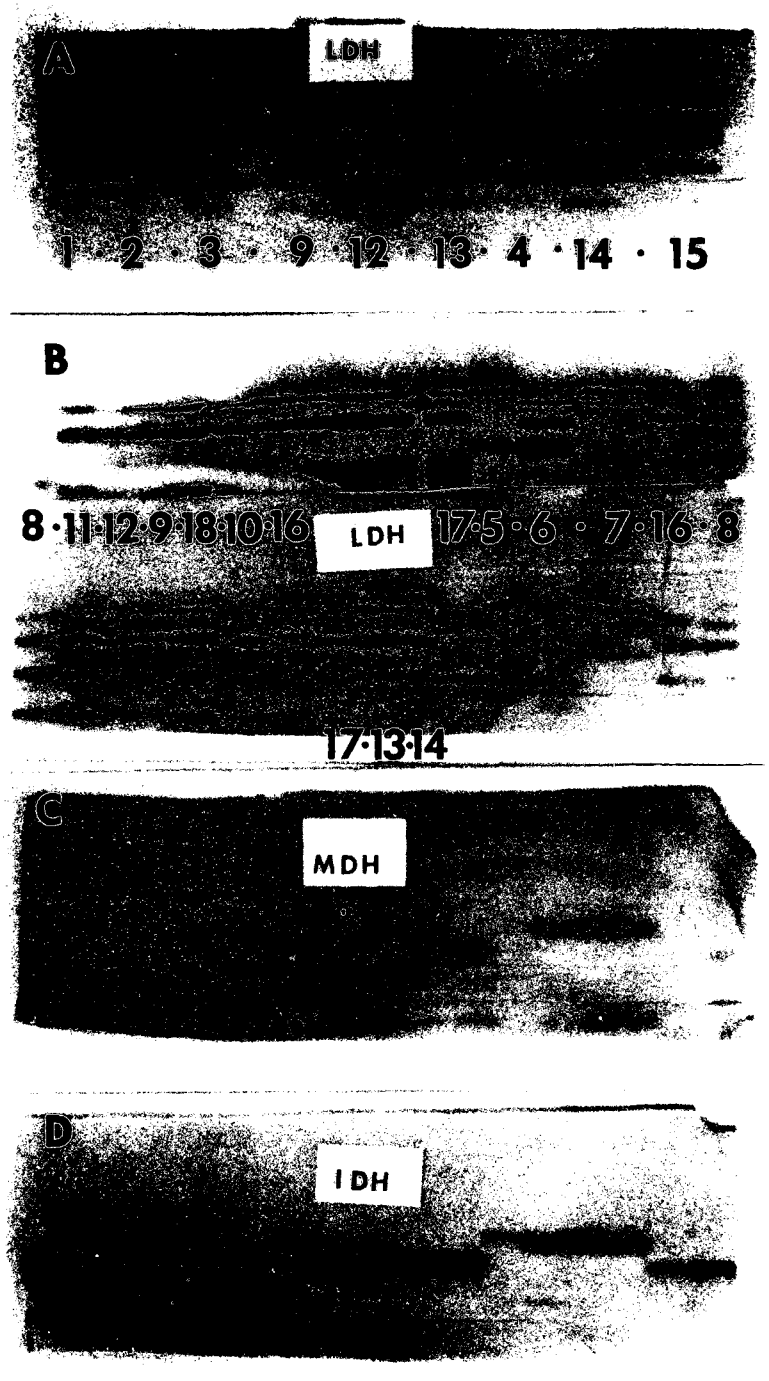
VIRUS INFECTIVITY STUDIES

Some viruses have exquisite specificity for their species of origin. Polioviruses and certain herpesviruses are among two groups that conform to this observation. Polioviruses have a specificity for cells of primate origin while certain serological types of the herpesviruses are species specific. For this reason, Poliovirus Type I (Mahoney 1954)

Fig. 19. Representative isozyme mobility patterns of various cell extracts and media when stained for 3 enzymes: LDH, MDH and IDH. Gels A, lower B, C and D were run in the Ridgway (79) buffer system. Gel illustrated by upper B was run in the Clayton-Tretiak (20) buffer system.

