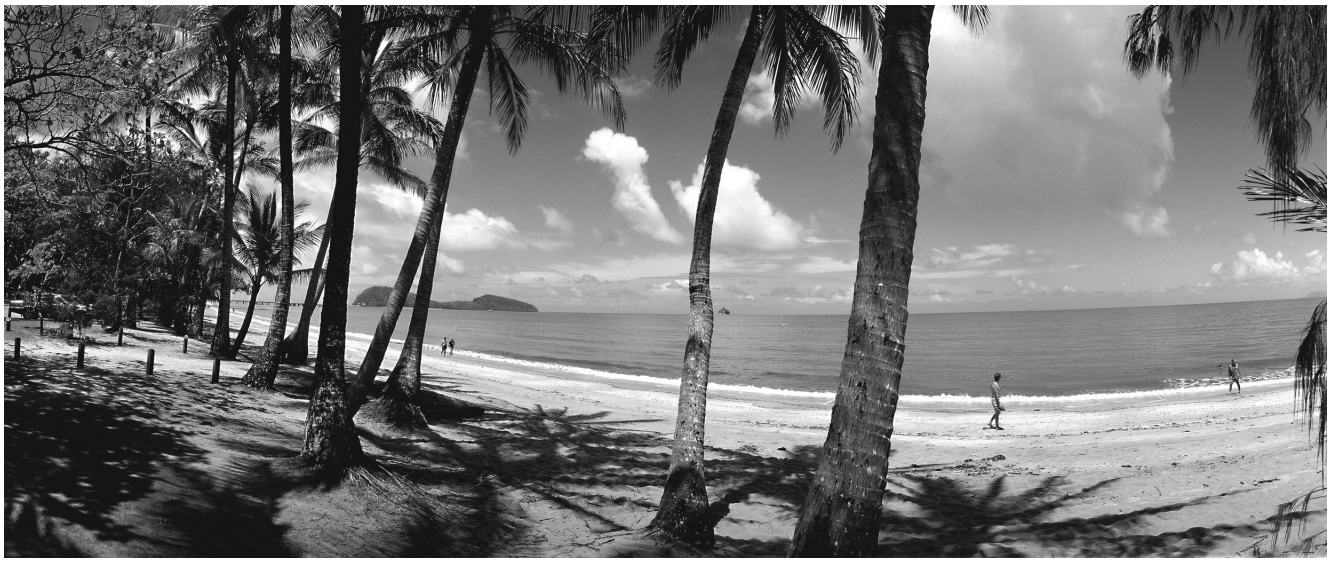
WELCOME TO CAIRNS

On behalf of the Organising Committee I would like to offer delegates to the 10th International EBV conference a big Aussie welcome. This year for the first time, The Herpesvirus Workshop will immediately follow our meeting. While most of the general format of previous meetings has been preserved, we have introduced a few innovations. Firstly, all abstracts have been reviewed and assessed by a series of panels independent of the organisers. Secondly, you will notice that there has been a major effort to showcase the next generation of investigators. Thirdly, two topics on which there are a variety of opinions have been selected as the basis of two Workshops. Here, the discussion Chair will give a brief introduction and then develop the issues with the help of the day's session Chairs and you, the delegates. The expectation is that these Workshops will become a forum for lively debate. And fourthly, the poster presentations, while remaining an important feature of the meeting, will be promoted as both a scientific and social occasion by introducing food and wine to complement viewing and conversation.

Although the formal science of the meeting will be of primary importance, not far behind will be an opportunity to absorb some of the atmosphere of Cairns in the company of your colleagues. We have organised a full day trip to the reef and the Symposium Dinner will be held at the Tjapukai Cultural Park. Of course, those of you who intend to make a holiday of it, Cairns is the ideal starting point to explore north Queensland. The world heritage listed Daintree Rainforest is an easy drive to the north. To the west, you can take the Kuranda Scenic Train to ascend the Kennedy Range through hand hewn tunnels and past magnificent waterfalls on the way to Kuranda. The social program will provide an opportunity to visit some of the spectacular scenery surrounding Cairns.

The Organising Committee would like to thank the sponsors and those who have contributed to the planning of this meeting. We sincerely hope that everyone has tremendous fun.

Tom Sculley
on behalf of the EBV2002 Organising Committee



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Associate Professor Gillian Bushell	Dr Ihor Misko
Professor Kenzo Takada	Dr Tony Simmons

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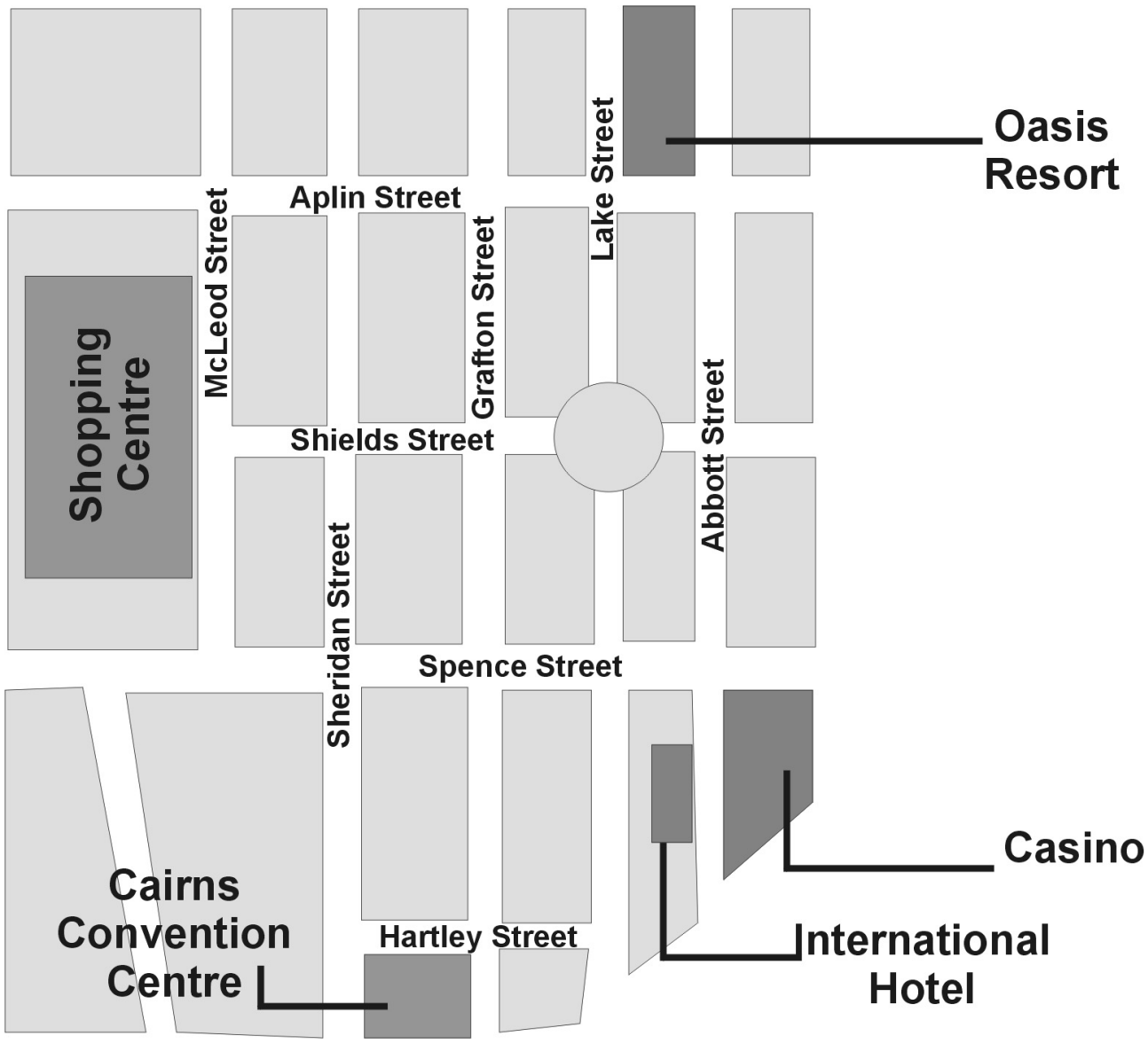
Herpesvirus Workshop

The Atlantic Foundation

SESSION CHAIRS

Session 1 Burkitt's Lymphoma	Paul Murray	Ingemar Ernberg
Session 2 Transformation	Tom Sculley	Clare Sample
Session 3 NPC	Maria Lung	Jen-Yang Chen
Session 4 Malignancy	Sam Choon-kook	Richard Ambinder
Session 5 Immunosurveillance	Kai-Tai Yao	Maria Masucci
Session 6 Disease Associations	Hans Wolf	Sofia Haryana
Session 7 Primary Infection	Andy Morgan	John Sixbey
Session 8 Infection and Reactivation	Bill Sugden	David Rowe
Session 9 Hodgkin's Disease	Jaap Middeldorp	Paul Farrell
Session 10 Latency	Martin Rowe	Ann Ricksten
Session 11 Epithelial Tumours	Joseph S. Pagano	Shih-Tung Liu
Session 12 Viral Replication	George Miller	Ya Cao
Session 13 Immune Mechanisms	Andrew Hislop	Scott Burrows
Session 14 Comparative Systems	Fred Wang	Takeshi Sairenji
Session 15 Oncogenesis/Cell Cycle	Nancy Raab-Traub	Richard Longnecker
Session 16 Herpesvirus and Transplantation	Dorothy Crawford	Alan Rickinson

CAIRNS MAP


Oasis Resort
Aplin Street
McLeod Street
Lake Street
Grafton Street
Shopping Centre
Shields Street
Abbott Street
Sheridan Street
Spence Street
Casino
Cairns Convention Centre
Hartley Street
International Hotel

WEDNESDAY 17TH JULY

Session 1 Burkitt's Lymphoma

8:30	CELLULAR PROTEIN INTERACTIONS WITH EBNA1	**L. Frappier**
9:00	CELLULAR PATHWAYS TARGETED BY EBV IN BURKITT LYMPHOMA	**I. K. Ruf**
9:15	SH2D1A IS EXPRESSED IN EBV POSITIVE GROUP I BL LINES AND IS DOWN-REGULATED IN PARALLEL WITH SHIFT TO THE IMMUNOBLASTIC PHENOTYPE	**N. Nagy**
9:30	EBNA-1 IS REQUIRED FOR SURVIVAL OF BURKITT'S LYMPHOMA CELLS AND INHIBITS APOPTOSIS IN THE ABSENCE OF THE VIRAL GENOME	**G. Kennedy**
9:45	REGULATION OF THE RUNX (AML) TRANSCRIPTION FACTORS FOLLOWING EBNA-2 ACTIVATION	**L. Spender**

Session 2 Transformation

10:30	EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN-6 CO-LOCALIZES WITH THE SURVIVAL OF MOTOR NEURON (SMN) PROTEIN	**T. Sculley**
10:45	EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 5 INHIBITS PRE-mRNA CLEAVAGE AND POLYADENYLATION	**J. Flodin**
11:00	IDENTIFICATION AND CLONING OF A NOVEL CHROMATIN ASSOCIATED PROTEIN PARTNER OF EBNA2	**B. Kwiatk-owski**
11:15	EBNA-3 FAMILY PROTEINS TARGET GENE-SILENCING COMPLEXES	**M. Allday**
11:30	NEGATIVE REGULATION OF MITOTIC KINASE CDC2 BY EPSTEIN-BARR VIRUS EBNA2 PROTEIN	**C-S Lin**
11:45	METHYLATION-DEPENDENT BINDING OF EBNA2 TO THE SURVIVAL MOTOR NEURON PROTEIN (SMN)	**F. Grässer**

Session 3 NPC

1:30	TUMOR-SUPPRESSIVE ACTIVITY LOCALIZED TO CHROMOSOME BAND 11Q22-23 IN NASOPHARYNGEAL CARCINOMA	**L. Lung**
1:45	INTERFERON REGULATORY FACTOR 7 HAS ONCOGENIC PROPERTIES AND IS EXPRESSED IN HUMAN NASOPHARYNGEAL CARCINOMA	**L. Zhang**
2:00	EXPRESSION OF A FUNCTIONAL C-KIT RECEPTOR IN A SUBGROUP OF EBV-ASSOCIATED NASOPHARYNGEAL CARCINOMAS	**P. Busson**
2:15	EPIGENETIC AND GENETIC INACTIVATION OF *BLU*, A TUMOR SUPPRESSOR GENE CANDIDATE ON THE 3P21 LOCUS, IN NASOPHARYNGEAL CARCINOMA (NPC)	**Q. Tao**
2:30	A RECOMBINANT MVA-EBNA1/LMP2 VACCINE TO BOOST T CELL RESPONSES AGAINST EBV-POSITIVE NASOPHARYNGEAL CARCINOMA	**G. Taylor**
2:45	CLONING AND ANALYZING THE REGULATORY REGION OF THE GENE SPECIFICALLY EXPRESSED IN MOUSE NASOPHARYNX	**K. Yao**

Session 4 Malignancy

3:30	THE ACTION OF EBNA-1 AS AN ONCOGENE *IN VIVO*	**P. Tsimbouri**
3:45	ACTIVATION OF THE PI3-KINASE/AKT PATHWAY BY EBV LATENT MEMBRANE PROTEIN 2A	**J. Harrelson**
4:00	VP22 BASED GENE THERAPY FOR EPSTEIN-BARR VIRUS ASSOCIATED MALIGNANCIES	**R. J. Jones**
4:15	INDUCTION OF C-MET PROTO-ONCOGENE BY EBV LMP-1 AND THE CORRELATION WITH CERVICAL LYMPH NODE METASTASIS OF NASOPHARYNGEAL CARCINOMA	**T. Yoshizaki**
4:30	EBV LATENCY CONTROL AS A TUMOR RISK FACTOR	**I. Ernberg**
4:45-5:30	**Workshop 1 - Does EBV cause Tumours ?**	**L. Young Discussion Chair**

7:00pm – 9:00pm **Poster Session 1 - Cairns International Hotel (Dinner served)**

Presenters (odd numbered) should be at their posters between 7:00 – 8:00pm

THURSDAY 18TH JULY

Session 5 Immunosurveillance

8:30	EPSTEIN-BARR VIRUS AND B CELL LYMPHOMAS : AN UPDATE	**A. Rickinson**
9:00	INDUCTION OF MHC CLASS-I AND -II RESTRICTED EPITOPE PRESENTATION BY UREA-TREATED BZLF1 PROTEIN: A NOVEL TECHNOLGY FOR THE DETECTION OF PROTEIN-SPECIFIC CYTOTOXIC T-CELLS	**B. Bauer**
9:15	PROTEASOME INHIBITORS RECONSTITUTE THE PRESENTATION OF CTL EPITOPES IN EPSTEIN-BARR VIRUS ASSOCIATED TUMORS	**R. Gavioli**
9:30	EPSTEIN-BARR VIRUS INHIBITS THE DEVELOPMENT OF DENDRITIC CELLS BY PROMOTING APOPTOSIS OF THEIR MONOCYTE PRECURSORS IN THE PRESENCE OF GM-CSF AND IL-4	**V. Levitsky**
9:45	EBNA-2 DEPENDENT TRANSCRIPTIONAL ACTIVATION OF HERV-K18 SUPERANTIGEN	**N. Sutkowski**

Session 6 Disease Associations

10:30	INCREASED VIRAL LOAD OF EBV AS WELL AS KSHV IN THOSE AT RISK OF KAPOSI'S SARCOMA (KS)	**D. Whitby**
10:45	*IN VIVO* TRANSCRIPTION OF THE EPSTEIN-BARR VIRUS (EBV) BAMHI-A REGION WITHOUT (RK-) BARF0 PROTEIN EXPRESSION IN EBV-ASSOCIATED DISORDERS	**J. van Beek**
11:00	ACTIVATION OF TRAFs SIGNALING PATHWAY IN EBV-INFECTED T CELL: IMPLICATION FOR HEMOPHAGOCYTIC SYNDROME	**Ih-Jen Su**
11:15	THE β-CATENIN PATHWAY IS ACTIVATED IN EBV TYPE III LATENCY	**J. Shackelford**
11:30	IMMUNODOMINANT CD4 T CELL RESPONSE TO THE EBV LYTIC ANTIGEN BHRF1 IN DR*0401 INDIVIDUALS EVIDENCED BY IMMUNOMAGNETIC SORTING WITH MHC CLASS II TETRAMERS	**E. Houssaint**
11:45	NOVEL *IN VIVO* BINDING SITE FOR C-MYC IN THE EBER-LOCUS SUGGESTS SPECIFIC ROLE FOR EBV IN TUMORIGENESIS	**H. H. Niller**

Session 7 Primary Infection

1:30	EPSTEIN-BARR VIRUS mRNA EXPORT FACTOR EB2 IS ESSENTIAL FOR PRODUCTION OF INFECTIOUS VIRUS	**H. Gruffat**
1:45	ANALYSES OF THE ACQUISITION, PERSISTENCE, AND COMPARTMENTALIZATION OF EBV STRAINS USING A HETERODUPLEX TRACKING ASSAY	**D. Sitki-Green**
2:00	MULTIPLE EBV INFECTIONS IN HEALTHY INDIVIDUALS	**D. Walling**
2:15	CHARACTERIZATION OF EPSTEIN-BARR VIRUS-INFECTED GERMINAL CENTER B-CELLS DURING INFECTIOUS MONONUCLEOSIS	**J. Kurth**
2:30	PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) EXPRESS RECOMBINASE ACTIVATING GENES (RAG) 1 AND 2 DURING EBV-INDUCED INFECTIOUS MONONUCLEOSIS (IM)	**R. S. Scott**
2:45	DEVELOPING A POLYTOPE VACCINE FOR EPSTEIN-BARR VIRUS ASSOCIATED INFECTIOUS MONONUCLEOSIS	**M. Bharadwaj**

Session 8 Infection and Reactivation

3:30	MECHANISM OF REACTIVATION OF EPSTEIN-BARR VIRUS FROM LATENCY	**H. Bryant**
3:45	MOLECULAR DIAGNOSIS OF EPSTEIN-BARR VIRUS (EBV) REACTIVATIONS IN HEALTHY EBV CARRIERS	**W. J. Jabs**
4:00	PHOSPHATIDYLINOSITOL 3- KINASE ACTIVITY DETERMINES SENSITIVITY OF BURKITT'S LYMPHOMA CELLS TO B CELL RECEPTOR -MEDIATED EBV ACTIVATION	**D. Iwakiri**
4:15	IMMUNE ACTIVATION CORRELATED WITH CLINICAL EVENTS IN ACUTE INFECTIOUS MONONUCLEOSIS	**H. Williams**
4:30	EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 INDUCES AND CAUSES RELEASE OF FIBROBLAST GROWTH FACTOR-2	**J. Pagano**
4:45-5:30	**Workshop 2 - Is an EBV vaccine plausible?**	**R. Khanna Discussion Chair**

7:00pm – 9:00pm **Poster Session 2 - Cairns International Hotel (Dinner served)**

Presenters (even numbered) should be at their posters between 7:00 – 8:00pm

SATURDAY 20TH JULY

Session 9 Hodgkin's Disease

8:30	EBV AND EPITHELIOID MALIGNANCIES	**K. Takada**
9:00	CONTRIBUTION OF THE FAS/FAS LIGAND PATHWAY TO IMMUNE ESCAPE OF EBV-INFECTED HODGKIN/REED-STERNBERG CELLS	**A. Dutton**
9:15	CELL-FREE EBV IS DETECTED IN PATIENTS WITH EBV(+) HODGKIN'S LYMPHOMA (HL) YEARS IN ADVANCE OF DIAGNOSIS	**R. Ambinder**
9:30	INTRACLONAL HETEROGENEITY SUGGESTIVE OF AN ONGOING MUTATIONAL PROCESS CHARACTERIZES THE LATENT MEMBRANE PROTEIN-1 GENE OF HIV-RELATED HODGKIN'S DISEASE	**R. Dolcetti**
9:45	EPSTEIN-BARR VIRUS (EBV)-SPECIFIC CYTOTOXIC T CELLS (CTL) TO DELIVER TRANSGENIC IL-12 TO EBV-POSITIVE HODGKIN'S DISEASE	**H. J. Wagner**

Session 10 Latency

10:30	THE EPSTEIN BARR VIRUS RK-BARF0 INDUCES DEGRADATION OF SPECIFIC INTERACTING CELLULAR PROTEINS	**N. Raab-Traub**
10:45	NOVEL CELLULAR TARGETS OF EBV ENCODED, TRANSFORMATION ASSOCIATED PROTEINS	**L. Szekely**
11:00	EPSTEIN-BARR VIRUS EBNA2 BLOCKS NUR77 MEDIATED APOPTOSIS	**J. M. Lee**
11:15	IDENTIFICATION OF EBV-INDUCED GENES: ROLE OF LMP1 AND NF-κB	**E. Cahir-McFarland**
11:30	AN ANALYSIS OF EBV LATENT MEMBRANE PROTEIN 2A (LMP2A) LIPID RAFT LOCALIZATION AND FATTY-ACID MODIFICATION	**R. Katzman**
11:45	EBNA1 PARTITIONS PLASMIDS IN YEAST BY ATTACHING TO EBP2 ON MITOTIC CHROMOSOMES	**P. Kapoor**

Session 11 Epithelial Tumours

1:30	THE EBV DNA POLYMERASE PROCESSIVITY FACTOR, BMRF1, ACTIVATES EXPRESSION OF GASTRIN	**S. Kenney**
1:45	INHIBITION OF FAS-MEDIATED APOPTOSIS IN EBER-EXPRESSING EPITHELIAL CELLS	**A. Nanbo**
2:00	ARE CLONALITY DETERMINATIONS IN EBV-ASSOCIATED EPITHELIAL CELL MALIGNANCIES AN ARTIFACT OF LMP-2A DIRECTED EPISOME SELECTION?	**C. A. Moody**
2:15	GENES ASSOCIATED WITH GENETIC SUSCEPTIBILITY TO NASOPHARYNGEAL CARCINOMA ARE LOCATED PRIMARILY WITHIN THE HLA-A LOCUS IN TAIWANESE	**C-C. Lu**
2:30	THE PARADOXICAL ROLE OF LMP1 IN CARCINOGENESIS	**J. B. Wilson**
2:45	A COMPARISON OF INFECTION OF CELLS VIA LYMPHOCYTE AND EPITHELIAL RECEPTORS	**L. M. Hutt-Fletcher**

Session 12 Viral Replication

3:30	**HENLE LECTURE** REGULATION OF EXPRESSION AND MECHANISM OF ACTION OF THE EPSTEIN-BARR VIRUS LYTIC CYCLE ACTIVATOR, ZEBRA	**G. Miller**
4:30	INHIBITION OF THE EBV LYTIC CYCLE SWITCH PROTEIN ZTA	**A. Sinclair**
4:45	THE ZINC FINGER E-BOX-BINDING PROTEIN, ZEB, NEGATIVELY REGULATES THE LYTIC SWITCH BZLF1 GENE PROMOTER OF EPSTEIN- BARR VIRUS.	**R. Kraus**
5:00	THE EPSTEIN-BARR VIRUS ZEBRA PROTEIN ACTIVATES TRANSCRIPTION FROM THE EARLY LYTIC F PROMOTER BY BINDING TO A PROMOTER-PROXIMAL AP-1-LIKE SITE	**H. Zetterberg**
5:15	CHARACTERIZATION OF DOMAINS OF THE EB2 PROTEIN REQUIRED FOR mRNA EXPORT: NLS, NES, RNA-BINDING AND INTERACTION WITH REF AND TAP	**E. Hiriart**

