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Deregulation of calcium fluxes in HTLV-I infected CD4-positive T-cells plays a major role in malignant transformation

Haidar Akl[1], Bassam Badran[1], Nabil El Zein[2], Gratiela Dobirta[1], Arsène Burny[1], Philippe Martiat[1]

[1]Experimental Hematology, Universite Libre de Bruxelles, Institut Jules Bordet 127 Boulevard de Waterloo, 1000 Bruxelles, Belgium, [2]Department of Oncology, Laboratory of Pediatric Oncology, Hôpital des Enfants, Brussels, Belgium

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1. ABSTRACT

The CD4+ T-cell malignancy induced by human T-cell leukemia virus type 1 (HTLV-I) infection and termed; Adult T-cell Leukemia lymphoma (ATLL), is caused by defects in the mechanisms underlying cell proliferation and cell death. In the CD4+ T-cells, calcium ions are central for both phenomena. ATLL is associated with a marked hypercalcemia in many patients. The consequence of a defect in the Ca^{2+} signaling pathway for lymphocyte activation is characterized by an impaired NFAT activation and transcription of cytokines, chemokines and many other NFAT target genes whose transcription is essential for productive immune defense. Fresh ATLL cells lack the TCR/CD3 and CD7 molecules on their surface. Whereas CD7 is a calcium transporter, reduction in calcium influx in response to T-cell activation was reported as a functional consequence of TCR/CD3 expression deficiency. Understanding these changes and identifying the molecular players involved might provide further insights on how to improve ATLL treatment.

2. INTRODUCTION

The Ca^{2+} ion is a second messenger responsible for regulating a diverse array of physiological processes (1). These processes range from muscle contraction to synaptic transmission, and from cellular proliferation to apoptosis (2). Ca^{2+} signals are generated by elevations in cytoplasmic Ca^{2+} that in turn activate downstream effectors including Ca^{2+}-sensitive phosphatases, kinases, and proteases. In T-cells, the signaling cascade initiated by antigen binding to the TCR activates phospholipase Cγ, generating IP3 which induces Ca^{2+} release from the endoplasmic reticulum (reviewed in (3-5)). The resulting elevation of cytoplasmic Ca^{2+} is shaped by complex processes involving not only release of Ca^{2+} from the ER, but also extracellular Ca^{2+} uptake through channels in the plasma membrane and uptake of Ca^{2+} by mitochondria. The result is a remarkable variety of cytoplasmic Ca^{2+} response patterns, including transient elevation of Ca^{2+}, repetitive Ca^{2+} spikes (oscillations) or sustained Ca^{2+} elevation (6). Different patterns of Ca^{2+} elevation enable this single

second messenger to encode a wide range of cellular processes. Large transient Ca^{2+} elevations preferentially activate NFkB and JNK, whereas NFAT is preferentially activated by sustained Ca^{2+} oscillations. In lymphocytes, information encoded by the frequency amplitude and shape of Ca^{2+} oscillations differentially regulates transcription factors (7, 8). Since calcium-signaling regulates specific and fundamental cellular processes, it represents the ideal target of viral proteins, in order for the virus to control cellular functions and favor its persistence, multiplication and spread. A detailed analysis of reports focused on the impact of viral proteins on calcium-signaling has shown that virus-related elevations of cytosolic calcium levels allow increased viral protein expression (HIV-1, HSV-1/2), viral replication (HBx, enterovirus 2B, HTLV-1 $p12^{I}$, HHV-8, EBV), viral maturation (rotavirus), viral release (enterovirus 2B) and cell immortalization (EBV). Interestingly, virus-induced decreased cytosolic calcium levels have been found to be associated with inhibition of immune cells functions (HIV-1 Tat, HHV-8 K15, EBV LMP2A). In human T-cell leukemia virus type 1 (HTLV-I) infections, viral proteins such as $p12^{I}$ and $p13^{II}$ are able to modulate intracellular calcium-signaling to control cell viability. Adult T-cell leukemia/lymphoma (ATLL) is a malignancy slowly emerging from (HTLV-I)-infected mature $CD4^{+}$ T-cells. Fresh ATLL cells lack TCR/CD3 and CD7 molecules on their surface. In this review we made an effort to understand how HTLV-I induces Ca^{2+} influx alterations and changes in immunophenotype, how this could be related or not to the leukemogenic process