Key to cells, cell lines and media:

1. LAH (medium)
2. FCS (medium)
3. JT-5-77 SG (bovine salivary gland)
4. JT-3-4-5-6-77 SG (pooled bovine SG from primary culture)
5. JT-4-6-77 SG " "
6. JT-1-3-5-77 SG " "
7. Bovine salivary gland tissue homogenate
8. Human foreskin tissue homogenate
9. Hep-2 (human)
10. HEK "
11. OV(H)Tr (ovine transformed passaged in hamsters)
12. OSG-17 (ovine transformed without passage in hamsters)
13. Calf B Sg Tr (bovine transformed-parent cell line)
14. Calf B SC-2 (" " -single cell isolation 2)
15. Calf B SC-4 (" " -single cell isolation 4)
16. 390K (bovine kidney)
17. Vero (African Green Monkey kidney)
18. H7BSG (unknown origin)


A
LDH
1 · 2 · 3 · 9 · 12 · 13 · 4 · 14 · 15
B
8 · 11 · 12 · 9 · 18 · 10 · 16
LDH
17 · 5 · 6 · 7 · 16 · 8
17 · 13 · 14
C
MDH
D
IDH

was used to infect various cell lines. Several cultures of purported bovine origin (JT-3-77 SG5A, BT-40, JT-1-77 SG1A) were refractory to replication of the virus as evidenced by lack of cytopathology after prolonged exposure to virus. Transformed cell lines of purported ovine and bovine origin and lines of primate origin were lytically infected by this virus. Among the susceptible lines tested were Calf B Sg Tr, Calf B Sg Tr SC-2, 390K, OSG-17, OV(H)Tr, H7BSG (origin unknown), HEK (human) and Hep-2 (human).

A herpesvirus of bovine origin, infectious bovine rhinotracheitis (IBR Colorado), was used for the "reverse" experiment. Cultures of purported bovine origin which had been refractory to poliovirus (JT-3-77 SG5A, BT-40, JT-1-77 SG1A) in the previous experiment were lytically infected by IBR virus. Cytopathic effect (CPE) included the rounding of cells characteristic of herpesviruses in all three of the susceptible cultures. The aforementioned lines which had been susceptible to replication of Poliovirus Type I proved refractory to IBR in every case.

THE GIEMSA MARKER

Review of the literature of primate karyologies disclosed reports of the existence of a well-known marker chromosome of cercopithecid monkey cells (19, 68). This marker is also evident in three presumably unrelated human cell lines, HEK, HBT-3 and HBT-39B. Comparison of G-banding patterns of these latter cell lines with the larger Calf B Sg Tr cell line marker (illustrated in Figure 20), revealed the presence of nearly identical banding patterns.

Fig. 20. Enlargement of the larger marker of the Calf B Sg Tr cell line karyotype. The marker is G-banded.

CHAPTER 4

DISCUSSION

WC-DERIVED TISSUES IN CULTURE

Evidence derived from the culture of 18 primary cell cultures derived from tissues of 7 calves suggests that transformation may not be a reproducible sequelae of long-term in vitro cultivation of WC-derived tissues. This statement must be made with reservations. That these tissues were indeed representative of the disease known as WCS rests ultimately on the subjective interpretation of J. K. Ward, D.V.M., who is credited with first reporting the condition (97). For this reason the criteria whereby an individual is included in that set of animals known as "weak calves" may have undergone subtle interpretative changes through the years as observation has been tempered by accumulated experience and data. Therefore, the possibility exists that these tissues bear little resemblance to the tissues that were reported to have been transformed by a virus suspected as the etiologic agent of WCS (46, 47). Another qualifying factor may have been that the weather during gestation was not unduly harsh and thus did not contribute a stress factor that is thought to play a role in the increased incidence of WCS (97). Finally, the Gordian knot presented by any attempt to describe WCS as a disease rests on the fact of whether WCS is a distinct clinical entity with a defined etiology or merely a nonspecific collection of circumstances affecting newborn calves.

The fact that cytopathology is evident in some of the cell lines but not in all suggests that an agent of a microbiological nature may be causing these changes. The relationship of the pathobiology associated with WCS to the initiation or progression of these cellular changes is not yet understood. The possibility that these agents are merely adventitious may not be discounted. Cytotoxicity as distinguished from CPE could not be ruled out as being responsible for cellular degeneration of lines approaching the 5th passage level.

