The National research program on AIDS
(Extramural research projects)

Project

PATHOLOGY, CLINIC AND THERAPY OF AIDS
Scientific Coordinator: Stefano VELLA

Projects financed № 89
A HISTOGENETIC MODEL FOR HODGKIN’S DISEASE (HD) DEVELOPMENT IN HIV-POSITIVE (HIV⁺) AND HIV-NEGATIVE (HIV⁻) INDIVIDUALS

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The tumor cells in most cases of HD have been recently recognized to originate from the B-cell lineage, but their precise differentiation stage is not fully clarified. Here we report data suggesting a histogenetic model for HD development in HIV⁺ and HIV⁻ patients.

Expression of BCL-6, a transcription factor expressed in germinal center (GC) B-cells, and CD138/syndecan-1 (syn-1), a proteoglycan associated with post-GC, terminal B-cell differentiation, has been assessed by immunohistochemistry (IHC) on paraffin-embedded and frozen tissues from 76 classic HD (CHD) (49 HIV⁻ CHD and 27 HIV⁺ HD). The phenotype of Reed-Sternberg (RS) cells has been correlated to the host's HIV status, expression of EBV-encoded latent membrane protein 1 (LMP1) by the tumor clone, and phenotype (CD4, CD8, CD40L) of microenvironmental T-cells, as assessed by IHC and flow-cytometry.

The tumor cells of a fraction of HIV⁻ CHD (27/49; 55%) including 6 LMP1⁺ cases displayed the BCL-6⁻/syn-1⁺ phenotype of post-GC B-cells, whereas another fraction of HIV⁻ CHD (22/49; 45%) including 6 LMP1⁺ cases was constituted by a mixture of tumor cells reflecting the GC (BCL-6⁺/syn-1⁻) or post-GC (BCL-6⁻/syn-1⁻) phenotypes. BCL-6⁻/syn-1⁺ tumor cells of HIV⁻ CHD were mostly found surrounded by T-cells expressing CD40L, consistent with the observation that CD40 signaling downregulates BCL-6 expression. On the other hand, most HIV⁺ HD cases (25/27, 92.6%) expressed LMP1 and displayed the BCL-6⁺/syn-1⁺ phenotype thus reflecting post-GC B-cells. Although BCL-6⁻/syn-1⁺ RS cells of HIV⁺ HD expressed CD40, they were not surrounded by CD40L reactive T-lymphocytes which, in HIV⁻ HD, are thought to regulate the disease phenotype through CD40/CD40L interactions.

These data indicate that the phenotype of tumor cells of HIV⁻ CHD appears to be modulated by the surrounding cellular background, particularly CD40L reactive T-cells. Conversely, RS cells of virtually all HIV⁺ HD (92.6%) express LMP1, which, being functionally homologous to CD40, may contribute to the modulation of the HIV⁺ HD phenotype.

This work has been supported by ISS, II Programma nazionale di ricerca sull’AIDS 1998 - Progetto “Patologia, clinica e terapia dell’AIDS”. Proposta di Ricerca N° 13
PROPOSAL N. 17
Cellular drug-resistance to antiretroviral drugs: biological and clinical aspects.
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2. UNESCO Center c/o "IRCCS L. Spallanzani", Rome
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The main and the final aim of the project is to verify whether cellular factors, other than viral factors, may be involved in the failure of treatment of HIV infection. We start to verify whether the prolonged treatment with nucleoside analogue (NA) or with HIV- proteinase inhibitor (IP) may induce cellular factors responsible for a development of the resistance to antiviral drugs or to see whether the already known mechanisms of cellular resistance may influence the antiviral activity of some specific drugs. To this regard it has been recently demonstrated that HIV-1 IP are substrate for the P-glycoprotein (Pgp) a transmembrane efflux pump that actively extrude a variety of unrelated drugs. In order to study the influence of this glycoprotein on antiviral activity of proteinase inhibitor, we used a cellular line expressing high level of Pgp (CEMVBL) and the parental cell line (CEM) as control. Cells were infected with HIV-1 and treated with different concentrations of Saquinavir and Indinavir. The results indicated that in cells expressing high level of Pgp the antiviral activity of these drugs is reduced (p<0.05) when compared to parental cell line. The phenomenon can be reverted by the use of an inhibitor of Pgp activity. These results suggest that Pgp is responsible for the reduced antiviral activity of IP in this cell line. In order to study this phenomenon in vivo we start to examine whether PBL from HIV infected patients undergoing different drug regimens (NA± IP for at least 5 months) could express detectable amount of Pgp. Preliminary results indicate that only in PBL from patients treated with a combination that includes an IP, a significant percentage of expression of Pgp could be recorded (CTR=0.9±1.6; NA+IP=6.7±5.2; NA alone=1.8±2.3). Interestingly, the data show that the main cells expressing such protein are represented by monocytes. The clinical significance of such observation is however still to be elucidated. As far as the effects induced by NA on cellular metabolism are concerned, we are currently trying to select resistant cells to NA. Briefly. CEM are cultured in the presence of increasing concentrations of the following drugs: ddl, d4T and 3TC. Periodically the cytotoxic activity of the drug is evaluated to see whether the cells have acquired resistance. Preliminary results indicate that cells resistant to the cytotoxic activity of ddl, 3TC, d4T can be obtained by this experimental approach. Interestingly cells after prolonged treatment with d4T show a higher degree of resistance versus AZT than versus d4T. Studies are in progress to further elucidate the mechanism involved in the acquisition of this type of cellular resistance.
ACTIVATION OF CD40 FAVORS THE GROWTH AND VASCULARIZATION OF KAPOSI'S SARCOMA.

