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Review article

Immortal porcine lymphoblastoid cell lines: interest for veterinary and medical research

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Summary — Immortal lymphoblastoid cell lines of B and T lineages have been derived from blood cell cultures of Duroc, Miniature (histocompatible) or unknown porcine breeds. The lymphoblast immortalization has been putatively attributed to an oncogenic virus belonging either to the *Herpesviridae* or to the *Retroviridae* groups. But a definitive proof of a viral etiology is still lacking, impeding some therapeutic use of tissue culture products secreted by these cell lines. Nevertheless, immortal cell lines have many advantages over finite ones, owing to their ease of maintenance in simple media and to the acquisition of an infinite lifespan. The different cell lines are mainly used to develop the hybridoma technology in this species for cytogenetics and xenogenic research.

pig / lymphoblastoid / hybridoma / cytogenetics / xenotransplantation

Résumé — Lignées lymphoblastoïdes porcines immortelles : intérêt pour la recherche vétérinaire et médicale. *Les lignées lymphoblastoïdes disponibles actuellement ont été obtenues à partir de cultures de cellules sanguines de porcs de races Duroc, Miniature (histocompatible) ou inconnue. L'immortalisation du lymphoblaste a été attribuée à l'intégration dans le génome cellulaire d'un herpès ou d'un rétrovirus oncogène, sans qu'à l'heure actuelle aucune démonstration définitive d'une étiologie d'origine virale n'ait été fournie. Cette incertitude sur le mécanisme d'immortalisation entrave d'éventuelles utilisations thérapeutiques de produits obtenus à partir de ces cellules. Néanmoins, ces lignées offrent de nombreux avantages, comme leur culture dans un milieu relativement simple et leur propriété de croissance illimitée. Les lignées lymphoblastoïdes sont utilisées principalement pour développer la technologie des hybridomes dans l'espèce porcine, ainsi qu'en cytogénétique et en xénotransplantation.*

porc / lymphoblastoïde / hybridome / cytogénétique / xénotransplantation

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INTRODUCTION

The main cellular constituents of blood, lymphocytes, are easy to obtain in large quantities by venipuncture and are among the most common cell types used by cell biologists and biochemists (Alberts *et al,* 1989; Adams, 1990). At the onset of culture, the lymphocyte is in general a quiescent cell and requires mitogenic stimulation, usually sustained by lectins, to undergo at most a few cell divisions before dying (Adams, 1990). This lymphocyte population can be maintained in active growth for a few days constituting a primary culture. The use of porcine lymphocytes in primary culture was proposed in 1968 by Forsdyke, despite obvious disavantages, such as the frequent necessity to slaughter pigs and the between-animal variability in growing blood cells. Sometimes a fraction of the dividing cells can be subcultured *in vitro* for a limited number of cell generations before dying out. This cellular subpopulation constitutes a finite cell line (Freshney, 1992). The selection of pig T cell lines maintained in active growth by supplementation of the growth medium with an antigen (Canals *et al,* 1992; Dillender and Lunney, 1993) and of cloned gamma delta T cell lines (Grimm *et al,* 1993) have been reported recently. The selection of immortal lymphoblastoid cell lines has been initiated partly to circumvent the difficulties of primary lymphocyte culture. Immortal cell lines (also referred to as established or continuous) have many advantages, including the acquisition of an infinite lifespan and the ease of maintenance in simple media retaining the ability to multiply at low densities (1–2 cells/ml) which facilitates their isolation as single cells (clone). The acquisition of an infinite lifespan implies a deregulation of the mitotic cycle enabling a majority of the cell

population to by-pass the quiescent stage (G0) of the mitotic cycle (Adams, 1990; Freshney, 1992). The immortalization mechanism requires aberrant gene expression due to either a physical rearrangement of the lymphocyte genome by chromosome translocations, improper packaging of the genomic DNA or to the integration into the cellular genome of oncogenic sequences of viral or cellular origin (Alberts *et al,* 1989; Adams, 1990). The successful development of immortal lymphoblastoid cell lines (Hammerberg *et al,* 1985, 1988; Kaeffer *et al,* 1990; Yang *et al,* 1992a) arises from the application in pigs of a general cell culture method to grow lymphocytes and from the search for porcine oncogenic retroviruses.

This review will present the rationale sustaining the development of these lymphoblastoid cell lines and the main applications derived thereof to the veterinary and medical fields.

LYMPHOBLASTOID CELL LINES: THEIR GROWTH CONDITIONS, SELECTION, AND THE ESTABLISHED COLLECTIONS

Construction of the growth medium

Historically, different media have been developed for specific cell lines in order to obtain optimal growth or in an attempt to grow cells in defined media without the addition of serum (Freshney, 1992). The Rosswell Park Memorial Institute-1640 medium (RPMI-1640) was originally described by Moore *et al* (1967) to cultivate normal human leukocytes and is used as a base line medium to propagate porcine lymphocytes. Alternatively, the Dulbecco's Modified Eagle Medium (DMEM) was chosen as base line medium by Grimm *et al* (1993) to establish T lymphocyte cell lines.