6:30-10:30 **Symposium Dinner - Tjapukai Cultural Park**

Bus pick up at the Oasis Resort at 6:30pm

SUNDAY 21ST JULY

Session 13 Immune Mechanisms

8:30	EPSTEIN-BARR VIRUS GP42 OCCURS AS A TYPE II MEMBRANE PROTEIN AND AS A SOLUBLE SECRETED PROTEIN AND INHIBITS MHC CLASS II RESTRICTED T CELL ACTIVATION	**E. Wiertz**
8:45	BZLF1 INHIBITS THE ABILITY OF LMP1 TO UPREGULATE EXPRESSION OF MHC IN LYTIC CYCLE	**M. Rowe**
9:00	IMMUNIZATION WITH EPSTEIN-BARR VIRUS-SPECIFIC PEPTIDE PULSED-DENDRITIC CELLS INDUCED FUNCTIONAL CYTOTOXIC T CELL IMMUNITY AND TUMOR REGRESSION IN PATIENTS WITH NASOPHARYNGEAL CARCINOMA	**C. L. Lin**
9:15	DIFFERENTIAL RECOGNITION OF EBNA1 BY HUMAN T AND B-CELL RESPONSES: IMPLICATIONS FOR ANTIGEN-PROCESSING AND PRESENTATION IN VIVO.	**J.Middeldorp**
9:30	GENERATION OF IMMUNOTHERAPEUTIC CTL POPULATIONS SPECIFIC FOR LMP2 BY RETROVIRAL TRANSDUCTION OF THE TCR.	**R. Orentas**
9:45	CYTOLYTIC $CD4^+$ T CELL CLONES SPECIFIC FOR EBNA 1 INHIBIT EPSTEIN-BARR VIRUS-INDUCED B CELL PROLIFERATION	**S. Nikiforow**
10:00	EBNA1 SPECIFIC $CD4^+$ TH1 CELLS KILL EBV ASSOCIATED BURKITT'S LYMPHOMA THAT EVADES $CD8^+$ CTL RECOGNITION	**C. Münz**

Session 14 Comparative Systems

10:45	GAMMAHERPESVIRUS CAPSID STRUCTURE AND PROTEIN COMPOSITION: COMPARISON OF RHESUS MACAQUE RHADINOVIRUS (RRV) AND KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV)	**D. Kedes**
11:00	THE COMPLETE GENOMIC SEQUENCE OF THE FIRST EPSTEIN-BARR-RELATED HERPESVIRUS NATURALLY INFECTING A NEW WORLD PRIMATE: A DEFINING POINT IN THE EVOLUTION OF ONCOGENIC LYMPHOCRYPTOVIRUSES	**P. Rivailler**
11:15	TUMORIGENESIS AFTER EXPERIMENTAL INFECTION IN AN EPSTEIN-BARR VIRUS PRIMATE ANIMAL MODEL	**F. Wang**
11:30	COMBINATION OF EPSTEIN-BARR VIRUS-ENCODED BARF1 GENE AND H-*RAS* IS NECESSARY TO TRANSFORM A PRIMARY MONKEY EPITHELIAL CELL	**G. Cabras**
11:45	CHARACTERIZATION OF CIS AND TRANS-REQUIREMENTS FOR KSHV LATENT ORI FUNCTION	**R. Renne**
12:00	NEW DATABASE OF ANNOTATIONS OF VIRAL GENOMES: HERPESVIRUS SECTION	**M. Borodovsky**

Session 15 Oncogenesis/Cell Cycle

2:00	LMP1 STIMULATION OF THE PI3-KINASE PATHWAY ACTIVATES DIVERSE CELLULAR TARGETS SUCH AS AKT AND THE SMALL RHO GTPASES	**C. Dawson**
2:15	C/EBPa MEDIATES ZTA-INDUCED CELL CYCLE ARREST	**G. Hayward**
2:30	HUMAN CYTOMEGALOVIRUS pp71 DEGRADES THE Rb TUMOR SUPPRESSORS THROUGH A PROTEASOME-DEPENDENT, UBIQUITIN-INDEPENDENT MECHANISM, AND STIMULATES CELL CYCLE PROGRESSION.	**R. Kalejta**
2:45	LMP1 SIGNALS FROM AN INTRACELLULAR COMPARTMENT	**N. Lam**
3:00	SELF-SUSTAINING LMP1 EXPRESSION VIA IL-6 INDUCTION OF STAT 3 AND STAT5	**S. Hayward**

Session 16 Herpesvirus and Transplantation

3:45	POST TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS ARE FREQUENTLY DERIVED FROM B CELLS CARRYING RANDOMLY MUTATED OR NON-FUNCTIONAL IMMUNOGLOBULIN GENES	**A. I. Bell**
4:00	RECURRENCE OF HIGH VIRUS LOAD AND LYMPHOPROLIFERATIVE DISEASE (LPD) AFTER SUCCESSFUL TREATMENT WITH HUMANIZED ANTI-CD20 IN PEDIATRIC LIVER TRANSPLANT RECIPIENTS.	**B. Savoldo**
4:15	PORCINE CYTOMEGALOVIRUS IN PIGS BEING BRED FOR XENOGRAFT ORGANS: PROGESS TOWARDS CONTROL	**J. F. Fryer**
4:30	THE SEARCH FOR PORCINE GAMMA-HERPESVIRUSES AND IMPLICATIONS FOR XENOTRANSPLANTATION: CHARACTERISATION OF THE NOVEL PORCINE LYMPHOTROPIC HERPESVIRUSES (PLHV-1, -2 AND -3)	**M. Goltz**
4:45	STEM-CELL TRANSPLANT RECIPIENTS NOT RECOVERING EBV-SPECIFIC IMMUNITY ARE AT RISK TO DEVELOP HIGH LEVEL EBV VIRAL LOAD AND LYMPHOPROLIFERATIVE DISEASE	**P. Meij**
5:00	PARTIALLY HLA-MATCHED ALLOGENEIC CYTOTOXIC T CELLS FOR THE TREATMENT OF EBV-POSITIVE POST TRANSPLANT LYMPHOPROLIFERATIVE DISEASE	**T. Haque**

ORAL SESSIONS ABSTRACTS

CELLULAR PROTEIN INTERACTIONS WITH EBNA1

M. N. Holowaty[1], M. Zeghouf[2], V. Athanasopoulos[1], J. Greenblatt[2] and L. Frappier[1]

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EBNA1 plays several roles in latent EBV infection, including the replication and segregation of the viral episomes, transcriptional activation and regulation of viral latency genes, and escape of host immune detection due to the failure of EBNA1 to be processed by the proteasome. All of these functions likely involve specific interactions with cellular proteins. A few cellular proteins that bind EBNA1 have been identified using yeast two-hybrid screens, but these cellular proteins cannot account for all of the functions of EBNA1. We have recently taken two additional approaches to profile cellular proteins that bind EBNA1. First, we identified by mass spectrometry the cellular proteins that are specifically retained on an EBNA1 affinity column, and eliminated those that occur through nucleic acid and non-specific charge interactions. This approach revealed a previously unknown interaction between EBNA1 and the nuclear ubiquitin-specific protease, USP7 (also called HAUSP), a known target of herpes simplex virus. This interaction suggests that EBNA1 might avoid targeting to the proteasome due to the removal of conjugated ubiquitin from EBNA1, and/or that EBNA1 might affect the turnover of cellular proteins by sequestering USP7. Secondly, we adapted a tandem affinity purification (TAP) approach for mammalian cells and used it to purify TAP-tagged EBNA1 from human cells in complex with associated proteins. This approach confirmed the interaction with USP7. The current state of our understanding of the EBNA1-USP7 interaction will be discussed.

NOTES:

CELLULAR PATHWAYS TARGETED BY EBV IN BURKITT LYMPHOMA

I. K. Ruf and J. T. Sample
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Tumorigenic potential of the Burkitt lymphoma (BL) cell line Akata is dependent upon the restricted latency program of EBV, which is characterized by expression of EBNA-1, EBER-1 and EBER-2 (EBERs) and low levels of the *Bam*HI A rightward transcripts. Expression of the EBERs in the context of EBV-negative Akata cells enhances tumorigenic potential, however, this effect is only partial relative to EBV infection, suggesting that additional genes are required to fully restore tumorigenicity. We have previously demonstrated that EBV likely contributes to tumorigenicity through the protection of cells from c-MYC-induced apoptosis under growth-limiting conditions. Our recent data suggests that this is accomplished via a translational down-regulation of c-MYC expression. Consistent with the failure of EBERs to fully restore tumorigenic potential, EBER expression has no effect on c-MYC and consequently is unable to measurably protect cells from apoptosis following serum withdrawal. These findings implicate the coordinate action of several viral gene products as well as the targeting of multiple cellular pathways by EBV to promote lymphomagenesis. We have utilized a transcriptional profiling approach to identify cellular genes and pathways targeted by EBV infection, and specifically by expression of the EBERs. We have identified a number of genes involved in cellular antiviral response pathways as being downregulated in EBV-positive cells due to expression of the EBERs. We have also found that EBV mediates additional effects on the expression of these and other genes which can not be reconstituted by expression of the EBERs in EBV-negative cells. We are currently investigating the mechanisms utilized by EBV to mediate these effects as well as their significance to EBV persistence and tumorigenesis in BL.

NOTES:

SH2D1A IS EXPRESSED IN EBV POSITIVE GROUP I BL LINES AND IS DOWN-REGULATED IN PARALLEL WITH SHIFT TO THE IMMUNOBLASTIC PHENOTYPE

N. Nagy[1], A. Maeda1[2], K. Bandobashi[1], L. L. Kis[1], J. Nishikawa[1], P. Trivedi[3], K. Takada[4], G. Klein[1] and E. Klein[1]

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The SH2 domain containing SH2D1A protein has been characterized in relation to the X-linked lymphoproliferative disease (XLP). Mutations in this gene are believed to be the cause of the disease.

Considering that XLP manifests mainly after EBV infection and that B cells are the major targets of EBV infection, we addressed the question whether SH2D1A is expressed in certain B cell subsets and whether EBV infection affects SH2D1A expression.

We did not find any normal B cell population that expressed the SH2D1A protein. However, SH2D1A was detected in 7/9 EBV positive group I Burkitt lymphoma lines, but not in the 11 EBV positive group II/III lines tested. The 9 EBV negative BL lines were also devoid of SH2D1A.

Motivated by these differences, we studied the impact of EBV and of the cellular phenotype on SH2D1A expression. Induction of immunoblastic markers on the group I BL cells by LMP1 transfection or CD40 ligation was accompanied by down-regulation of SH2D1A. It seems therefore that the presence of EBV and the phenotype of the cell together regulate SH2D1A expression in the BL cells. It is possible that SH2D1A is expressed in a narrow window of B cell development represented by germinal center cells.

NOTES:

Session 1: Burkitt's Lymphoma

EBNA-1 IS REQUIRED FOR SURVIVAL OF BURKITT'S LYMPHOMA CELLS AND INHIBITS APOPTOSIS IN THE ABSENCE OF THE VIRAL GENOME

J. Komano, G. Kennedy and B. Sugden

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Epstein-Barr virus (EBV)-encoded nuclear antigen-1 (EBNA-1) contributes both to the replication of EBV DNA and to transcription of most viral genes expressed during latent infection of cells infected *in vitro*. However, its contribution to the highly evolved Burkitt's lymphoma tumor cells has been uncertain. We have demonstrated that introduction of a dominant, negative derivative of EBNA-1 inhibits proliferation in 10- of 11- normal- and tumor-derived EBV-positive B cells, but has no effect on 6 EBV-negative lymphoma cells. This inhibition of proliferation depends on the efficiency with which the dominant, negative derivative of EBNA-1 is expressed. EBNA-1's function is, therefore, required for proliferation of EBV-positive cell lines. Surprisingly, we find that inhibition of EBNA-1's function induces apoptosis of EBV-positive cells measured morphologically, by detection of active caspase 3, and by binding of annexin V to intact cells. In agreement with these findings, EBNA-1, in the absence of other EBV genes, also prevents apoptosis induced by p53 in two cell lines, SAOS-2 and VM-10, by 50% ($p \leq 0.01$). These findings indicate that EBNA-1 itself can inhibit apoptosis. Given that EBNA-1 is expressed in all EBV-associated malignancies and is now shown to be required for the proliferation of multiple EBV-infected B cell lines, inhibitors of EBNA-1 should constitute potential treatments for EBV-associated malignancies.

NOTES:

REGULATION OF THE RUNX (AML) TRANSCRIPTION FACTORS FOLLOWING EBNA-2 ACTIVATION

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Novel targets of EBNA-2 were identified by microarray expression profiling of the conditional EBNA-2 cell line EREB2.5. The transcription factor AML-2 (RUNX3) was induced following estrogen activation of EBNA-2. This was independently confirmed by RT-PCR and induction occurred both in the presence and absence of protein synthesis inhibitors indicating that AML-2 is potentially a direct target of EBNA-2. AML-2 protein induction in EREB2.5 cells was observed 4 hours after estrogen addition.

AML-2 was also induced following EBV infection of primary B cells. Quiescent B cells initially expressed high levels of another member of the AML family, AML-1, but this protein was rapidly downregulated upon infection as the levels of AML-2 increased. Analysis of Burkitt Lymphoma (BL) and lymphoblastoid cells lines showed that they also exhibit reciprocal expression of AML proteins. EBV negative and Group I BL expressed predominantly AML-1 while Group III BL and LCLs expressed AML-2. This is the first example of a transcription factor that distinguishes between the different phenotypes.

We are currently investigating how EBV induces AML-2 and whether AML-2 has a role in immortalisation. In addition we are investigating whether expression of either AML-1 or AML-2 may contribute to the BL Group I/III phenotypes.

NOTES:

EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN-6 CO-LOCALIZES WITH THE SURVIVAL OF MOTOR NEURON (SMN) PROTEIN

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The EBNA-6 protein is essential for Epstein-Barr virus induced immortalization of primary human B-lymphocytes *in vitro*. Previous studies have shown that EBNA-6 acts as a transcriptional regulator of viral and cellular genes and a number of functional domains of the 140 KDa EBNA-6 protein have been characterized. In this study, fusion proteins of EBNA-6 with green fluorescent protein (GFP) have been used to characterize its nuclear localization and organisation within the nucleus. EBNA-6 associates with nuclear structures, and in immunofluorescence demonstrates a punctate staining pattern. Herein we show, that the association of EBNA6 with these nuclear structures was maintained throughout the cell cycle and with the use of GFP-E6 deletion mutants, that the region amino acids 626-940 of EBNA-6, contains a domain that can influence the association of EBNA6 with these nuclear structures. Co-immunofluoresence and confocal analyses demonstrated that nuclear SMN (survival of motor neurons protein) co-localizes to the EBNA-6 containing nuclear structures and that EBNA-6 appeared to influence the amount of SMN present in the nuclei of cells.

NOTES:

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EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 5 INHIBITS PRE-mRNA CLEAVAGE AND POLYADENYLATION

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The long-standing suspicion that Epstein-Barr virus nuclear antigen 5 (EBNA5) is involved in transcription regulation was recently confirmed by the observation by several groups that EBNA5 co-operates with EBNA2 in the activation of the LMP1 promoter. In attempts to elucidate the molecular basis for the EBNA5-mediated enhancement of EBNA2 transactivation, we obtained evidence of an additional function of EBNA5: at high but still biologically relevant levels, EBNA5 acted as a repressor of gene expression by interfering with the processing of pre-mRNA. Transient transfections with reporter plasmids revealed that EBNA5 repressed reporter mRNA and protein expression in the cytoplasm, but did not lower the steady state level of reporter RNA in the total cellular RNA fraction. Using the RNase protection assay with a probe comprising the pre-mRNA cleavage and polyadenylation site, EBNA5 was found to inhibit 3´-end cleavage and polyadenylation of pre-mRNAs from the reporter plasmids investigated. The effect of inhibitory levels of EBNA5 on chromosomal genes was examined in transient transfections by expression profiling using a cDNA microarray panel containing 588 genes. Of the 145 panel genes expressed in the cells only 2 were repressed showing that EBNA5 can inhibit expression of genes in the chromosomes. Thus, a regulatory mechanism must exist in the cells that confers specificity to the selection by EBNA5 of target genes for repression.

NOTES:

IDENTIFICATION AND CLONING OF A NOVEL CHROMATIN ASSOCIATED PROTEIN PARTNER OF EBNA2

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A novel human protein interacting with EBNA2 was identified from a yeast two-hybrid screen using the divergent region of EBNA2 as bait. Co-immunoprecipitation experiments confirmed the association observed in yeast cells. The protein, FOE, for "friend of EBNA2," contains 1227 amino acids and has an arginine rich motif, bipartite nuclear localization signal and a nuclear export sequence as well as three highly acidic domains and a coiled-coil domain. FOE is expressed in all hematopoetic and other cell lines surveyed as a 130kD doublet band. Biochemical fractionation and imaging by indirect immunofluoresence show that FOE is predominantly nuclear with a fraction distributed in cytoplasmic speckles. A major portion of FOE is associated with the nuclear matrix, with the remainder associated with soluble chromatin. In the nucleus FOE is excluded from nucleoli and from regions rich in heterochromatin as determined by DAPI staining. During metaphase, FOE is excluded from mitotic chromosomes and overall levels of FOE decrease. Chromatin immunoprecipitation shows targeting of FOE to episomal constructs containing the EBNA2 responsive Tp promoter. FOE is a newly identified member of the EBNA2 complex that is a component of both actively transcribing chromatin and the nuclear matrix.

NOTES:

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EBNA-3 FAMILY PROTEINS TARGET GENE-SILENCING COMPLEXES

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EBNAs 3A and 3C can repress transcription from their own common promoter and repress transcription when targeted to DNA by a Gal4 DNA-binding domain.

EBNA3C can recruit HDAC1 and Sin3A and also binds to the co-repressor CtBP via a PLDLS motif. Here we show that EBNA3A also binds CtBP *in vitro* and *in vivo* via a novel, non-consensus, bipartite motif. In this ability to bind CtBP, both EBNA3A and 3C resemble Hairless, which binds dCtBP, acts as a transcriptional repressor and antagonises the *Drosophila* Notch signalling pathway. There is a very good correlation between the ability of these two viral oncoproteins to bind CtBP and their ability to co-operate with (Ha-)ras in the immortalisation and transformation of primary rodent fibroblasts. We also show that both EBNA3A and EBNA3C exist in very high MW complexes in LCLs and are co-immunoprecipitated from these cells with human polycomb group proteins (and proto-oncogenes) RING1 and BMI1. This may in part depend on the ability of CtBP to bind Human polycomb protein-2 (HPC2), which in turn can associate with RING1 and BMI1. These polycomb complexes are involved in cell cycle-associated gene repression and epigenetic gene silencing. The functional significance of these interactions will be discussed.

NOTES:

NEGATIVE REGULATION OF MITOTIC KINASE CDC2 BY EPSTEIN-BARR VIRUS EBNA2 PROTEIN

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The Epstein-Barr virus (EBV) EBNA2 protein is essential for EBV to immortalize primary B-cells. It is considered as a potential oncogenic viral gene. Expression of EBNA2 in Burkitt's lymphoma (EMBO, 1996, 15: 375-382; J. Gen. Virol., 1996, 77: 227-237) and several adherent cell lines (J. Mol. Biol., 2000, 303:7-23) leads to growth retardation. It is of interest to study how EBNA2 retards cell growth. Here we show that EBNA2 can regulate scheduled cell cycle progression. We found that cyclin B1-Cdc2 kinase activity was suppressed in EBNA2-expressing cells. Flow cytometric analysis also showed delay of mitosis entry in these cells. Suppression of Cdc2 activity was, at least partly, due to hyperphosphorylated tyrosine 15 on this kinase. It is consistent with the results of much more inhibitory phosphorylated Cdc25C in EBNA2-expressing cells. Hypophosphorylated Cdc25C reactivates Cdc2 activity by dephosphorylating Cdc2 at Y15. EBNA2 also stimulated p53 phosphorylation at serines 15 and 20, which in turn activated p53 activity and induced cyclin-dependent kinase inhibitor $p21^{WAF1}$. These data suggest that EBNA2 can affect cellular kinases or phosphatases, which play an important role in cell cycle regulation.

NOTES:

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METHYLATION-DEPENDENT BINDING OF EBNA2 TO THE SURVIVAL MOTOR NEURON PROTEIN (SMN)

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We have previously shown that EBNA2 and SMN cooperate in the activation of the viral LMP1-promoter by EBNA2 releasing SMN from an inactive DP103/SMN complex (Voss et al., J.Virol. 75:11781,2001). We now demonstrate that EBNA2 binds SMN directly through methylated arginine residues within the arginine-glycine repeat region encompassing aa 337-354 of EBNA2. In vitro studies show that binding of SMN to EBNA2 is critically dependent on the presence of methylated arginine residues as inhibition of methylation of EBNA2 abrogates binding. Surprisingly, the deletion of the RG-repeat was previously shown to stimulate transcriptional activation of the LMP1 promoter as compared to EBNA2-wt but to strongly reduce transformation of B-lymphocytes in vitro (Tong et al., J.Virol. 68:6188, 1994). The increase in transactivation by EBNA2-delRG is dependent on the presence regulatory elements different from RBPJk-binding sites within the LMP1-promoter as EBNA2-delRG stimulated a promoter construct featuring only 12 RBPJk-sites to the same extend as EBNA2-wt. In contrast, the five-fold increase in stimulation of the LMP1-promoter by EBNA2-delRG over EBNA2-wt was further increased two-fold through co-expression of SMN. Our results thus suggest that SMN, an essential factor for cell survival, plays a dual (positive as well negative) role in the co-regulation of LMP1 gene expression by EBNA2.

NOTES:

TUMOR-SUPPRESSIVE ACTIVITY LOCALIZED TO CHROMOSOME BAND 11Q22-23 IN NASOPHARYNGEAL CARCINOMA

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The molecular genetic basis of nasopharyngeal carcinoma (NPC) is still not clear. Which tumor suppressor genes (TSGs) contribute to the development of the cancer is under study. Obtaining functional evidence for tumor suppression is useful before embarking on the tremendous task of identifying TSGs with a role in the cancer.

Microcell-mediated chromosome transfer techniques were utilized to transfer an intact human chromosome 11 into HONE1 nasopharyngeal carcinoma cells. Tumor-suppressive hybrids were established. Upon injection into athymic nude mice small tumors developed only after a long latency period. Excised tumors were used to establish tumor segregant cell lines. Using microsatellite allelotyping and fluorescent *in situ* hybridization techniques, we were able to narrow down the critical tumor suppressive regions that distinguish the non-tumorigenic hybrids as compared to their paired tumorigenic tumor segregants. The critical region responsible for tumor suppression in the somatic cell hybrids was, thus, narrowed down from a 28 megabase region to 2.2 megabases.

It is now clear that several chromosomes contain tumor suppressive regions critical to the development of NPC. Chromosome 11 harbors at least two critical regions. Results of these studies will be presented.

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Cheng Y, Chakrabarti R, Garcia-Barcelo M, Ha TJ, Srivatsan ES, Stanbridge EJ, and Lung ML. Mapping of nasopharyngeal carcinoma tumor suppressive activity to a 1.8 megabase region of chromosome band 11q13. *Genes, Chromosomes, and Cancer* 33: 1-7, 2002.

NOTES:

INTERFERON REGULATORY FACTOR 7 HAS ONCOGENIC PROPERTIES AND IS EXPRESSED IN HUMAN NASOPHARYNGEAL CARCINOMA

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Interferon regulatory factor 7 (IRF-7) is implicated in the regulation of Epstein-Barr virus (EBV) latency as well as activation of a subset of type I IFN genes upon viral infection. Because the expression of IRF-7 is closely associated with a transformation state of EBV, whether IRF-7 has oncogenic properties is examined. IRF-7 caused growth transformation of NIH3T3 cells as assayed by focus formation as well as anchorage-independent growth. Latent membrane protein 1 (LMP-1), the principal EBV oncoprotein, and IRF-7 had an additive effect on the growth transformation of NIH3T3 cells. Moreover, IRF-7-expressing NIH3T3 cells formed tumors in athymic (nu/nu) nude mice. We further discovered that IRF-7 is expressed in all (n=14) human nasopharyngeal carcinoma (NPC) specimens examined as well as in NPC passaged in nude mice. Thus, IRF-7 has oncogenic properties and may be involved in the pathogenesis of human NPC.