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CD40 is a 50 kD member of the tumor necrosis factor/nerve growth factor receptor superfamily involved in regulation of cell survival, proliferation and differentiation. Although CD40 is expressed by several tumor lines and is upregulated on tumor vascular endothelium, its role in tumor biology is still unclear. In the present study, we investigated the role of CD40 in the growth and vascularization of Kaposi's sarcoma (KS). In vitro, stimulation of CD40, using anti-CD40 agonistic antibody or the soluble form of its ligand CD154, induced migration of KS cells and inhibited vincristine-induced apoptosis. Similarly, the engagement of CD40 on endothelial cells resulted in cell contraction, migration and prevention of serum withdrawal-induced apoptosis. To understand the biological relevance of CD40 in vivo, KS cells were engineered to express and release a soluble form of CD40 (KS-sCD40) able to disrupt CD40-CD154 interactions. SCID mice subcutaneously injected with KS-sCD40 cells developed tumors that were significantly smaller than those induced by control cells (KS-neo). In addition, KS-sCD40 tumors showed several areas of necrosis, diffuse presence of apoptotic cells and poor vascularization. In contrast, KS-neo tumors showed few or absent areas of necrosis and apoptosis and intense vascularization. In addition, anti-CD40 antibodies stimulated neo-angiogenesis in a subcutaneous murine Matrigel. These observations provide strong evidence that CD40 supports tumor cell survival, growth and neo-vascularization of Kaposi's sarcoma.

Proposal N° 30
PROTOCOL DO-ART. DIRECTLY OBSERVED ANTI-RETROVIRAL THERAPY.

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Background: Adherence is a critical variable for the success of anti-retroviral therapy. Complicated therapeutic daily schedules are often needed and reduce adherence. We evaluated in a prospective, clinically based study, a simple once-a-day regimen with didanosine (300 mg) + lamivudine (300 mg) + efavirenz (600 mg).

Results: Patients could be either naive for antiretroviral therapy or pre-treated with NRTIs and PIs, all of them were naive for NNRTIs. Fifty-two patients were enrolled, 54% of them were naive. Their mean age was 35.1 years and 72% of them were males. At baseline the median HIV-RNA was 88,279 copies/ml (range from 1,330 to 750,000) and the mean CD4 T-cell count was 324 cells/µl (range from 5 to 760). After one month of therapy the median HIV-RNA value dropped to 717 copies/ml (range from <50 to 67,200 , with all patients but two below 5000 copies/ml). Among naive patients 53% had a viral load below 400 copies/ml and 35% below 50 copies/ml. The same figures among experienced patients were 38% and 23%. Preliminary results indicate that after 4 month of therapy almost all patients were below the limit of detection of the test (50 copies/ml).

Three patients interrupted therapy because of hepatitis, rash or gastro-enteric intolerance to ddl.

Conclusions: Once-a-day therapy is well accepted by patients. The regimen we are studying reduces the pill burden and the stress of a complicated daily schedule. It shows a high potency, inducing a median reduction of viral load greater than 2 logs and the complete suppression of viral replication in half of the patients after a single month of therapy. Preliminary results indicate that the treatment retains a marked antiviral efficacy on the longer period, too. Treatment was generally well tolerated.

Proposta n. 34
VEGF-C AND VEGF-D PARTICIPATE TO THE ANGIOGENESIS OF KAPOSI'S SARCOMA (KS) AND ACTIVATE KS SPINDLE CELLS

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Vascular Endothelial Growth Factors (VEGF) represent a family of glycoproteins which primarily activate an angiogenic program in endothelial cells, by acting through three different tyrosine kinase receptors: VEGFR-1, -2, and -3. The first identified member has been VEGF-A, which is pivotal in angiogenesis associated to Kaposi's sarcoma (KS) (Cornali et al., Am. J. Pathol. 149, 1851, 1996; Masood, et al. Proc. Natl. Acad. Sci. USA 94, 979, 1997; Samaniego et al, Am. J. Pathol. 152, 1433, 1998). VEGF-C and -D share respectively 30 and 40% homology in aminoacid sequence with VEGF-A and are able to activate VEGFR-2 and VEGFR-3. In this study we evaluated the biological response of KS cells to VEGF-C and D, using an immortal cell line derived from a KS lesion (KS IMM), which retains most features of the parental tumor and can induce KS-like sarcomas when injected subcutaneously in nude mice. We show that VEGFR-3 and VEGFR-2 were expressed by KS IMM cells grown in vitro and in vivo. In vitro, VEGF-C and VEGF-D induced the tyrosine phosphorylation of VEGFR-2, a receptor also for VEGF-A, as well as that of VEGFR-3. The activation of these two receptors in KS IMM cells was followed by a dose-responsive mitogenic and motogenic response. The stimulation of KS IMM cells with a mutant VEGF-C unable to bind and activate VEGFR-2 resulted in no proliferative response and in a weak motogenic stimulation, suggesting that VEGFR-2 is essential in trasducing a proliferative signal and cooperates with VEGFR-3 in inducing cell migration. VEGF-C and -D activated also the proliferation, and the in vitro angiogenesis, whereas only VEGF-C the migration. KS-IMM, as well as other KS cell lines tested, did not produce VEGF-C and D in basal condition or after inflammatory cytokine stimulation. Our data add new insights on the pathogenesis of KS, suggesting that the involvement of endothelial growth factors may not only determine KS-associated angiogenesis, but also play a critical role in controlling KS cell growth and/or migration and invasion by a paracrine mechanism.
PHENOTYPIC ANALYSIS OF CD4+ AND CD8+ T CELLS DURING TREATMENT OF HIV INFECTION WITH COMBINATION ANTIRETROVIRAL THERAPY ASSOCIATED OR NOT WITH LOW DOSE INTERLEUKIN-2


Di.M.I. and * Di.M.I.Psi.Crim. - University of Genova and **Molecular Virology Unit - ABC, Genova, Italy.

Aim of the present study was the multiparametric evaluation of the effects of low dose interleukin-2 (IL-2) on immunological reconstitution in HAART treated HIV+ patients. 14 subjects (11 males and 3 females, age 18-50) with CD4 counts between 50 and 500 cells/µl have been enrolled and randomized into two groups of 7 patients each to receive: A) conventional combination therapy (two reverse transcriptase inhibitors + one protease inhibitor); B) conventional combination therapy associated with low dose subcutaneous IL-2 (500,000 IU/sqm, 5 days a week for 6 months). Results are reported in the table as mean values.