Additional metabolites are always supplied to RPMI-1640 to promote lymphocyte growth (Adams, 1990). First of all, L-glutamine is an essential-amino acid which, unlike most other amino acids, is rapidly inactivated *in vitro* (Griffiths, 1992). The concentration usually chosen is 2 mM, which allows an active growth for 3 to 5 d (Griffiths, 1992).

Pyruvate is always added to grow pig lymphocytes at a 1 mM concentration as an immediate precursor for biosynthetic processes requiring acetyl-coenzyme A (Maurer, 1992).

Cells are usually grown in an atmosphere containing 5% carbon dioxide and at 37°C to mimic mammalian internal body conditions. Nevertheless, some immortal B cell lines like L14 can be propagated without carbon dioxide at the porcine internal body temperature (39°C; Kaeffer *et al,* 1990).

Different serum sources have been used to grow pig lymphocytes, first porcine serum, assuming that homologous serum is better for cellular growth at the onset of culture (Hammerberg *et al,* 1985), or horse serum, because of its lower cost and excellent reputation for growth promotion (Hammerberg *et al,* 1988; Adams, 1990). Fetal calf serum is a more expensive source of serum but, in my opinion, is more widely available and of consistent quality (Kaeffer *et al,* 1990). In my laboratory, serum is heat-inactivated to reduce the risk of mycoplasma contamination and to inactivate the complement system that may interfere in virus or cytotoxicity assays, even though such practices may destroy some growth factors (Adams, 1990).

Antibiotics are usually added to prevent common microbial contaminations because, even though their surface effects on cells or their metabolization are not well known, they are considered negligible (Paul and Beswick, 1982).

2-β-Mercaptoethanol (50 μM) is incorporated into growth medium as a reducing agent and is known to enhance the transformation of lymphoid cells by Abelson murine leukemia virus (Rosenberg *et al,*

Table I. Immortal peripheral blood lymphoblastoid cell lines of B and T lineages.

Line	*Pig status*	*Main characteristics of cell lines*	*References*
P-SC(1)	Outbred pig 1	B cell secreting monomeric IgA and μ heavy chain	Hammerberg *et al,* 1985
P-16(2)	Oubred pig 2	B cell secreting monomeric IgA and μ heavy chain	Hammerberg *et al,* 1985
A4	Duroc pig, 4 months	B cell secreting low amount of immunoglobulins	Hammerberg *et al,* 1988
L14	Histocompatible miniature boar (haplotype d/d), 5 months post-weaning	Non-secreting B cell (expression of membrane IgM)	Kaeffer *et al,* 1990*
L23	"	B cell secreting IgM	Kaeffer *et al,* 1990
L52	"	B cell secreting IgM	Kaeffer *et al,* 1990
L35	"	T cell expressing CD2a antigen	Kaeffer *et al,* 1990
L45	"	T cell expressing CD2a antigen	Kaeffer *et al,* 1990
PBL-1	Outbred pig 1	B cell	Yang *et al* (1992a) cited by Kirk *et al* (1993)
PBL-2	Oubred pig 2	B cell	Yang *et al* (1992a) cited by Kirk *et al* (1993)

* The cell lines described in this publication are deposited to the European Collection of Animal Cell Culture, Division of Biology, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, SP4 OJG, UK.

1975) and considered as a potential mitogen for mouse B cells (Goodman and Weigle, 1977). Addition of 2-β-mercaptoethanol was described as a prerequisite to isolate immortal B cell lines from blood provided that the peripheral blood mononuclear cells are kept in culture over a long period of time (Hammerberg *et al,* 1985). Only one B cell line (A4, table I) has been rendered independent of 2-β-mercaptoethanol (Hammerberg *et al,* 1988). B and T cell lines described by Kaeffer *et al* (1990) require 2-β-mercaptoethanol for optimal growth. More recently, Grimm *et al* (1993) showed that the selection of gamma delta T cell lines did not have to include this reducing agent in the growth medium.

Addition of non-essential amino acids is sometimes made to avoid cellular starvation by lack of a proper amino acid supply (Maurer, 1992).

To summarize, RPMI-1640 medium supplemented with L-glutamine (2 mM), pyruvate (1 mM), 10% serum (horse or fetal calf) and some antibiotic cocktail is the minimum medium to establish lymphoblastoid cell

lines. Addition of 50 μM 2-β-mercaptoethanol is advisable and I believe that the addition of a cocktail of non-essential amino acids is superfluous. In our laboratory, an adaptation of L231 (subline of L23, table I) to serum-free medium has been easily carried out (Kaeffer and Bottreau, unpublished experiments) with Ultraculture, a product purchased from Biowhittaker (Fontenay-sous-Bois, France) supplemented with previously given concentrations of L-glutamine, pyruvate and 2-β-mercaptoethanol. Even though not all components of this medium are known, it is a first step toward growth in a completely defined medium, solving definitively the problem of serum quality, and paving the way to determine the growth medium requirements of lymphoblastoid cells and to the recovery of their secreted products.

