Preliminary Characterization of a New Type of Viruses Isolated from Paediatric Neuroblastoma and Non-Hodgkin’s Lymphoma: potential Implications for Aetiology

Ugo Rovigatti°, Albert Tam@, Andrea Piccin°, Renato Colognato° and Bernard Sordat#

°University of Pisa Medical Schhol & Retrovirus Center, Pisa, IT;@ Genelabs Inc., Redwood City, CA, USA; #ISREC, Lausanne, CH Tel (UR) +39-328-915327 Fax +39-050-560276 E-mail: urovigatti@biomed.unipi.it

Summary:
This study presents data on a new type of Reoviridae, initially isolated from paediatric neuroblastoma, which we have also partially characterized from two cases of Burkitt’s lymphoma. Although this was not directly addressed by additional and specific epidemiological studies, population mixing (particularly in the neuroblastoma-clustering episode) could have been relevant. Both types of viruses appear to belong to the Reoviridae family, but several features – biological behaviour, electron microscopy, molecular sequencing etc.- distinguish them from Classical Reoviridae. Also in view of extensive epidemiological work, which suggests the potential association/relevance of still-unknown infectious agent(s) in childhood leukaemia/lymphoma and in paediatric neuroblastoma, this new type of viruses warrants further consideration and future studies.

Introduction:
Etiological clues about the origins of children leukaemia and paediatric tumours have been sought for several years and evidence has been presented for association with: 1. ionising radiation; 2. non-ionizing radiations; 3. chemical carcinogens; 4. prenatal genetic alterations and 5. infectious agents. Epidemiological studies by the group of L. Kinlen in the past fifteen years have clearly shown that population mixing can dramatically increase childhood leukaemia incidence, thus suggesting that an infectious agent associated with such population mixings may be responsible (Kinlen 1989) (Kinlen 1995). However, attempts by R. Jarrett’s and M. Greaves’s groups to isolate specific viruses of the Herpes or Polyoma Virus family from affected children ended up with negative results (MacKenzie, Perry et al. 1999; MacKenzie, Gallagher et al. 2001)). Furthermore, the work of M. Greaves’s has well documented that chromosomal translocations, which are typical/specific for particular childhood leukaemia, are present not only in the leukaemic clones at diagnosis, but also and already at the time of birth (Greaves 2002; Greaves and Wiemels 2003). This indicates that some of the chromosomal translocations have a prenatal origin and that leukaemic cells with identical molecular translocations (i.e. identical fusion points) can spread into homozygous monochorionic twins through placental anastomoses (thus appearing as inter-twin “metastases”). However, these data on leukaemia-specific translocations occurring in utero must also take into account a triggering event at the time of leukaemia-onset (diagnosis), since such prenatal aberrations are at least 100 times more frequent (at birth) than the disease itself (Greaves and Wiemels 2003). What this trigger may be has been debated in the past few years, as a specific infectious virus appears to be more likely from Kinlen’s epidemiology (Kinlen 1995) (Kinlen 2004), while the previously mentioned negative results (although focused only on two specific virus families) and more recent publications by Greaves suggest instead an ‘unspecific infection event’ (Greaves 2002; Greaves and Wiemels 2003). In the past several years, we have isolated and partially characterized a new type of viruses from paediatric Neuroblastoma and Non-Hodgkin’s Lymphoma (Rovigatti 1992; Rovigatti 1997). In view of the described debate on leukaemia/paediatric tumour aetiology, their initial identification and characterization as well as potential implication for aetiology will be presented here.

Materials and Methods:

Patients: The initial MFV isolation has been previously described (Rovigatti 1992). Additional isolates were obtained from two neuroblastomas tumours and one primitive neuroectodermal tumour studied at Zurich Children’s Hospital (Kispi, Zurich, CH) (Rovigatti 1998). Two additional patients (from Kispi and from Lucern Hospital) diagnosed Non-Hodgkin’s Lymphoma (NHL) of the Burkitt’s type (BL) were studied, initially as controls. In both cases, diagnosis was confirmed by presence c-MYC rearrangement involving 8;14 and 2;14 chromosomal translocations, respectively (Rovigatti 1998) (Rovigatti 2000-B).

Tumour cell cultures and Virus isolation. Tumour biopsies were obtained immediately after surgery. The Neuroblastoma/Lymphoma cultures were started as previously described (Rovigatti 1992). Electron Microscopy was initially performed by negative staining, as it was previously described (Rovigatti 1992; Rovigatti 1997). Ultra-thin section staining was subsequently extensively performed and the majority of sections and particles (for both NB and NHL) have been characterized in this way. A Focus Formation Assay (FFA) was set up, by infecting normal human embryonic fibroblasts (WI-38, IMR-90, etc.) with ultra-filtered supernatants obtained from primary cultures of paediatric tumours (Rovigatti 1998). Number of micro-foci (appearing on the monolayer) were scored 10-15 days after infection.