3. THE HTLV-I ACCESSORY PROTEIN P12 (I) AND CALCIUM

Encoded by both singly spliced mRNA pX (ORF) 1 and doubly spliced mRNA pX-rex- (ORF) 1, p12 (I) plays a critical role in the establishment of HTLV-I infection and optimal infectivity *in vivo* (9) and of quiescent primary lymphocytes (10) HTLV-1 p12 (I) is a small hydrophobic protein, which contains four proline-rich SH3 domain binding motifs associated to regulation of signal transduction. The protein associates with the 16 kDa subunit of the vacuolar H^{+}-ATPase, binds to IL-2 receptor β and γ chains and has been shown to enhance papillomavirus E5 transforming ability (reviewed by (11)). P12 (I) which is localized to the endoplasmic reticulum and to the golgi, interacts with calreticulin and calnexin. It increases calcium release following protein kinase C activation by phorbol myristate acetate (PMA) leading to NFAT translocation into the nucleus (12). The nuclear translocation of NFAT boosts the T-cell proliferation and survival.

p12 (I) has been recently demonstrated to promote cell-to-cell viral spread by inducing LFA1 clustering on T-cells via a calcium-dependent mechanism (13). Several Ca^{2+} regulated proteins are affected by p12 (I)-dependent increase of $(Ca^{2+})_{cyt}$, including the transcriptional co-activator, p300 (14, 15). Moreover, the HTLV-I regulatory protein P12 (I) activates NFAT nuclear translocation and NFAT directed transcription (16, 17). Overexpression of calreticulin was shown to block p12 (I)-dependent activation by preventing calcium-release from the ER and calcium entry through the plasma membrane (18). Interestingly, a new study showed that p12 (I) decreases NFAT (nuclear factor of activated T-cells) activation upon engagement of TCR/CD3 with anti-CD3 antibody by inhibiting LAT phosphorylation and, consequently, the phosphorylation of phospholipase C-gamma 1 and Vav (19). There is no contrast between the recent study and the previously reported positive effect of P12 (I) on NFAT activation following PMA stimulation, since, PMA stimulation bypasses TCR ligation. Thus, p12 (I) has contrasting effects on TCR signaling: it down-regulates TCR signaling in a LAT-dependent manner on the one hand, and on the other, it increases calcium release in a LAT-independent manner (20). Overall, p12 (I) is an essential HTLV-I factor for the establishment of a persistent viral infection. Moreover, p12 (I) downmodulates the expression of class I major histocompatibility complex (MHC) molecules (21). MHC suppression might help infected cells to escape the immune surveillance.

In addition to p12 (I), the HTLV-I accessory protein p13 (II) appeared to be implicated in Ca^{2+}-signaling regulation. It was suggested that it acts through the formation of a channel, giving rise to a rapid flux of Ca^{2+} across the inner mitochondrial membrane (22).

4. TCR/CD3 SIGNAL TRANSDUCTION AFTER HTLV-I INFECTION

The specific recognition of antigen/MHC complexes by the TCR/CD3 complex leads to the phosphorylation - of an immunoreceptor tyrosine based activation motif (ITAM), present in the cytoplasmic domains of the CD3 subunits (23-28). At least four different signal transduction pathways are activated after the tyrosine phosphorylation of the ITAMs, including: the PLCγ1 pathway, the ras-mitogen activated protein kinase (MAPK) pathway, the phosphoinositol 3 kinase (PI3K) pathway, and the inducible T cell tyrosine kinase (Itk) pathway.

Activation of the PLCγ1 pathway leads to the generation of inositol 1,4,5 triphosphates (IP3) and diacylglycerol (DAG), which induce a calcium influx and activate PKC, respectively. This pathway converges on the activation of Ca^{2+}-dependent calcineurin phosphatase activity, which in turn, when complexed with calmodulin and cyclophilin, acts on the cytoplasmic component of the NFAT (Nuclear factor of activated T-cells) family of transcription factors (29-31).