Selection

Immortal lymphoblastoid cells can be derived from bulk culture of peripheral blood cells without exogenous mediators (Grimm *et al,* 1993) or with 2-β-mercaptoethanol (Hammerberg *et al,* 1985, 1988). Approximately 1 immortal B cell line can be derived per 2 x 10^7 blood cells in culture as reported by Hammerberg *et al* (1985, 1988). This spontaneous transformation constitutes a problem of background to verify viral oncogenesis by a biological assay. The *in vitro* selection scheme proposed by Kaeffer *et al* (1990) aims to isolate immortalized cells following retrovirus integration. Phytohemagglutinin has been temporarily supplied to maintain cells in active mitogenesis and promote the chronic infection of lymphocytes by replicating viruses. The addition of 5-azacytidine to the growth medium helps promote the expression of genes silenced by hypermethylation (Jones, 1985). This drug is a nucleotide analogue of cytosine base whose N in position 5 forbids the methylation by cellular methylases (Razin and Riggs, 1980). A significant advantage of 5-azacytidine over other nucleotide analogues is its ability to induce heritable hypomethylation (Razin and Riggs, 1980). The drug 5-azacytidine has been widely used in molecular genetics to induce differentiation of mouse fibroblasts (Hsiao *et al,* 1984) and allow expression of murine type-C proviruses in non-permissive cell lines. These proviruses had been maintained inactive following an extensive methylation of the viral gene (Harbers *et al,* 1981). We should keep in mind however that with 5-azacytidine any oncogene might be reactivated as well as any latent viral genome (Razin and Riggs, 1980).

Established collections of immortal lymphoblastoid cell lines of B and T lineages

In mammalian blood, lymphocytes represent 20–50% of the total cellular population. They display 2 strikingly different morphologies; the majority are small mature cells containing a large, spherical nucleus surrounded by a thin layer of non-granular cytoplasm and the minority are large immature cells containing a large nucleus and an abundant cytoplasm (Stevens and Lowe, 1992). The latter is the morphology displayed by the immortal cell lines derived from some porcine blood cultures summarized by the term 'lymphoblastoid' (Hammerberg *et al,* 1985). These cells are growing in suspension, frequently as small clumps of 10–50 cells (Hammerberg *et al,* 1985; Kaeffer *et al,* 1990). As shown in figure 1, neighboring cells in a clump are bound by cellular junctions suggesting that an active cellular cooperation may exist during selection for drug resistance of mutant lymphoblastoid cells.

To demonstrate the property of infinite lifespan, many authors maintain their cell

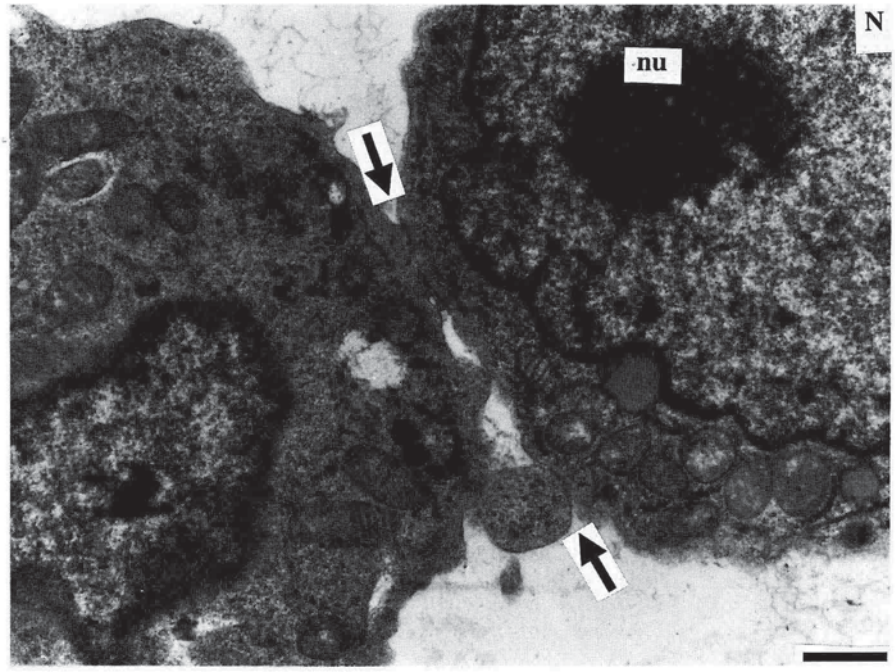

Fig 1. Electron micrograph of 2 typical immortal lymphoblastoid cells showing that adjacent cells in a clump are bound by cellular junctions (between arrows). The main characteristics of lymphoblastoid cell morphology can be seen such as large nucleus (N) containing a prominent nucleolus (nu). The cytoplasm contains numerous mitochondria. Bar represents 1 μm.

lines in active growth for at least 100 passages (1 passage represents a population doubling). The lymphoblastoid cell lines reported in table I have all been claimed to survive 100 passages over 20 months (Hammerberg *et al,* 1985) and over 12 months (Kaeffer *et al,* 1990). The description of these lines by Grimm *et al* (1993) does not mention long-term propagation in culture to demonstrate the property of infinite lifespan but it seems reasonable to postulate from their description that this property could be established. A relevant growth rate parameter to compare these lines would be their doubling times (Freshney, 1992). Unfortunately, a direct comparison between cell line doubling times is impossible, due to divergent definition between authors, but to give a close estimate, the L14 cell line doubling time is 23 h (Kaeffer *et al,* 1990).