Molecular Biology: Gel electrophoresis, Southern, Northern blotting were performed as previously described (Rovigatti, Mirro et al. 1984; Rovigatti, Watson et al. 1986). Initial cloning was performed by construction of a cDNA library from SK-N-SH cells acutely infected by MFV and employing the vector λgt11 (in collaboration...
with Genelabs Inc., Redwood City, California). Probes were obtained by retro-transcribing the same mRNA extracted from SK-N-SH cells acutely infected with MFV: it was reasonably assumed – from massive cytopathic effects elicited in 48-72 hours and from running the extracted RNA on 2.2 agarose gels- that the majority of mRNA produced had viral origin. Several clones were isolated and three studied in more detail. Automatic sequencing with universal primers was performed at Genelabs employing ABI sequencers. This initial sequencing has allowed us to design additional primers for RT-PCR screening of three gene-segments (Rovigatti, Afanasyeva et al. 1998) (Rovigatti 2000-B) (Rovigatti U. 2002) and further cloning and sequencing. Briefly, retro-transcribed (Promega Biotech) RNA from infected cells was amplified employing such primers and then inserted into PCR product specific vectors (Qigen, Inc., Germany). The inserts were sequenced with Pharmacia Automatic sequencers (ALF-RED or ALF-EXPRESS), by employing universal primers, or MFV specific primers. Sequence analysis was performed with Dnasis programs (Contig Manager, etc.) in MacIntosh environment and with DNAassist programs in Windows environment.

Results:
By employing the previously described RT-PCR and Focus Formation Assay (FFA), a few supernatants (from neuroblastomas and neuroectodermal tumours) appeared to be strongly positive in comparison with all the controls from normal tissue and additional human tumours, which were negative. Table 1 summarizes the results for Focus Formation Assay, RT-PCR and electron microscopy. In addition to neuroectodermal tumours, two cases of Non-Hodgkin’s Lymphoma of the Burkitt’s type (BL), were scored as highly positive by RT-PCR and by FFA (Table 1). In both cases, diagnosis was confirmed by presence of chromosomal translocations 8;14 and 2;14, respectively, both involving c-MYC. Electron microscopy has documented in both cases the presence of virus particles extremely similar in shape and cellular localization (but with some difference) to the original MFV isolate (see Fig. 1).

FIG.1 Electron Microscopy of MFV and MFV-related viruses (MFRV). 1A. Neuroblastoma cells containing MFRV’s in cytoplasm (magnif. 15K). 1B. Diffuse spreading pattern of MFRV’s from BL (magnif. 5K). 1C. MFV original identification (magnif. 350K). 1D MFVRV’s from NB (magnif. 300K). 1E MFRV’s from BL (magnif. 175K)

TABLE 1.

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<th>Test</th>
<th>Contr.</th>
<th>Virus NB</th>
<th>Virus BL</th>
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<tr>
<td>Focus Formet. Assay (FFA)</td>
<td>Not Detect</td>
<td>++++</td>
<td>+++</td>
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<tr>
<td>RT-PCR assay(MFV primer)</td>
<td>Not Detect</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Electron Microsc. (negative &amp; post.)</td>
<td>Not Detect</td>
<td>++++</td>
<td>+++</td>
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Comparison of these two isolates from BL patients with original MFV led to identification of clear differences:

1. MFV and NB viruses are typically confined inside a minority (3-5%) of cells of NB cultures/cell lines (Fig. 1A). To the contrary, the two lymphoma isolates (BL) tend to spread and destroy the whole culture or cell line (typically, >90% of infected cultured cells show virus particles) (Fig. 1B).
2. The diameter of viruses isolated from NB and neuroectodermal tumours (MFV and additional isolates) was consistently smaller (approximately 65-73 nm, Fig. 1D) in comparison with the isolates from BL (85-92 nm, Fig. 1E).
3. Inside infected-transformed cells, the two isolates behave differently, as MFV and other viruses from neuroectodermal tumours are typically restricted inside membranous cytoplasmic structures (similar to
virus factories, see Fig. 1A), while the BL isolates seem to spread throughout the inner cellular space (i.e., nucleus and cytoplasm, see Fig. 1B).

After the initial cloning (in collaboration with Genelabs Inc., Redwood City, CA), analysis of the retrieved sequences indicated a good homology with human Reoviridae family (75-85% homology at the nucleic acid level). In particular, three segments were studied in more detail: one structural gene (σ3) and two non-structural/regulatory genes (σNS and µNS). The similarity with classical Reoviridae-3 (Dearing strain) for two isolates—one from NB and one from BL—for a shorter sequence of the µNS gene is shown in Fig. 2.