NOTES:

EXPRESSION OF A FUNCTIONAL C-KIT RECEPTOR IN A SUBGROUP OF EBV-ASSOCIATED NASOPHARYNGEAL CARCINOMAS

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In a previous publication, Stem Cell Factor (SCF) transcripts were identified as overexpressed mRNAs in NPC specimens (Xie et al., J. Cancer res. Clin. Oncol. 126: 400-406, 2000). These data prompted us to investigate the expression of the c-KIT molecule in NPCs. c-KIT, which is the cognate SCF receptor, was detected by immunohistochemistry in 30 to 50% of NPC biopsies. It also was detected by Western blot and immunoprecipitation in C17, a xenografted NPC tumor line. The c-KIT protein expressed by C17 cells had a low apparent molecular weight (135 Kd) below the range of variations resulting from usual post-translational modifications (145-160 Kd). Phosphorylation of the C17 c-KIT protein was only apparent under stimulation with exogenous SCF. Both SCF transcripts – produced by alternative splicing of exon 6 – were detected by RT-PCR in C17 material, supporting the hypothesis of an autocrine stimulation loop involving the SCF/c-KIT system. Our present objectives are 1) to investigate possible c-KIT mutations in C17 and other NPC specimens and 2) to assess the role of the C-KIT/SCF system in C17 tumor growth. Specific inhibitors of c-KIT tyrosine-kinase activity are currently in late stages of clinical development and might become useful in the subgroup of c-KIT-positive NPCs.

NOTES:

EPIGENETIC AND GENETIC INACTIVATION OF *BLU*, A TUMOR SUPPRESSOR GENE CANDIDATE ON THE 3P21 LOCUS, IN NASOPHARYNGEAL CARCINOMA (NPC)

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Loss of heterozygosity at the 3p21 locus is very common in NPC. *BLU* is a candidate tumor suppressor gene (TSG) located in this region. The *BLU* promoter and its first exon are located within a CpG island. The epigenetic and genetic alterations of *BLU* in NPC were studied. In NPC cell lines, the expression of *BLU* was down-regulated and correlated with promoter hypermethylation. Hypermethylation was further detected in 17/28 (61%) of primary tumors. In normal nasopharyngeal tissues, *BLU* was expressed and unmethylated. Furthermore, homozygous deletion of the *BLU* gene was found in 6/28 (21%) cases, but not in any normal DNA. Hypermethylation and homozygous deletion was mutually exclusive. Hypermethylation of another TSG, *RASSF1A* which is located immediately downstream of *BLU,* was also examined. Hypermethylation of *RASSF1A* was detected in 13/26 (50%) cases. No correlation between the hypermethylation of these two TSGs was observed. Treatment of NPC cell lines with 5-aza-2'-deoxycytidine activated *BLU* expression along with the demethylation of the promoter. These data imply that *BLU* is a TSG candidate for NPC, being disrupted in 82% of NPC tumors by either epigenetic or genetic mechanisms. Inactivation of this putative TSG is therefore involved in NPC pathogenesis.

NOTES:

A RECOMBINANT MVA-EBNA1/LMP2 VACCINE TO BOOST T CELL RESPONSES AGAINST EBV-POSITIVE NASOPHARYNGEAL CARCINOMA

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Of the EBV antigens expressed in nasopharyngeal carcinoma (NPC), the C-terminal half of the nuclear antigen EBNA1 is a rich source of CD4+ T cell epitopes, and the latent membrane protein LMP2 contains several CD8 epitopes relevant to HLA alleles common in South East Asia. Here, we report the construction of a replication-defective vaccinia virus (based on the Modified Vaccinia Ankara MVA) carrying sequences derived from a Chinese EBV strain and expressing, as a fusion protein, the C-terminal half of EBNA1 joined to full length LMP2. This fusion protein lacks both the transactivating capacity of wild type EBNA1 and, by mutation of key tyrosine residues, the tyrosine kinase pathway interactions of LMP2. The endogenously expressed EBNA1/LMP2 protein is processed efficiently for recognition by CD8 T cell clones reactive to different epitopes derived from the EBNA1 or LMP2 areas of the molecule. Furthermore, dendritic cells infected with MVA-EBNA1/LMP2 *in vitro* can reactivate these same CD8 T cells from the memory pool of EBV-immune donors, as well as memory T cells to CD4 epitopes within the EBNA1 sequence. By eliciting linked CD4 and CD8 T cell responses *in vivo*, such a construct may have value as an adjunct to conventional therapies for NPC.

NOTES:

CLONING AND ANALYZING THE REGULATORY REGION OF THE GENE SPECIFICALLY EXPRESSED IN MOUSE NASOPHARYNX

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The carcinogenic potential of EBV has been attributed to the latent membrane protein (LMP1) so far, but attempts to construct LMP1-transgenic mice have not offered any strong evidence that LMP1 could induce NPC itself *in vivo* yet. Hence, one of our goals is to construct a LMP1-transgenic mouse model, in which transferred LMP1 will be strictly expressed in nasopharynx, and ultimately induce NPC in the transgenic mouse. In light of the fact that *PLUNC* is specifically expressed in mouse nasopharynx, thereof promoter region, designated *PLUNC-p,* was cloned with Genome walking technique, and was registered in GenBank as AF225964. The results of luciferase reporter gene assay showed that *PLUNC-p* possesses epithelial specific promoter activity *in vitro.* The core region of *PLUNC-p* was identified within 200 bp upstream from the transcription start site by serial deletion analysis. The result of transgenic Xenopus Laevis demonstrated that *PLUNC-p* could strictly direct green fluorescent protein to be expressed in branchial arches and epidermis in Xenopus Laevis embryos, further confirming the property of specific regulation of *PLUNC-p in vivo*. These results shed light on constructing the LMP1 transgenic mice model of nasopharyngeal carcinoma.

NOTES:

THE ACTION OF EBNA-1 AS AN ONCOGENE *IN VIVO*

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Epstein-Barr virus nuclear antigen-1 (EBNA-1) plays a key role in the maintenance of the viral genome and is consistently expressed in all the EBV associated tumour types. We have shown that expression of EBNA-1 in transgenic mice predisposes the mice to B cell lymphoma (Wilson *et al*., 1996, EMBO J., 15, p3117). We are currently analysing how EBNA-1 may act as an oncogene *in vivo*. We have found that it is redundant with *Bcl-2* in transgenic crossbreed experiments in the assay of tumour latency. In accordance with this observation we have noted up-regulation of the anti-apoptotic gene *Bc-lx$_L$*. Furthermore, spleen and BM cell cultures derived from EμEBNA 1 mice show a greater capacity for cultured growth compared to controls, most notably in the presence of IL-2 (Tsimbouri *et al.,* Oncogene, in press). We are currently extending our analysis of the EBNA-1 transgenic lymphocytes in response to IL-2.

Using macroarrays for differential gene expression, our preliminary studies suggest that expression of several immediate early response genes may be influenced by EBNA-1. We are expanding this analysis using microarrays with a view to correlating the observed gene expression profile with the observed phenotype.

NOTES:

ACTIVATION OF THE PI3-KINASE/AKT PATHWAY BY EBV LATENT MEMBRANE PROTEIN 2A

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Expression of EBV latent membrane protein 2A (LMP2A) activates the serine/threonine kinase Akt via phosphatidylinositol-3-kinase (PI3K) in the human keratinocyte cell line, HaCaT, and in EBV-infected B lymphocytes. Furthermore, LMP2A expression inhibits differentiation of HaCaT cells and induces colony formation in soft agar in a PI3K-dependent manner. Akt signaling downstream of PI3K can inhibit apoptosis in various contexts and may promote cell cycle progression. To determine if Akt is activated in EBV-associated malignancies, samples of EBV-negative and EBV-positive Hodgkin's Disease (HD) and nasopharyngeal carcinoma (NPC) were tested for activated Akt. Activated Akt was occasionally detected in Reed-Sternberg (R-S) cells in EBER-negative Hodgkin's lymphomas but was readily detected in most of the R-S cells in EBER-positive HD samples. Activated Akt was not detected in any of the infiltrating lymphoid cells. Activated Akt was also detected in the C15 NPC tumor, in which transcription of the LMP2A gene is detected. These data suggest that LMP2A is expressed in HD and NPC and that one consequence of this expression is activation of Akt. LMP2A-mediated activation of Akt in epithelial cells and the *in vitro* effects on potential Akt targets will be presented.

NOTES:

VP22 BASED GENE THERAPY FOR EPSTEIN-BARR VIRUS ASSOCIATED MALIGNANCIES

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EBNA1 is essential for the maintenance of the episomal EBV genome in virus-infected cells and thus provides an ideal target for developing new approaches to the treatment of EBV-associated tumours. We have used a dominant negative form of EBNA 1 comprising the DNA binding and dimerisation domains fused to the trafficking protein VP22 of HSV in a recombinant adenovirus-based delivery system to target EBV-infected cell lines. Targeting of both EBV-positive epithelial cell lines and cell lines stably expressing EBNA1 with the VP22-dnEBNA1 fusion protein resulted in profound cell cycle arrest and extensive cell death. This toxicity was associated with the processing and breakdown of both wild-type endogenous EBNA 1 and the VP22dnEBNA1 fusion protein, presumably as a result of the dominant negative destabilising the wild-type protein. Similarly, targeting of LCLs with the VP22-dnEBNA1 fusion protein also resulted in marked growth inhibition, cell cycle arrest and loss of EBNA1. These data confirm our previous work on the cytotoxic potential of EBNA1 and confirm that EBNA1 targeting is a viable approach for the treatment of EBV-associated malignancies.

NOTES:

INDUCTION OF C-MET PROTO-ONCOGENE BY EBV LMP-1 AND THE CORRELATION WITH CERVICAL LYMPH NODE METASTASIS OF NASOPHARYNGEAL CARCINOMA

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Nasopharyngeal carcinoma (NPC) is highly metastatic tumor. Up-regulation of cell motility is essential for metastatic potential. c-Met proto-oncogene is a high affinity receptor for hepatocyte growth factor (HGF). We observed close association of c-Met expression with cervical lymph node metastasis ($p=0.0272$) in 39 NPCs. EBV encoding latent membrane protein-1 (LMP-1) is a primary oncogene and is suggested to enhance the metastatic property of NPC. Previously, we reported that LMP-1 enhanced the motility of Madin-Darby canine kidney (MDCK) epithelial cells which was mediated by activation of Ets-1 transcription factor. Therefore, we examined the interrelationships of LMP-1, Ets-1, and c-Met. In immunohistochemical studies, the expression of LMP-1, Ets-1, and c-Met correlated significantly with each other in NPC (LMP-1 versus Ets-1: $p<0.0001$; Ets-1 versus c-Met: $p=0.0012$; LMP-1 versus Met: $p=0.0005$). Transfection of LMP-1 expressing plasmid in MDCK cells induced c-Met protein expression. The c-Met protein was also induced by Ets-1 expression, and induction of c-Met by LMP-1 was suppressed by introducing a dominant negative form of Ets-1 in LMP-1 expressing MDCK cells. These results suggest that LMP-1 induces c-Met through the activation of Ets-1, which may contribute in part to the highly metastatic potential of NPC.

NOTES:

EBV LATENCY CONTROL AS A TUMOR RISK FACTOR

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EBV genomes are found in B cells in peripheral blood, with the highest frequency in resting latency 0/latency I type memory B cells and IgA-positive B-cells, cells which rarely are represented in EBV-positive lymphomas. In order to better understand the link between latently infected cells and lymphoma we do long term follow up of several cohorts of patient groups with high lymphoma risk: HIV-carriers and BMT-transplanted children, characterizing the virus host interaction and study aspects of the latency switch *in vitro*. The latent membrane protein (LMP)2A serves as a molecular scaffold to recruit both B cell tyrosine kinases and C2/WW/Hect domain E3 protein-ubiquitin ligases, thereby promoting Lyn and Syk ubiquitination in a fashion that may contribute to a block in B cell signaling. LMP2A may also subvert physiologic mechanisms in the regulation of epithelial cell signaling. Regulation of viral latency depends on interaction of EBNAs with cellular transcription factors. We are analyzing the transcriptional effects of EBNA 1 together with cellular transcription factors. Finally, studies of LMP 1 suggest a modulation of apoptosis which depends on the types of stimuli. These results will be discussed in relation to loss of control of the latent infection.

NOTES:

EPSTEIN-BARR VIRUS AND B CELL LYMPHOMAS : AN UPDATE

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EBV is linked to three histologically and clinically distinct types of B cell malignancy, Burkitt's Lymphoma (BL) , Hodgkin's Disease (HD) and Post-transplant Lymphoproliferative Disease (PTLD). The observation that these three tumours usually present with different patterns of EBV latent gene expression implies that the viral contribution to lymphomagenesis may be different in each case. In particular it is not clear whether the virus' B cell growth transforming function, which is the main driver of early onset PTLD, plays any role in the pathogenesis of the other malignancies. Two recent findings shed new light on this question. Firstly, Ig genotyping of tumour cells in monoclonal cases of PTLD shows that growth-transforming infections in vivo often involve post-germinal center B cells with non-antigen-selected or sterile IgH genes, similar to the genotypes reported in the malignant cells of EBV-positive HD. Secondly, a subset of endemic BL tumours do not show the classical restriction of virus latent gene expression involving Qp promoter usage; instead they use the transformation-associated Wp promoter and appear to have been selected from a growth-transformed progenitor by pressure for specific downregulation of the nuclear antigen EBNA2. Both findings suggest that there may be more common ground between the pathogeneses of EBV-positive B cell malignancies than hitherto imagined.

NOTES:

INDUCTION OF MHC CLASS-I AND -II RESTRICTED EPITOPE PRESENTATION BY UREA-TREATED BZLF1 PROTEIN: A NOVEL TECHNOLGY FOR THE DETECTION OF PROTEIN-SPECIFIC CYTOTOXIC T-CELLS

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Several techniques, such as ^{51}Cr-release assays, ELISPOT analysis, FACS and tetramer technologies have been developed to evaluate specific cytotoxic T-cell (CTL) responses. Most of these methods use peptide-pulsed antigen-presenting cells for T-cell stimulation, and thus are limited by HLA-restriction of epitope presentation.

In our studies, we tested the potency of urea-treated proteins to get access to the MHC class-I and -II processing and presentation pathway. Here we analyzed the capacity of urea-denatured Epstein-Barr virus (EBV) BZLF1 protein to stimulate $CD4^+$ and $CD8^+$ T-cells from EBV-positive, HLA B8-positive donors. BZLF1 includes an epitope, which is recognized by CTL of HLA B8-positive, EBV-infected individuals. PBMC of several donors were stimulated with various concentrations of urea-treated BZLF1, and frequencies of INF-γ producing cells were determined at different time points post stimulation using ELISPOT-analysis. PBMCs of HLA B8-positive donors showed substantially increased frequencies of IFN-γ producing cells, when compared to that of HLA-B8 negative persons. Cell depletion studies and FACS analysis indicate, that both $CD4^+$ and $CD8^+$ T-cells contributed to INF-γ production.

These results suggest treatment with urea to be a suitable technology to direct polypetides towards the MHC-I and MHC-II pathway, thus providing a useful tool for the detection of protein-specific CTL.

NOTES:

PROTEASOME INHIBITORS RECONSTITUTE THE PRESENTATION OF CTL EPITOPES IN EPSTEIN-BARR VIRUS ASSOCIATED TUMORS

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Epstein-Barr virus (EBV) infected cells and EBV-associated tumors may evade CTL-recognition by defective antigen processing, resulting in poor presentation of CTL epitopes. Since the proteasome is the major source of MHC class I presented peptides, we analyzed the effect of proteasome inhibitors on the expression of surface HLA class I, and the generation of EBV-derived CTL epitopes presented by the HLA-A2 and HLA-A11 alleles. Treatment with covalent and reversible inhibitors of the proteasome partially reduced the total and allele specific expression of surface HLA class I in EBV-carrying lymphoblastoid cell lines (LCLs). The expression of HLA-A2 was also decreased by treatment with leupeptin and bestatin, while HLA-A11 expression was affected by treatment with phenantroline. In spite of their general inhibitory effect on HLA class I expression, all proteasome inhibitors tested enhanced the presentation of two subdominant HLA-A2 epitopes from the EBV latent membrane proteins (LMP)1 and LMP2, while the presentation of the immunodominant HLA-A11-restricted epitope from the EBV nuclear antigen (EBNA)4 was inhibited by MG132 and lactacystin and increased by ZL_3VS. Treatment with ZL_3VS restored the presentation of endogenously expressed EBNA4 in one HLA-A11-positive Burkitt's lymphoma cell line. These findings suggest that specific inhibitors of the proteasome may be used to increase the antigenicity of virus infected and malignant cells that are per se inefficient in the generation of particular CTL target epitopes.

NOTES:

EPSTEIN-BARR VIRUS INHIBITS THE DEVELOPMENT OF DENDRITIC CELLS BY PROMOTING APOPTOSIS OF THEIR MONOCYTE PRECURSORS IN THE PRESENCE OF GM-CSF AND IL-4

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Epstein-Barr virus (EBV) is a tumorigenic human herpesvirus that persists for life in healthy immunocompetent carriers. The viral strategies that prevent its clearance and allow reactivation in the face of persistent immunity are not well understood. Here we demonstrate that EBV infection of monocytes inhibits their development into dendritic cells (DCs) leading to an abnormal cellular response to granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4 and apoptotic death. This pro-apoptotic activity was not affected by UV-inactivation and was neutralised by EBV antibody positive human sera indicating that binding of the virus to monocytes is sufficient to alter their response to the cytokines. Experiments with the relevant blocking antibodies or mutated EBV strains lacking either the EBV envelope glycoprotein gp42 or gp85 demonstrated that interaction of the trimolecular gp25/gp42/gp85 complex with the monocyte membrane is required for the effect. Our data provide the first evidence that EBV can prevent the development of DCs through a mechanism that appears to bypass the requirement for viral gene expression and suggest a new strategy for interference with the function of DCs during the initiation and maintenance of virus specific immune responses.

NOTES:

EBNA-2 DEPENDENT TRANSCRIPTIONAL ACTIVATION OF HERV-K18 SUPERANTIGEN

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Superantigens are microbial proteins that vastly over-stimulate the immune system. We have recently shown that infection with Epstein-Barr virus (EBV) activates a superantigen that is encoded within our genes, but normally remains dormant. We have discovered that EBV transactivates the human endogenous retrovirus HERV-K18. The *env* gene contains superantigen activity, defined by the stimulation of TCRBV13 specific T cell hybridomas, and the strong polyclonal activation of peripheral blood T cells. Functional T cell activation studies using LCL transformed by deletion mutant EBV, indicated that superantigen activity was associated with the latent cycle transactivators. We show here that EBV transactivation of HERV-K18 transcription is dependent upon the latent gene EBNA-2. We have developed a semi-quantitative RT-PCR assay for the detection of HERV-K18 read-through transcripts. These transcripts were increased in the Burkitt lymphoma cell line BL41 after infection with B95-8 virus, but not EBNA-2 deficient P3HR1 virus. Transfection of EBNA-2 restored HERV-K18 transcription. Furthermore, HERV-K18 transcription was downregulated after EBNA-2 removal in LCL transformed by recombinant EBV carrying an estrogen regulatable EBNA-2. We propose that the immune stimulation elicited by the HERV-K18 superantigen after EBV infection may play a role in EBV biology and related diseases.

NOTES:

INCREASED VIRAL LOAD OF EBV AS WELL AS KSHV IN THOSE AT RISK OF KAPOSI'S SARCOMA (KS)

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KS patients, versus others with AIDS, are at higher risk for immunoblastic lymphoma, a malignancy linked to EBV. We postulated that poor control of both KSHV and EBV may increase KS risk.

From our cohort of 245 homosexual men in New York (NYC) and Washington, evaluated annually (1982-2000), we compared 24 incident KS cases (all pre-HAART) to 48 controls matched for CD4 count, HIV and KSHV antibody (K8.1 EIA) on the visit before KS (a median of 312 days). KSHV and EBV viral loads in plasma and PBMC were determined by real-time PCR.

KS cases and controls did not differ (chi-sq, Wilcoxon rank sum) on age (median 41 vs 42), race (92% white), city (54% vs 46% NYC), smoking, nitrite inhalant use, K8.1 titer (1:256 vs 1:200), LNA-IFA titer (1:4800 vs 1:1600), or HIV load (4.6 vs 4.4 log10, p=0.52). KSHV PCR detection (33% vs 3%, p=0.008) and load (median 7 vs 1 copies) were higher in cases. Interestingly, EBV load also was higher in KS cases than controls (225 vs 75 copies, p=0.03).

Our data suggest that KS occurs in those unable to control both KSHV and EBV, corroborating the higher immunoblastic lymphoma risk for KS patients.

NOTES:

IN VIVO TRANSCRIPTION OF THE EPSTEIN-BARR VIRUS (EBV) BAMHI-A REGION WITHOUT (RK-) BARF0 PROTEIN EXPRESSION IN EBV-ASSOCIATED DISORDERS

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We investigated the *in vivo* expression of the BamHI-A rightward transcripts (BARTs) of Epstein-Barr virus (EBV) and the encoded BARF0 protein in EBV-associated malignancies. RT-PCRs for different BART splice variants and both NASBA and RT-PCR specific for the BARF0 open reading frame (ORF) were used. We found abundant transcription of BARTs in EBV-associated Hodgkin's lymphomas, Burkitt's lymphomas, T cell non-Hodgkin's lymphomas, posttransplant lymphoproliferative disorders, AIDS-related lymphomas and gastric and nasopharyngeal carcinomas. Using RISH, BARTs were detected within the neoplastic cells of these malignancies. BARTs encoding RK-BARF0 ORF were not detected. BARTs actually harboring BARF0 ORF were detected only in specimens containing relatively many EBV-positive cells. New BARF0 monoclonal antibodies efficiently recognized prokaryotic and eukaryotic recombinant BARF0. However, no BARF0 protein was not detected in any clinical samples nor in EBV-positive cell lines, despite positivity for BARTs by RISH and/or RT-PCR/NASBA. Using immunoblot, no antibodies against Baculovirus-expressed BARF0 were detected in the sera of patients with various EBV diseases and healthy EBV carriers. Thus, BARTs containing the BARF0 ORF are expressed *in vivo*, but BARF0 protein cannot be detected and may at best be only marginally expressed. Conclusion: BARF0 protein is unlikely to play a role *in vivo* in EBV-positive malignancies.

NOTES:

ACTIVATION OF TRAFs SIGNALING PATHWAY IN EBV-INFECTED T CELL: IMPLICATION FOR HEMOPHAGOCYTIC SYNDROME

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The Epstein-Barr virus (EBV) can infect T cells, leading to chronic active infection, or T cell lymphoma. The most characteristic clinical features of these EBV-associated T lymphoproliferative disorders is the frequent hemophagocytic syndrome (HS), characterized by macrophage activation and a cytokine storm. Tumor necrosis factor (TNF)-alpha is a major cytokine responsible for the development of HS We previously demonstrated the activation of TNF-alpha in EBV-infected T cells. In recent studies, we further demonstrated that EBV-LMP-1 is the only EBV gene product responsible for TNF upregulation. In order to investigate whether TNF receptor-associated factor (TRAFs) superfamily is involved in EBV-infected T cell lymphomas, we studied the expression of TRAF2, TRAF3, and TRAF5 in T cell lines and T cell lymphoma tissues. In EBV-infected or LMP-1-expressed T cell lines, but nor in B cell lines, TRAF2,and TRAF5, but not TRAF3, are upregulated. In EBV-infected lymphoma tissues, the same findings were observed. Interestingly, the expression pattern of TRAFs in EBV-infected T lymphoma cells showed aggregation or “dot-like” expression of TRAFs, distinct from that in EBV-negative T lymphoma cells which showed a “diffuse” cytoplasmic pattern. The same observation was observed in EBV-infected RS cells in Hodgkin’s disease. Therefore, the TRAF pathway appears to be activated in EBV-infected T cells, which will lead to subsequent activation of cytokines, particularly TNF-alpha, explaining the pathogenesis of EBV-associated hemophagocytic syndrome.

NOTES:

THE β-CATENIN PATHWAY IS ACTIVATED IN EBV TYPE III LATENCY

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EBV infection generates three latency types, each with a different spectrum of protein expression that is associated with different malignancies. In type III latency, exemplified by EBV lymphoproliferative diseases, all latent viral proteins are expressed, compared to type I latency, where chiefly EBNA1 is functional.

Recently it was shown that β-catenin plays a major role in signaling as a partner of T-cell Factor (TCF) to activate transcription. Mutations in β-catenin are common in a variety of cancers.