Some karyotype analyses have been reported by Kaeffer *et al* (1990) concluding to a near diploidy of L14, L23, L35, L45 and L52 cloned cell lines and representative photographs of karyotypes were published for L142 and L231 sublines by Kaeffer *et al* (1991). Only the L52 line proved to be unique for its diploid chromosome formula and relevant to cytogenetic studies (Yerle *et al,* 1993).

Lymphoblastoid cells growing in culture can be distinguished by the presence of antibodies (B cells) or characteristic surface or cytoplasmic antigens (T cell types or B cell). A considerable amount of collabora-

tive work has been undertaken with monoclonal antibodies directed to surface or cytoplasmic cellular proteins used as markers to correlate porcine lymphocyte classification with the definition of the 'cluster of differentiation (CD)' available to classify rodents and human lymphocytes (Lunney, 1993).

The immortalized lymphoblastoid cells established by Hammerberg *et al* in 1985 and 1988 are of B origin (table I). The P-SC(1) or P-16(2) uncloned cell lines secrete a whole monomeric IgA molecule and a μ chain (Hammerberg *et al,* 1985). In addition, all P-SC lines (named after the morphology of P-SC(1)) secrete an interleukin-1-like factor (Hammerberg *et al,* 1988). Among the cell lines of B lineage (table I) described by Kaeffer *et al* (1990), the L23 and L52 sublines produce immunoglobulins of the μ isotype but it should be underlined that the epitope recognized by anti-μ chain K513D MAb is absent on these molecules. The L14 cell line harbors membrane-bound IgM molecules with low if any secretion of IgM molecules. Apart from the independence of the A4 cell line to the 2-β-mercaptoethanol requirement (Hammerberg *et al,* 1988), 2 major differences between the B cell lines were described by Hammerberg *et al* (1985) and Kaeffer *et al* (1990): 100% of the cells of P-SC(1) or P-16(2) harbor class II antigen, a percentage which is only of 10–20% for L14, L23 and L52; and the levels of immunoglobulin products in culture supernatant are apparently lower for P-SC lines (Hammerberg et al, 1985) comparatively to L23 and L52 (Kaeffer *et al,* 1990). In immunology, a subline derived from P-16 cell line proved to be invaluable for the construction of heteromyeloma (Yang *et al,* 1992b) and a subline selected from L23 line is convenient to obtain intraspecies hybridoma (Kaeffer *et al,* 1991).

Recently, new lymphoblastoid cell lines of B lineage have been succintly described by Kirk *et al* (1993) who cited Yang *et al* (1992a) as originators of these lines (table I). More extensive descriptions of the properties of these lines are being considered for publication (De Buysscher, University of North Carolina, personal communication). The PBL1 and PBL2 are B cells selected along with a pre-B cell line, named PNL, derived from lymph node. Each line is derived from a different outbred pig and expresses class I and II major histocompatibility complex antigens (Kirk *et al,* 1993).

The immortal L35 and L45 cell lines belong to the T lineage by expressing the CD2a molecules detected with MSA4 monoclonal antibody (Kaeffer *et al,* 1990; Lunney, 1993). As neither CD4 nor CD8 molecules have been detected on these cells, this marker of differentiation is associated with the beginning of T cell commitment. To my knowledge, no attempt has been made to detect the expression of gamma/delta receptors by these cells and no publication reports on the use of these cell lines. Cloned T cell lines selected by Grimm *et al* (1993) express a gamma/delta receptor.

Apart from these lymphoblastoid cell lines (table I), 3 immunoblast cell lines are described by Kadota *et al* (1988) isolated from neplastic tissues scattered in muscular masses of a 6-month-old crossbred hog. A line named 'Cell 2', which produced IgM molecules, has been maintained some time in culture (Kadota *et al,* 1988). The destiny of these apparently immortal cell lines remains obscure.

In conclusion, the rather vague terminology 'lymphoblastoid' is the outcome of a tacit choice made by 2 independent groups of scientists (Hammerberg *et al,* 1985; Kaeffer et al, 1990). We have to hope that further characterization of these B lines will be performed in order to connect their differentiation stages to the molecular steps of the B lymphocyte differentiation (Rolink and Melchers, 1991).