Cytogenetic analysis revealed normal chromosome complements for all parameters tested. However, several interesting features of the bovine karyotype were demonstrated. Most remarkable among these features is the apparent inability to demonstrate constitutive heterochromatin associated with the X chromosome. Since constitutive heterochromatin has been observed in the centromeric regions of chromosomes in all mammals and birds (except possibly macrochromosomes) thus far studied, it is noteworthy that none can be demonstrated in the bovine X by the technique used (99). Hansen (37) has previously made this observation using another variation of the C-banding method. The Y chromosome appears to have a species of heterochromatin which may be identical with the constitutive heterochromatin of the autosomes. Although this heterochromatin stains less intensely than the constitutive heterochromatin coincident with the centromeres of the autosomes, this effect may result from diminished amounts being present. These interpretations are reinforced in part by the observations of Schnedl et al. (89) that the centromeric regions of cattle, goat and sheep

chromosomes bind anti-5-methylcytosine. The short arm of the cattle Y chromosome also bind anti-5-methylcytosine whereas the centromere of the X chromosome of cattle remains nearly unstained.

Another feature of the bovine karyotype which deserves comment is the lack of staining of all the centromeric regions of the autosomes by the G-banding technique. The G-banding technique also demonstrates that the autosomes may be truly telocentric since there is a lack of positive or pale staining bands in the paracentromeric regions where short arms would occur if the elements were acrocentric.

The biological significance of these features are not readily apparent. As noted in the Introduction, Robertsonian-type translocations would be viable rearrangements since no essential genes are lost. Robertsonian translocations would facilitate karyotypic evolution in the direction of biarmed chromosomes. In the sheep (Ovis aries), a closely related ungulate, it is interesting that there are 54 pairs of chromosomes but 6 pairs are biarmed (31). There are many examples in the literature which demonstrate similar tendencies toward centric fusion in animals having acro- and telocentric chromosomes. Nelson-Rees, Kniazeff and Darby (67) reported an increase in numbers of atelocentric chromosomes in cell cultures of bovine origin. They advanced the hypothesis that the debut and accumulation of centric fusion products could be used as an index of the age of the cell line. Alterations first appeared in cell lines at the 10th through 15th passage level. In light of this information it is not surprising that centric fusion products were not observed in WC-derived cell

lines since at this writing, none have as yet been cultured beyond the 9th passage level.

These studies demonstrate that cytogenetically the chromosome complements of the weak syndrome calves do not appear to have visible chromosome abnormalities. These observations do not rule out the possibility that alterations may have occurred. Sizable deletions or reciprocal exchanges would not be visualized using conventional techniques. Also, it is conceivable that chromosomal changes at the molecular level which could represent massive alterations in the genotype may not be detected by G-banding techniques. Cytogenetic analysis would also fail to detect any of the regulatory changes that could be postulated to have occurred as a result of the milieu surrounding the birth of a weak calf. The inherent limitations of cytogenetic techniques should not however obscure their value as a powerful diagnostic tool; a feature which will hopefully become evident later in this discussion.

These studies also point up the remarkable stability of primary bovine tissue in culture.

ANALYSIS OF THE CALF B SG TR CELL LINE

The demonstration that the karyotype of the Calf B Sg Tr cell line was not interpretable by comparison to normal G-banded bovine chromosomes necessitated alternative hypotheses for the changes being observed. As previously stated, the first hypothesis entertained was that structural rearrangements had occurred at sometime during the sequences leading to transformation. In Robertsonian translocations in humans, and several

that have been noted in cattle (34, 35, 77), the rearranged chromosomes retained the banding patterns of the chromosomes from which they were derived. These rearrangements only involve two elements at most. Meiotic synapsis would present an obstacle to fertility (i.e., the ability to form a viable diploid zygote) in those individuals carrying these translocations. This of course is not a problem in a tissue culture system or even in a tumor. Evidence is accumulating that structural rearrangements have indeed occurred in such long-lasting transplantable animal tumors as the Ehrlich mouse ascites tumor, the Yoshida rat tumor, the Dunning rat leukemia and the canine venereal tumor mentioned previously (70). This evidence is based on inability to match up banding patterns.