Here, comparing genetically identical lymphoblastoid cell lines with type I and type III latency phenotypes (Sav I and Sav III) we show in latency III β-catenin is accumulated compared to latency I in which only trace amounts of β-catenin are detectable. Using a luciferase reporter construct with TCF binding sites we show β-catenin/TCF transcriptional activity is significantly elevated in type III latency.

EBNA2 is required for EBV transformation of lymphocytes. To assess the possibility that EBNA2 may be involved in the activation of β-catenin, we studied DG75/TA and DG/TA-EBNA2 cell lines. In cells expressing EBNA2, β-catenin was accumulated compared to the control cells.

Thus activation of β-catenin is implicated in type III latency and is likely to be involved in EBV lymphoproliferative diseases.

NOTES:

IMMUNODOMINANT CD4 T CELL RESPONSE TO THE EBV LYTIC ANTIGEN BHRF1 IN DR*0401 INDIVIDUALS EVIDENCED BY IMMUNOMAGNETIC SORTING WITH MHC CLASS II TETRAMERS

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The CD4 T cell response to EBV lytic antigens is poorly characterized, mainly due to the presumably low frequency of EBV-specific CD4 T cells in the peripheral blood of seropositive donors. Human MHC class II tetramers provide a means to detect and characterize such rare T cells. Along a screening of responses to lytic and latent EBV antigens within synovial- and PBL-derived CD4 T cell lines, we identified an HLA-DR*0401-restricted epitope derived from BHRF1, a viral protein produced during the early stages of the lytic cycle. By means of magnetic beads coated with recombinant BHRF1/DR*0401 complexes, we sorted out BHRF1-specific T cells from all the DR*0401 donors studied. In vitro, BHRF1-specific CD4 T cell clones killed DR*0401 B-LCLs and showed a Th1 cytokine profile, thus suggesting their direct contribution to the control of EBV replication.

This study documents for the first time the existence of a dominant CD4 T cell response against an EBV lytic antigen. This CD4 T cell epitope could be of potential interest for the design of immunotherapeutic approaches targeting frequent Th responses to EBV.

NOTES:

NOVEL *IN VIVO* BINDING SITE FOR C-MYC IN THE EBER-LOCUS SUGGESTS SPECIFIC ROLE FOR EBV IN TUMORIGENESIS

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The two Epstein-Barr virus (EBV) encoded small RNAs (EBER) have been implicated in EBV related tumorigenesis. Furthermore, the EBER-region has been shown to participate in the nuclear matrix attachment function for the viral episome. To more understand both functions we wished to examine with nucleotide resolution in vivo protein binding at this locus. Therefore, we undertook a comprehensive analysis of the EBER locus through genomic footprinting in a panel of cell lines representing latency classes I and III. Several novel transcription factor binding sites were discovered. An E-box element, located approximately 130 nucleotides upstream of the start site of EBER1 transcription, was strongly protein protected in all cell lines studied. Electrophoretic mobility shift and transient transfection experiments showed that the E-box element is bound by c-Myc in vitro and is activated by c-Myc in vivo. A model for the specific role of EBV as a cofactor in lymphomagenesis is presented.

NOTES:

Session 7: Primary Infection

EPSTEIN-BARR VIRUS mRNA EXPORT FACTOR EB2 IS ESSENTIAL FOR PRODUCTION OF INFECTIOUS VIRUS

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The splicing machinery which positions a protein export complex near the exon-exon junction mediates nuclear export of mRNA generated from intron-containing genes. Many EBV early and late genes are intronless and an alternative pathway, independent of splicing, must export the corresponding mRNA. Since the EBV EB2 protein induces the cytoplasmic accumulation of intronless mRNA, it is tempting to speculate that EB2 is a viral adapter involved in the export of intronless viral mRNA. If this is true, the EB2 protein is essential for the production of EBV infectious virions. To test this hypothesis, we generated an EBV mutant in which the BMLF1 gene encoding the EB2 protein has been deleted (EBV-$_{BMLF1\text{-}KO}$). Our studies show that in effect, EB2 is necessary for the production of infectious EBV, and its function cannot be transcomplemented by a cellular factor. In the EBV-$_{BMLF1\text{-}KO}$ 293 cells, *oriLyt*-dependent DNA replication was greatly enhanced by EB2. Accordingly, EB2 induced the cytoplasmic accumulation of a subset of EBV early mRNA coding for essential proteins implicated in EBV DNA replication during the productive cycle. Two herpesvirus homologues of the EB2 protein, the HSV1 protein ICP27 and, the HCMV protein UL69, only partly rescued the phenotype of the EBV-$_{BMLF1\text{-}KO}$ mutant, indicating that some EB2 functions in virus production cannot be transcomplemented by ICP27 and UL69.

NOTES:

ANALYSES OF THE ACQUISITION, PERSISTENCE, AND COMPARTMENTALIZATION OF EBV STRAINS USING A HETERODUPLEX TRACKING ASSAY

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EBV persists in memory B-cells that can reactivate in the oral lymphoid tissue resulting in the release of virus into the oral cavity. The relationship of the virus found in the peripheral blood and the oral cavity was investigated through the identification of strain variants present in the two body compartments in hairy leukoplakia (HLP) subjects, and asymptomatic carriers. A heteroduplex tracking assay (HTA) was developed in this study that can identify the seven distinct forms of LMP1 and estimate their relative abundance. Analyses revealed different strain profiles in the HLP and throat washing in comparison to the peripheral blood from the same patient. Strain profiles were also determined for asymptomatic carriers over time. These subjects exhibited a dynamic multi-strain profile where strains appeared to increase and decrease in relative abundance and cycle between compartments. Ongoing studies include the characterization of strains in infectious mononucleosis patients. These studies provide clear evidence for compartmentalization and for the transmission between blood and oropharynx.

NOTES:

MULTIPLE EBV INFECTIONS IN HEALTHY INDIVIDUALS

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EBV persistently infects more than 90% of the human population. Infection with multiple EBV genotypes is routinely observed in immunocompromised persons, but multiple EBV infections of healthy individuals are believed to be uncommon. In this study, 9 healthy individuals were examined to determine the prevalence of multiple EBV infections in saliva and blood over multiple time points. Distinct EBV genotypes were identified by strain-defining, nucleotide sequence variation in the EBV LMP-1 gene, as detected by nested PCR amplification, cloning of the amplification products, and sequencing of multiple clones per specimen. Multiple EBV infections were demonstrated in 2 of the 9 individuals studied, as defined by one or more of the following three criteria: 1) simultaneous detection of two or more EBV genotypes in a single saliva and/or blood specimen. 2) detection of different EBV genotypes in saliva and blood specimens obtained simultaneously from a single individual. 3) temporal changes in the population of EBV genotypes detected in sequential saliva and/or blood specimens obtained from the same individual. These results demonstrate that EBV infection of healthy individuals with multiple genotypes is not uncommon and may be a normal aspect of EBV biology. These data may have implications for EBV vaccine development.

NOTES:

CHARACTERIZATION OF EPSTEIN-BARR VIRUS-INFECTED GERMINAL CENTER B-CELLS DURING INFECTIOUS MONONUCLEOSIS

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During infectious mononucleosis (IM) Epstein-Barr virus (EBV) infects directly naive and germinal center (GC) and/or memory B-cells leading to expansion of infected (post) GC B-cells. No evidence was obtained supporting the origin of EBV-positive memory B-cells from infected naive B-cells passing through GCs (as suggested for EBV persistence). However, as EBV-infected cells are occasionally detected in GCs, we characterized such cells by micromanipulation and PCR for rearranged V genes. Among EBV-carrying cells located inside GCs, expansion of cells with mutated V gene rearrangements (characteristic for GC and memory B-cells) was observed. Members of individual clones were detected inside different GCs and in the interfollicular region. Whereas clonally related EBV-negative GC cells showed ongoing V gene mutation (a hallmark of GC B-cells) EBV-positive clones lacked such intraclonal diversity. Thus, EBV-infected cells do not participate in the GC reaction even if located in functional GCs, supporting the idea that GC passage of EBV-harbouring naive B-cells plays no role for the generation of infected memory B-cells during IM. Rather, EBV-positive GC B-cells may represent memory B-cells passing through GCs without participating in the GC reaction and/or directly infected GC B-cells that silenced somatic hypermutation and thus the normal GC differentiation pathway, while continuing to proliferate.

NOTES:

PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) EXPRESS RECOMBINASE ACTIVATING GENES (RAG) 1 AND 2 DURING EBV-INDUCED INFECTIOUS MONONUCLEOSIS (IM)

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Aberrant V(D)J recombination has a well-documented role in tumorigenesis. Normally expressed early in B-cell development and in germinal center B-cells, RAG has not been detected in PBMC. Using EBV-GFP, we infected RAG-negative BL cells and showed immediate RAG mRNA induction in the fluorescent cell subset. To determine if RAG induction *in vitro* reflects what occurs after infection of mature B-cells *in vivo,* we examined PBMC from 26 IM patients. Using real-time PCR to correlate viral DNA load with RAG RNA levels, we demonstrated RAG1 in PBMC from 42%(11/26) of patients and RAG2 in 8%(2/26). Median EBV copy number/μg cellular DNA was 266, 243, 125, 34 and 4 at diagnosis, 1, 2, 6, and 24 months respectively, with RAG detection generally coinciding with maximal EBV levels. Absence of RAG1 in convalescent samples and healthy controls is consistent with reports that RAG is not expressed in PBMC. We propose EBV has usurped cellular mechanisms that edit, via secondary recombination, faulty B-cell receptors to guarantee appropriate survival signaling and its own life-long persistence within the host cell. While enhancing odds of EBV persistence, the same molecular mechanism brings the risk of auto-antibody production, exuberant B-cell proliferation driven by self-antigen, and diverse genetic lesions underlying lymphomagenesis.

NOTES:

DEVELOPING A POLYTOPE VACCINE FOR EPSTEIN-BARR VIRUS ASSOCIATED INFECTIOUS MONONUCLEOSIS

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We have developed a polytope vaccine, containing multiple cytotoxic T lymphocyte (CTL) epitopes for Epstein-Barr Virus (EBV)-associated infectious mononucleosis (IM). During this development we studied some of the following issues pertaining to the formulation of a polytope vaccine 1) CTL response to EBV epitopes in humans; 2) comparison of CTL response to latent vs lytic epitopes; 3) processing of the polytope by human cells. A hierarchy of CTL responses was established in acute IM and in healthy seropositive donors and the relative immunogenicity of these epitopes assessed in transgenic mice. Further we have also tested the polytope formulation *in vitro* on human cell lines for processing and presentation of epitopes and on PBMCs from EBV seropositive individuals for induction of a CTL memory response. Some of our findings will be presented.

NOTES:

MECHANISM OF REACTIVATION OF EPSTEIN-BARR VIRUS FROM LATENCY

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EBV reactivation in Burkitt's lymphoma cell lines in response to BCR stimulation offers an opportunity to identify the biochemical mechanisms of latency and reactivation of a human herpesvirus. For the first time, biochemical modification of transcription factors that can bind to the important promoter elements has been identified and correlated with the promoter activity. Four promoter elements are essential for reactivation and one sequence mediates a repression of the promoter. A cyclosporin A sensitive dephosphorylation of MEF2D can be observed 10 minutes after BCR cross linking and this correlates with the induction that occurs through the ZI promoter elements. Inactivity of the Zp promoter in latency correlates with deacetylated histones on the Zp promoter. HDAC7 was the most readily detected class II HDAC in Akata cells and Raji cells harbouring latent EBV. Reactivation from latency involves histone acetylation at Zp, mediated primarily through factors associated with ZI elements. The eventual decline in Zp activity was delayed by acyclovir or PAA, indicating that a late viral gene modulates the switch off of the Zp promoter. The results provide a coherent biochemical picture of the biochemical mechanism of reactivation of EBV.

NOTES:

MOLECULAR DIAGNOSIS OF EPSTEIN-BARR VIRUS (EBV) REACTIVATIONS IN HEALTHY EBV CARRIERS

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Reactivation of EBV's lytic cycle and synthesis of viral particles is necessary for infection of new B lymphocytes as well as for interindividual transmission. EBV replication was shown to periodically occur in the mucosa-associated lymphoid tissue, but not in the peripheral blood, of healthy EBV carriers. Recent studies have emphasized the association of increases in latently infected peripheral B cells (viral load) with replicative activity at remote sites. We here propose that EBV reactivation is characterized by a short period of plasma viremia resulting from remote viral replication but not from replicative activity in peripheral B lymphocytes, followed by a substantial increase in viral load.

Highly sensitive real-time PCR assays were used to detect and quantitate EBV genomes in the peripheral blood of 23 blood donors over a period of 15 months. Individuals were monitored every 2–4 months, at least 3×/year. Individuals with a stable viral load did not show viremia or serologic evidence of reactivation at any time. Six individuals had a period of viremia typically prior to a significant change in viral load. In addition, all donors with serologic tests suggestive of EBV replication once exhibited significant increases in viral load. On the other hand, individuals were always negative for ZEBRA mRNA.

NOTES:

PHOSPHATIDYLINOSITOL 3- KINASE ACTIVITY DETERMINES SENSITIVITY OF BURKITT'S LYMPHOMA CELLS TO B CELL RECEPTOR - MEDIATED EBV ACTIVATION

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B cell receptor (BCR) cross-linking leads to proliferation and differentiation of primary B-lymphocytes through the intracellular signal transduction. In Burkitt's lymphoma (BL) cell line Akata, anti-immunoglobulin (Ig) treatment stimulates BCR signaling and induces lytic program of EBV effectively. However, other BL cell lines including Daudi are less responsive to anti-Ig treatment. To assess the difference of signal transduction between Akata and Daudi cells, we analyzed BCR-mediated signaling induced by anti-Ig treatment. Our results revealed that activation of mitogen activated protein kinase (MAPK) and phosphatidylinositol 3'- kinase (PI3K) pathways were significantly repressed in Daudi cells compared to Akata cells. Since treatment with specific inhibitors of both pathways resulted in inhibition of EBV activation in Akata, PI3K and MAPK pathways are supposed to be required for BCR-mediated EBV activation. Furthermore, simultaneous treatment with anti-Ig and PI3K activator resulted in activation of PI3K and MAPK pathways and induction of lytic program in Daudi cells. These results suggest that activity of PI3K pathway determines sensitivity of BL cell lines to BCR-mediated EBV activation.

NOTES:

IMMUNE ACTIVATION CORRELATED WITH CLINICAL EVENTS IN ACUTE INFECTIOUS MONONUCLEOSIS

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Infectious Mononucleosis (IM) is the most common prolonged illness in University students, and is caused by delayed primary infection with Epstein-Barr Virus (EBV). Clinical features are thought to be caused by extensive T cell activation, however the molecular mechanisms underlying this are unclear. SAP, (SLAM associated protein), which is mutated in the inherited form of fatal IM, X-linked lymphoproliferative syndrome (XLPS), is a key regulator of lymphocyte activation via signals from cell surface 2B4 and Surface Lymphocyte Activation Marker (SLAM). Our aim was to follow T cell activation via this SAP/SLAM/2B4 pathway in IM, and correlate results with clinical features.

At diagnosis SAP, 2B4 and SLAM were significantly upregulated on both CD4 and CD8 T cells ($p<0.05$); expression fell over the course of IM, but 2B4 and SLAM remained elevated on CD8 cells at 40 days post diagnosis. The percentage of lymphocytes expressing CD8 was significantly higher in severe compared to mild cases of IM ($p=0.004$), and increased expression of 2B4 on CD8 cells correlated with increased viral load ($p< 0.05$). We suggest that T cells expressing 2B4 and SLAM are responsible for the clinical features of IM, but control of activation is maintained by parallel increased expression of SAP.

NOTES:

EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 INDUCES AND CAUSES RELEASE OF FIBROBLAST GROWTH FACTOR-2

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We have shown that the EBV oncoprotein, LMP1, induces a constellation of cellular tumor-invasiveness factors. Fibroblast growth factor (FGF)-2 is an angiogenic factor as well as a mitogenic protein. Although FGF-2 does not contain a hydrophobic signal sequence for secretion, FGF-2 is known to be released extracellularly. Here we show first that LMP1 induces in epithelial cells the expression of FGF-2 mRNA and protein through both LMP1 CTAR 1 and 2 *via* NF-κB signalling. Moreover, in contrast to the inducing agent, PMA, LMP1 induced the release into medium of the 18 kDa isoform of FGF-2 protein. LMP1 also promoted release of FGF-2 from 293 cells, which do not contain endogenous FGF-2, when cotransfected with FGF-2 expression plasmid. Finally, release of FGF-2 was partially suppressed by ouabain, which inhibits the activity of Na^+/K^+-ATPase α1 subunit, but not by Brefeldin A, which inhibits the classical ER/Golgi-dependent secretory pathway. The release of 18 kDa FGF-2 was almost completely inhibited by IκBα (S32A/S36A). The results suggest that extracellular release of FGF-2 protein is independently mediated by NF-κB signalling, not simply a consequence of induction itself.

NOTES:

EBV AND EPITHELIOID MALIGNANCIES

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EBV is associated with various epithelioid malignancies, including nasopharyngeal carcinoma (NPC), lympthoepithelioma-like carcinomas of the salivary glands, thymus, lungs and stomach, and some cases of gastric adenocarcinoma of ordinary histology. More recently, the association of EBV has been extended to breast carcinoma and hepatocellular carcinoma, in which a fraction of carcinoma cells are infected with EBV. Oropharyngeal epithelial cells are considered to be a site of EBV replication. This is clearly represented in the tissue of oral hairy leukoplakia, which is often found in AIDS patients.

In spite of frequent detection of EBV in various epithelioid tissues, epithelial cells have displayed a remarkable resistance to EBV infection *in vitro*. The use of recombinant EBV carrying a selectable marker has enabled this difficulty to be overcome. EBV-infected cell clones could be obtained from most carcinoma cell lines examined, and it was found that cell-to-cell contact was an efficient mode of EBV infection. Utilizing the soluble form of the gp350 protein and an anti-CD21 antibody, it was found that there are two distinct pathways for EBV infection of epithelial cells, a gp350-dependent pathway and a gp350-independent pathway, and at least in the latter, infection is mediated by a receptor other than CD21.

The majority of EBV-infected cell clones invariably express EBNA1, EBER, BARF0 and LMP2A, but not other EBNAs and LMP1. In these cells, only the Q promoter is used for transcription of EBNA, even in the early stage of EBV infection. This contrasts with EBV infection of B-lymphoma cells, in which the C/W promoter is active and all 6 EBNAs are expressed in the early infection stage.

EBV-infected cell clones, including those derived from primary culture cells had higher proliferation rates, cell saturation density and clonability in soft agarose then non-infected cell clones. Moreover, EBV-infected cells became resistant to induction of FAS-mediated apoptosis. Transfection of the EBER gene into EBV-uninfected epithelial cells conferred all the activities mentioned above. Although LMP1 is very important for immortalization of B-lymphocytes, it is not expressed in EBV-associated gastric carcinoma and about half of the cases of NPC. EBER appears to be a universal oncogene in both epithelioid and lymphoid malignancies.

NOTES:

CONTRIBUTION OF THE FAS/FAS LIGAND PATHWAY TO IMMUNE ESCAPE OF EBV-INFECTED HODGKIN/REED-STERNBERG CELLS

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EBV can be detected in the Hodgkin/Reed-Sternberg (HRS) cells in a proportion of Hodgkin's disease (HD) cases. Despite the fact that EBV-infected HRS cells could represent potential targets for EBV-specific CTLs, clearly *in vivo* such responses are either absent or insufficient to prevent tumour growth. We have examined the contribution of the Fas/FasL pathway to immune escape of EBV-infected HRS cells.

Firstly, we tested whether expression of Fas ligand by HRS cells may confer protection from CTLs by deletion of anti-tumour lymphocytes (tumour 'counter-attack'). Using a combination of approaches, including immunohistochemistry, western blotting and laser capture microdissection assisted-single cell RT PCR, we firstly confirmed that not only HD-derived cell lines, but also HRS cells from biopsies, express FasL irrespective of the presence of EBV in tumour cells. Using chromium release assays we have also examined the ability of FasL-expressing HD-derived cell lines to kill a variety of Fas-expressing targets, including EBV-specific CTLs.

Secondly, despite their close proximity to FasL-positive CTLs, HRS cells express Fas but are resistant to Fas-mediated killing. Here we show that both EBV-positive and EBV-negative HRS cells express the cellular FLICE-like inhibitory protein (cFLIP), an endogenous inhibitor of Fas-induced apoptosis and that this can contribute to resistance to CTL killing.

NOTES:

CELL-FREE EBV IS DETECTED IN PATIENTS WITH EBV(+) HODGKIN'S LYMPHOMA (HL) YEARS IN ADVANCE OF DIAGNOSIS

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Excess sera from the US Armed Forces HIV testing program was retrieved from the Department of Defense Serum Repository for 156 personnel who subsequently developed HL including 41 with EBV(+) tumor cells (identified by in situ hybridization or immunohistochemistry showing EBV gene expression in tumor cells) as well as from control personnel matched for age, gender, race and time of blood draw. TaqMan real-time PCR was used to detect EBV DNA in the archived sera. In patients who developed EBV(+) HL, specimens collected as many as 7 years in advance of diagnosis showed the presence of viral DNA. Compared to matched healthy controls, cases with EBV(+) HL were 21-fold more likely to have had EBV detected in serum 2 years before diagnosis (Mantel-Haenszel OR: 21, 95% CI: 2.3-192.7). In contrast, in patients with EBV(-) HL, viral DNA was detected only occasionally in prediagnostic sera, and the frequency of detection did not differ from that of matched healthy controls (Mantel-Haenszel OR: 0.5, 95% CI: 0.11-2.19). In all cases of EBV(+) HL, there was an upward trend in the quantity of viral DNA detected in the years preceding diagnosis, while in patients with EBV(-) HL and controls the presence of EBV DNA in sera was usually transient. The presence of viral DNA in serum may mark an early event in the development of EBV(+) HL.

NOTES:

Session 9: Hodgkin's Disease

INTRACLONAL HETEROGENEITY SUGGESTIVE OF AN ONGOING MUTATIONAL PROCESS CHARACTERIZES THE LATENT MEMBRANE PROTEIN-1 GENE OF HIV-RELATED HODGKIN'S DISEASE

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In the present study, we show that latent membrane protein-1 (LMP-1) deletions are more prevalent in Italian HIV-related Hodgkin's disease (HIV-HD) than in reactive lymphadenopathies from HIV^+ patients of the same area (28/30, 93% vs. 14/27, 51.9%; p=0.0006). Moreover, comparative analysis of paired neoplastic and non-neoplastic tissues showed that LMP-1 deletion mutants accumulate in HIV-HD lesions. To better understand the origin and biologic significance of these deletion mutants, the LMP-1 gene was subcloned and sequenced in 6 HIV-HD cases. The analysis showed a marked degree of LMP-1 intraclonal heterogeneity in all cases, consistently with an ongoing mutational process targeting the LMP-1 gene, whereas only limited sequence variations was observed in a polymorphic region of the EBNA-2 gene. Of note, the features of LMP-1 mutations were similar to those affecting immunoglobulin genes in germinal center B cells. Considering the histogenetic origin of Reed-Sternberg cells, our findings are consistent with the possibility that, in HIV-HD, LMP-1 may be targeted by an hypermutation process that may contribute to the enhanced generation of LMP-1 deletions in this setting. Furthermore, the high frequency of non-synonymous substitutions observed in most cases is consistent with a possible selection *in vivo* of LMP-1 protein variants with altered functional properties.