LYMPHOBLAST IMMORTALIZATION: THE ONCOGENIC VIRUS HYPOTHESIS

In 1988 Hammerberg *et al* reported that immortal lymphoblastoid cell lines could be derived from long-term culture of the peripheral blood mononuclear cells of 80% of the pig sampled provided the growth medium is supplemented with 2-β-mercaptoethanol. This percentage is similar to the percentage of humans whose peripheral blood cells gave rise to immortal lymphoblastoid cell lines of B lineage when passaged in cultures subsequent to a chronic infection by Epstein-Barr virus, a *Herpesviridae* (Hammerberg *et al,* 1985; Cho and Louie, 1992). In pigs the herpes family is represented only by *Suid herpesvirus* type 1 and 2 (Roizmann *et al,* 1992); the lack of an oncogenic herpes virus and the absence of reports about the occurrence of such viruses might explain why the hypothesis of involvement of a herpes virus in the lymphoblast immortalization is considered obsolete.

Considerable data are available to suggest the involvement of retroviruses in the lymphoblast immortalization, even though a definitive demonstration between the occurrence of retrovirus particles and the immortalization mechanism is still lacking. Retroviruses are RNA-containing viruses which can integrate the host genome by a reverse transcription of their genomic RNA into a DNA molecule through the enzymatic activity of a virus-coded enzyme: the reverse transcriptase (Pastoret and Portetelle, 1990; Lueders, 1991). Porcine genome and other mammalian genomes are known to contain many retrovirus-like sequences (Benveniste and Todaro, 1975; Lueders, 1991), some of which might be reactivated by exposure to chemicals to give rise to infectious viral particles (Moennig *et al,* 1974).

The involvement of retroviral gene was originally suggested by Head *et al* (1974) to explain the occurrence of hereditary lymphosarcoma in some Large-White animals (McTaggart *et al,* 1979) bearing a recessive autosomal gene (McTaggart *et al,* 1971, 1979). Histological data on lymphosarcoma in swine with genetic or viral etiology describe the disease as multicentric with very rare occurrence of hard-tissue tumors (Head *et al,* 1974; Kadota and Nakajima, 1988). From different epidemiological surveys, we know that retrovirus infection leading to malignant lymphomas in domestic pig is a rare event regarding the low frequency of lymphosarcoma attributed to retrovirus: 4–20 per million swine slaughtered in England, Italy, and Japan (Andersen and Jarrett, 1968; Marcato, 1987; Hayashi *et al,* 1988). Even during the experimental induction of cancer with 90 strontium-fed minipigs, the development of various kinds of myeloma, osteosarcoma or sarcoma has been only putatively attributed to retroviral gene expression without any success at isolating an oncogenic virus (Frazier, 1985).

Nevertheless, several cell lines have been established from organs of leukemic or apparently healthy pigs and found to produce retroviral particles described as either type-A (Kadota *et al,* 1988), type-C (Woods *et al,* 1973; Moennig *et al,* 1974; Bouillant *et al,* 1975, 1981; Lieber *et al,* 1975; Frazier *et al,* 1979; Kodama *et al,* 1981) or retrovirus-like (Hammerberg *et al,* 1988). It has been demonstrated that a porcine type-C retrovirus isolate can silently infect the kidney cell line (Moennig *et al,* 1974) or the swine-testis-Iowa pig cell line (Lieber *et al,* 1975). As initially described by Stewart *et al* (1972) for the murine retrovirus, porcine retrovirus gene expression can be enhanced when chronically infected cells are grown in the presence of 5-bromo-2'-deoxyuridine (Moennig *et al,* 1974; Bouillant *et al,* 1975). In 1981, Kodama *et al* established an epithelial-like cell line called 'Shimozuma' isolated from the spleen of a pig suffering from malignant lymphoma. Shimozuma cells spontaneously produce a number of type-C parti-

cles referred to as porcine retrovirus Tsukuba-1 (Suzuka *et al,* 1985). Unintegrated genome of Tsukuba-1 virus is partly cloned (Suzuka *et al,* 1986). Reverse transcriptase activity of the virion has been characterized (Suzuka *et al,* 1985) and monoclonal antibodies against reverse transcriptase of Tsukuba-1 virus have been raised (Suzuki *et al,* 1988). To my knowledge, this is the most recent progress made by Japanese scientists with Tsukuba virus.

Two years after Suzuki's article, Kaeffer *et al* (1990) reported the results of an injection of Shimozuma cells into histocompatible miniature pigs and an *in vitro* selection scheme to isolate previously described immortal cell lines. The authors referred to a mouse model (Rapp and Todaro, 1980), to suppose that the chronic and persistent infection state of Shimozuma cells by Tsukuba-1 retrovirus might be favorable to the accumulation of replication-competent virus variants which could be unravelled *in vivo*. The injection of the Shimozuma cells into recipient pigs triggered a non-neoplastic syndrome (Kaeffer *et al,* 1990). Since then, we have reproduced this non-neoplastic syndrome in inbred swine following an injection of Shimozuma cells or B1 cells (parental cell line of the lymphoblastoid cell lines (Kaeffer *et al,* 1990)), but *in vivo* experiments on such a vague syndrome have now stopped (Kaeffer and Bottreau, unpublished observations).