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<th>Treatment A</th>
<th>Months</th>
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<tr>
<td>CD4 (%)</td>
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<td>CD4/CD45 RO (%)</td>
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<tr>
<td>CD4/CD45 RA (%)</td>
<td>8</td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>46</td>
</tr>
<tr>
<td>CD8/CD28 (%)</td>
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</tr>
<tr>
<td>Viral load (copies/ml)</td>
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# Number of subjects with values under the detection limit

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<td>CD8 (%)</td>
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<td>CD8/CD28 (%)</td>
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# Number of subjects with values under the detection limit; *P < 0.05 and **P < 0.01 vs. baseline

Results indicate that the association of low dose IL-2 with antiretroviral treatment leads to a significant and long lasting increase in CD4 cells number concomitant with low viral load levels. Non integrated HIV-DNA was frequently detected in patients undetectable viremia.

Project N° 43
AN 18 MONTHS CLINICAL AND IMMUNO-VIROLOGIC CHARACTERIZATION OF THE EFFICACY OF STAVUDINE, LAMIVUDINE AND INDINAVIR IN PEDIATRIC HIV INFECTION

Alessandra Vigano*, Len Dally, Dorella Bricalli, Natasca Sala, Maria Pirillo, Marina Saresella, Daria Trabattoni, Stefano Vella, Mario Clerici and Nicola Principi.

Clinical, virologic and immunologic parameters were analyzed in 25 HIV vertically-infected children, previously antiretroviral-experienced (AZT, ddI, ddC), protease inhibitor naive, with clinical symptoms (CDC class A, B, C) and evidence of immunosuppression (CDC class 2, 3), to establish efficacy of an 18 months treatment with stavudine, lamivudine and indinavir. At 1, 6, 12 and 18 months the proportions of patients with HIV-RNA < 400 copies/ml were: 94%, 100%, 94%, 87% in Immunologic Class 2 and: 50%, 67%, 67%, 72% in Immunologic Class 3. At 12 months, the median CD4+ count and percent increased significantly in both CDC groups, but to greater extent in Immunologic Class 3. In the 12-to-18 months period there was no significant changes within the groups. In both CDC groups there was a steady increase in the proportion of children with positive delayed type hypersensitivity (DTH) skin tests and in number of positive DTH skin tests. Immunologic Class 2 children were more likely to develop a positive DTH response and a greater number of positive DTH responses. Lymphocyte proliferative response to recall antigens improved significantly in all patients. The rate of increase in positive tests was faster in Immunologic Class 2 than in Immunologic Class 3 children. During the 18 months of treatment only minor clinical events occurred and neither disease progression nor opportunistic infections were observed. Potent antiretroviral therapy achieves a sustained benefit in HIV-infected children but immune reconstitution may be better warranted in children with less advanced disease.

Proposta di ricerca n. 45
CD10 (neutral endopeptidase 24.11 or NEP), is a 100 kDa type II integral membrane protein. Although the neutral endopeptidase activity of CD10 is well documented, its function in the physiology of CD10-expressing lymphoid cells is poorly understood. However, some evidence obtained on mature B cells suggests that CD10 expression may be related to apoptosis: 1) CD10 is found on germinal center B cells that are particularly prone to apoptosis; 2) Burkitt’s lymphoma cells, which readily undergo spontaneous apoptosis both in vivo and in vitro, express abundant surface CD10; 3) B cells from lymphoblastoid B cell lines transfected with c-myc-carrying episomes, concomitantly acquire the capacity to express CD10 and an increased propensity to spontaneous apoptosis in vitro; 4) B cells induced into apoptosis by HIV infection in vitro express CD10.

In the present study, we investigated whether the correlation between apoptosis and CD10 expression noticed in B cells was also true for T cells. We found that mature T cells induced into apoptosis in a variety of manners in vitro or spontaneously undergoing apoptosis in vivo invariably expressed CD10. Cells from continuous T cell lines became CD10-positive when induced into apoptosis by HIV infection and exposure to CD95 mAb, etoposide or staurosporin. Inhibitors of caspases blocked apoptosis and CD10 expression. Both CD4-positive and CD8-positive T cells purified from normal peripheral blood expressed CD10 upon apoptotic induction. The apoptosing cells newly synthesized CD10 since its expression was inhibited by exposure to cycloheximide and CD10 mRNA became detectable by RT-PCR in T cells cultured under conditions favoring apoptosis. In order to demonstrate CD10 on T cells apoptosing in vivo, lymphonode and peripheral blood T cells from HIV-positive subjects were employed. These suspensions were comprised of a substantial although variable proportion of apoptosing T cells, which consistently expressed CD10. In contrast, CD10 positive as well as spontaneously apoptosing T cells were virtually absent in peripheral blood from normal individuals. Collectively, these observations indicate that CD10 may represent a reliable marker for identifying and isolating apoptosing T cells in vitro and ex vivo and possibly suggest novel functions for surface CD10 in the apoptotic process of lymphoid cells.

Proposta n. 46
MOLECULAR MECHANISMS INVOLVED IN THE PATHOGENESIS OF AIDS RELATED BURKITT'S LYMPHOMAS AS SUGGESTED BY IMMUNOGLOBULIN VARIABLE REGIONS ANALYSES.

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³Divisione di Anatomia Patologica, Ospedale Sant’Andrea, La Spezia, Italy.