Alternatively, inability to interpret the banding patterns of the transformed karyotype imposed the necessity of proving (by ways other than banding) that the cells were, in fact, of bovine origin.

Isozyme mobility patterns revealed that all transformed lines purportedly derived from tissues of cattle and sheep afflicted with WCS were inaccurately specified. Comigration with isozymes of ungulate origin could not be demonstrated for any of the transformed lines. Comigration of isozymes from the transformed lines with those of human and nonhuman primate lines could be demonstrated. Isozyme comigration may not necessarily indicate identity.

Isozyme analysis as applied in these studies suffer from any number of limitations. A few examples from among these drawbacks are: (1) diseased tissues can present isozymes with altered mobilities, (2) epigenetically modified isozymes would migrate differently from

counterparts that were not altered, (3) heterogeneous populations of cells or tissues can produce isozymes which are the allelic products of one locus, i.e., the locus is polymorphic, (4) polymorphisms within a species may mimic interspecific differences in isozyme mobilities but with increasing numbers of loci the probability of inaccurate specification becomes lessened, (5) rates of synthesis and degradation of a particular enzyme may add variability to isozyme migration patterns, (6) mutations in vitro may result in isozymes with altered mobilities and (7) the manner of obtaining the cellular homogenates would fail to distinguish alternate forms of an enzyme at various subcellular compartmentalizations. In addition since transformed cells are thought to more nearly represent embryonic cells than normal differentiated tissues, it may be possible to demonstrate different isozyme patterns for normal and neoplastic tissues (107).

These studies tried to address some of these points by using differentiated tissues where possible (bovine parotid and submaxillary salivary glands, and human foreskin) to offer comparison with isozyme patterns of transformed lines. A transformed bovine line was not available for study nor was there a source of primary African Green Monkey tissue.

Virus infectivity studies provided an elegant demonstration of species specificity using Poliovirus Type I (Mahoney) and IBR (Colorado), a herpesvirus of cattle. These studies showed the transformed cells to be of primate origin in every case. The assumption inherent in the studies is that viruses can infect only those cells which have receptors specific for them. The argument may be advanced that the

transformation process may expose or eliminate receptors that would confer susceptibility or refractoriness to infection. Subsequent replication of a virus would thus be allowed or disallowed in each respective case. However, the probability seems remote that these events (gain or loss of receptors) would occur simultaneously for two viruses differing widely in species of origin.

The "Giemsa" marker is the designation that Nelson-Rees, Flandermeyer and Hawthorne (68) assigned to a marker chromosome characteristic of HeLa cells. This marker appeared slightly different in such presumably unrelated human cell lines as HEK, HBT-3 and HBT-39B but always resembled the well-known marker chromosome first described in cells derived from cercopithecid monkeys (19). The literature reference noted above (68) illustrates a G-banded Giemsa marker for the HBT-39B cell line which is virtually identical with the larger of the G-banded marker chromosomes of the Calf B Sg Tr cell line (Fig. 20). Both of these markers were easily the most noteworthy features of the karyotype. They eluded identification because little thought was given to the possibility that the Calf B line might be from a species other than bovine. The reason(s) for lack of staining on the q arms of these markers is not known since they fail to stain with G-, C- and R-banding procedures.

Nelson-Rees, Flandermeyer and Hawthorne (68) point out that the Giemsa marker could only be observed in HEK cells that had undergone considerable passages, while those HEK lines of low passage exhibited the marker in less than 2% of the metaphases. This observation may explain why these markers escaped notice in early karyotype analysis

of the Calf B cell line, i.e., the contaminating cell was from a cell line of low passage number. Other explanations may be found in the observations that highly contracted chromosomes would obscure the achromatic region of these markers, the markers may have been absent in the particular cells chosen for study and the possibility that the Calf B cell line did not originate as a contaminant.

Taken together then: (1) inability to match the G-banded Calf B Sg Tr karyotype with the normal G-banded bovine karyotype, (2) isozyme mobilities in starch gel electrophoresis, (3) patterns of viral infectivities and replication and (4) the presence of the Giemsa marker support the conclusion that the Calf B Sg Tr cell line in particular, and the transformed cell lines of purported bovine and ovine origin in general, are all now primate cell lines.