NOTES:

EPSTEIN-BARR VIRUS (EBV)-SPECIFIC CYTOTOXIC T CELLS (CTL) TO DELIVER TRANSGENIC IL-12 TO EBV-POSITIVE HODGKIN'S DISEASE

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Adoptive immunotherapy with EBV-specific CTL is effective for the prophylaxis and treatment of EBV-induced lymphoma in immunosuppressed patients. In EBV-positive Hodgkin's disease EBV-specific CTL function may be impaired by tumor-derived immunosuppressive factors, such as the TGF-β or Th2-promoting cytokines and chemokines such as IL-13 and TARC, which may suppress Th1 cellular function. Thus, methods are warranted to genetically modify T cells so that they retain their function, activity and ability to proliferate *in situ* when they enter the Hodgkin tumor site. IL-12 is a pivotal Th1 cytokine with potent anti-tumor activity. However, its use in human clinical trials has been severely limited by its systemic toxicity. We hypothesize that activation-dependent expression of transgenic IL-12 by EBV-specific CTL, could allow delivery of high doses of IL-12 to tumor sites, without systemic toxicity. EBV-specific CTL were transduced with a retrovirus vector encoding IL-12 (Flexi-IL12). Flexi-IL12-transduced CTL stably produced high amounts of biologically active IL-12, resulting in an elevated production of Th1 cytokines, such as INF-γ and TNF-α and decreased production of the Th2 cytokines, IL-4 and IL-5. EBV-specific Flexi-IL12-transduced CTL may not only be able to destroy the Th2 microenvironment supporting Hodgkin tumor cells, but also retain their anti-tumor specificity and function *in vivo*.

NOTES:

THE EPSTEIN BARR VIRUS RK-BARF0 INDUCES DEGRADATION OF SPECIFIC INTERACTING CELLULAR PROTEINS

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Yeast two hybrid analysis of the RK-BARF0 peptide identified several cellular proteins including the Notch family proteins, Notch3 and Notch4, a factor that influences epithelial cell growth, epithelin, the phospholipid enzyme, scramblase, and the human i-mfa domain containing protein, HIC. These proteins interact with RK-BARF0 in vivo as detected by co-immunoprecipitation. The interaction with RK-BARF0 significantly enhanced the proteosomal degradation of Notch and epithelin but not scramblase or HIC.

As an indirect marker for RK-BARF0 expression, the levels of endogenous unprocessed Notch were determined in EBV positive and negative lymphoid cell lines. Unprocessed Notch was detected at high levels in BJAB, BL30, and BL41, but was detected at low levels in Raji, HR-1, B95, or the EBV converted forms of BL30 and BL41 cells. Two antisera were prepared to peptides from the amino and carboxy regions of BARF0 and shown to specifically identify transfected RK-BARF0 but did not consistently identify related proteins in EBV+ cell lines or the C15 tumor. The low levels of Notch in EBV infected cells suggests that an EBV protein such as RK-BARF0 induces Notch degradation. The absence of detectable protein may indicate that the BARF0 ORF is not expressed in vivo. Alternatively, the effects of RK-BARF0 on proteosomal-mediated protein degradation may result in rapid turnover of the RK-BARF0 protein.

NOTES:

NOVEL CELLULAR TARGETS OF EBV ENCODED, TRANSFORMATION ASSOCIATED PROTEINS

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Our group uses two alternative strategies to identify novel targets of EBV encoded transforming proteins:

(1) yeast two-hybrid system screening combined with in vitro and in vivo validation

(2) suppressive-subtractive hybridisation PCR to find EBV induced genes

Using the former method we identified two novel partners of EBNA3A: a new uridin kinase/uracil phosphoribosyl transferase and the arylhydrocarbon receptor subunit p38, also known as Xap2, a protein that is a binding partner of HBV X-antigen. We found that EBNA-5 binds p14ARF. EBNA-5 can inhibit ARF induced apoptosis and growth arrest. EBV infection of B-lymphocytes induces ARF expression. Transfection of ARF into ARF negative cells that express wtp53 and hdm2 leads to the formation of extranucleolar inclusions that are surrounded by PML bodies and proteasomes. Co-expression of EBNA-5 promotes the formation of the inclusions with concomitant accumulation of p53, hdm2, ARF and EBNA5 in these nuclear bodies. Our data suggest that EBNA5 participates in the regulation of degradation of the components of ARF-p53-hdm2 pathway. Looking for genes that are specifically induced by EBV in type I Burkitt lymphomas using SSH-PCR, we identified a cellular oncogene that is highly expressed in all BLs but its expression is greatly diminished in endemic BLs that lost the virus. The gene is silent in normal centroblasts and centrocytes. If the expression of this gene is necessary for BL development and its activation requires an independent secondary event then its induction by the virus in endemic BLs may explain how EBV contributes to BL development.

NOTES:

EPSTEIN-BARR VIRUS EBNA2 BLOCKS NUR77 MEDIATED APOPTOSIS

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Epstein-Barr virus (EBV) infection in vitro immortalizes primary B cells. EBNA2 is an EBV encoded transcriptional transactivator that mimics the effects of activated Notch signaling and is essential for this proliferative response. An assay using Sindbis virus (SV) as a cell death inducer revealed that, like Notch, EBNA2 also has anti-apoptotic activity. We show that Nur77 is a mediator of SV induced cell death and that EBNA2 anti-apoptotic activity results from interaction with Nur77. EBNA2 colocalized with Nur77 in transfected cells and coprecipitated with endogenous Nur77 in IB4 B cells. EBNA2 binds to Nur77 through sequences in the EBNA2 aa 123-147 conserved domain and an EBNA2 mutant unable to bind Nur77 also lost the ability to protect cells from SV induced apoptosis. EBNA2 exerted its anti-death function by retaining Nur77 in the nucleus and preventing Nur77 from targeting mitochondria in response to apoptotic stimuli. NotchIC was found to act similarly to EBNA2 in preventing Nur77 cytoplasmic relocalization. Thus EBNA2 mimicry of NotchIC extends to a cell survival function mediated through Nur77 targeting.

NOTES:

IDENTIFICATION OF EBV-INDUCED GENES: ROLE OF LMP1 AND NF-κB

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To investigate the contribution of NF-κB and latent membrane protein 1 (LMP1) to EBV-induced gene expression, we used transcriptional profiling. We assessed mRNA abundance in Burkitt's lymphomas (BLs), BLs infected with EBV, lymphoblastoid cells (LCLs), BLs with inducible LMP1 expression, and an LCL with conditional NF-κB inhibition. Of the 8844 detectable cDNAs, 5585 gave signals in 90% of the arrays. Expression of 2721 cDNAs was higher in LCLs than in BLs and were classified as EBV-induced. LMP1 expression in BL induced 1504 cDNAs of which 933 were also EBV-induced. 1219 of the LMP1-induced cDNAs were NF-κB-dependent in at least one experiment and 266 were NF-κB-dependent in all tests. The intersection of EBV-induced, LMP1-induced and NF-κB-dependent genes identified 200 cDNAs including many that affect transcription, survival, metabolism, cytokines, and signal transduction. A separate analysis, using hierarchical clustering, identified 31 genes that are co-ordinately EBV-induced, LMP1-induced, and NF-κB-dependent. These included genes that regulate NF-κB activity (IκBα, NFκB2, TRAF1, c-rel, NFκB1, TANK, IκBε), adhesion (ICAM1, cytohesin1, B7-1, CD83), and apoptosis (c-IAP2, A20, Bfl-1, Fas). Other genes that clustered with this group, such as ERK3 and MCM2, may be important in EBV-induced transformation.

NOTES:

AN ANALYSIS OF EBV LATENT MEMBRANE PROTEIN 2A (LMP2A) LIPID RAFT LOCALIZATION AND FATTY-ACID MODIFICATION

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Latent infection of B cells by EBV results in the expression of a specific set of latent viral proteins including the transmembrane protein LMP2A. *In vitro,* LMP2A constitutively localizes to lipid rafts. LMP2A abrogates B Cell Receptor (BCR) signaling by blocking lipid raft recruitment of the activated BCR. LMP2A blocks the accumulation of tyrosine phosphorylated proteins, calcium mobilization, and EBV lytic activation from B Cells after lymphocyte stimulation. LMP2A also provides a survival signal in LMP2A expressing lymphocytes, perhaps substituting for the tonic signal provided by the BCR in resting B cells and during B cell development.

Lipid rafts appear to function as sorting sites for proteins involved in BCR signal transduction; therefore, the significance of LMP2A localization to lipid rafts was explored. Evidence presented here suggests that LMP2A alters the lipid raft localization of several signaling proteins. Furthermore, LMP2A is post-translationally fatty-acid modified by palmitic acid. Deletion and site-directed mutagenesis of LMP2A was performed to determine the sites of LMP2A modification. Experiments on LMP2A lipid raft localization provide a broader biochemical understanding about the affinity of transmembrane proteins or receptors to lipid rafts and the maintenance of EBV latency.

NOTES:

EBNA1 PARTITIONS PLASMIDS IN YEAST BY ATTACHING TO EBP2 ON MITOTIC CHROMOSOMES

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EBNA1 governs the segregation of latent EBV genomes by mediating their attachment to the host mitotic chromosomes. Mitotic chromosome attachment appears to involve the interaction of the EBNA1 DNA binding domain with the EBV segregation element and the interaction of the EBNA1 GR-rich region (amino acids 325-376) with human EBP2 on mitotic chromosomes. We have reconstituted EBV plasmid segregation in budding yeast and shown that it has all of the same protein and DNA sequence requirements as in human cells. To further investigate the segregation mechanism we have used this yeast system to examine the functional contribution of EBP2. We find that EBP2 attaches to yeast chromosomes in mitosis and is required for EBNA1 to attach to the chromosomes. The chromosome attachment and EBNA1 binding domains of EBP2 are separable and these two domains are required and sufficient for EBNA1-mediated plasmid segregation and EBNA1 chromosome attachment. A fusion protein containing the EBP2 chromosome attachment and EBNA1 DNA binding domains also binds mitotic chromosomes and efficiently partitions EBV plasmids. The data indicate that the attachment of EBNA1 to mitotic chromosomes is critical for EBV plasmid segregation in yeast, as it is in humans, and that is the function of EBP2.

NOTES:

THE EBV DNA POLYMERASE PROCESSIVITY FACTOR, BMRF1, ACTIVATES EXPRESSION OF GASTRIN

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Some gastric carcinomas contain the EBV genome, but it is not clear how EBV infection contributes to the development of this malignancy. The gastrin peptide hormone, which is normally expressed only in gastric G cells, is a growth factor for gastrointestinal cells. Over-expression of gastrin is thought to contribute to the development of certain GI malignancies, including gastric carcinoma. We have previously shown that the BMRF1 gene product transcriptionally activates the promoter of the early EBV gene, BHLF1. Using microarray technology, we discovered that the BMRF1 gene product induces expression of gastrin in telomerase-immortalized keratinocytes. This result was confirmed by northern blot analysis. Co-transfection of gastrin promoter constructs with a BMRF1 expression vector also increased gastrin promoter activity, and this effect required two GC-rich motifs in the gastrin promoter which function as binding sites for the cellular ZBP-89 transcription factor. Furthermore, BMRF1 activated transcription of the early viral gene, BHLF1, through a ZBP-89 binding motif. Thus BMRF1 transcriptional effects may be mediated through an interaction with ZBP-89. In addition, our results suggest that the lytic form of EBV infection in gastric epithelium could potentially promote the development of gastric carcinoma though a paracrine mechanism involving increased release of gastrin.

NOTES:

INHIBITION OF FAS-MEDIATED APOPTOSIS IN EBER-EXPRESSING EPITHELIAL CELLS

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We succeeded in infecting human epithelial cells *in vitro* and demonstrated that EBV infection induces growth acceleration in epithelial cells. The next question is what EBV product is responsible for this. In studies of Burkitt's lymphoma (BL) cells, we have found that EBER plays important roles in the malignant conversion of BL cells. In this study we assessed the roles of EBER in epithelial cells using the Intestine 407 cell line, which is derived from human intestinal cells and is known to be susceptible to induction of Fas-mediated apoptosis. We transfected the EBER plasmid into Intestine 407 cells and isolated EBER-expressing cell clones. These cell clones were treated with an anti-Fas antibody in the presence of cycloheximide for 16 h and subjected to apoptosis assay by flow cytometry. The results indicated that apoptosis was induced in neomycin-resistant gene-transfected control cell clones, whereas EBER-expressing cell clones were resistant to apoptosis. Furthermore, EBER-expressing cell clones had enhanced population doubling and grew to attain a highly increased saturation density. Our findings suggest that EBER plays a role in the development of EBV-associated epithelioid malignancies.

NOTES:

ARE CLONALITY DETERMINATIONS IN EBV-ASSOCIATED EPITHELIAL CELL MALIGNANCIES AN ARTIFACT OF LMP-2A DIRECTED EPISOME SELECTION?

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Assignment of a causal role to EBV in cancers is based in part on the perception they are clonal expansions of a single EBV-infected cell. Upon entry, linear EBV circularizes by homologous recombination of terminal repeats(TR), producing fused termini of unique length for each circularization event. Expansions of one infected progenitor contain fused TRs of common lengths, a finding used to implicate virus in tumor initiation. After infection of cultured epithelial cells, what was expected to be a polyclonal outcome appeared clonal by termini analysis. When cells exposed to EBV-*neo* recombinants were maintained under antibiotic pressure, infections initially polyclonal evolved to predominance by single episomes. Because circularization allows LMP2A expression from exons at either end of the genome, we tested whether transcription of this spliced mRNA is influenced by intervening TRs. By quantitative RT-PCR of BL lines, LMP2A mRNA varied inversely to TR number. A similar pattern was observed in cell clones of infected epithelial cell line CCL20.2. Given the growth advantage provided epithelial cells by LMP2A, clonality determinations based on EBV termini may be spurious, homogeneous episomal populations reflecting instead selection for optimal TR number. Under this scenario EBV may infect established tumors, facilitating progression not initiation, a view compatible with viral presence in diverse malignancies.

NOTES:

GENES ASSOCIATED WITH GENETIC SUSCEPTIBILITY TO NASOPHARYNGEAL CARCINOMA ARE LOCATED PRIMARILY WITHIN THE HLA-A LOCUS IN TAIWANESE

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Numerous studies have indicated specific HLA haplotypes and genes within the HLA complex associated with NPC. To localize the genetic susceptibility gene, the HLA-A, -B, and -A2 subtypes were examined for their association to NPC. Consistent with previous reports, the frequencies of HLA-A2(OR = 2.50, *pc* value = 0.031) were significantly higher in patients with NPC than in healthy controls. Two-locus analysis indicated that A2(+)B46(+) individuals are at greater risk of NPC than A2(+)B46(−) populations. This however, may be due to the close linkage of these two genes. Moreover, A2(+)B38(+) individuals were at higher risk than A2(−)B38(−) individuals; A2 and B38 are not genetically linked. These findings suggest that B38 or B46 alone cannot confer a high risk of NPC, but that in conjunction with A2, being B38(+) or B46(+) greatly increases the risk. None of A2 subtypes identified was significantly associated with NPC. Microsatellite marker D6S211 close to HLA-A, was analyzed for its association with NPC. Allele 4 of D6S211 was statistically significantly associated with NPC (OR = 2.99, *pc* = 0.044). These results strongly support the hypothesis that genes associated with genetic susceptibility to NPC are located within the HLA-A locus.

NOTES:

THE PARADOXICAL ROLE OF LMP1 IN CARCINOGENESIS

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The role of LMP1 in predisposing epithelial cells to malignancy *in vivo* was investigated using the PyLMP1 transgenic mouse model, expressing LMP1 in the epidermis (Wilson *et. al.,* 1990. *Cell* **61**: 1315-1327). Skin chemical carcinogenesis studies revealed that LMP1 acts as a tumour promoter, augmenting the action of TPA (Curran *et. al.*, 2001 *Cancer Res* **61**:6730-6738). Treated PyLMP1 mice develop significantly more small papillomas than their wild type siblings. However, we have modelled the rate of lesion expansion and have found that the rate of growth of papillomas in PyLMP1 mice is significantly lower than that of controls. Also, LMP1 does not contribute to the conversion of papillomas to carcinomas.

The INK4a locus encodes two tumour suppressor genes, $p16^{INK4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in the mouse) and is regularly deleted in nasopharyngeal carcinomas (chromosome 9p21). The interplay between LMP1 and the INK4a locus was investigated using PyLMP1:INK4a null mice (Serrano *et. al*., 1996 *Cell* **85**:27-37). With carcinogen treatment, it was found that loss of the INK4a locus overcomes the LMP1 mediated inhibition of papilloma size expansion. Furthermore, LMP1 and loss of the INK4a locus co-operate strongly during carcinogenesis, leading to large lesions which have a high rate of conversion to carcinoma.

NOTES:

A COMPARISON OF INFECTION OF CELLS VIA LYMPHOCYTE AND EPITHELIAL RECEPTORS

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Infection of lymphocytes and epithelial cells involves different glycoproteins and receptors. B cell attachment requires gp350 and CR2. Entry requires interaction between gHgLgp42 and HLA class II mediated by gp42. Epithelial cell attachment requires gHgL and soluble gHgL could bind specifically to a gHgL receptor (gHgLR). Entry requires gHgL alone and is class II-independent. Viruses from B cells (B-EBV) and epithelial cells (E-EBV) differ in gp42 content which influences tropism. The relative contributions of receptors to infection by the two viruses were examined in gHgLR+ epithelial cells engineered to express CR2 and class II, phenotypes that likely exist *in vivo*. CR2/gp350 mediated high levels of binding of both viruses. Addition of class II enhanced infection by E-EBV more than B-EBV, facilitating infection of 100% of cells. Antibody mapped to the membrane proximal region of gH neutralized as a Fab fragment and indicated B-EBV used gHgL complexes only, whereas E-EBV used both gHgL and gHgLgp42. In the absence of CR2 B-EBV binding remained high. Infection was better with than without class II, but in both cases was low. E-EBV bound poorly to gHgLR, but infected better than B-EBV. This suggests use of gHgLR for attachment compromises participation of gH in fusion.

NOTES:

REGULATION OF EXPRESSION AND MECHANISM OF ACTION OF THE EPSTEIN-BARR VIRUS LYTIC CYCLE ACTIVATOR, ZEBRA

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The contributions of Werner Henle to EBV research are monumental; they include development of serologic methods to characterize the association of EBV with different diseases and demonstration of lymphocyte immortalization by the virus. Before working on EBV Werner Henle made fundamental discoveries about basic mechanisms of viral persistence and reactivation. In this Henle lecture we will address some of the numerous unresolved questions about lytic cycle reactivation of EBV. Do the many inducing stimuli converge on a final common pathway for reactivation? What role do chromatin repression and CpG methylation play in the regulation of BZLF1? Are there specific repressors of Zp? What is the reason that mutants in ZEBRA's basic domain fail to disrupt latency? What is the functional role of phosphorylation of ZEBRA protein? How does ZEBRA distinguish its numerous functions as transcriptional activator of early viral genes, viral replication protein and repressor of early expression of late viral genes? What is the mechanism of synergy between ZEBRA and Rta? How do cell and virus background modulate ZEBRA expression and action? Understanding the answers to these questions will eventually lead to a deeper understanding of the biology of EBV, of the problem of viral latency and reactivation, and of control of eukaryotic gene expression.

NOTES:

INHIBITION OF THE EBV LYTIC CYCLE SWITCH PROTEIN ZTA

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The switch between EBV latency and entry into the productive cycle of viral replication is governed by the function of the bZip protein (Zta) encoded by the EBV gene BZLF1. Our biophysical analyses demonstrate that the dimerisation domain of Zta contains a coiled-coil motif which is unusually short (aa 194 to 221) and of low thermal stability compared with other members of the bZip family[1].

We present evidence that a short synthetic peptide of coiled-coil sequence is able to disrupt the formation of full-length Zta DNA-complexes by inhibiting Zta dimerisation. This molecule has a similar IC50 for all characterised sequence variants within the coiled-coil region of Zta suggesting that it has the potential to form the basis of a therapy directed against all isolates of EBV.

We also present new evidence that although the previously defined short coiled-coil interface contributes to Zta function *in vivo* and *in vitro*, an additional 21 amino acids, located C-terminal to this motif is required for full Zta function. This region is unique to Zta and its interplay with the coiled-coil is under further investigation.

[1]Hicks, Balesaria, Pandaya, Woolfson and Sinclair, Journal of Virology 75: 5381-5384, 2001.

NOTES:

THE ZINC FINGER E-BOX-BINDING PROTEIN, ZEB, NEGATIVELY REGULATES THE LYTIC SWITCH BZLF1 GENE PROMOTER OF EPSTEIN-BARR VIRUS.

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Epstein-Barr virus (EBV) is a human herpesvirus capable of establishing a latent state in B-lymphocytes. One gene product of EBV, called BZLF1 (also termed Zta), can autoactivate its promoter as well as initiate the switch from latency to a productive infection. Previously, the Mertz laboratory identified a negative *cis*-acting element, called ZV, within the BZLF1 promoter. ZV is located at nts –17 to –12, contains the sequence 5'-CAGGTA-3', and binds the sequence-specific cellular factor termed ZVR. Based upon sequence-binding specificity, we postulated that ZVR may be ZEB, (zinc finger E-box binding factor) or a related zinc finger/homeodomain family member. Immunoshift assays indicated that ZVR cross-reacts with antibody to δEF1, the chicken homologue to ZEB, as does ZEB, obtained from whole-cell extracts of 293 cells overexpressing ZEB. Competition EMSAs showed that ZEB binds to the ZV element with the same binding specificity as ZVR. Overexpression of ZEB repressed Zta induction of the wild type BZLF1 promoter 3-to-4 fold in both ZVR-positive DG75 cells and ZVR-negative, MCF-7 cells, while it repressed Zta induction only two-fold in a mutant promoter unable to bind ZEB. Thus, we conclude that the previously identified cellular repressor ZVR is the zinc finger E-box protein ZEB. Further, we identified a ZEB binding site within the promoter of the BRLF1 lytic gene of EBV. We postulate the ZEB likely plays an important role in regulating the life cycle of EBV.

NOTES:

THE EPSTEIN-BARR VIRUS ZEBRA PROTEIN ACTIVATES TRANSCRIPTION FROM THE EARLY LYTIC F PROMOTER BY BINDING TO A PROMOTER-PROXIMAL AP-1-LIKE SITE

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The ZEBRA protein encoded by the Epstein-Barr virus (EBV) genome activates a switch from the latent to lytic gene expression programme of the virus. ZEBRA, a member of the basic leucine zipper family of DNA-binding proteins, is a transcriptional activator capable of inducing expression from several virus lytic cycle promoters by binding to activator protein 1 (AP-1) -like sites. The Epstein-Barr virus *Bam*HI F promoter, Fp, was for some time believed to initiate EBNA1-specific transcription in EBV-transformed latent cells. More recent data, however, show that Fp is an early lytic promoter and that the dominant EBNA1 gene promoter in latent cells is Qp, located about 200 bp downstream of Fp. In the present investigation we confirm that Fp displays the characteristics of a lytic promoter. Fp is down-regulated in latently EBV-infected cells in reporter plasmids which carry Fp regulatory sequences upstream of position -136 and down to +10 relative to the Fp transcription start site (+1). We show that the repression of Fp in latent stages of infection can be abolished by ZEBRA, and demonstrate that ZEBRA activates Fp through a direct interaction with an AP-1-like site at position -52/-46 in the promoter-proximal Fp region.

NOTES:

CHARACTERIZATION OF DOMAINS OF THE EB2 PROTEIN REQUIRED FOR mRNA EXPORT: NLS, NES, RNA-BINDING AND INTERACTION WITH REF AND TAP

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The Epstein-Barr virus protein EB2, product of the BMLF1 gene, is an essential early EBV gene required for the production of infectious viral particles (Gruffat et al, Session 07). The EB2 protein shares properties with mRNA export factors. EB2 induces the cytoplasmic accumulation of viral mRNAs, it binds RNA *in vivo* although no EB2-specific sequences have been identified and it shuttles between the cytoplasm and the nucleus. In this report, we have characterised the EB2 sequences required for nucleo-cytoplasmic shuttling, RNA binding and REF interaction. We have localised several Nuclear Localisation Signals (NLS) and the Nuclear Export Signal (NES) in the N-terminal part of the protein. This NES is different from the double NES motif previously described by Chen et al. (1), which in our experiments can be deleted without affecting EB2's shuttling. We have also found that the EB2 protein interacts both *in vitro* and *in vivo* with REF, a cellular factor directly involved in the nuclear export of mRNA and we have mapped the interaction domains both in EB2 and REF. RNA-bound EB2 could therefore recruit the mRNA export factor REF, and probably also TAP, to export viral mRNA.

(1). Chen et al (2001) Virology, 288, 119-128.