We have determined the sequence of rearranged Ig VH and VL genes in 7 cell lines derived from BL (PA628, HBL2, ZIN, LAM, BRG-M, HBL1, AS283). All samples displayed somatically mutated Ig V genes with evidence of antigen stimulation and, in some instances, of antigen selection (BRG-M and HBL-1). Percent of Ig VH mutations varied from 2% to 10% with evidence of mutations occurring after neoplastic transformation. In two cases (LAM and ZIN), striking differences were seen in the number of somatic mutations present in the Ig heavy and light chain. The frequency of VH mutations of LAM cells was $8 \times 10^{-2}$ whereas the VL gene was virtually unmutated ($0.3 \times 10^{-2}$). Similarly, frequency of VH mutations of ZIN was $6 \times 10^{-2}$ against a frequency of the VL gene of $0.6 \times 10^{-2}$. These differences might be explained with a secondary rearrangement occurred in the germinal center mediated by reactivation of RAG genes. We speculated that in the germinal center reaction the activation of the mutational machinery might cause the generation of non functional Ig genes. An example of such event come from the cell line AS283 in which an extensive deletion of the rearranged VH3 gene is present making this rearrangement non functional. As expected this cell line does not express surface Ig, although the presence of a functional VK gene was detected. This finding raises the question of how the cells may survive without Ig molecules being expressed. One possibility is that EBV (which infect this cell line) might have determined the survival of the cells. Indeed, EBV LMP2A gene has been shown capable of substituting sIg signals. Of note, LAM cell line is an EBV+ but we have evidence of late infection of EBV as determined by the presence of multiple EBV fused termini.

The present data suggest that antigen stimulation may represent an important step in the pathogenesis of BL. In fact, reactivation of RAG genes might mediate c-myc translocations in mature B cells. Rescue of B cells might be mediated by (auto)antigens and/or in some instance by EBV.

Abstract proposta N.46
STRATEGIES TO INDUCE EFFICIENT CTL RESPONSES FOR THE CONTROL OF EBV-ASSOCIATED MALIGNANCIES.

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University of Ferrara, Italy.

CTL responses directed to Epstein-Barr virus (EBV) tumor-associated antigens are not adequate to control EBV-associated tumor development. A possible clue to the failure to achieve an efficient immune control of EBV-associated tumors may be found in the poor immunogenicity of EBV-derived epitopes. Indeed, we demonstrated that the LMP2-derived, HLA-A2-associated CLGGGLTMV (CLG) epitope is not well presented at the cell surface of EBV-infected cells. We identified that poor binding was due to the lack of proper non-anchor residues necessary for the formation of stable HLA-A2/peptide complexes. To define CLG analogues with higher immunogenicity we synthesized and tested CLG-derived peptides carrying single or combined amino acid substitutions to increase HLA/peptide stability. Among the analogues tested we identified two peptides which, compared to the natural epitope, showed higher affinity for HLA-A2 molecules, and produced stable complexes. These peptides demonstrated a potent, specific stimulatory capacity and could be used for selective in vitro amplification of CTL precursors that can be transferred to the patients, or alternatively to pulse selected APC to increase the in vivo CLG-specific CTL response. In addition, among the analogues tested we characterized peptides with null activity. These peptides in combination with the natural weak agonist induces strong CTL responses directed against the natural epitope.

The definition of highly immunogenic synthetic peptides or of particular peptide combinations may be useful for the development of new strategies for CTL-based cancer therapies directed against weak natural epitopes expressed in tumor cells.

Proposta n.51.
A RANDOMIZED PHASE II TRIAL TO COMPARE THE TOXICITY, TOLERABILITY AND ACTIVITY OF 2-DRUG COMBINATIONS OF THE NUCLEOSIDE ANALOGUE REVERSE TRANSCRIPTASE INHIBITORS (NRTI) LAMIVUDINE (3TC), ZIDOVUDINE (ZDV) AND 1592U89 (ABACAVIR) WITH OR WITHOUT PROTEASE INHIBITOR NELFINAVIR (VIRACEPT-NFV) (PENTA 5)

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Di Rossi, L. Chieco-Bianchi.
Istituto di Oncologia, Padova.

In 1990 on the behalf of the European Union, Paediatric European network Treatment AIDS (PENTA) was established. The MRC HIV Clinical Trial Centre in London, and the ANRS/INSERM Trial Centre in Paris are the coordinating centre. The network is also sponsored by the Italian Institute of Health, and by similar program in Spain, Belgium and Germany.

So far 3 trials have been completed and two are ongoing, one of them Penta 5.
The objectives of Penta 5 trial are:

Primary:
1) to compare the activity, tolerability and toxicity of 3 different 2-drugs RTI combinations in children taking either NFV or NFV/placebo.
2) to compare the activity, tolerability and toxicity of NFV and NFV/placebo in children taking one of the three 2-drugs RTI combinations.

Secondary:
1) to describe the effect on viral resistance, CD4 cell count and clinical progression in the three RTI groups and the NFV/NFV placebo groups.
2) to compare the plasma viral load as measured by HIV RNA in the 1592U89 containing arms with that in the non-1592U89 arm in children taking NFV or NFV placebo.

The Penta 5 enrolment finished in march 1999. 132 children have been enrolled in 14 months meeting easily the initial target of 120. In Italy a total of 39 children were enrolled: 22 in A arm and 17 in arm B.

In the last 18 months the DSMC met three times and suggested to continue the blind part of the study. The drugs, particularly 1592U89, seems very well tolerated. The study will end in October 1999.

Proposta N° 53
CEREBROVASCULAR COMPLICATIONS AND HIV INFECTION: CLINICAL AND LABORATORY EVALUATION.

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1st Chair of Neurology, University of Milan; 1st and 2nd Department of Infectious Diseases, Luigi Sacco Hospital, Milan.

Several mechanisms are potentially involved in the genesis of vascular damage in HIV infection. Recent studies point on the frequent appearance of vascular complications in patients treated with protease inhibitors. In this study we are collecting data about all HIV-infected patients who present acute cerebrovascular manifestations, either ischemic or hemorrhagic. At present 11 patients (10 men, 1 woman, age range 29-57 yr) have been observed. Of the, 2 had a subdural hematoma, 2 an intraparenchymal hematoma, 3 a transient ischemic attack, 4 an ischemic stroke. Ten of these patients were under combined treatment with nucleoside analogues and 1 or 2 protease inhibitor, whereas only one of them was treated with nucleoside analogues alone. Both patients with cerebral hemorrhage died after the event; one patient had a second stroke while in treatment with salicylates. CD4+ levels did not differ significantly between subgroups (according to the vascular event). Platelet counts were low in 3 patients (2 with cerebral hemorrhage and 1 with TIA); standard coagulation parameters were normal in all patients. Positivity for anti-cardiolipin antibodies was detected in only 1 patient with subdural hematoma. These preliminary data suggest a relevant association between administration of protease inhibitors and acute cerebrovascular events, ischemic as well as hemorrhagic. The impact of antiretroviral therapy with circulating factors potentially affecting hemostasis is under evaluation.