To demonstrate the direct involvement of retroviral particles in the lymphoblast immortalization, an optimization of the assay of porcine reverse transcriptase enzyme activity for detection and quantitation of virions was needed (Kaeffer *et al,* 1990) and a first attempt in that direction was made by Phan-Thanh *et al* (1990, 1992). The design of a biological assay to detect the oncogenicity of the retroviruses produced by the lymphoblastoid cell lines described by Kaeffer *et al* (1990) is in progress by using transformation of primary porcine lymphocytes (Kaeffer and Bottreau, in preparation). My working hypothesis is that the host range of these retroviruses includes B cells and some phenotype of T cells harboring CD2a antigen (Kaeffer *et al,* 1990). From coculture experiments between L231 fusion partners and splenocytes or by the infection of splenocytes with 0.2 or 0.45 μm filtered tumor cell culture supernatants (Kaeffer *et al,* 1991; Kaeffer and Bottreau, unpublished results), immortalization of porcine lymphocytes is rare suggesting that the putative oncogenic virus is produced at a very low level; difficulties encountered during concentration and purification attempts of the virus suggest that it is highly labile. In the future, more emphasis should be given to research using the polymerase chain reaction to improve detection of these viruses taking advantage of the already available techniques in veterinary diagnostic medicine (Belák and Ballagi-Pordány, 1993) or using a method that may permit the identification of uncharacterized viral nucleic acid sequences (Mack and Sninsky, 1988).

MAIN APPLICATIONS TO VETERINARY AND MEDICAL RESEARCH

Hybridoma technology

Somatic cell fusion with a tumor cell line to perpetuate individual lymphocytes in tissue culture has been developed by Köhler and Milstein (1975) for mouse B cells allowing the establishment of continuous cell lines producing specific monoclonal antibodies (hybridomas). Hybridoma technology has been rapidly improved in Balb/c mouse to generate fusion partners which do not secrete immunoglobulins and retain a high proliferation rate (Shulman *et al,* 1978). The technology has found a wide application in the veterinary field using a fusion partner constructed with murine myeloma giving

rise to heterohybridoma (reviewed by Groves and Tucker, 1989) or with lymphoblastoid cell lines selected for deficiency in hypoxanthine phosphoribosyl transferase (HPRT) activity to obtain pig–pig hybridoma. I shall successively introduce the achievements of interspecies or intraspecies hybridoma technology.

Construction of interspecies hybridoma producing specific monoclonal antibody is well documented in the pig. Murine fusion partners Sp-2/0 Ag14 (Raybould *et al,* 1985) or P3-NSI/1-Ag4-1 (Buchegger *et al,* 1987) have been directly fused with splenic B lymphocytes to generate some hybridomas. Creation of heteromyeloma was later developed to improve the fusion efficiency. Such fusion partners are constructed from P3X63-Ag8.653 murine myeloma or 7E2 murine x bovine heteromyeloma by fusion with porcine lymphocytes (Greenlee *et al,* 1990) and subsequent selection to derive HM-1 and HM-2 fusion partners, respectively. Three additional fusion partners were constructed by fusing successfully HM-2 cells and porcine lymphocytes (HM-3) and 2 others obtained by fusing HM-3 cells with porcine lymphocytes. Another approach was chosen to construct a PM-1 fusion partner by fusing the porcine lymphoblastoid cells derived from P-16 cell line (table I) with murine myeloma P3X63-Ag8.653 (Yang *et al,* 1992b). To my knowledge none of these fusion partners is in general use or widely available.

Following the discovery of Köhler and Milstein (1975), the generation of intraspecies hybridomas was demonstrated in the rat (Galfrè *et al,* 1979), human (Croce *et al,* 1980; Olsson and Kaplan, 1980), bovine (Olobo and Black, 1990) and chicken (Nishinaka *et al,* 1991) depending on the tumor cell lines available. In the pig, the first fusion partners were described by Kaeffer *et al* (1991) taking advantage of lymphoblastoid cell lines derived from inbred miniature pig (Sachs *et al,* 1976). Since 1976, strains of miniature pigs that are homozygous for a different allele of the major histocompatibility locus have been developed from 2 independently established herds of minipigs breeding either the Vita Vet Lab Mini-pig which had its origin in Florida Swamp pigs in 1948 (Epstein and Bichard, 1986) or the Minnesota Miniature pig which was developed at the Hormel Institute from 1949 using several feral strains and imported animals from Guam (Epstein and Bichard, 1986). The lymphoblastoid cell lines are from a d/d haplotype animal and derivation of B sublines deficient in HPRT activity can be easily realized (Kaeffer *et al,* 1991).