INTER- AND INTRASPECIES CONTAMINATION OF CELL CULTURES

The former observations raise the question of whether all or some of these transformed lines originated as interspecies contaminants or were contaminated during subsequent handling and passage. The fact that three different people reportedly were able to observe transformation associated with WC-derived tissues lends credibility to the idea that cell line contamination may have occurred at a later time (46, 47, 101). With particular reference to the Calf B Sg Tr cell line, it should also be noted that a number of people have handled and passaged this cell line since inception. The possibility that this investigator may have inadvertently introduced the contaminating cell is also a very real one.

To the contrary, other arguments point toward the suggestion that these lines may have originated as cell line contaminants. First among these is that the 390K cell line was not recovered from storage for isozyme analysis until the problem with the Calf B line was evident. The possibility for contamination at the hands of this investigator was lessened by acute awareness of the implications of the experiment. Isozyme analysis could have been run on the frozen cells, but since they represented the last remaining copy, it was deemed more necessary to establish a large fresh pool for subsequent analyses. Therefore, the time in culture did expose them to the danger of cross-contamination in spite of added vigilance.

Another pertinent point is that since isozymes from both OSG-17/OVTr and OV(H)Tr comigrated with isozymes from cells of human origin, contamination probably occurred prior to passage in hamsters. The lines were presumably kept separate after the inoculation. Inoculation of a sheep cell line that produced a tumor in hamsters was reported (46). If the above cell lines are one and the same, then it may be said that contamination of the ovine line was a very early event in its history.

Documented cases of inter- and intraspecies contamination involving cells commonly used in the laboratory are becoming legion. Nelson-Rees and Flandermeyer (69) have presented data which indicates that in an 18 month period of monitoring cell line purity, 41 of 253 cultures (16%) were not as purported. These sobering statistics emphasize the need to continually monitor cell lines for purity in an attempt to avoid publication of data derived from the use of erroneously specified cell cultures.

These studies have demonstrated the use of a few techniques which can be used to distinguish interspecies contamination. Karyology through the use of chromosome banding techniques offer a valuable tool in cases of intraspecies contamination. In other words, cell line purity can be evaluated in cases where the donor's species and sex may be identical or obscured because of changes inherent in long-term-cultivation of cells. Other monitoring techniques include immunofluorescence and cytotoxicity tests.

Most recently, Nelson-Rees and Flandermeyer have become involved in a controversy with R. S. Chang (17) concerning their indictment of the Chang liver cell as a HeLa contaminant. The controversy is interesting because the cells apparently elaborate liver-specific enzyme activities and yet appear to contain a complex of HeLa marker chromosomes, exhibit a G6PDH mobility characteristic of HeLa cells (along with 7 other enzyme loci) and lack a Y chromosome (Henrietta Leaks, alias Helen Lane, was the donor of the cervical carcinoma from which the HeLa line was derived).

The seriousness of the charge is profound in light of the perspective that no other human liver line exists in spite of repeated attempts to initiate other such lines. As noted in the Introduction, the special advantages offered by the use of "liver cell cultures" may be a moot point if the line is a strain of HeLa.

Other HeLa strains are known which elaborate a variety of tissue specific products. Genetic derepression is offered as the most trivial explanation, but in view of the large numbers of cell lines that have been indicted as HeLa strains, one wonders whether the HeLa banding

patterns, enzyme mobilities, lack of a Y chromosome, etc., may be the ultimate fate of all long-term human cell lines.

In any event, in spite of the inherent limitations of isozyme analysis in particular for detecting intraspecies contaminations it is probably untenable to suppose that cells from different species cannot be differentiated by this method. This suggestion would take on greater validity as the numbers of enzymes monitored increased.

CONCLUSION

Because all of the transformed cell lines of purported ovine or bovine origin turned out to be of nonhuman primate or human origin, these data seem to suggest that all of these cultures may have originated as interspecies contaminants. In any event, and in light of the discovery that these cell lines may all be of primate origin, the questions asked at the outset concerning the nature of the chromosome changes observed in the transformed cells may have been resolved in a way that was not anticipated.

These studies may also suggest on the basis of limited data that transformation is not a reproducible sequelae of long-term in vitro culture of WC-derived tissues when the above-mentioned observation is considered along with the fact that cultured cells derived from tissues of 7 different calves from the 1977 calf crop failed to transform. A larger data base derived from the establishment and culture of primary bovine tissues from the 1978 calf crop should provide additional information necessary to test this hypothesis.