Proposta n. 57
AIDS ABSTRACT No. 60

Immunostimulatory effects of Δ5-androsten-3β-7β-17β triol in HIV infection. Study on in vitro cultured lymphocytes CD4.


The scope of the study is to verify whether or not a metabolite produced physiologically by the adrenal gland, 5-androsten - 3β-7β-17β triol, that has antiviral and immunostimulatory effects in animals, has antiviral and immunomodulatory effects on the blood of HIV infected patients at various stages of the disease.

Lymphocytes taken from each patient will be incubated in a medium or a medium with Δ5-androstenetriol for 24 hours prior to stimulation, which will be produced by antigens and mitogens. Proliferation of the cells induced through stimulation with antigens will be measured by incorporating (3H) thymidine; interferon γ and IL-10 will also be measured in the medium with immuno-enzymatic techniques. Viral replication will be evaluated with a branched DNA (BDN) quantitative method. All these elements will be measured after 7 days in a culture. The study was conducted on 15 patients with lymphocytes CD4 < 200mm³ before treatment with antiviral drugs and after treatment for 3 months with AZT, 3TC and SAQUINAVIR. The results obtained in the 15 patients with lymphocytes CD4 < 200mm³ showed that the addition of Δ5-androstenetriol to the concentration of 10-8M was enough to produce an increase in the incorporation of thymidine and to stimulate the production of interferon gamma as well as reducing the production of IL-10, in 10 out of 15 experiments (P < 0.05). Also significant, was the reduction of the viral concentrations measured by quantitative branched DNA.

Similar cytokine changes were present in patients after antiviral treatment - actually the cytokine reaction was stronger and present in 12 out of 15 patients. Viral replication, already reduced through treatment, was further reduced through the addition of Δ5-androstenetriol. 5 out of 15 patients showed no signs of the virus and in another 5, the virus had been reduced.
QUANTIFICATION OF THE IMPACT OF HIV-1 REVERSE TRANSCRIPTASE AND PROTEASE MUTATIONS ON THE EFFICACY OF RESCUE HAART.

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The impact of mutations in the HIV-1 reverse transcriptase (RT) and protease (PR) on the reduction of efficacy of the rescue therapy (administered on a clinical basis) was retrospectively determined in 48 AIDS patients failing HAART. A novel score calculation system based upon RT and PR mutations was developed to quantify the reduction in rescue treatment efficacy due to drug resistance. Since no patient had previously received NFV, this PR inhibitor (PRI) was pivotal in all rescue HAART schedules. In addition, all patients were naive for non-nucleoside analog RT inhibitors (NNRTI), including EFV. Patients were given one of the following rescue therapies: i) NFV+EFV+d4T (n=15); ii) NFV+SQV+3TC+d4T/AZT (n=13); iii) NFV+d4T+ddI/3TC/ddC (n=15); iv) miscellaneous treatments (NFV+SQV+ddI, 2 patients; NFV+SQV+d4T, 2 patients and NFV+SQV+d4T+ddI, 1 patient). Responders were patients showing a drop in HIV-1 RNA level \(\geq 0.5 \log_{10}\) after 3 months of therapy. RT and PR were retrospectively analyzed on baseline plasma samples for mutations conferring drug(s) resistance. After 3 months of rescue therapy, reduction in HIV-1 viral load was significantly different between group i) and groups ii) and iii) of patients (median decrease in viral load 1.93 \(\log_{10}\), 0.45 \(\log_{10}\) and 0.47 \(\log_{10}\), respectively). Interestingly, at baseline in no patient d4T-resistant strains were detected, despite prolonged administration of this NRTI. In contrast, mutations known to confer reduced susceptibility to NFV were detected in 42/48 patients (87.5\%) naive for NFV. No patient showed the presence of HIV-1 strains resistant to EFV. All of non-responders (n=20) harbored HIV-1 strains with mutations causing resistance to one or more drug(s) of the rescue HAART schedule. A significant inverse correlation between reduction in viral load and reduction in therapy efficacy due to drug-resistance as determined by the score calculation system was found \((r=0.62)\). With respect to treatment failure, a cut-off value of 36\% reduction in therapy efficacy showed a positive predictive value (capacity to detect failure of rescue treatment) of 81.2\% and a negative predictive value (ability to detect successful treatment) of 75.8\%. In conclusion: i) a significant correlation between the level of drug-resistance as determined by the novel score calculation analysis of RT and PR mutations and reduction in virologic response to rescue HAART was shown; ii) the superior performance of NFV+EFV+d4T combination treatment was likely to be due to the more favourable drug-resistance profile detected in this patient cohort.

Proposta n. 65
VIROLOGICAL AND IMMUNOLOGICAL PARAMETERS IN HIV+ PATIENTS TREATED WITH HAART+IL-2 VS HAART ALONE.

Centro di Riferimento Oncologico, Aviano (PN) and ICGEB, Trieste.