In pigs, as well as in other mammals, the HPRT locus has been located on the X chromosome (Leong *et al,* 1983; Melton *et al,* 1984). According to a comparison between 6-thioguanine and 8-azaguanine drugs to select for mutant lymphoblasts with an HPRT deficiency, which was in favor of the former (Evans and Vijayalaxmi, 1981), Kaeffer *et al* (1991) report the selection of 2 potent fusion partners (sublines L142 and L231) which can double in a relatively short period of time in a medium supplemented with 5 μg 6-thioguanine/ml and which remain near diploid. As reported for human lymphoblast mutants, these authors did not demonstrate any gross chromosomal abnormality nor any loss of the X chromosome except for the occurrence of a double minute chromosome associated with L142 genetic material (Kaeffer *et al,* 1991). Double minute chromosomes are known to be either associated with DNA amplification correlating with multi-drug resistance (Roninson *et al,* 1984) or *c-myc* oncogene amplification (Schimke, 1988). The double minute chromosomes present in L142 cells correlate probably with 6-thioguanine resistance of the fusion partner as they were progressively lost from some hybrid genomes in the absence of the drug selective pressure (Kaeffer *et al,* 1991).

Fusions have been performed with leukocytes from the spleen, the gut *lamina propria* or the mesenteric lymph nodes with the aim

of perpetuating different B lymphocyte subsets secreting different immunoglobulins (either IgM, IgA or both with and without any activity in VELCIA; Kaeffer *et al,* 1991). The quasi-diploidy of the tumor cells and 21 hybridomas is maintained over numerous generations suggesting that the cells retain the accuracy of chromosome disjunction at mitosis and that, following fusion, the hybrids lost chromosomes at random to restore a stable diploidy retaining the cellular or viral genes involved in oncogenesis and an active HPRT gene. These results are similar to the situation found in human for cell lines transformed by Epstein-Barr virus (Chiorazzi *et al,* 1982; Cho and Louie, 1992).

Consequently, a direct use of these fusion partners to generate routinely stable hybridoma is as a chimera. If the generation of hybrid cells is easy and is not dependent on strict physico-chemical handling conditions, the secretion of M immunoglobulin by sublines L142 and L231 as well as the instability of the hybrids which retained only a near-diploid chromosome formula were published as disadvantages of our fusion partners (Kaeffer *et al,* 1991), which make them unsuitable for widespread use. Over 2 years, new fusions performed in my laboratory with both fusion partners demonstrated that only L231 was of value to generate hybrids and this cell line has been introduced to the European Collection of Animal Cell Culture (1993). New mutants have been derived from L231, which do not secrete detectable level of immunoglobulins, but with a weak growth potential precluding a widespread use for fusion (Kaeffer and Bottreau, unpublished results). As the capacity of the fusion partners to shed retrovirus particles precludes their use for prophylactic or therapeutic purposes (Kaeffer *et al,* 1991; Osther *et al,* 1991), most of my research effort has been made on the oncogenic virus issues. The problem of the stability of hybrids in culture may also find a natural solution through the use of Ultra-culture medium to grow both fusion partner and hybridoma; it has been reported with murine hybridoma that growth in this medium stabilized their properties, especially the ability to retain additional chromosome (Kessler *et al,* 1993).

An encouraging outcome of intraspecies hybridoma research concerns the value of S2.2 hybridoma (ECACC N° 9200117) as a source of porcine type A immunoglobulins which has been confirmed by recent works in molecular biology (Butler, University of Iowa, personal communication).

Research on porcine cellular immune response

A natural use of immortal lymphoblastoid cell lines is to explore the porcine cellular immune response against pathogenic microorganisms. A first attempt in that direction is to study the T cell responses of pig against *Pestivirus* (hog cholera virus); L14 cells have been used as target-cell line following an infection by a mutant (Ms205) of pseudo-rabies virus expressing the surface protein of hog cholera virus (Kimman *et al,* 1993). Among other immunological abnormalities, the hog cholera virus causes a depletion of B lymphocytes in the circulatory system and lymphoid tissue (Kimman *et al,* 1993). The interest of L14 in such studies is bound to their B lineage and their infinite lifespan. The infected L14 cells bearing the envelope glycoprotein of hog cholera virus were not lysed by lymphocytes of pigs immunized by hog cholera virus (Kimman *et al,* 1993) leading to the conclusion that E1 envelope glycoprotein of this virus is not an important antigen recognized by polyclonal proliferating T cells. These workers generated indirect information about the permissivity of L14 cell line to infection by pseudo-rabies virus and its non-permissivity for an infection by hog cholera virus (Kimman *et al,* 1993).

Xenogenic research

Xenotransplantation is the use of animal organs as a substitute for human organ donation. The animal species can be concordant (namely non-human primates) or discordant (mainly pig) species (Kirk *et al,* 1993). Pig lymphoblastoid cell lines PLN, PBL-1 and PBL-2 along with primary culture of pig lymphocytes have been actively used to explore the extent of cytotoxic and natural killer activity of human peripheral blood cells against porcine antigens (Kirk *et al,* 1993). This work leads to the conclusion that human T cells are capable of recogizing and proliferating to porcine cells in a similar manner to their response to allogeneic cells (Kirk *et al,* 1993). Lymphoblastoid cell lines as well as heterohybridomas generated with PM-1 fusion partner have been proposed for general use in xenogenic research (Yang *et al,* 1992b).