NOTES:

EPSTEIN-BARR VIRUS GP42 OCCURS AS A TYPE II MEMBRANE PROTEIN AND AS A SOLUBLE SECRETED PROTEIN AND INHIBITS MHC CLASS II RESTRICTED T CELL ACTIVATION

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Epstein-Barr virus persists lifelong in infected hosts. In particular the large number of viral proteins that is expressed during primary and recurrent lytic infection provides a vast array of potential targets for anti-viral immunity. The fact that EBV nevertheless persists for life indicates that the virus has acquired effective ways to elude the host immune system.

The lytic phase protein gp42, encoded by the BZLF2 orf, functions as a co-receptor for viral entry into B-cells. Gp42 binds to HLA class II molecules. Although this interaction is well documented, it is unknown whether endogenous synthesis of gp42 in infected cells influences antigen presentation by MHC class II molecules. Here, we demonstrate that endogenously expressed gp42 inhibits HLA class II-restricted activation of T-helper cells. Gp42 does not cause down-regulation of class II/ peptide complexes. Rather, gp42 associates with class II molecules at their various stages of maturation. Gp42 occurs as a full length type II trans-membrane protein and as a truncated soluble protein that results from co- or post-translational cleavage, presumably by a signal peptidase. The soluble protein is secreted and is sufficient to block T-cell activation.

In conclusion, EBV gp42 acts as an inhibitor of antigen-specific, HLA class II-restricted T-cell immunity by virtue of its class II-binding properties.

NOTES:

BZLF1 INHIBITS THE ABILITY OF LMP1 TO UPREGULATE EXPRESSION OF MHC IN LYTIC CYCLE

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The growth-transforming properties of EBV are associated with efficient presentation of antigen to immune-effectors by latently-infected lymphoblastoid cells. This is important for limiting the potentially pathogenic effects of EBV infection in the healthy host. We now show that, in common with other herpesviruses which employ numerous mechanisms for immune evasion to facilitate virus persistence, EBV lytic cycle is associated with reduced expression of MHC. Flow cytometry analysis of BZLF1-positive cells showed MHC class I to be reduced by 75-80% and MHC class II by about 50% relative to latently infected cells. This is paradoxical since LMP1, which can upregulate MHC class I and II, is expressed in lytic cycle in B cells. Downregulation of MHC is an early event in lytic cycle which is not blocked by culturing cells in acyclovir, and it persists into late lytic cycle as shown by 2-colour flow cytometry for MHC and viral capsid antigen. While BZLF1 did not directly affect MHC expression, we demonstrated that BZLF1 completely inhibited the ability of LMP1 to upregulate MHC. The precise mechanism of this inhibition remains to be resolved, but we have shown that it is not primarily due to any effect upon activation of NFκB.

This work was funded by the Medical Research Council and the Wellcome Trust, London.

NOTES:

IMMUNIZATION WITH EPSTEIN-BARR VIRUS-SPECIFIC PEPTIDE PULSED-DENDRITIC CELLS INDUCED FUNCTIONAL CYTOTOXIC T CELL IMMUNITY AND TUMOR REGRESSION IN PATIENTS WITH NASOPHARYNGEAL CARCINOMA

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Nasopharyngeal carcinoma (NPC) is a common neoplasm in southeastern Chinese and is associated with Epstein-Barr virus (EBV) infection. Dendritic cells are professional antigen-presenting cells that have been exploited to induce anti-tumor immunity both *in vitro* and *in vivo*. In this study, autologous monocyte-derived dendritic cells were cultured from patients with advanced NPC, matured with cytokine, and pulsed with (depending on patients' HLA type) HLA-A1101-, A2402-, and B40011-restricted epitope peptides from EBV latent membrane protein-2 (LMP2), a protein expressed in tumor cells. Dendritic cells were injected into one of the inguinal lymph nodes under ultrasonographic guide. Sixteen patients were studied, all with local recurrence or distant metastasis following conventional therapies. Each patient received four injections at weekly intervals. Post-immunization CTL responses were assayed by epitope-specific interferon-γ production and cytotoxicity. Clinical responsiveness was regularly monitored. Results showed that epitope-specific CTL response were elicited or boosted in nine patients receiving HLA–A1101 or A2402-restricted peptides, with a higher frequency seen in A1101-responsive patients. The frequency of epitope-specific CTLs in blood was increased 2 weeks after the first injection, sustained for at least 3 months, but declined to pre-vaccination levels after 6 months. CTLs harvested at 3-month post-vaccination from A1101-responsive patients possessed significant cytolytic capacity against target cells, which was not detectable in A2402 patients. Two A1101-responsive patients had partial tumor reduction three months after vaccination, coincident with an increase in HLA-A1101 epitope-specific CTLs. These findings suggest that intranodal injection of dendritic cells primed with synthetic LMP2 epitopes is tolerable in patients with advanced NPC, and epitope-specific CTL immunity could be enhanced and were accompanied by tumor regression in selected patients. Approaches leading to stronger and more sustained EBV-specific CTL responses, therefore, have therapeutic potential in the context of NPC.

NOTES:

DIFFERENTIAL RECOGNITION OF EBNA1 BY HUMAN T AND B-CELL RESPONSES: IMPLICATIONS FOR ANTIGEN-PROCESSING AND PRESENTATION IN VIVO.

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In healthy carriers EBNA1 is a dominant target for IgG-antibodies and $CD4^+$ T-cells but is minimally recognised by CD8+ T-cells. Using overlapping peptides, the epitopes involved in these responses were fine-mapped using PEPSCAN and IFNγ-ELISPOT assays and localised to functional domains on the EBNA1 protein.

Antibody epitopes dominantly located in the Gly-Ala repeats, in a region spanning AA390-450 proximal to the DNA binding-dimerization (core) domain and in the C-terminus acidic repeat (AA620-641). Further fine-mapping revealed small 3-D conformational epitopes on the surface of the core domain excluding regions involved in DNA-binding and dimerization. Few epitopes mapped to the Gly-Arg rich regions flanking the Gly-Ala repeat. Multiple CD4-epitopes were identified, mainly locating in the core domain (AA460-607) and its proximal end (AA430-460) and not at all in the Gly-Ala repeat or the extreme C-terminus. Only three CD8+ T-cell epitopes were identified at AA73-81, 410-419 and 528-537 respectively.

The data suggest that EBNA1 is presented as multimeric DNA-bound antigen to B-cells and processed as exogenous antigen by APC's for T-cell presentation, thereby revealing hidden epitopes in the stable DNA-binding core domain. The Gly-Ala repeats are not processed into MHC-I/-II epitopes. Multimeric EBNA1-DNA complexes may provide a T-cell independent antigen for B-cell stimulation.

NOTES:

GENERATION OF IMMUNOTHERAPEUTIC CTL POPULATIONS SPECIFIC FOR LMP2 BY RETROVIRAL TRANSDUCTION OF THE TCR.

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EBV-associated Hodgkin's disease (HD) does not express the immunodominant EBNA-3 latency antigens, the primary target of CTL lines used in the immunotherapy of PTLD. HD does express latency membrane protein (LMP)-2, which is recognized, with a lesser magnitude, by the cellular immune response in half of seropositive volunteers (Lee, S.P. et al., 2000). To develop an immunotherapeutic strategy to treat EBV-associated HD, CTL clones specific for an HLA-A2 restricted peptide from LMP2 (LLWTLVVLL) were generated, the TCR-alpha and -beta chains cloned, transferred to a retroviral expression vector, and then used to transduce activated PBMC. A fully functional TCR was transferred as demonstrated by cytolytic activity and gamma-IFN secretion. To produce a TCR-expression vector that transfers the ability to lyse endogenously expressed LMP2, as demonstrated by B-LCL lysis, TCRs from two CTL clones, CS1C7 (HLA A23-restricted, PYLFWLAAI-specific) and NB20 (HLA A2-restricted, CLGGLLTMV-specific) have been molecularly cloned and introduced into retroviral TCR expression constructs. Re-cloning of transduced PBMC has demonstrated that a wide range of lytic activity among individual CTL is generated, arguing that the optimal therapeutic benefit with transduced lymphocytes would be gained by first selecting lymphocyte subsets that best support functional expression of the transduced TCR.

NOTES:

CYTOLYTIC CD4$^+$ T CELL CLONES SPECIFIC FOR EBNA 1 INHIBIT EPSTEIN-BARR VIRUS-INDUCED B CELL PROLIFERATION

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In the absence of immunosurveillance, EBV-infected B cells generate neoplasms *in vivo* and transformed cell lines *in vitro*. We previously demonstrated *in vitro* that memory CD4+ T cells were necessary and sufficient to prevent proliferation of newly EBV-infected B cells.(1) Here we show that CD4+ T cell clones specific for the latent EBV antigen EBNA1 are also capable of preventing proliferation of B cells from MHC Class II-matched donors. Clones were generated by limiting dilution of CD4+ T cells alternately exposed to LCLs and dendritic cells expressing EBNA1. They secreted IFNγ upon MHC Class II-mediated recognition of freshly infected B cells as early as 4 days after exposure to EBV. EBNA1-specific clones inhibited proliferation of EBV-infected B cells with an efficacy similar to that of mixed CD4+ T cells. They lysed LCLs via a perforin-independent mechanism. Once specifically activated, they effected "bystander" killing of non-MHC-matched EBV-infected B cells. An EBNA1-specific CD4+ T cell clone's ability to recognize an LCL correlated with its ability to exert immune control over newly EBV-infected B cells from the same donor. Since EBNA1 is the only viral protein expressed in every form of EBV-related malignancy and evades recognition by CD8+ T cells, EBNA1-specific CD4+ T cell clones are candidates for use in immunotherapy against multiple neoplasms.

1. Nikiforow, S., K. Bottomly, and G. Miller. 2001. CD4+ T-cell effectors inhibit Epstein-Barr virus-induced B-cell proliferation. J Virol. **75:**3740-52.

NOTES:

EBNA1 SPECIFIC CD4$^+$ TH1 CELLS KILL EBV ASSOCIATED BURKITT'S LYMPHOMA THAT EVADES CD8$^+$ CTL RECOGNITION

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Herpesviruses have evolved with the human immune system and developed various immune evasion strategies. Epstein-Barr virus infects B cells at different stages of their development and of different antigen presentation ability. Infected germinal center B cells support latent EBV infection with a small subset of latent EBV antigens expressed. Of this origin, Burkitt's lymphoma presents the most extreme case of EBV immune evasion. The only expressed EBV antigen, EBNA1, prevents its own presentation onto MHC class I and this processing pathway is in addition generally disabled in these cells by downregulation of TAP. We found the first T cell response that directly recognizes this tumor despite its immune evasion mechanisms. We could demonstrate that Burkitt's lymphoma cells cause EBNA1 specific CD4$^+$ T cells to secrete IFNγ and kill via Fas/FasL interaction. This suggests that the human immune system has adapted to control this state of latent EBV infection by substituting CD8$^+$ with CD4$^+$ T cell recognition. Endogenous processing of EBNA1 onto MHC class II and cytolytic function of EBNA1 specific CD4$^+$ T cells can target the EBV associated malignancy Burkitt's lymphoma. Since EBNA1 specific CD4$^+$ Th1 cells can be consistently found in healthy EBV carriers at high frequency in peripheral blood, we suggest that they mediate an important supportive role for EBV specific T cell immunity as well as direct protection against EBV associated lymphomas.

NOTES:

GAMMAHERPESVIRUS CAPSID STRUCTURE AND PROTEIN COMPOSITION: COMPARISON OF RHESUS MACAQUE RHADINOVIRUS (RRV) AND KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV)

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Kaposi's sarcoma (KS), the most common AIDS-associated malignancy, is caused by the gammaherpesvirus, KSHV. We have previously isolated KSHV capsids and analyzed their protein composition and structure. However, extension of this work and study of capsid assembly has met with considerable difficulty due to the exceedingly low yields of KSHV in *in vitro* culture systems. In contrast, RRV, the closest known phylogenetic relative to KSHV, displays robust lytic phase growth in cell culture and, thus, holds promise as a model system.

We now report the isolation and characterization of RRV capsids. As with other herpesviruses, RRV lytic replication results in three distinct (A, B and C) capsid species. We have identified the structural protein composition of each. Furthermore, employing electron cryomicroscopy and computer-aided reconstruction, we have determined the structures for RRV B and C capsids to less than 30 Å, while refining that of A capsids to 17Å--the highest resolution reached to date for any gammaherpesvirus capsid. Taken together with results of our earlier KSHV studies, our data indicate a high degree of similarity between the capsids of the two viruses and argue for the use of RRV as a potentially powerful model to study gammaherpesvirus structure and assembly.

NOTES:

THE COMPLETE GENOMIC SEQUENCE OF THE FIRST EPSTEIN-BARR-RELATED HERPESVIRUS NATURALLY INFECTING A NEW WORLD PRIMATE: A DEFINING POINT IN THE EVOLUTION OF ONCOGENIC LYMPHOCRYPTOVIRUSES

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The complete genomic sequence has been derived for the marmoset LCV (Callithricine herpesvirus-3, CalHV-3), the first Epstein-Barr-related herpesvirus found naturally infecting a New World primate. In contrast to Old World LCVs, the marmoset LCV gene repertoire is different from EBV. Sixteen EBV genes are absent from the marmoset LCV genome, and the marmoset LCV encodes 8 unique genes, identified as C0 to C7, that have no homology with known cell or viral genes. As a prototype for New World LCVs, the marmoset LCV provides the basis for defining groups of LCV genes based on their evolutionary pattern: 1) the universal herpesvirus genes, 2) the ancestral LCV-specific genes, ie those present in all LCVs, that can be divided into conserved and divergent subgroups, and 3) the acquired LCV-specific genes, ie those found in Old World LCVs but absent in the marmoset LCV. These different groups reflect the dynamic evolution of the LCV genera and provide clues as to how specific viral genes may contribute to pathogenesis of EBV infection.

NOTES:

TUMORIGENESIS AFTER EXPERIMENTAL INFECTION IN AN EPSTEIN-BARR VIRUS PRIMATE ANIMAL MODEL

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To develop an experimental model for AIDS-associated, EBV-positive lymphomas, primary infection with the EBV-related rhesus lymphocryptovirus (LCV) was studied in immunocompetent or SHIV 89.6P infected, immunosuppressed rhesus macaques. Primary LCV infection after oral inoculation of immunocompetent hosts was characterized by an acute viremia and seroconversion followed by asymptomatic LCV persistence. Immunosuppressed macaques infected orally with LCV failed to develop an LCV-specific humoral response, viremia was more pronounced, but there was no evidence of lymphoproliferation. Additional immunosuppressed macaques were challenged intravenously with 10^8 autologous, LCV-transformed B cells. These immunosuppressed animals also failed to seroconvert, but in these instances there was evidence for lymphoproliferation. One animal was euthanised after 2 months for a nosocomial infection and had infiltration of LCV-infected lymphocytes in multiple organs. A second animal developed a large, rapidly growing submandibular mass after 5 months, and pathological studies confirmed an LCV-positive, malignant lymphoma. These studies demonstrate that this LCV isolate is tumorigenic in vivo and provide an experimental model for studying LCV-induced oncogenesis in a natural host. SHIV-infected hosts are relatively resistant to LCV-induced oncogenesis suggesting that non-CD4+ T cell dependent immune responses can be important for control and prevention of EBV-induced malignancies.

NOTES:

COMBINATION OF EPSTEIN-BARR VIRUS-ENCODED BARF1 GENE AND H-*RAS* IS NECESSARY TO TRANSFORM A PRIMARY MONKEY EPITHELIAL CELL

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Epstein-Barr virus (EBV) immortalizes *in vitro* both primary lymphocytes and epithelial cells. The oncogenic activity of EBV in B lymphocytes is well documented, but little is known in epithelial cells. We previously showed that transfection of BARF1 gene encoded by EBV genome in the monkey kidney primary epithelial cells led to continuous cell growth (*Wei et al., 1997, Oncogene, 14:3073-3082*). For immortalization of human epithelial cells, SV40 large-T alone was not sufficient but required the co-expression of telomerase gene (*Hahn et al.,Nature,1999, 400, 464-468)*. However the role played by telomerase in EBV oncogenesis is not understood. We show here that BARF1 gene transfection led to immortalization of primary epithelial cells through telomerase activation. The telomerase expressing immortalized cells however did not induce any tumor in nude mice. In these cells, c-myc and Max genes involved in the activation of telomerase were highly expressed. The expression of additional Ras gene in these cells accelerated the cell growth and created a malignant transformed cell line. We thus could establish an *in vitro* experimental model for studying the oncogenic role of EBV.

NOTES:

CHARACTERIZATION OF CIS AND TRANS-REQUIREMENTS FOR KSHV LATENT ORI FUNCTION

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KSHV/HHV-8 is associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. The latency-associated nuclear antigen (LANA) is a multifunctional protein expressed in all KSHV-associated malignancies.

LANA is required for maintenance of episomal DNA in dividing cells and regulates transcription. Co-localization studies suggest that LANA tethers the episome to chromosomes during mitosis. In support of this model a LANA binding site has been identified within the terminal repeat (TR) and a chromatin interaction domain was mapped within the N-terminus of LANA.

By performing short-term replication assays in epithelial- and endothelial-derived cells, we demonstrate for the first time that de novo synthesis of TR containing plasmids is dependent on the presence of LANA. We map minimal cis-acting sequences within TR and show that the DNA binding domain is required for its replication activity and by itself partially supports replication. We also identify a second LANA binding site in TR that binds LANA cooperatively. These data show that LANA, like EBNA-1, supports DNA replication, and genome segregation. In addition, our data demonstrate that all required cis-elements for oriP function are located within a single TR suggesting that the putative ori of KSHV is structurally remarkably different from those of other γ-herpesviruses.

NOTES:

NEW DATABASE OF ANNOTATIONS OF VIRAL GENOMES: HERPESVIRUS SECTION

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More than a thousand genomes of eukaryotic viruses currently available in GenBank were analyzed by the new gene finding program GeneMarkS EV. The predicted genes were compiled into a database (http://opal.biology.gatech.edu/GeneMark) with an interactive interface. The predictions were compared with the GenBank annotation and the comparison results are cited in the database gene records. The database design allows the user to further explore newly predicted proteins by BLASTP and SMART searches. One of the most interesting sections of the database is the section of herpesviruses. While 757 gene predictions matched the annotated genes in nine human herpesvirus genomes, we have identified 49 protein-coding regions previously not annotated as genes. Several new predictions have already been included in the RefSeq database, the part of GenBank annotated by the National Center for Biotechnology Information. The GeneMarkS EV program is the new version of the GeneMarkS program (Besemer et al., 2001) developed for analysis of newly sequenced genomes of eukaryotic viruses.

1. Besemer J., Lomsadze A. and M. Borodovsky (2001) GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions, *Nucleic Acids Research,* 29, 2607-2618.

NOTES:

LMP1 STIMULATION OF THE PI3-KINASE PATHWAY ACTIVATES DIVERSE CELLULAR TARGETS SUCH AS AKT AND THE SMALL RHO GTPASES

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Phosphatidyinositol-3-kinase (PI3-kinase) mediates a range of effects in response to extracellular stimuli including cell growth, differentiation and survival. Like TNF-α and CD40L we have shown that LMP1 also activates the PI3-kinase pathway. LMP1 was found to stimulate PI 3-kinase activity in both Rat-1 fibroblasts and in human epithelial cells (SCC12F) inducing activation of c-Akt, a downstream target of PI3-kinase. This activity required the cytoplasmic tail of LMP1, as cross-linking of a rat CD2-LMP1 chimaera was sufficient to induce c-Akt activation. Microinjection of LMP1 mutants with a GFP-tagged PH domain of GRP1 (a PIP3 binding protein) identified CTAR1 as the domain of LMP1 responsible for PI3-kinase activation. Treatment of LMP1-expressing epithelial cells with the PI3-kinase inhibitor LY294002 resulted in decreased phosphorylation of c-Akt and reduced cell survival. Similar treatment of Rat-1 fibroblasts or SCC12F cells expressing LMP1 not only reversed the transformed morphological phenotype of these cells, but also resulted in apoptotic cell death. The ability of LMP1 to induce actin stress-fibre formation was also dependent on PI3K activation. These data implicate PI3-kinase activation in LMP1-induced phenotypic effects and suggest that this pathway contributes to the oncogenicity of this molecule.

NOTES:

C/EBPa MEDIATES ZTA-INDUCED CELL CYCLE ARREST

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The EBV ZTA transactivator is known to arrest the cell cycle at G_0/G_1 but the exact molecular mechanism is unknown. We found that ZTA coprecipitates with endogenous C/EBPa in extracts made from IgG cross-linked Akata cells. In reporter assays, ZTA cooperated with C/EBPa to up-regulate the C/EBPa promoter. Activation of C/EBPa was confirmed in D98/HR1 cells treated with TPA where only cells expressing ZTA induced C/EBPa. C/EBPa has a recognized role in cell differentiation where it arrests the cell cycle at G_1 by activating the $p21^{CIP-1}$ promoter, stabilizing $p21^{CIP-1}$ and directly inhibiting cdk2 and cdk4. Expression of a $p21^{CIP-1}$ p-luciferase reporter was activated synergistically by ZTA plus C/EBPa. To further examine the role of ZTA in cell cycle arrest, we constructed a recombinant adenovirus vector expressing ZTA. Immunofluorescence assays performed on human fibroblasts infected with Ad-ZTA revealed that cells expressing ZTA induced endogenous C/EBPa and $p21^{CIP-1}$ proteins and failed to enter S-phase as measured by BrdU incorporation and by FACS analyses. Furthermore, ZTA was unable to induce either $p21^{CIP-1}$ or G1 arrest in MEF cells derived from a C/EBPa knockout mouse. We conclude that C/EBPa is an essential player in ZTA-induced cell cycle arrest.

NOTES:

HUMAN CYTOMEGALOVIRUS pp71 DEGRADES THE Rb TUMOR SUPPRESSORS THROUGH A PROTEASOME-DEPENDENT, UBIQUITIN-INDEPENDENT MECHANISM, AND STIMULATES CELL CYCLE PROGRESSION.

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The human cytomegalovirus pp71 protein stimulates the cell cycle by degrading the hypophosphorylated forms of the Rb family of tumor suppressors (Rb, p107 and p130) in a proteasome-dependent manner. At least two functional domains are required for these processes, an LxCxD motif that is essential for both Rb family degradation and cell cycle stimulation, and a SOCS-box that is necessary for degradation and contributes to the cell cycle effect. pp71 interacts with the hypophosphorylated forms of all three Rb family members both *in vitro* and *in vivo*. In addition, pp71 binds to elongin B and elongin C, and is the first example of a SOCS-box protein that directs the degradation of the Rb family. Although the majority of proteins degraded by the proteasome are polyubiquitinated, and the elongins are components of ubiquitin ligase complexes, the pp71-mediated degradation of the Rb family occurs without detectable polyubiquitination. Furthermore, our preliminary evidence indicates that the degradation proceeds in the absence of a functioning ubiquitinating system. Thus, pp71 degrades the Rb family through a proteasome-dependent, ubiquitin-independent mechanism, and represents a new means by which a viral protein attacks the Rb family to alter cell cycle progression.

NOTES:

LMP1 SIGNALS FROM AN INTRACELLULAR COMPARTMENT

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LMP1 is an oncoprotein important for EBV-mediated B cell proliferation. LMP1 functionally mimics CD40 and signals in a ligand independent manner. We are characterizing the intracellular trafficking of LMP1. LMP1 at the plasma membrane was monitored by its accessibility to cleavage by chymotrypsin in intact cells. Interestingly, the maximal fraction of LMP1 accessible to chymotrypsin in intact cells differed dramatically from cell line to cell line, with 20% in 721 cells, 3 to 5% in 293 cells, and no more than 1% in BJAB cells. A mutant of LMP1 designated the "trileucine mutant", which had a potential dileucine-based motif disrupted by leucine-to-alanine substitutions at aa 362,363,364, was not accessible to digestion by chymotrypsin in 293 cells, even though the assay can detect as little as 0.1% of LMP1 being cleaved. The trileucine mutant activated NF-kB and JNK as efficiently as wild-type LMP1 did, indicating that localization of LMP1 to the plasma membrane is not required for its activation of these two signaling pathways. The conclusion drawn from both chymotrypsin assays and the studies of the trileucine mutant was supported by examination of cells with confocal microscopy which showed that substantial amounts of LMP1 colocalized with TRADD and TRAF3 in perinuclear structures. A study using electron microscopy is underway to test these biochemical results independently.