CHAPTER 5

SUMMARY

Cytogenetic studies were done on a variety of tissues of bovine origin. Tissues derived from cattle tentatively diagnosed at necropsy as being afflicted with WCS as well as tissues thought representative of the normal condition were analyzed by various cytogenetic techniques and compared. Application of conventional Giemsa staining methods revealed that chromosomes in tissues from both sources lacked any obvious structural rearrangements and appeared cytogenetically normal. In addition, C- and G-banding analysis did not reveal any chromosomal aberrations in tissues up to the 9th level of passage. The C-banded bovine chromosomes presented an exceptional karyotype by comparison to most mammals in that constitutive heterochromatin was not demonstrated in the bovine X chromosome, but the Y chromosome appears to have a species of heterochromatin which may be identical with the constitutive heterochromatin of the autosomes. Transformation was not observed in any of the WC-derived tissues.

A cell line (Calf B Sg Tr) thought to have transformed as a consequence of the pathobiology associated with WC-derived tissues was also analyzed by the above-mentioned cytogenetic techniques. The karyotype of this cell line presented with multiple biarmed chromosomes in contrast to the usual telocentric morphology of the normal bovine autosome. C-banding analysis revealed the presence of telomeric and interstitial constitutive heterochromatin in some elements. These features are not normally found in the bovine karyotype.

Induction of G-bands produced patterns that were not interpretable by comparison with normal bovine G-banded patterns. The possibilities of structural rearrangements or cell line contamination were considered.

Isozyme analysis revealed that 6 of the Calf B Sg Tr cell enzymes tested did not migrate with enzymes from normal bovine or primary cell culture tissues. Subsequent analysis of other ovine and bovine transformed lines thought to have originated by a similar series of events as the Calf B line revealed that they were of primate origin in every case.

Viral infectivity studies using poliovirus and a herpesvirus of bovine origin corroborated the suggestion that the transformed cell lines were in every case primate or nonhuman primate cells.

A review of the literature revealed the existence of a Giemsa marker first reported in cercopithecid monkeys and present in some cell lines of human origin which was nearly identical in G-banding pattern to the larger marker of the Calf B karyotype.

Taken in total these studies suggest the hypothesis that transformation may not be a reproducible sequelae of long-term in vitro culture of WC-derived tissues. Studies of cell cultures derived from tissues of calves thought to be afflicted with WCS from the 1978 calving season are currently in progress to further test this hypothesis.

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APPENDIX

Culture Media Formulations

NCTC 135

NCTC 135	9.4 g
TDW	1.0 l
FCS	% concentration
$NaHCO_3$	1.0 g
L-Glutamine	0.1 g
Antibiotic stock*	1.0 ml/l

Minimal Essential Medium (MEM)

MEM	9.6 g
TDW	1.0 l
FCS	% concentration
$NaHCO_3$	1.0 g
Antibiotic stock*	1.0 ml/l

Medium 199 (M-199)

M-199	9.9 g
TDW	1.0 l
FCS	% concentration
$NaHCO_3$	1.5 g
L-Glutamine	0.1 g
Antibiotic stock*	1.0 ml/l

*see next page

Antibiotic Stock

Penicillin G 100X

100,000 units/ml = 1,000,000 u/10 ml

1,000,000 u/1595 u/mg = 626.96 mg in 10 ml

weigh 0.627 g for 10 ml (100X)

Streptomycin 100X

100,000 ug/ml = 1.0 g/10 ml (100X)

1. Weigh 1.0 g strep + 0.627 g pen and dissolve in 10.0 ml TDW
2. Dilute 1.0 ml (100X) in 1000 ml medium

L-Glutamine

MW 146 g/mole (M)

0.2 M = 29.2 g (200 mM)

Make 100 ml at 100X concentration

1. Weigh 2.92 g and dissolve in 100 ml TDW (100X)
2. Filter sterilize
3. Dilute 1.0 ml (100X solution) in 100 ml medium (= 2 mM)
4. Store prepared powder at 5 - 7^{o}C
5. Store stock solution at 0^{o}C