The aim of this study is to evaluate the virological and immunological parameters in HIV+ patients treated with HAART alone or in combination with IL-2. We have already enrolled 12 pts in HAART +IL-2 (6MUl/day) group (gI) and 10 in the hAART alone group (gII). Inclusion criteria were CD4≥200/mmcr and HIV viremia >500 copies/ml. The virological and immunological data have been analyzed at t=0, 2, 4, 12, 24 and 48 weeks of treatment. A novel therapeutic schedule including 9 MIU/day of IL-2 has also been started. Pretreatment blood virus levels were 68 ± 130 x 10^3 copies/ml in gI and 50 ± 70 x 10^3 copies/ml in gII. After 24 weeks viremia was < 500 copies/ml in all the patients, while after 48 weeks viremia rose <500 copies/ml in 3 patients in gI and in 2 in gII. Before treatment, proviremia (qPCR) was 5660 ± 4990 copies /1 million CD4 in gI and 23980 ± 53000 copies in gII. After 24 weeks of treatment, proviremia was significantly reduced in both groups (1896±1970 in gI, 5485±9700 in gII, p<0.05) and this decrease was also maintained after 48 weeks (1550±1930 in gI and 1859±1970 in gII). The decrease of proviremia remains statistically significant even when calculated as proviral copies/ mmc of blood. In both groups of patients, CD4 cell numbers increased significantly after treatment. In gI CD4 cell numbers were 329±151/mmcr (t=0), 504±88 (24 w) and 575±205/mmcr (48w), while in gII they were 311±93/mmcr (t=0), 469±157/mmcr (24w) and 502±204/mmcr (48w). In gI CD4 naive cells (45RA+CD62L+) statistically significantly increased from 148±72/mmcr (t=0), to 257±110/mmcr (t=24w), reaching 330±120/mmcr at the end of therapy; in gII we found only a moderate increase of naive cells, from 143±84/mmcr (t=0) to 230±115/mmcr (t=24w) and to 238±90/mmcr (48w). Treatment including IL-2 was associated with a statistically significant increase of IL-16 production, as measured in culture supernatants from stimulated PBMCs and in the cytoplasm of CD8+ lymphocytes (flow cytometric assay).

Proposal № 68
A STUDY OF INDINAVIR PHARMACOKINETICS USING A POPULATION APPROACH.

University of Genova and Brescia, Oncology Center of Aviano, Hospitals of Monza and Ferrara, ISS-Rome.

Background: The study had the purpose of characterizing indinavir pharmacokinetics and the relationship between indinavir pharmacokinetic parameters and a number of demographic and physiopathologic parameters in a cohort of HIV infected patients.

Methods: Seventy-three patients were enrolled in 11 centres. Thirty-eight of the patients were observed in the ISS-IP-1 study.
Blood samples for indinavir PK were drawn at random following a steady-state administration (800mg q8h). A mean of 7 samples/patient (517 samples) were analysed. Blood samples were drawn in several occasions for some patients as follows (occasion/patient): 2/21, 3/13, 4/8 and 5/4. The data were analysed using a population approach (NONMEM) with a one compartment pharmacokinetic model parameterized in absorption rate constant (ka), clearance/F (Cl/F) and volume of distribution/ (V/F). Interindividual (IIV) and interoccasion (IOV) variabilities (IIV) in CL/F and V/F were fit with an exponential model. Residual variability fitted best with a slope-intercept error model. The influence of the following covariates on CL/F and V/F was tested: age weight, gender, tobacco smoking, risk factor, ALT, serum creatinine, total bilirubin, albumin, CD4+ lymphocytes, diarrhea, wasting syndrome, site, concomitant administration of fluconazole, TMP-SMX, phenobarbital, benzodiazepines, methadone, diuretics and gastric acid secretion inhibitors.

Results: The final model is the following:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CL/F</th>
<th>V/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.79</td>
<td>31.3</td>
</tr>
<tr>
<td>IIV</td>
<td>55%</td>
<td>47%</td>
</tr>
<tr>
<td>IOV</td>
<td>19%</td>
<td>25%</td>
</tr>
</tbody>
</table>

Residual variability: slope: 0.25 intercept: 0.127

The following covariates resulted significantly associated (p < .05) with CL/F: concomitant administration of phenobarbital (n=5, +64%) and observation at the Oncology Center of Aviano (n=10, +184%)

Discussion: Although a few covariates were significantly associated with low concentrations (higher CL/F) of indinavir, the interindividual variability unexplained on the basis of covariates remained extremely high. On the other hand, the variability in the same individual (IOV) was relatively low. These results suggest that monitoring of indinavir plasma concentration, with subsequent personalization of therapy based on such concentrations, may have an application in everyday clinical practice.

Proposta No.: 71
RITONAVIR TROUGH CONCENTRATION AS A PREDICTIVE FACTOR OF VIROLOGIC RESPONSE IN HIV-INFECTED CHILDREN.

1st Institute of Infectious Diseases c/o G. Gaslini Institute – University of Genoa.

Background: It is subject of debate whether therapeutic drug monitoring should be introduced in clinical practice for protease inhibitors. More pharmacodynamic data are needed to address this issue. We studied a number of factors, including ritonavir plasma levels, in their capability of predicting response to therapy in a small cohort of pediatric patients.

Methods: Eleven HIV-infected children tolerating ritonavir (in combination with 2 NRTIs) were studied. Demographic parameters were: age [median, (range)]: 10 (2-13) years, weight: 26 (10-38) kg, body surface area: 0.93 (0.47-1.21) m², body mass index: 16.8 (12.7-20.2), baseline CD4+: 137 (2-1390) cells/µl and %CD4+: 9.5 (0.4-32.4), and baseline viral load: 5.15 (4.30-6.18) log copies/ml. All patients had received intensive prior treatment with NRTIs but were protease inhibitor naive. Underlying therapy was modified upon ritonavir initiation in 5 patients. Ritonavir dose was 318 (266-409) mg/m² BID. Ritonavir peak (3.5 hours following administration) and trough (predose) plasma levels were determined by HPLC in one occasion at steady-state following a morning dose. Time normalized AUC below baseline (NAUC) of viral load was tested at 24 weeks of follow-up vs. demographic parameters and ritonavir levels by linear regression. The influence of underlying therapy (modified vs. not modified) was tested with the Mann Withney U test.

Results: Median NAUC was -0.25 log copies/ml/month. Only one patient had an increase in viral load of 0.37 log copies/ml/month. In the remaining patients NAUC ranged from -0.01 to -1.25 log copies/ml/month. Ritonavir peak and trough were 14.9 (3.2-31.4) and 5.0 (0.1-15.6) mg/L, respectively. Ritonavir trough was the only factor which appeared associated with NAUC (r=.54, p=.087).