$CD4^{+}$ T-cells from patients with ATLL are routinely characterized as having a $CD3^{-}$ or $CD3^{low}$ phenotype (32-34). Experimental infection of $CD4^{+}$ T-cells with HTLV-I and HTLV-II (35, 36) has also been associated with defects in TCR/CD3 expression and function. HTLV-I was found to ultimately downregulate *CD3*γ, *CD3*δ, *CD3*ε, and *CD3*ζ gene transcripts leading to a $TCR/CD3^{-}$ surface phenotype after 200 days of *in vitro* infection. Recently, we have investigated the sequence of gene transcription; a decrease of *CD3*γ followed by the

subsequent progressive reduction in *CD3*δ, then *CD3*ε and *CD3*ζ mRNA has been observed (37). Alternatively, a reduction in calcium influx in response to T-cell activation was reported as a functional consequence of TCR/CD3 expression deficiency (38).

5. NFAT AND HTLV-I INDUCED CD4+ CELL TRANSFORMATION

The Nuclear factor of activated T-cells (NFAT) represents a family of transcription factors involved in the transcriptional regulation of cell surface receptors (TCR/CD3), cytokines (IL-2), as well as other transcription factors. NFAT proteins are translocated from the cytoplasm to the nucleus in response to Ca^{2+} mobilization and are returned to the cytoplasm when the Ca^{2+} signal is terminated (30, 39). NFAT proteins consist of at least five structurally related proteins, whose activity is regulated by a calcium-dependent phosphatase, calcineurin (40). After activation by calmodulin, calcineurin dephosphorylates the NFAT family members, allowing their translocation to the nucleus to activate genes controlled by NFAT (41-45). It has been shown that calcineurin activation is critical for the maintenance of the leukemic phenotype *in vivo* (46). Previous studies have shown that Tax with TPA or ionomycin (47) or P12 (I) (48) activates the Il-2 expression through NFAT in Jurkat T-cell line. However, it should be noted that relatively few HTLV-I-infected T-cell lines express significant levels of IL-2 (49).

6. HTLV-I SILENCING AND CALCIUM

ATLL cells are characterized by a silenced HTLV-I provirus. Of note, The 5' LTR is frequently deleted or methylated, whereas the 3' LTR invariably remains intact in all cases of ATLL (50). However, It has been demonstrated that epigenetic mechanisms are responsible for HTLV-I-genes transcriptional silencing (51). DNA hypermethylation and histone modifications of the 5'-LTR appear to be important mechanisms by which HTLV-1 gene expression is repressed during viral latency (51, 52).

Eukaryotic DNA is packaged within the nucleus through its association with histone proteins, forming the fundamental repeating unit of chromatin, the nucleosome. Chromatin modifiers mobilize or eject nucleosomes and histone modifying complexes that add covalent modifications to histones (53). These post-translational modifications include, but are not limited to, acetylation, phosphorylation, methylation, ubiquitinylation and sumoylation (54). Some modifications, including acetylation and phosphorylation, are reversible and dynamic, and are often associated with inducible regulation of individual genes. In contrast, histone methylation appears to be more stable and seems to be involved in the cellular memory of the transcriptional status by fixing the chromatin organization in a heritable manner. It has been demonstrated that histone tails play a role in the folding process of the chromatin (55). Histone hyperacetylation is most commonly associated with active transcription (56). Acetylation of histones may augment transcription by neutralizing the positive charge on lysines, thus reducing histone association with negatively charged DNA. Addition of the acetyl groups to histones also decreases compaction between histones within nucleosomal arrays. Furthermore, acetylated lysines may provide a platform for the recruitment of other transcription factors that aid in gene expression (57).

The development of ChIP analysis allowed revealing the *in vivo* presence of Tax, a variety of ATF/CREB and AP-1 family members, and both p300 and CREB-binding protein (CBP) at the HTLV-I promoter (58). Moreover, Histones H3 and H4 were acetylated at specific sites at the level of the proviral genome and especially at the active viral promoter. Histone acetylation has been shown to play a pivotal role activating HTLV-I transcription *in vitro*. In addition, histone deacetylases (HDAC) are present at the promoter and inhibition of their activity using trichostatin A (TSA) was correlated with increased viral mRNA expression (59). Furthermore, a physical interaction between Tax and HDAC1 has been reported (60). Another HDAC inhibitor FR901228 induced apoptosis of Tax expressing and non-expressing HTLV-I infected T-cell lines and selective apoptosis of primary ATLL cells, especially those of patients with acute ATLL (61). However, the NF-κB inhibitor IkappaBalpha, constitutively phosphorylated in various HTLV-1-infected T-cell lines and ATLL-derived cell lines positively regulates cellular homeodomain-containing transcription factors through cytoplasmic sequestration of HDAC1 and HDAC3 (62). Indeed, histone H1 represses p300 acetyltransferase activity at the promoter of HTLV-I. Interestingly, Tax prevents that histone H1 negative effect without eliminating p300 from HTLV-I promoter (63). The coactivator-associated arginine methyltransferase 1 (CARM1), which methylates histone H3 and other proteins such as p300/CBP enhanced transcriptional activity of the HTLV-I LTR through direct interaction with Tax (64). A recent study has presented arginine methylation at histone H3R2 as a control mechanism of H3K4 trimethylation thus providing an insight into its function on chromatin (65).