PBS pH 7.2

131.625 g NaCl (0.15 M) or 0.875% saline

375.0 ml (0.133 M) $Na_2HPO_4 \cdot 7H_2O$ (or 0.0033 M after dilution)

14,625 ml TDW

1. Prepare 0.133 M $Na_2HPO_4 \cdot 7H_2O$ by dissolving 35.65 g/l
2. Adjust pH with 1 N HCl or 1 N NaOH

10X Pd

NaCl	160.0 g
KCL	4.0 g
Na_2HPO_4	23.0 g
or $Na_2HPO_4 \cdot 7H_2O$	43.4 g
KH_2PO_4	4.0 g
Glass distilled water	2.0 l

Sterilize by autoclaving at 121°C, 15 psi
for 15 min

Preparation of Trypsin

I. 0.25% Trypsin Solution

2.5 g trypsin
1000 ml Pd (100 parts 10X Pd stock + 900 parts TDW)

Mix for 1 h at room temperature

Filter sterilize

Dispense in either:

100 ml aliquots in tissue culture clean milk dilution bottles or,

5 ml aliquots in tissue culture clean screw cap test tubes

Tilt at a 30-45° angle and freeze at -20°C

The shelf life of frozen trypsin solution is short so do not make up more than a month's supply. Once a bottle or tube is thawed, use immediately. Do not refreeze.

II. Trypsin-Versene Solution

NaCl	8.0	g
KCl	0.4	g
Glucose	1.0	g
Trypsin	1.5	g
TDW	1000	ml
Versene (EDTA)	0.2	g
$NaHCO_3$	0.58	g

Add all ingredients except Versene and $NaHCO_3$ to TDW. Stir 45 min at room temperature. Add Versene and $NaHCO_3$, stir 15 min longer. Filter sterilize. Dispense in tissue culture clean tubes in 5 ml quantities and freeze.

phenol red indicator (optional)
1 mg/100 ml w:v
Use 10 ml/1000 ml

Adjust pH with 1 N NaOH to 7.2 - 7.4

Stock Giemsa

1. Add 0.5 g Giemsa powder to 33.0 ml glycerol; heat @ 60°C for 2 h
2. Cool slightly then add 36 ml absolute methanol and mix
3. Store in dark at least one week prior to use

Stock Leishman (Harleco #252)

1. Dissolve 100 mg in 50 ml methanol
2. Let stand overnight then filter and store for at least 2 weeks at room temperature
3. Store in foil covered bottle

Gurr's Buffer (pH 6.8)

1. Place tablet in large beaker and mash with stirring rod
2. Add 1 l H_2O then stir with magnetic stirrer until dissolved

Colcemid Stock Solution

1. Dilute stock colcemid (100 µg/ml) 1:19(v:v) in M-199 with Hanks' BSS (GIBCO)
2. Stock solution concentration = 5.0 µg/ml

Chromosome Medium

Medium 199 with Hanks' Base Salt Solution (GIBCO)	11.1 g/l
$NaHCO_3$	0.35 g/l
FCS (GIBCO)	20% (V:V)
Heparin (preservative free)	50 units/ml
sodium salt (Sigma)	311.0 mg/l
Penicillin	100 units/ml
Streptomycin	100 µg/ml
or gentamycin	50 µg/ml
Phytohemagglutinin (Wellcomes) (or as recommended by company)	1 vial/l

1. Do not use Earle's because salt concentration is too high resulting in pH problems.

2. If making up a 1 l volume from scratch; make up 1 l then remove 200 ml....so that a final volume of 1 l with 20% FCS can be obtained. This is done because the PHA is made up for a 1 l volume.

3. Heparin is added after the FCS.

Hanks' BSS 10X 1 L

Solution A:

NaCl	80.0 g
KCl	4.0 g
$Na_2HPO_4 \cdot 7H_2O$	1.13 g
KH_2PO_4	0.6 g
Glucose	10.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g

Solution B:

$CaCl_2 \cdot 7H_2O$	1.76 g
or $CaCl_2$ anhydrous	1.4 g

Procedure for Preparation of Hanks' BSS

1. If making 10X solution do not mix Solutions A and B....precipitate will result
2. Hydrated forms preferred
3. To use: 0.03% $NaHCO_3$ in 1X medium.
4. Add 40 ml of 0.5% (or 0.2 g) phenol red