Conclusions: Our observation of a higher decrease in viral load in patients with higher ritonavir trough concentration warrants further pharmacodynamic studies of protease inhibitors in pediatric patients.

Proposta No.: 71
EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF CHEMOKINE RECEIVER CXCR4 AND SDF1α LIGAND IN CULTURED TYPE I RAT ASTROCYTES, CORTICAL NEURONS AND CEREBELLAR GRANULE CELLS

Proposal N° 77

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Infection of HIV-1 is frequently associated with neuronal death responsible for AIDS dementia complex (ADC). The current opinion supports the hypothesis that an indirect mechanism exists to explain the neuronal cell death and that gp120 is one of the main candidates as mediator of HIV-1 neurotoxicity. We demonstrated that the treatment of rat cortical neurons, in presence of cocultures of type I astrocytes, with recombinant HIV-1gp120 induced neuronal damage. However the site of interaction of gp120, on neurons and astrocytes, where cellular death is mediated is still unknown. It has been demonstrated that some members of the chemokine receptors may act as coreceptors, with the CD4, to allow the entry of HIV-1 into the cells. These observations suggest a potential role for these proteins in the pathogenesis of HIV-1 and prompted us to study their production in the CNS cells. We studied the localization of chemokine receptors in the adult rat brain and we demonstrated that the CXCR4 and CCR5 receptors are expressed in different areas. Moreover we demonstrated, by RT-PCR, northern blotting and immunofluorescence the expression of CXCR4 and SDF1, its natural ligand, in type I rat astrocytes, cerebellar granule cells and cortical neurons. We observed that LPS and PMA treatment induces down regulation of CXCR4 transcription whereas the SDF1α messenger is up-regulated by LPS and unmodified by PMA. Chemokine signalling is linked to the generation of transient elevations of cytosolic Ca²⁺ level. To determine if the CXCR4 receptor expressed on glial and neuronal cells is functionally related to this pathway, we studied the modification of intracellular calcium concentration, by microfluorometric analysis, after stimulation with hSDF1α. Our results put in evidence that SDF1 stimulation induces a cellular response in astrocytes and cortical neurons whereas no signal was evident when cerebellar granule cells was stimulated.

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THE MOLECULAR PROFILE OF HHV-8+ PRIMARY EFFUSION LYMPHOMA REVEALS HISTOGENETIC AND PATHOGENETIC FEATURES OF THE DISEASE.

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Primary Effusion Lymphoma (PEL) is characterized by consistent infection of the tumor clone by HHV-8 and by selective tropism for the serous body cavities. Here we aimed at refining the histogenesis and pathogenesis of PEL. Although PEL fails to express conventional B-cell markers, immunogenotypic studies have confirmed its B-cell derivation. In particular, PEL harbors genotypic markers denoting B-cell transition through the germinal center (GC), including mutations of the S' non-coding regions of the BCL-6 proto-oncogene and somatic mutations of immunoglobulin gene hypervariable (IgV) regions. The rate and characteristics of BCL-6 S' mutations in PEL are similar to those of other lymphomas known to derive from the GC, whereas the molecular pattern of Ig somatic hypermutation is consistent with antigen stimulation and selection. These observations suggest that PEL derives from a GC-related B-cell but do not discriminate between GC and post-GC B-cells. Conversely, the post-GC nature of PEL cells is documented by the following phenotypic features: i) positive expression of CD138/syndecan-1 and co-expression of the c-MET tyrosine kinase and its ligand HGF, which, throughout the spectrum of mature B-cells, selectively cluster with the pre-terminal stages of B-cell differentiation; ii) absent expression of BCL-6 protein, which is expressed by GC B-cells but is absent in post-GC B-cells. In vitro stimulation of PEL cells with HGF induced a rapid increase of phosphorylation of the MET 145 kD E-chain, confirming functional integrity of the MET/HGF loop. Because HGF induces proliferation and motility, the MET/HGF signal pathway may affect the mitogenic and motogenic properties of PEL.

Apart from histogenetic relevance, molecular analysis of PEL bears also pathogenetic implications. In particular, the association of PEL with recurrent chromosomal alterations (trisomy 12, trisomy 7, abnormalities of 1q25-q27) suggests that PEL development requires genetic lesions of cellular loci and that HHV-8 infection, though an absolute requirement for the disease, is not sufficient for PEL development.

Finally, although EBV frequently infects PEL cells, the precise pathogenetic contribution of the virus to PEL development remains unclear. Infact, PEL cells fail to express the LMP-1 and EBNA-2 viral antigens which are transforming for B-cells. Also, in contrast to other EBV-infected lymphomas, molecular analysis of EBV heterogeneity has failed to reveal a preferential clustering of specific EBV variants with PEL cells.

Overall, these data indicate that: i) PEL is histogenetically related to germinal center, antigen experienced B-cells; ii) the pathogenesis of PEL involves alterations of cellular genes in addition to infection by HHV-8; iii) the pathogenicity of EBV, if any, does not proceed through the most common pathways associated with other types of AIDS-related lymphomas carrying EBV infection.