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<th>FIGURE 2: comparison of sequences (Micron NS-µNS- gene) from Reoviridae3, MFV and one isolate from Burkitt’s lymphoma (BL). Divergence from Reo is approximately 20%.</th>
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<tbody>
<tr>
<td><strong>MFV</strong></td>
</tr>
<tr>
<td><strong>σNS</strong></td>
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<tr>
<td><strong>µNS</strong></td>
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<td><strong>σNS</strong> (comparison)</td>
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Discussion and Conclusions:

Our preliminary findings of a new type of human viruses in paediatric malignancies, belonging to the Reoviridae family, open several questions. The original virus, MFV, was isolated from a situation of so-called ‘cancer-clustering’ of paediatric tumours with epicentre in Morgan City (Louisiana) and an excess of cases (approximately 10 fold over the estimated National incidence for NB). Morgan City had experienced –just before the study period (1986-88)- population increases (2-3 folds) and mixings, due to construction of a new industrial setting (E. Fontham, LSU, personal communication). No extensive epidemiological investigation was performed in this case, nor for the two Burkitt’s lymphoma patients (one was the daughter of recent immigrants from Turkey). The most extensive epidemiological work on paediatric malignancies has focused on childhood leukaemia/lymphomas, which are approximately 50 fold more frequent than paediatric neuroblastoma (Kinlen 1998). Very recent epidemiological studies, however, indicate that neuroblastoma behaves similarly to childhood leukaemia/lymphoma, thus suggesting that immune mechanism(s) and an infectious agent may play a role in neuroblastoma as well (Menegaux, Olshan et al. 2004). In our studies, MFV or MFV related viruses were detected by several assays and isolated from neuroectodermal tumours as well as two (of two tested) Non-Hodgkin’s lymphomas of Burkitt’s type. Evidence against trivial contaminations in these cases are: 1. The independent isolation in four different laboratories (New Orleans, Paris, Zurich and Pisa), where similar viruses were not grown at that time and in different time-frames; 2. Experimental evidence of cellular transformation in vitro and tumorigenesis in vivo, induced by these viruses; 3. Data indicating the different behaviour of MFV and other MFRV’s in comparison with classical Reoviridae in terms of infection and virus reproduction on normal embryonic human cells; and 4. Different sequences in comparison with other MFRV’s and with non-pathogenic Reoviruses, at least in the three segments-genes that have been more extensively analysed so far (see Fig. 2). Although an evaluation of the role played by these viruses awaits 1. a more complete/refined analysis of their genome (i.e., by complete sequencing) and 2. a more widespread and systematic study of paediatric cases for detection/characterization of similar MFRV’s isolates, a possible role for this type of viruses in human and animal cancers can be hypothesized also in view of important observations:

Similar viruses (sometimes referred to as ‘endogenous Reoviruses’) are extensively widespread throughout animal kingdom (an also in plants). E. Gateff isolated a Reovirus from haemopoietic neoplasms of Drosophila and showed that it induced similar neoplasms, if injected in Drosophila larvae, but not in the adult flies (Haars, Zentgraf et al. 1980) (Gateff 1980). Similar viruses also appear to be linked to inherited Drosophila mutations (Louis, Lopez-Ferber et al. 1988) (Lopez-Ferber, Vyvurnes et al. 1989).

Burkitt’s Lymphomas in Western Countries are associated with EBV infection in only approximately 30% of cases. Tumours from the two BL cases here reported did not show any evidence of EBV infection. Furthermore, also in African Burkitt’s, similar Reoviridae viruses were described by British and Australian studies (often, more frequently than EBV: (Bell, Massie et al. 1964; Bell, Massie et al. 1966; Bell 1967). The Fourth COMARE report on the excesses of childhood leukaemia/lymphoma cases in Sellafield suggested that normal hygienic conditions were lacking during the initial building of the Sellafield nuclear plant, thus possibly initially targeting the childhood population, among which leukaemia/lymphoma cases were diagnosed (Bridges B.A. 1996). Furthermore, one of the most dramatic examples of leukaemia/lymphoma clustering was described in the Chicago suburb of Niles over 40 years ago: in this episode several cases of
leukaemia/lymphoma were diagnosed among children who had attended the same parochial school. Interestingly, many children were –in the same period and group- affected by rheumatic disorders (Schwartz, Greenspan et al. 1963) (Greaves 1997).

**In conclusion:**
We have isolated from both paediatric neuroblastoma’s and childhood lymphoma’s new types of Reoviridae, which appear related, although distinct from Classical Reoviridae and with distinct biological, structural and molecular features between themselves. The potential relevance of such infectious agents in paediatric malignancies pathogenesis warrants further investigations on these and similar viruses.

**References**