Several studies have elucidated a correlation between Ca^{2+} signaling and chromatin remodeling. The results of Tarkka *et al*, suggested that nucleotide and Ca^{2+} binding may be important for H1-mediated chromatin changes (66). Protein kinase C (PKC)/ras and Ca^{2+}-mediated membrane signaling has been shown to regulate hSWI/SNF chromatin remodeling during T-lymphocyte activation (67). The chromatin remodeling enzymes of class II histone deacetylases (HDAC4) regulate SRF activity in a Ca^{2+}-sensitive manner. Under basal conditions the transcription activity of SRF is negatively controlled by HDAC4 and this is reversed upon activation of Ca^{2+}/CaMK signaling (68).

7. HTLV-I INFECTED CELLS: CD7, CALCIUM PATHWAYS AND APOPTOSIS

The CD7 antigen is a 40-kDa cell surface glycoprotein, belonging to the immunoglobulin (Ig) gene superfamily, and is one of the earliest antigens to appear on cells of the T-lymphocyte lineage. It is found on

thymocytes and mature T-cells and is the most reliable clinical marker of T-cell acute lymphocytic leukemia. The *CD7* gene is localized on chromosome 17 (69). The gene comprises 4 exons that span a 3.5 kb band and a promoter with no TATA boxes (70). Whereas, the function of CD7 is not yet fully understood, T-cells of patients with severe combined immunodeficiency do not express *CD7* (71) and show defective T-cell proliferative responses to mitogens; however these T-cells were found to provide help for the differentiation of normal B cells into Ig-secreting cells. Abundant circulating B cells were detected. These findings supported the idea that the CD7 deficiency was related to a defect in T-cell precursors, and that CD7 plays an essential role not only in T-cell interactions but also in certain aspects of T-cell/B-cell interaction during early lymphoid development. It was reported that CD7 delivers a proapoptotic signal during galectin-1-induced T-cell death (72). Furthermore, $CD4^+ \cdot CD7^-$ leukemic T-cells from patients with Sezary syndrome are protected from galectin-1-triggered T-cell death (73).

7.1. CD7 and ATLL

It has been shown that fresh ATLL cells are lacking CD7 at their surface (74). Later on, immunohistochemical studies in ATLL showed that the lack of CD7 expression was a typical characteristic of the malignant lymphocytes (75). It has also been shown that survival of acute myeloid leukemia cells requires phosphatidylinositol 3-kinase (PI3K) activation (76). Moreover, activation of PI3K/Akt signalling is involved in fibroblast Rat-1 transformation by HTLV-I (77). Interestingly, antibody ligation of CD7 leads to association with phospho-inositol 3-kinase and phosphatidylinositol 3,4,5-triphosphate formation in T lymphocytes (78). Furthermore, functional association of CD7 with PI3K has been reported (79). Our observations indicate that CD7 disappearance parallels PI3K activation and phosphorylation of Akt and Bad (80).

7.2. CD7 and calcium influx

It has been reported that mitogenic activation of T-cells increased CD7 cell surface expression (81). Ware *et al* showed later that induction of a transmembrane calcium flux generates signals that lead to CD7 gene transcription (82). Moreover, treatment with either a Ca^{2+} ionophore (A23187) or a cAMP analogue, dibutyryl cAMP (Bt,cAMP), stimulated CD7 expression on the surface of T lymphocytes, by increasing the steady-state-specific mRNA levels (83). Furthermore, ligation of CD7 with anti-CD7 mAb induces transmembrane Ca^{2+} flux in T and natural killer (NK) cells. The antithymocyte globulin (ATG)-Fresenius (ATG-F) enhanced acetylcholine (ACh) release, likely through transient increases in intracellular Ca^{2+} ((Ca^{2+}) (i)) mediated by CD7, which led to declines in intracellular ACh content (84).