NOTES:

SELF-SUSTAINING LMP1 EXPRESSION VIA IL-6 INDUCTION OF STAT 3 AND STAT5

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We previously showed that STAT3 and STAT5 are constitutively activated in NPC cells and that these STATs in turn activate expression from the EBV Qp and LMP1 TR promoters. In normal signaling, STATs are only transiently activated. To investigate how constitutive STAT activation is sustained in epithelial cells, we examined STAT phosphorylation in the paired cell lines CNE2 and CNE2-LMP1 and HeLa and HeLa-EBV. A significant increase in the activated forms of STAT3 and STAT5 were detected in CNE2-LMP1 and HeLa-EBV compared to the parental cell lines. No change was observed in STAT1 or STAT6 phosphorylation. Treatment with IL-6 increased activated STAT3 levels in CNE2 and conversely, treatment of CNE2-LMP1 with anti-IL-6 neutralizing antibody abolished STAT3 activation. Expression of gp130, a signal transducer for IL-6 was detected in all epithelial lines tested. We suggest that EBV infection of an epithelial cell containing activated STATs would permit LMP1 expression which would in turn establish a positive feed-back loop of IL-6 induced STAT3 and STAT5 activation, LMP1 expression and viral genome persistence.

NOTES:

POST TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS ARE FREQUENTLY DERIVED FROM B CELLS CARRYING RANDOMLY MUTATED OR NON-FUNCTIONAL IMMUNOGLOBULIN GENES

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Post-transplant lymphoproliferative disease (PTLD) arises from the expanded pool of Epstein-Barr virus-infected B cells found in the immunocompromised host. Identifying the precise cellular origin of these tumours may shed light on their pathogenesis, particularly since in immunocompetent individuals EBV is preferentially carried within the memory (as opposed to naive) B cell subset. In this study PCR amplification of the IgH locus from 13 EBV-positive PTLDs identified 11 monoclonal lesions where the IgH genotype of the tumour could be unequivocally identified. Two tumours had a naive B cell genotype and another two showed patterns of IgH mutation typical of antigen-selected memory cells; all four expressed EBNA2 and LMP1, markers of the EBV-transformed state. However the majority of tumours, including some which were EBNA2, LMP1-positive and others where these antigens had been down regulated, arose from cells with randomly mutated or functionally-inactivated IgH sequences, some of which even showed evidence of ongoing somatic mutation. Hence the range of target cells giving rise to PTLD is unexpectedly broad. The frequent involvement of B cells carrying IgH mutations normally incompatible with survival *in vivo* highlights a common link between the pathogenesis of PTLD and that of other EBV-associated B cell malignancies

NOTES:

RECURRENCE OF HIGH VIRUS LOAD AND LYMPHOPROLIFERATIVE DISEASE (LPD) AFTER SUCCESSFUL TREATMENT WITH HUMANIZED ANTI-CD20 IN PEDIATRIC LIVER TRANSPLANT RECIPIENTS.

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The humanized anti-CD20 monoclonal antibody (Rituximab) that induces in-vivo complement-mediated killing of B-cells is successful for the treatment of EBV-driven LPD occurring in solid organ transplant recipients. At our institution, one child with EBV-related mononucleosis-like syndrome and four children with biopsy-proven polymorphic EBV-LPD 0.8 to 8 years after liver transplant were treated with Rituximab (375mg/m^2, 4 doses at weekly intervals). Treatment was well tolerated and complete remission was achieved by all children. After Rituximab, B-lymphocytes were undetectable in the peripheral blood and EBV-load (6,161-59,3414 copies/μg DNA at diagnosis) decreased in all children to undetectable levels and remained low for the following 5/6 months. However, in three children the high virus load recurred (2,720-49,471 copies/μg DNA) when B-cells recovered, and LPD recurred in two of them. The frequency of EBV-specific precursors assessed by Elispot-assay did not improve after Rituximab treatment, suggesting that restoration of cellular immunocompetence is crucial for the long-term control of EBV-mediated proliferation. Adoptively-transferred EBV-specific cytotoxic T-cells (CTL) may be able to maintain the clinical remission, while the transient control of disease obtained with Rituximab allows time for the ex-vivo generation of CTL. We are currently investigating their efficacy, safety and persistence in a phase I dose escalating protocol.

NOTES:

PORCINE CYTOMEGALOVIRUS IN PIGS BEING BRED FOR XENOGRAFT ORGANS: PROGESS TOWARDS CONTROL

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Xenotransplantation raises concerns about potential zoonotic transmission of viruses including porcine cytomegalovirus (PCMV), an endemic infection of pigs, thought to be transmitted peri- and post-natally and *in utero*. Human CMV is frequently transmitted in allotransplantation causing end-organ disease and graft rejection. Using qualitative and quantitative competitive PCR methods we examined the prevalence, quantity and organ distribution of PCMV in a range of samples from hDAF (CD55) transgenic pigs and investigated when virus is acquired.

We identified PCMV in a range of organs in both adult pigs and piglets including the spleen and potential xenograft organs. In comparison with adult pigs, virus was more widely disseminated and viral loads were significantly higher in piglets. Examination of fetal spleens failed to identify evidence of transplacental infection and prospective monitoring of two litters showed that infection occurred in the post-natal period. In hysterotomy-derived barrier-reared piglets we found no evidence of *in utero* or post-natal infection, despite sows testing PCMV DNA positive.

Our findings demonstrate that while PCMV is a common infection in these transgenic pigs, it could potentially be eradicated from pigs being bred for xenotransplantation. Additionally pigs could be quality controlled by testing spleen from individual sentinel animals if clinical trials proceed.

NOTES:

THE SEARCH FOR PORCINE GAMMAHERPESVIRUSES AND IMPLICATIONS FOR XENOTRANSPLANTATION: CHARACTERISATION OF THE NOVEL PORCINE LYMPHOTROPIC HERPESVIRUSES (PLHV-1, -2 AND -3)

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Xenotransplantation research is focussed on the use of pigs as donors of organs for human transplantation. Therefore, attempts are presently made to breed pathogen-free pigs for xenotransplantation. For this purpose, valid monitoring methods as well as comprehensive knowledge of porcine microorganisms is needed . During the last 4 years, three novel porcine gammaherpesviruses have been discovered by our group. We designated them as porcine lymphotropic herpesviruses 1 -3 (PLHV-1, -2 and -3).

Recently, PLHV-1 has been detected at high copy numbers in animals suffering from post-transplant lymphoproliferative disease (PTLD). While human PTLD is an EBV-associated complication following clinical allotransplantation, porcine PTLD is a disease recently described in pigs undergoing experimental allogeneic hematopoietic stem cell transplantation.

Here we summarize our knowledge about the gene content and tropism of the three PLHV viruses, present the results of transcription analysis and discuss the implications of our findings for xenotransplantation.

NOTES:

STEM-CELL TRANSPLANT RECIPIENTS NOT RECOVERING EBV-SPECIFIC IMMUNITY ARE AT RISK TO DEVELOP HIGH LEVEL EBV VIRAL LOAD AND LYMPHOPROLIFERATIVE DISEASE

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EBV-induced lymphoproliferative disease (LPD) is a serious complication of allogeneic stem-cell transplantation (allo-SCT). Recently it has been described that EBV reactivation is a frequent event after allo-SCT, however only patients receiving a T-cell depleted allo-SCT and with a high viral load (≥ 1000 geq/ml) appeared to be at risk developing LPD. Since EBV-specific T-cells are important in controlling EBV infection, the aim of this study was to evaluate whether the presence or absence of EBV-specific T-cells would be associated with viral reactivation and LPD. EBV-specific immunity was analysed using tetramer staining on $CD8^+$ T-cells. Tetramers used: A*0201-GLC/CLG/LLD, B*0702-RPP/VPA, B*0801-RAK/FLR/QAK and B*3501-EPL/HPV/YPL. In 56 patients blood samples were taken 2, 3, 6, 9, 12, 18 and 24 months after transplantation.

No difference was found in the presence or absence of tetramer-binding T-cells between patients with or without EBV reactivation. However, significantly less (p=0,01) tetramer-binding T-cells were detected in patients with a viral load of <1000 geq/ml as opposed to patients with a viral load of ≥ 1000 geq/ml. Furthermore, no EBV-specific T-cells were detected in patients developing LPD. The presence of EBV-specific T-cells in the graft appeared not to be protective. These results indicate that the development of EBV-specific T-cells after allo-SCT may protect against uncontrolled EBV reactivation with high viral load and against the development of LPD.

NOTES:

PARTIALLY HLA-MATCHED ALLOGENEIC CYTOTOXIC T CELLS FOR THE TREATMENT OF EBV-POSITIVE POST TRANSPLANT LYMPHOPROLIFERATIVE DISEASE

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Epstein-Barr virus (EBV)-associated post transplant lymphoproliferative disease (PTLD) is a common complication of bone marrow and solid organ transplantation and is often fatal. Since tumour growth results from inadequate T cell control of latent EBV, novel immunotherapeutic approaches to treatment are being pioneered[1].

In a phase I/II trial, 10 patients with progressive PTLD unresponsive to conventional therapy were treated with partially HLA matched allogeneic EBV-specific cytotoxic T lymphocytes (CTL) grown from healthy blood donors. Of the 7 patients who completed the treatment, 5 attained complete remission and 2 patients showed no clinical response. One patient showed a partial response after 2 infusions. No graft-versus-host disease or allo-specific antibodies were detected, and graft function improved in 5 cases. Tumour responses were primarily seen in those with early, localised, polyclonal disease and when PTLD developed following primary EBV infection. EBV load in peripheral blood fell to undetectable levels in all those who showed a response, but was more variable in the non-responders[2].

We show that the use of partially HLA matched CTL grown from unrelated donors is safe and effective. Our approach has the advantage of providing CTL for immediate use, and opens up the possibility of establishing banks of CTL to treat other infectious and neoplastic diseases in a large number of patients.

1. Rooney et al. *Lancet* 1995; 345: 9-13.
2. Haque et al. *Lancet* 2002; *In press.*

NOTES:

POSTER SESSIONS ABSTRACTS

Poster: 1

IN SEARCH OF EBV BZLF1 INTERACTORS: *DROSOPHILA* AS A MODEL SYSTEM

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The immediate-early protein BZLF1 is known to interact with a variety of cellular proteins. These protein-protein interactions are presumably important for efficient viral replication and survival. To investigate these interactions, we have expressed the BZLF1 gene in the *Drosophila* eye, which yields an extraordinary mutant phenotype, and have conducted genetic screens to search for dose-sensitive second-site modifiers of the BZLF1 eye phenotype. The genetic screens involved crossing mutagenized wild-type male flies to females harbouring a dose-sensitive BZLF1 eye phenotype, and screening the resulting progeny for alterations of the eye phenotype. Genetic enhancers of the BZLF1 eye phenotype correspond to genes whose wild-type function normally inhibits BZLF1 activity, while modifiers that suppress the phenotype correspond to genes whose wild-type function normally aids BZLF1 activity. The genetic modifiers isolated were then characterized for chromosome position and gene identification. Human homologs of the identified genes will then be ascertained and studied in regards to EBV biology.

NOTES:

Poster: 2

FUNCTIONAL COMPARISON OF THE BZIP PROTEINS OF EBV AND HHV8

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EBV and HHV8 encode gene products that display structural and functional similarities to the cellular bZip family of transcription factors. Zta, encoded by the BZLF1 gene of EBV, is a transcription factor required to mediate the switch between latency and the productive cycle. In contrast, the function of K–bZip, encoded by the K8 gene of HHV8, is less well defined.

We have undertaken a structural and functional comparison of these two proteins. Analysis of the potential of the bZip regions to fold as coiled coil structures suggests that K-bZip will form a stable coil between amino acid residues 185 and 213. This overlaps with the region previously demonstrated to contain the dimerisation domain (amino acid residues 190 to 237). In contrast, Zta forms a weaker dimerisation interface. We are further characterising the dimerisation domain of K-bZip using *in vitro* and *in vivo* assays with domain swap mutations. We have already established that K-bZip does not interact with a DNA sequence motif recognised by Zta; specific target sequences for K-bZip are being sought using binding site selection assays.

NOTES:

Poster: 3

QUANTIFICATION OF BZLF1, BALF5 AND BLLF1 MESSENGER-RNA FOR MONITORING OF EPSTEIN-BARR VIRUS (EBV) REACTIVATION

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Reactivation of Epstein-Barr virus in latently infected individuals during immunosuppression is associated with severe complications e.g. after organ transplantation.

Currently, quantification of viral DNA is used to monitor EBV reactivation in immune compromised patients. As early diagnosis of viral replication is essential for anti-viral therapy we established real-time RT-PCR protocols using TaqMan technology to sensitively quantitate several viral transcripts expressed at different times of the lytic cycle, namely BZLF1, an immediate early transactivator that is responsible for the transition from latency to lytic replication, the DNA-polymerase BALF5 and the major viral glycoprotein gp350/220 (BLLF1).

As only few transcripts of Epstein-Barr virus are spliced, RNA-isolation was optimized to eliminate contaminating DNA. Preparations were shown to be DNA-free for up to 10^6 copies of RNA. With our RT-PCR systems it is possible to detect at least 10 copies of DNA or 500 copies of RNA as shown with serial dilutions of DNA-plasmids or in vitro transcribed RNA, respectively. All steps of the protocol including RNA-isolation, reverse transcription and TaqMan PCR are highly reproducible.

Preliminary data on screening of healthy carriers as well as patients under immunosuppression will be presented.

NOTES:

Poster: 4

BENZIMIDAVIR (1263W94) ALTERS THE CELLULAR PATTERN OF THE LARGE SUBUNIT OF EBV RIBONUCLEOTIDE REDUCTASE

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Ribonucleotide reductase (RR) plays an essential role in the *de novo* synthesis of DNA in all living organisms by catalyzing the conversion of all four ribonucleotides to the corresponding deoxyribonucleotides. RR1 (large) and RR2 (small) subunits of the enzyme are highly conserved among the alpha- and gammaherpesviruses, and key catalytic residues are readily identifiable. These facts suggest that active viral RR might be important for the normal progression of the cytolytic cycle. Here we report on the effect of the antiviral compound 1263W94 on the expression and the cellular pattern of EBV RR1. BORF2, the gene that encodes EBV RR1, appears to be an early gene based on the kinetics of RNA and protein accumulation. The protein is expressed as a filamentous network in the cytoplasm at early times after viral reactivation and peaks at about 24 hours coinciding with the peak of DNA replication. Treatment with 1263W94 affected neither the kinetics nor the level of the RR1 mRNA; however, it resulted in a delay and overall reduction in EBV RR1 protein expression, as well as in changes of the RR1 pattern from filamentous to speckled. These results suggest that the filamentous network created by EBV RR1 is important for its function in the viral cytolytic cycle.

NOTES:

Poster: 5

MARIBAVIR (1263W94) CHANGES SUBCELLULAR LOCALIZATION OF THE EBV DNA PROCESSIVITY FACTOR EA-D

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Benzimidazole β-L-riboside 1263W94 has been shown previously to inhibit replication of Epstein-Barr Virus (EBV). The compound inhibits the appearance of the hyperphosphorylated form of the EBV DNA processivity factor, EA-D, during viral reactivation in latently infected Akata cells. In studying the mechanism of action and possible targets of the drug, we examined the subcellular localization of EA-D during viral reactivation in Akata cells in the presence or absence of the drug. The results indicate that upon reactivation and up to 48 hours afterward EA-D localized both in the nucleus and cytoplasm; however at 48 hours it is detected mainly in cytoplasm. Drug treatment caused drastic changes in the EA-D localization pattern; at 24 hours after reactivation, EA-D was located mainly in the cytoplasm forming in some instances dense bodies. This pattern coincides temporally with the loss of the hyperphosphorylated form of EA-D detected by immunoblotting. Separation of the nuclear and cytoplasmic fractions revealed that indeed the hyperphosphorylated form of EA-D is located only in the nucleus and almost disappears after 1263W94 treatment. In contrast, the hypophosphorylated form is detected in both the nucleus and the cytoplasm and is not affected significantly by the compound. Thus, our results imply a link between the phosphorylation status of EA-D and its function in the cell.

NOTES:

Poster: 6

INVESTIGATION ON THE EXPRESSION OF SOLUBLE GP25 BY BACULOVIRUS

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GP25 is an envelope glycoprotein of EBV and can form complexes with GP85 and GP42. Sequence comparison of GP25 revealed that it had 80 % homology with gL of *Herpesvirus papio* and 65 % homology with gL of *Callilrichine herpesvirus 3*. However, no tertiary structure of any soluble GP25 or other homologs has ever been determined. We attempted to produce soluble GP25 employing the Bac-to-Bac baculovirus expression system. We have engineered both gp25 and an expression cassette of enhanced green fluorescence protein (EGFP) into the pFastBac DUAL transfer vector. Two different signal peptides derived from GP25 itself and GP67 of baculovirus separatly cloned into the system. We could identify and select the Sf9 insect cells containing the recombinant of interest with EGFP by fluorescence microscopy. The potential secondary structure and expression of GP25 will be discussed.

Remark: # These two authors contributed equally. * Corresponding authors

NOTES:

Poster: 7

EPSTEIN-BARR VIRUS IMMEDIATE EARLY PROTEIN BZLF1 ACTIVATES EXPRESSION OF NEUROPEPTIDE GALANIN

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Oral hairy leukoplakia is hyperproliferative epithelial lesion on the tongue that contains the lytic form of EBV infection. The effect of the EBV immediate-early protein, BZLF1, on host cell gene expression in telomerase-immortalized keratinocytes was examined by microarray analysis. BZLF1 dramatically increased the mRNA level of the neuropeptide, galanin, and this effect was confirmed by northern blot analysis, as well as immunocytochemistry. Galanin, a 29-amino acid neuropeptide, is over-expressed in small cell lung carcinomas, as well as certain breast cancers, and induces cell growth of small cell lung cancer cells. It has been suggested that galanin acts as a paracrine growth factor for certain cancers. Immunohistochemical analysis of oral hairy leukoplakia (OHL) tissue samples showed increased galanin expression in cells expressing BZLF1. We suggest that BZLF1-induced activation of galanin expression may contribute to the hyperproliferative nature of OHL and possibly play a role in the pathogenesis of certain EBV-positive tumors.

NOTES:

Poster: 8

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE EPSTEIN-BARR VIRUS PROTEASE

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Herpesvirus proteases may constitute a new antiviral target because they are essential for the production of new virions during the lytic phase of the viral infection. The protease domain of the assemblin-protease protein of EBV (EBV-PR) has been expressed in *E. coli*. As for other herpesviruses, it forms a monomer-dimer equilibrium in solution (Buisson, *J. Mol. Biol.* 2001)

Here, we report the structure of the EBV-PR by X-ray crystallography to 2.3 Å resolution after inhibition of the protease with diisopropyl-fluorophosphate. The overall structure of EBV-PR shows a high degree of conservation of the structure throughout the herpesvirus family. Nevertheless specific folding of the EBV-PR has been identified within the substrate binding site and the dimer interface. We also demonstrated *ex vivo* by molecular sequencing in EBV-infected cell lines and peripheral mononuclear cells (PBMC) that the EBV-PR gene is well conserved between EBV1 and EBV2 subtypes or within different strains of the same subtype. By RT-PCR, all the cell lines but not the PBMC showed an expression of EBV-PR mRNA (4.5 to 7 log10 copies µg/RNA).

These results could lead to the development of new antiviral strategies at least to inhibit the lytic phase of the EBV infection;

NOTES:

Poster: 9

EPSTEIN-BARR VIRUS-PRODUCTIVELY INFECTED LYMPHOCYTES IN TONSILS

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The findings that Epstein-Barr virus (EBV) particles were detected in the salivas and EBV-infected cells were observed in the peripheral blood lymphocytes of seropositive persons, indicate that one of the replication sites of EBV may be in an oral cavity of seropositive individuals. To clarify this, the tonsils of chronic tonsillitis patients were used. The expression of EBV transcripts EBER1, EBNA2, LMP1, LMP2a and BZLF1 was examined in RNA from mononuclear cells that were isolated from 15 tonsil specimens by RT-PCR. EBER1 and BZLF1 transcripts were detected in 100% and 42.9% respectively of the tonsil specimens. ZEBRA and VCA were observed in the same tonsil specimens in which BZLF1 transcripts were detected by immunohistochemistry, using serial sections of formalin-fixed, paraffin-embedded tonsils. Although ZEBRA- and VCA-positive cells were failed to specify the type of cells, these cells most resembled lymphocytes in morphology. By DNA-DNA in *situ* hybridization using BamHI W as a probe, BamHI W-positive cells were found to correspond to lymphoid cells and localized in the same area as ZEBRA- and VCA-positive cells. These findings indicate that EBV replication may occur in tonsillar lymphocytes, and tonsillar lymphocytes may be one of the EBV replication sites and a reservoir for EBV in normal individuals.

NOTES:

Poster: 10

ACTIVATION OF THE EPSTEIN-BARR VIRUS LYTIC CYCLE BY THE LATEX OF THE PLANT *EUPHORBIA TIRUCALLI*

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Endemic Burkitt's lymphoma (eBL) is the most common childhood cancer in sub-Saharan Africa. Infection with Epstein-Barr virus and the occurrence of holoendemic malaria are two agents that are co-factors in the development of eBL. However, exposure to the plant *Euphorbia tirucalli* has also been proposed to be a co-factor in the development of eBL. Extracts of the milky latex of the plant *E. tirucalli* contain 4-deoxyphorbol ester, a compound similar to 12-O-tetradecanoylphorbol-13-acetate (TPA). In our study, we tested whether the unpurified latex from *E. tirucalli* could activate EBV lytic cycle in the Jijoye BL cell line. We found that treatment of Jijoye cells with latex diluted up to10^{-5} in culture media resulted in induction of the viral lytic cycle and production of infectious virions. Co-treatment with a protein-kinase C inhibitor blocked this lytic cycle induction, indicating that E. tirucalli stimulated induction occurs through the PKC pathway, analagous to TPA. These studies demonstrate that very low levels of unpurified latex can induce EBV reactivation and support the hypothesis that exposure to *E. tirucalli* plays a causal role in the genesis of BL.

NOTES:

Poster: 11

BZLF1 AUTO-REGULATES ITS OWN ACTIVITY BY TRANSLOCATING NF-κB TO THE NUCLEUS

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We have previously shown that the BZLF1 gene product interacts with, and is inhibited by, the NF-κB family member p65. However the effects of BZLF1 on NF-κB activity have not been studied. Using an adenovirus vector expressing the BZLF1 gene product, we found that BZLF1 increased the amount of NF-κB DNA binding, and that this effect was due to a decreased level of the cytoplasmic inhibitor of NF-κB, IκB-α. Although IκB-α protein stability is commonly regulated by phosphorylation, we found that inhibition of the proteasome failed to restore IκB-α protein levels, and instead BZLF1 decreased the level of IκB-α RNA. Since IκB-α transcription is regulated by NF-κB, we determined if other NF-κB responsive genes were activated or repressed in the presence of BZLF1. Several different NF-κB responsive promoters were inhibited by BZLF1 expression, in spite of the increased levels of nuclear NF-κB binding activity. NF-κB expression inhibited the ability of transfected BZLF1 to induce viral replication, confirming that NF-κB inhibits BZLF1 transcriptional function. Our results are consistent with a model in which BZLF1 inhibits IκB-α transcription, resulting in increased NF-κB in the nucleus, which then serves to negatively regulate BZLF1 transcriptional activity and promote transition to the late stage of viral gene expression.