Cytogenetics and genetic engineering

Historically, complex chromosome abnormalities and sequential evolution of karyotype in a porcine cell line producing a retrovirus were described by Strandström *et al* (1974). Extensive karyotypic analyses have been performed by Kaeffer *et al* (1990) on lymphoblastoid cells, partly to exclude the possibility that lymphoblastoid cell lines isolated from the peripheral blood cell cultures were not some cloned offspring of the heteroploid Shimozuma cells.

In 1990, the L14, L45 and L52 cell lines were given to the laboratory of Vaiman and Frelat at CEA, Fontenay-aux-Roses (France) for extensive cytogenetic analyses by flow cytometry. The flow cytogenetic technique allows the detection of numeral and structural aberrations (Schmitz *et al,* 1992a). A major advantage of lymphoblastoid cell lines is their growth in suspension, which is a rare property described once with the IB-RS-2 kidney cell line (Chapman and Ramshaw, 1972). The most surprising outcome of the flow karyotype analysis was that L52 cells displayed a normal male flow karyotype except for a slight difference for the peak containing chromosome 10 (Schmitz *et al,* 1992a, b). Consequently, the L52 line seems to retain the accuracy of chromosome disjunction at mitosis, a quite uncommon property for immortal cell lines (Alberts *et al,* 1989). A slight variation in the position of the peak containing chromosome 10, comparatively to pig standard flow karyotype, might reveal either some variability between breed or some alterations into the chromosome 10 due to the transformation. As such a variation has been found to be constant between the 3 lymphoblastoid cell lines supplied, it seems that a major alteration of the lymphoblast genome has been detected by this method (Schmitz *et al,* 1992a, b). Anyway, these results are not in favor of major structural rearrangements due to L52 transformation (Schmitz *et al,* 1992b). The L52 line has been proposed to purify by flow cytometry intact porcine chromosomes providing in addition a suitable source of porcine male chromosome (Schmitz *et al,* 1992a, b; Yerle *et al,* 1993). A very high-resolution flow karyotype of L52 is available, in which all peaks but one are superimposed on those of the standard porcine karyotype (Schmitz *et al,* 1992b; Yerle *et al,* 1993). A remarkable proposal made by Schmitz *et al* (1992b) is the use of L52 line to map the porcine genome, which would inevitably help clarify its immortalization process. The use of L52 chromosomes to construct a porcine standard flow karyotype by Yerle *et al* (1993) seems to be a first step in this direction.

In genetic engineering and independently of the previous works, some L52 cells have been transfected with a retroviral construction containing the bacterial gene (LacZ) fused to a nuclear location signal (nlslacZ) in order to generate cells bearing a reporter

gene that is easily detectable by its enzymatic activity. A potential use of the transfected L52 cells has been proposed as *in vivo* tracer cells for a gene therapy approach applicable to human disorders (Charreau *et al,* 1991). Some problems arose during the propagation of transfected-L52 cells; expression of the reporter gene slowly declined along with the cell generation (Grépinet, INRA-Nouzilly, personal communication). I think that this problem is the result of a deregulation of the cellular methylases following the incorporation of 5-azacytidine into the growth medium of L52. The activity of L52 methylases has not yet been assayed, but I believe that a deregulation of the cellular methylases might explain partly the difficulty in isolating any oncogenic virus.

CONCLUSION

Lymphoblastoid cell lines constitute a significant progress in pig research helping develop new approaches in the hybridoma technology, cytogenetic, immunology and xenotransplantation. The limitations encountered with the use of these cell lines are due to our ignorance of the mechanism involved in the immortalization process, which may be misleading for some biotechnological applications. As an example, the occurrence of retroviral particles putatively harboring oncogenic properties precludes the use of some of these cells to produce biological products for human use (Kaeffer *et al,* 1991; Osther *et al,* 1991). Establishment of new immortal cell lines is a time-consuming task-for research which should be reduced through a more extensive use of the services of the international cell culture collections. At a time when pig organs are offered for human graft (Niekrasz *et al,* 1992), it seems wise to remember that between 1970 and 1993, only few contributions to the American Type Culture Collection (1992) or to the European Collection of Animal Cell Cultures (1993) were made to improve the available pig cell lines in quantity (14 cell lines available), diversity (kidney, testis, peripheral blood and small intestine) and quality (House *et al,* 1988). From a general point of view, the importance of pig in biomedicine is due to its similarities to the human at physiological, anatomical and immunological levels. Minipigs have been proposed as animal models for studies of transplantation biology, because the major histocompatibility complex of this animal exhibits extensive similarities to its human counterpart in both structure and function (Gustafsson *et al,* 1990). Through research in cell biology, minipigs are now proposed as a model to improve *in vitro* methods to reconstruct and to propagate small intestinal epithelium (Kaeffer *et al,* 1993). The establishment and distribution through international collections of cell lines derived from different porcine tissues or breeds will benefit both xenograft research and other fields of porcine research dealing with its value as a world food resource.

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