7.3. Ca^{2+}, apoptosis and cancer

Altered activity or expression of specific Ca^{2+} channels and pumps might be an adaptive response or might offer a survival advantage, such as resistance to apoptosis. A reduction in the Ca^{2+} content of the ER would be expected to reduce sensitivity to apoptosis (85). Moreover, the anti-apoptotic protein Bcl2, which is commonly deregulated in cancer (86) appears to modulate IP3-receptor Ca^{2+} channel activity on the ER Ca^{2+} stores (87-89) and reduces luminal ER Ca^{2+} levels through Ca^{2+} leakage when overexpressed in MCF-7 breast cancer cells (90). The reduction in ER Ca^{2+} means that Ca^{2+} release is insufficient to produce apoptosis through excessive mitochondrial Ca^{2+} accumulation (91, 92). Such changes in apoptosis sensitivity and proliferation are likely to bestow tumor-promoting properties by giving the cell a growth advantage. Recent studies also show that BCL2 inhibits pro-apoptotic Ca^{2+} signals, without inhibiting Ca^{2+} oscillation signals associated with cell survival (93). So it is possible to modify one set of Ca^{2+} responses (for example, apoptosis) without altering others. This highlights the potential for certain Ca^{2+} channels or pumps to be targeted in some cancers to induce apoptosis, concomitantly normal cells and normal physiological responses will not be severely compromised.

7.4. The PI3K/Akt/Bad pathway alteration after HTLV-I infection and calcium

We have recently shown an altered PI3K/Akt/Bad pathway after HTLV-I infection (80). Akt is a serine (Ser)/threonine (Thr) protein kinase which plays an important role in controlling cell growth and apoptosis (94). Through its PH domain, Akt binds to PIP3, activating Akt by phosphorylation. Activated Akt targets the apoptotic pathway, the cell cycle, and transcriptional translational machinery. The proapoptotic protein Bad is one of the main targets of the phosphorylated Akt. Given the critical role of the PI3K/Akt pathway in homeostasis and cell growth, it is not surprising that constitutive activation of the pathway contributes to the pathogenesis of many types of cancer (95). Similarly, the disruption of PI3K/Akt signalling has been effective against highly invasive breast cancer cells (96). However, the pro-apoptotic function of Bad is regulated by the Akt-mediated phosphorylation at Ser112 and Ser136 preventing its binding to its antiapoptotic partner Bcl-xL. Akt phosphorylates Bad both *in vitro* and *in vivo* and blocks the Bad induced death of primary neurons in a site-specific manner (97). Global gene expression studies in Mantle Cell Lymphoma (MCL) have shown an overexpression of elements of the PI3K/Akt pathway. Furthermore, a constitutive activation of Akt and Bad contributes to the pathogenesis of MCL (98).

Upstream of Akt there is the phosphatidylinositol 3-kinase (PI3K), which activates Akt in part via activation of 3′-phosphoinositide-dependent kinase-1 (99). The PI3K is composed of two subunits: A regulator one (p85) and a catalytic one p110 (100). Phosphoinositide 3-kinase (PI3K) and its downstream target Akt are activated in response to cytokine receptors (101). The resulting activation of PI3K/Akt pathway prevents many cells from undergoing apoptosis (102-104). Other studies have shown that survival of acute myeloid leukemia cells requires PI3K activation (105). Activation of PI3K/Akt signaling is involved in fibroblast Rat-1 transformation by HTLV-I (106). Taken all together, PI3K/Akt signaling pathway may be implicated in the cell transformation induced by HTLV-I.