Proposta di Ricerca N° 85.
5’-NUCLEOTIDASES IN THE ACTIVATION OF NUCLEOSIDE ANALOGS

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5’-nucleotidases (NT) remove the phosphate group from the 5’ carbon atom of ribo- and deoxyribonucleoside monophosphates and produce nucleosides that leave the cell by facilitated diffusion across the plasma membrane. Nucleoside analogs (NA) exert their antiviral activity in the triphosphate form. The first step in their activation is the conversion to monophosphates by (deoxy)nucleoside kinases. NT counteract the reaction catalyzed by the kinases. However, NT may also contribute to NA activation by two mechanisms: 1) their direct phosphorylation in a phosphotransferase reaction where the phosphate removed from a physiologic substrate is transferred to the 5’C of the NA, 2) accumulation of the analog in the cellular nucleotide pool by preferential dephosphorylation of the physiologic substrates. We study the participation of two cytosolic nucleotidases - the high kₘ NT and the deoxynucleotidase- in the activation of NA. The high kₘ NT is the only cellular enzyme able to activate ddl to the nucleotide level. It does this in a phosphotransferase reaction using IMP as phosphate donor. This reaction is fairly inefficient. With the aim of finding conditions that improve the activation of ddl we have produced stable inducible cell lines transfected with the cDNA of the high kₘ NT. We found that when the enzyme level is twofold increased the phosphorylation of ddl increases about 20% relative to the control, whereas with higher NT levels (10-20 fold) the increased potential for ddl phosphorylation is counterbalanced by the depletion of IMP. A phosphotransferase activity has been reported also for the deoxyNT, the only known NT that prefers deoxy -5’-nucleotide substrates. We have cloned the cDNA and tested the phosphotransferase activity of the recombinant protein. No phosphotransferase activity was detected, although several purine nucleosides, including ddl, stimulated the nucleotidase reaction catalyzed by the enzyme. We are now testing if the deoxyNT contributes to NA activation by the second mechanism mentioned above. We will compare the substrate efficiency of AZT and D4T monophosphates relative to the physiologic pyrimidine substrates which we have already characterized kinetically.

Proposta n. 89
ANALYSIS OF T CELL RECEPTOR REPERTOIRE DURING HAART.


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The analysis of T cell receptor (TCR) repertoire during HAART showed that the immediate decline of viral load and the subsequent increase of CD4 lymphocytes were not followed by immediate modifications of TCR diversity. Even important augments of CD4 count (e.g., from 74/μl to 711/μl) were not followed by expansions of lymphocytes bearing specific TCRBV chains or by changes of TCR diversity. In most cases the monoclonal or polyclonal pattern of T lymphocytes observed before the beginning of the therapy is conserved after 6 months of treatment. This finding was found also a patient with viro-immunologic dissociation in which, however, the T cell repertoire was highly restricted in both CD4 and CD8 cells.

The therapy interruption was generally characterized by immediate T cell clonal expansions.

We are now evaluating the heterogeneity of different T cell subpopulations in those 13 patients (out of the 42 initially enrolled for this study) that are still under HAART. Only 6 showed a stable decrease of viral load and a constant increase of CD4 cell count and only one manifested a viro-immunologic dissociation. Therefore, we are following, during time, 17 further patients that started HAART about 9 mounts ago. Among they, there are two individuals who underwent surgical splenectomy and in which the TCR repertoires of splenic and circulating CD4+, CD8+, CD8+CD28-CD11b+ and CD8+CD28-CD11b- lymphocytes were very similar. HAART was successful in only one patient who showed, two month after splenectomy, a further increase of CD4 count (from 363/μl to 665/μl).

Proposta n. 90
PERSISTENT MONO/OLIGOCLONAL T-CELLS EXPANSIONS IN IDIOPATHIC CD4+ LYMPHOCYTOPENIA.

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The analysis of the T-cell diversity in HIV+ patients has been always complicated by the difficulty to discriminate between the effect of HIV and the one of opportunistic infections that may also have a profound impact in reshaping the repertoire. Thus, the availability of patients with severe CD4+ lymphocytopenia (ICL) that present the same immunodeficiency than the one caused by HIV, but in the absence of the viral agent, represents a new and important model for dissecting the causes that can influence repertoire modulation.

We identified two patients with ICL complicated by opportunistic infections that showed very low CD4 count (< 50/μl) since 1993, but manifested, over time, different outcomes. Sequential analysis of T-cell receptor (TCR) alfa/beta and gamma/delta T-cell repertoires demonstrated that the heterogeneity of T cells is constantly limited in both patients, but that the degree of disruption of T-cell repertoires seemed to influence the susceptibility to infections. In one patient, the expansion of TCRDV1+ lymphocytes, at the expense of other gamma/delta cells, together with the presence of mono or oligoclonal alfa/beta CD4 and CD8 cells appeared to make this individual unable to mount protective T-cell response to a Mycobacterium intracellulare infection. In the other patient, the initial presence of more diversified T-cell repertoires was followed by a broadening of TCR specificity and by a disease improvement. These results indicate that, as observed in HIV+ patients, the idiopathic loss of CD4+ lymphocytes could give rise to mono/oligoclonal T-cells expansions. Furthermore, it appears that the severity of T-cell repertoire disturbance could be more informative than CD4+ cells count as predictor of disease progression.

The recent recognizing of two further ICL patients and their immunological characterization could prove these conclusions.

Proposta n. 90
REGRESSION OF AIDS-RELATED KAPOSI'S SARCOMA FOLLOWING ANTIRETROVIRAL THERAPY WITH PROTEASE INHIBITORS: BIOLOGICAL CORRELATES OF CLINICAL OUTCOME.

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The clinical response of AIDS-related Kaposi's sarcoma (KS) to highly active antiretroviral therapy (HAART), a combination of HIV-1 protease and reverse transcriptase inhibitors, was studied in 11 patients, all but one with progressive KS. CD4+ cell counts, plasma HIV-1 RNA levels, and antibody titers to lytic ORF65 and latency-associated HHV-8 proteins were determined in sequential samples. Six complete and 3 partial clinical responses were achieved in a median time of 6 and 3 months, respectively, and confirmed after a median time of 16 months on HAART. Two patients showed disease progression. A consistent decrease in HIV-1 RNA levels, paralleled by an increase in CD4+ cell count, was observed in all patients who showed complete or partial clinical response; HIV-1 RNA levels remained persistently high in the two progressors, despite a change in HAART. HHV-8 antibody titers were generally higher in patients with mucosal/visceral involvement vs patients with limited disease; a decrease in ORF65 antibody titer was significantly associated with a clinical response. These results indicate that HAART is effective for AIDS-related KS; the clinical response correlates with a decrease in plasma HIV-1 RNA levels, an increase in CD4+ lymphocytes, and a decrease in antibodies to ORF65 HHv-8 protein.

Proposal n. 95