The Ca^{2+} influx alteration in HTLV-I-infected cells would thus reduce, credibly, the activity of the PLC. Indeed, PLC and PI3K compete for the same substrate, the phosphatidylinositol- (4,5) diphosphate (PIP2). The PLC transforms it into DAG and inositol (1,4,5) triphosphate (IP3). The PI3K transforms the PIP2 into phosphatidylinositol (3,4,5) triphosphate (PIP3). Given the Ca^{2+} influx perturbation observed in the HTLV-I-infected cells, we could imagine a deregulation of the balance between PLC and PI3K. On the other hand, it is well established that Ras activates PI3K. A mutation in Ras oncoprotein could be a second reason for the altered PI3K pathway after HTLV-I infection.

7.5. Galectins, CD7 and apoptosis

Galectins are a family of mammalian beta-galactoside- binding proteins that positively and negatively regulate T-cell death. Galectin-1 induces TCR apoptosis, but inhibits IL-2 production and cell proliferation (107). CD4CD7 leukemic T-cells from patients with Sezary syndrome (SS) are protected from galectin-1-triggered T-cell death. Expression of CD7 in CD7 HUT78 T-cells derived from a patient with SS rendered the cells susceptible to galectin-1-induced death (108). The deregulated expression of Galectin-3, which is expressed in various tissues and organs can result in tumor transformation and invasiveness, or confer propensity for tumor cell survival (109). Moreover, the majority of carcinomas express the galectin-3 protein (110). Increased expression of the LGALS3 gene was observed in human non small cell lung cancer, and it was suggested to play a role in the process of metastasis in this malignancy but not in small cell lung cancer (111).

8. CONCLUSIONS AND PERSPECTIVES

At this stage of our review, and also based on our own experiments (37, 80), we are confronted with facts and hypotheses. Here are the facts. HTLV-I *in vitro* infection of an IL-2 dependent CD4-positive cell line (WE 17/10) results in a rapid (10 weeks) silencing of the proviral genome. However, a process has been launched, which continues over a longer period of time (1–2 years), despite this silencing, which ultimately leads to immunophenotypic (progressive loss of CD3 and CD7) and functional changes (acquisition of IL-2 independence, deregulation of intracellular Ca^{2+} influx, activation of the PI3K pathway and blockade of pro-apoptotic signaling). It is worth reminding that these phenomenons are fully present only at the stage of CD7-negative phenotype. We can speculate that the proviral silencing, probably through epigenetic mechanisms (51), has emerged along the evolution as a way to escape immune surveillance. Now, we must face hypotheses. Several established observations first: the absence of the TCR/CD3 complex has been shown to abrogate Ca^{2+} intracellular influx, through abrogation of the possibility to activate the PLC□1 pathway, though maintaining Ca^{2+} oscillations necessary for cell survival (112). Changes of Ca^{2+} influx can induce chromatin remodeling, but cannot be responsible for proviral gene silencing, which occur much earlier. They could, however, activate other pathways. That this is the reason for the PI3K pathway to become constitutively activated is a pure speculation. On the other hand, Ca^{2+} influx abrogation is closely timely related with loss of CD7 expression (80) and there is a known relationship (113) between CD7 activation and increase in Ca^{2+} influx. Finally, the suppression of Ca^{2+} influx has been shown (114) to inhibit pro-apoptotic signals.

What can be held for sure is that HTLV-I infection initiates several processes that lead, after a long period of time, to a transformed phenotype: loss of dependence from growth factor (IL-2), resistance to apoptosis and growth advantage (80) ending up with a CD3-negative, CD7-negative monoclonal population. These processes go on despite early silencing of the proviral genes, which in turn, allows the cell to escape immune surveillance. In summary, all the conditions required to produce a T-cell malignancy are gathered.

The hypotheses, for which more work is needed, relate to two main topics: what is the way HTLV-I launches processes that persist and amplify after proviral silencing? Among the functional changes observed, which precedes which? Does one of the event cause another one, and in what order? Or are some of these changes occurring independently from some others? What would happen upon reactivation of Tax expression (by transfection) in these monoclonal T-cells? Understanding these changes, and their chronology, and identifying the molecular players involved may provide further insights into the process of transformation. This could allow finding ways to improve ATLL treatment, and target more specifically the malignant cells.

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Send correspondence to: Haidar Akl, Experimental Hematology, Universite Libre de Bruxelles, Institut Jules Bordet 127 Boulvard de Waterloo, 1000 Bruxelles, Belgium, Tel: 32-0-2 5413730, Fax: 32-0-2 5423453, E-mail: haidar.akl@ulb.ac.be

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