NOTES:

Poster: 12

THE EBV BRRF1 EARLY GENE PRODUCT IS REQUIRED FOR EFFICIENT LYTIC REPLICATION

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Lytic EBV infection requires BamHI R gene(s) (Feederle et al, EMBO J. 19:3080, 2000). However, the previously reported BRLF1-knock-out virus deleted not only the BRLF1 IE protein, but also the promoter of the BRRF1 early gene, and the vector used to "rescue" the phenotype contained both the BRLF1 and BRRF1 genes. Using an expression vector which makes the BRLF1 gene product only, we found that BRLF1 by itself induced only very low expression of either the BZLF1 IE gene, or the BMRF1 early gene, in 293 cells latently infected with BamHI R-deleted virus. Transfection of cells with a vector expressing BRRF1 only (a gift from Alain Sergeant) did not activate BZLF1 or BMRF1 expression. However, co-transfection with both the BRLF1 and BRRF1 vectors significantly increased expression of the BZLF1 and BMRF1 genes in cells infected with the BamHI R-deleted virus, although the level of BRLF1 was similar to cells transfected with the BRLF1 vector alone. BRRF1 did not enhance the ability of BRLF1 to induce lytic gene expression in 293 cells infected with wild-type virus. These results suggest that BRRF1 enhances the ability of BRLF1 to induce the lytic form of EBV infection.

NOTES:

Poster: 13

INDUCTION OF EPSTEIN-BARR VIRUS LYTIC REPLICATION BY HISTONE DEACETYLASE INHIBITOR

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Histone deacetylases (HDACs) are recruited to gene-specific promoters by interaction with Histone deacetylases. (HDACs) are recruited to gene-specific promoters by interaction with DNA-binding repressors such as YY1 and MeCP2, a 5-methyl cytosine binding protein in CpG methylated DNA. HDACs remove acetyl moieties from acetylated core histones and keep the inactive state of the promoter. Since EBV latency is likely to be regulated by repressors interacting with HDAC, TSA, a reversible HDAC inhibitor, is expected to alter expression of EBV genes and growth properties of latently infected cells. To analyse the effects of TSA on EBV lytic reactivation, the expression of lytic antigens were assessed by immunofluorescence assay and Western blot after TSA treatment in EBV transformed LCLs (SNU-20 and SNU-1103, latency 3) and BL cell line (Akata, latency 1). Expressions of EBV lytic antigens ZEBRA, EA-D, gp350 were enhanced by TSA in both latency I and latency III type cell lines suggesting that TSA is useful to activate EBV lytic replication.

This work was supported by grant No. R01-1999-00066 from the Basic Research Program of the Korea Science & Engineering Foundation.

NOTES:

Poster: 14

CPG METHYLATION INHIBITS SENSITIVITY TO LYTIC INDUCTION BY HISTONE DEACETYLASE INHIBITORS

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Histone deacetylase (HDAC) inhibitors like sodium butyrate induce lytic cycle gene expression in some EBV positive cell lines such as B95.8. Upon examination of the BZLF1 promoter region in these cell lines, little or no methylation was observed. In other EBV positive cell lines such as the Burkitt's lymphoma cell line Rael, the BZLF1 promoter region was found to be densely methylated. In these cells, HDAC inhibitors are unable to reactivate lytic gene expression. However, resistance to lytic activation in cells with dense CpG methylation by HDAC inhibitors could be reversed by pre-treatment with DNA methyltransferase inhibitors. Pretreatment of Rael cells with 5-aza-2'-deoxycytidine (AzadC) sensitized cells to lytic cycle induction by sodium butyrate and its derivative sodium phenylbutyrate. In the presence of AzadC, a dose-dependent increase in the number of cells expressing Zta was observed with increasing HDAC inhibitor concentrations. No Zta expression was observed in the absence of AzadC even at high concentrations of HDAC inhibitors.

NOTES:

Poster: 15

IDENTIFICATION OF THE ORIGIN OF LYTIC DNA REPLICATION OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

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Herpesviruses utilize different origins of replication during lytic versus latent infection. Latent DNA replication depends on the cellular DNA replication machinery, whereas lytic cycle DNA replication requires virally encoded replication proteins. In lytic DNA replication, the lytic origin (*ori-Lyt*) is bound by a virus-specified origin-binding protein (OBP) that recruits the core replication machinery. The *ori-Lyt* and an OBP of KSHV were unidentified. Here, we report that two regions in the KSHV genome, between K4.2 and K5 and between K12 and ORF71, were found to be able to serve as origins for lytic cycle-specific DNA replication. The two *ori-Lyt* domains share identical 500 bp sequence and additional similar 1.2 kb sequence. An initial mapping analysis suggests that the 1.7 kb DNA sequences is sufficient to act as a *cis* signal for replication. In addition, a KSHV-encoded bZip protein, namely K8, was found to bind to a KSHV sequence within the *ori-Lyt* by using a DNA binding site selection. The binding of K8 to this region was confirmed in cells using the chromatin immunoprecipitation (Chip) method. K8 protein displays significant similarity to the Zta protein of Epstein-Barr virus, which is known to be an OBP of EBV. This notion, together with the ability of K8 to bind to the KSHV *ori-Lyt,* suggest that K8 may function as an OBP in KSHV.

NOTES:

Poster: 16

ALTERATIONS OF GENE EXPRESSIONS IN NASOPHARYNGEAL EPITHELIAL CELLS BY THE EPSTEIN-BARR VIRUS ENCODED LATENT MEMBRANE PROTEIN 1

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Nasopharyngeal carcinoma (NPC) is prevalent in southern China and is closely associated with EBV infection. Among the latent EBV proteins expressed in NPC, LMP1 is detected in about two-third EBV positive NPC cases. Previous *in vitro* studies have shown that LMP1 possesses oncogenic properties as it can induce malignant transformation of certain rodent fibroblast cell lines as well as altering growth property and morphology of established epithelial cells. However, the pathological significance of LMP1 in nasopharyngeal epithelial cells is not defined yet. Expression of LMP1 induced specific gene expression including LAMC2 and ITGα6 genes in established nasopharyngeal carcinoma cell lines (Lo et al., Biochim Biophys Acta (2001) 1520:131-40). In this study, the effects of LMP1 expression in a SV40T immortalized nasopharyngeal epithelial cell line, NP69, were examined. The gene expression profiles of NP69 cells expressing LMP1 and control vector were compared by cDNA arrays. Twenty-eight genes were identified to be differentially expressed in LMP1 expressing NP69 cells. Majority of the identified genes are associated with cell growth, differentiation, cell shape, migration, invasion and angiogenesis. These results support the roles of LMP1 in promoting cell growth, migration and invasion in early pre-malignant nasopharyngeal epithelial cells.

NOTES:

Poster: 17

EXPRESSION OF INHIBITORS OF APOPTOSIS GENE FAMILIES (IAP) IN NASOPHARYNGEAL CARCINOMAS

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Nasopharyngeal Carcinomas (NPC) is an epithelial malignancy occurring highly frequently in Taiwan. Highly metastasis and Epstein-Barr virus-association make NPC distinct from other head and neck carcinomas. Unlike other carcinomas, NPC did not have frequent mutations in most well-defined oncogenes or tumor suppressor genes. In order to further reveal the cellular background of NPC micro-environment, expression of inhibitors of apoptosis gene families (IAP) was determined in NPC biopsies and other control samples. We performed real-time quantitative reverse transcription-polymerase chain reaction (RT-Q-PCR) to assess the expression levels of IAP gene families in NPC and NPC metastases. The nasopharyngeal lymphoid hyperplasia tissues (LH), nasal epithelial cells (Epi) and head and neck carcinoma biopsies (H & N) were selected as control tissues. Results of RT-Q-PCR revealed that the expression levels of survivin, HIAP-1 and HIAP-2 transcripts were elevated in NPC and NPC metastases compared to that of LH and Epi. Cells responsible for IAP expression were determined by RT-Q-PCR of paramagnetic bead-fractionated cells and by immunohistochemical staining of paraffin-embedded NPC sections. The role of IAP in NPC carcinogenesis will be discussed.

NOTES:

Poster: 18

EPSTEIN-BARR VIRUS INFECTION INDUCES A SIGNAL TRANSDUCTION PATHWAY FOR NASOPHARYNGEAL CARCINOMA PROLIFERATION INVOLVING UP-REGULATION OF NUCLEOLIN GENE EXPRESSION

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In the Epstein-Barr virus (EBV)-infected Nasopharyngeal carcinoma (NPC), especially the undifferentiated cases, the tumor masses usually grow faster and bigger than the EBV-free cases. This phenomenon is also found in the animal experiment. In order to observe the mechanism of EBV induction of tumor cell proliferation, we have infected and transfected NPC-TW04 cells with EBV and LMP-1 plasmid, separately, and observed the mRNA expressions of all possible signal transduction pathway related genes. It was found that the EGFR, Shc, Grb2, Ras (RREB), MAPKAPK3, casein kinase 2 (CK2) and nucleolin genes all showed increase of their mRNA expressions in the EBV-infected cells when compared with EBV-free cells. If antisense phosphorothioate oligonucleotide of A-RAF gene was transfected into both the EBV-infected and LMP-1 plasmid transfected cells, the protein expressions of A-RAF, CK2, and nucleolin were also clearly suppressed in both EBV-infected and LMP-transfected cells, but the effect of gene expressions is stronger in LMP-1 transfected cells. Transfection of EBNA-1 plasmid into the NPC cells showed no differential gene expression. In conclusion, the elevated nucleolin expression in the EBV-infected NPC cells may play an important role in enhancing EBV-infected NPC cell growth through its acceleration of cell cycle function.

NOTES:

Poster: 19

SCREENING A NATURAL PRODUCTS LIBRARY FOR POSSIBLE ENVIRONMENTAL ACTIVATORS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

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Kaposi's sarcoma-associated herpesvirus (KSHV) is thought to have co-evolved with the human species and infection has been reported in all human populations studied. However both the prevalence of KSHV and the incidence of Kaposi's sarcoma vary considerably geographically, being elevated in Mediterranean areas and sub-Saharan Africa. The co-factors for this variation in prevalence and pathogenesis of KSHV are unknown and are likely to include both genetic and environmental factors.

In order to search for possible environmental co-factors of KSHV infectivity and pathogenesis we have developed an in-vitro KSHV re-activation assay, using the latently infected primary effusion lymphoma cell line BCP-1 and real time Taqman quantitative PCR for KSHV and ERV-3 (a cell quantitation marker). This assay was used to screen approximately 5000 aqueous extracts from the NCI natural products library for environmental activators of KSHV.

We have identified 181 natural products that caused re-activation of KSHV *in vitro*. Further testing is in progress on 28 extracts that caused the highest levels of viral re-activation in the initial screen. We speculate that environmental exposure to such products may cause re-activation of KSHV in infected individuals, leading to increased transmission, prevalence and possibly disease.

NOTES:

Poster: 20

INDUCTION OF CYCLOOXYGENASE-2 BY LMP1 IS INVOLVED IN VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION IN NASOPHARYNGEAL CARCINOMA CELLS

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Cyclooxygenase-2 (COX-2) is an inducible form of COX and is overexpressed in diverse tumors. In biopsy specimens, LMP1-positive nasopahryngeal carcinoma (NPC) frequently expresses COX-2 whereas LMP1-negative NPC rarely expresses the enzyme. LMP1 induces COX-2 expression in EBV-negative epithelial cells in NF-κB-dependent manner. Increased production of prostaglandin E_2 suggests LMP1-induced COX-2 is functional. LMP1 also stimulates production of vascular endothelial growth factor (VEGF), a major angiogenic factor. A selective COX-2 inhibitor, NS-398, decreased VEGF production, suggesting that LMP1-induced VEGF production is mediated by COX-2. These results suggest that COX-2 induction by LMP1 may play a role in angiogenesis in NPC.

NOTES:

Poster: 21

CLINICO-PATHOLOGICAL FEATURES OF EPSTEIN-BARR VIRUS (EBV)-CARRYING GASTRIC ADENOCARCINOMAS IN THE NETHERLANDS.

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In this study, we analyzed EBV-carrying gastric adenocarcinomas in The Netherlands for their clinico-pathological features with an emphasis on survival to determine whether they comprise a different clinical entity.

EBV-status of the tumor of 553 cases, selected from the Dutch randomized surgical D1D2 trial (1989-1993)(Bonenkamp *et al.,* 1999), was determined by a recently evaluated PCR-EIA-based pre-screening method in combination with the EBER RNA in situ hybridization. The inclusion criteria from the original trial were used: surgery with curative intent and physically suitable for D1 and D2 dissection, patients younger than 85 years and no co-existing malignancies or previous gasterectomy for benign disease.

EBV prevalence of this Dutch cohort was 7.1%. EBV-carrying gastric adenocarcinomas were significantly more frequently observed in male ($p<0.0001$) and in younger patients ($p=0.023$), were mostly of the intestinal type ($p=0.046$) and located in the upper part of the stomach ($p<0.0001$). A significantly lower TNM-stage ($p=0.007$) was observed in the EBV-carrying patients, which was solely explained by less lymph node (LN) involvement ($p=0.015$) in these cases. Very interesting, less LN-involvement at presentation and during follow up ($p= 0.019$) resulted in a better prognosis as reflected by a significantly longer disease free period ($p=0.0182$) for these patients.

NOTES:

Poster: 22

MOLECULAR DIVERSITY UNDERLYING THE IgG AND IgA RESPONSE IN NASOPHARYNGEAL CARCINOMA: A COMPARISON OF CHINESE, INDONESIAN AND CAUCASIAN CASES

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The molecular diversity of IgG and IgA responses against EBV was studied in detail by EBV-VCA and EBV-EA specific immunoblot analysis in 40 healthy donors (HD), 15 non-NPC tumor patients (non-NPC) and 35 NPC patients of Chinese origin from Hong Kong and 107 HD, 5 non-NPC and 27 NPC patients of Javanese origin from Indonesia. Control serology was done by standard IFA and ELISA. HD and non-NPC from Hong Kong and Indonesia had a restricted IgG diversity with main responses to VCA-p18, VCA-p40, EBNA1 and frequently including ZEBRA, as observed in HD from other parts of the world (USA, Europe, Africa). Occasional (weak) IgA to EBNA1 or VCA-p18 was detected. Patients with stage-I NPC had a pattern similar as HD, mostly without IgA. NPC patients with stage II-IV/V had a significant enhanced IgG diversity, increasing with stage of disease. IgG and IgA diversity is clearly dissimilar in NPC patients of all stages. No correlation was found between standard serology and IgG/IgA diversity. Caucasian NPC cases (n=5) show very similar results.

The data imply that divers IgG and IgA responses are characteristic for NPC patients from all genetic origins and are triggered differentially. The findings may improve NPC-specific serodiagnosis.

NOTES:

Poster: 23

HIGH NUMBERS OF GRANZYME B/CD8 POSITIVE TUMOR INFILTRATING LYMPHOCYTES IN NASOPHARYNGEAL CARCINOMA BIOPSIES PREDICT RAPID FATAL OUTCOME IN PATIENTS TREATED WITH CURATIVE INTENT

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To determine if tumor-infiltrating lymphocytes (TIL) include activated CTLs and whether numbers of CTLs are related to clinical outcome, we enumerated numbers of activated TIL-CTLs and analysed expression of MHC-I and granzyme-B antagonist PI-9 in nasopharyngeal carcinoma (NPC).

We studied primary tumor biopsies of 43 Indonesian NPC patients (T_{1-3}, N_{1-3}, M_0) who were treated with curative intent by radiotherapy only. TIL-CTLs were detected using antibodies against granzyme B, CD8 and CD56. Expression of MHC-I was determined using antibodies against HLA-A, HLA-B/C and β_2-microglobulin. Expression of PI-9 was determined using a recently developed monoclonal antibody.

Activated CTLs (i.e. granzyme B and CD8 positive lymphocytes) were detected in all NPC biopsies (range 5-80%). High percentages (>25%) of granzyme B positive TILs were a strong predictor of rapid fatal outcome, independent of stage. Worse prognosis correlated with increasing percentages of activated CTLs. Absence of MHC-I heavy chain expression on tumor cells was observed in 11 of 31 evaluable cases and low levels in 7 additional cases. Expression of granzyme B antagonist PI-9 in tumor cells was detected in 3 cases.

Conclusion: Presence of many activated CTLs in NPC tumor biopsies is a strong and stage independent marker for rapid fatal clinical outcome.

NOTES:

Poster: 24

NASOPHARYNGEAL CARCINOMA RELATED GENES

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Epstein-Barr virus (EBV) is closely related to nasopharyngeal carcinoma (NPC), additionally, chemical carcinogens and genetic factors may also play important roles in NPC carcinogenesis. Recently, many attempts have been made, especially in China, to clarify the genes involved in NPC carcinogenesis. Zeng et al attempted to locate "susceptibility gene" in NPC by complex segregation analysis and the results indicate it might be located at 4p12 –q25. Li et al cloned and analyzed many putative tumor suppressor genes, such as BRD7, NAG6, NAG7, NAG11, NAG12 and UBAP1. BRD7 is also supposed to be NPC's " susceptibility gene". By using microarray or array filter, we also repeatedly detected down-regulated genes (TNF receptor-associated factor 5 (TRAF5), mitogen-activated protein kinase 12 etc., 18 genes) and up-regulated genes (B-raf proto-oncogene, retinoblastoma-like protein 2 etc., 10 genes) in NPC biopsy specimens. A down-regulated gene (*YH-1*) of NPC was also cloned and analyzed, and it is found to be highly homologous to mouse *PLUNC* gene. Also *TRK-T3,* a gene derived from the fusion of *NTRK1* and *TFG*, is also found in some NPC specimens, even though its incidence is not high (18%). The role of above-mentioned genes in NPC carcinognesis remains to be clarified in the future.

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Poster: 25

EXPRESSION OF NASOPHARYNGEAL CARCINOMA DERIVED LATENT MEMBRANE PROTEIN 1 INDUCED ATYPICAL HYPERPLASIA IN NASOPHARYNGEAL EPITHELIUM OF TRANSGENIC MOUSE

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Objective To establish nasopharyngeal carcinoma(NPC) derived latent membrane protein 1(N-LMP1) transgenic mouse line, and investigate the function of N-LMP1 in *vivo*, further elucidate the role of Epstein-Barr virus in the process of nasopharyngeal oncogenesis. **Methods** keratinocyte specific promoter EDL2 was linked to NPC-derived LMP1 by using the DNA recombinant techniques, then transgene EDL2-N-LMP1 was microinjected into the pronuclei of the mouse zygotes, these zygotes were transplanted into the pseudo-pregnant female mice. Expression of N-LMP1 and pathologic change in nasopharyngeal epithelium of founder mice were observed. **Results** There were 53 founder mice which were tested by PCR and Southern blot analysis. 6 of them were positive for gene integration, the positive ratio was 13.3%. Atypical hyperplasia in nasopharyngeal epithelium was found in one 4-month-old founder mouse. **Conclusion** Transgenic mice bearing N-LMP1 were constucted; N-LMP1 induced atypical hyperplasia of nasopharyngeal epithelium in the mouse, illustrating that the N-LMP1 gene alone might be sufficient to cause the precancerous lesion in mouse nasopharyngeal epithelium. This study provided a strong evidence to verify that EB virus is a key pathogen of NPC.

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Poster: 26

INTERACTION OF TUMOR CELLS, LEUCOCYTES AND EXPRESSION OF EBV LMP1 IN NASOPHARYNGEAL CARCINOMA

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The aim of study is to analyse the possible interaction of tumour cells with infiltrating T lymphocytes and function of LMP1 on apoptosis and p53 accumulation. Twenty-one markers were applied in 87 cases of NPC by immunohistochemistry and it was further assayed in vitro experiments.

Our findings indicate a correlation between proliferation (Ki 67) and expression of p53 in NPC cells. The p53 expression also significantly correlated to that of LMP1 and MMP9. The effect of LMP1 on the balance of Bcl-2 and Bax ratio was not observed. Neither Fas or Fas-L expression correlate with the expression of LMP1, p53 and Ki67.

The frequency of apoptotic cells in NPC, as analysed by TUNEL labelling, correlated to Fas-L and caspase-3 expression, and correlated inversely to LMP1, p53 and MMP 9 expression. No significant correlation was observed between apoptotic index and expression of Ki67, Bcl2, Bax or Fas.

CD8+ T cell infiltration was predominately seen in nests of cancer cells with strong EBV-LMP1 expression, but these CD8 + T cells showed low expression of CD25 and TIA-1, indicating that they were not activated. The infiltration of CD68+ macrophage in cancer nests was associated with high Bcl2 expression in the tumor cells. There was no correlation of nm-23 expression to LMP1 and MMP9.

Our observation suggests that LMP1+ NPC tumors can be heavily infiltrated by lymphocytes, which, however, do not appear to counteract tumor growth by cytoxicity as indicated by the low apoptotic index. The possible role of EBV LMP1 on p53 function was investigated.

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Poster: 27

CHARACTERIZATION OF EBNA-1 SUBTYPES IN NASOPHARYNGEAL CARCINOMA FROM TAIWAN BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

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Nasopharyngeal carcinoma (NPC) is associated with Epstein-Barr virus (EBV) in all parts of the world but vary in incidence between different population groups. High NPC incidence rates are seen among the Southern Chinese. EBV has been classified into five subtypes based on the amino acid sequences of EBNA-1, the only viral protein detected in all EBV-associated malignant tissues. To find out the EBNA-1 subtypes in Taiwan, we developed a simple genotyping method based on restriction-fragment length polymorphism (RFLP) with enzymes *Hinf*I and *Alu*I for cleavage of PCR products of the EBV genome. Of samples from 19 NPC biopsies, the EBNA-1 subtypes were confirmed by PCR-RFLP as well as sequencing analyses, with consistent results. V-val was the only subtype observed in the NPC samples investigated. These data suggest that V-val subtype may play a role in the pathogenesis of NPC in Taiwan. The results also demonstrated that PCR-RFLP is a sensitive and specific method for EBV genotyping.

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Poster: 28

EPSTEIN-BARR VIRUS ENCODED LATENT MEMBRANE PROTEIN 1 REGULATES Ig KAPPA EXPRESSED IN TUMOR CELLS

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A transforming gene, Tx, cloned from a nasopharyngeal carcinoma cell line CNE2, is expressed at lower level in Epstein-Barr virus negative cell line, but at much higher levels in EBV positive cell lines and planted nude mice, which implies that EBV might regulate the expression of Tx. By bioinformatics, Tx is found highly homologous with human immunoglobulin kappa light chain JC region, which only exists several base pairs changes. Therefore, Tx is an aberrant human Ig kappa light chain gene, which lacks the variable region.

LMP1, an onco-protein encoded by EBV, involves signal transduction via NF kappa B. We indicate that expression of Ig kappa is regulated by LMP1, which provides a basis for further research the LMP1 transactivating immunoglobulin through induction of NF kappa B activity.

Using the ESTs searching results of Tx, we select one EST AI569037, derived from four differentiation primary and metastasis squamous tumors which shows high scores (920) and high identities (98%), as a template to design a probe presenting C region of human Ig kappa light chain. Assayed by in situ hybridization, our data showed that epithelial carcinoma tissues such as cervical carcinoma, gastric carcinoma and colorectal carcinoma could express Ig kappa mRNA. In all, our data challenge classical immunology on immunoglobulin, furthermore, its expression is regulated by EB virus encoded latent membrane protein 1.

NOTES:

Poster: 29

ASSESSMENT OF FAS-L EXPRESSION IN NASOPHARYNGEAL CARCINOMA: ASSOCIATION WITH ADVANCED DISEASE STAGE AND LYMPH NODE METASTASIS

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Nasopharyngeal carcinoma (NPC) is an epithelial cancer with a high incidence in South-East Asia. How it escapes attack of host immune system is not fully understood. Recently, pieces of evidence show that Fas-ligand (Fas-L)-mediated apoptosis may involve in immune privilege of tumours. The purpose of this study was carried out to investigate whether there was any correlation between Fas-L expression and clinical stages in NPC. Assessment of Fas-L was examined by immunohistochemical methods in NPC specimens. Clinical staging of patients was conducted by using the 1997 American Joint Committee on Cancer/International Union Against Cancer staging system. A total of 80 patients were enrolled in this study. Forty-two of the 80 cases (52.5%) showed positive Fas-L expression including 0 of the 14 (0%) stage I NPC, 20 of the 35 (57.1%) stage II NPC, 7 of the 12 (58.3%) stage III NPC, and 15 of the 19 (78.9%) stage IV NPC ($p<0.05$). Eleven of the 27 (40.7%) T1, 20 of the 36 (55.6%) T2, 4 of the 6 (66.7%) T3, and 8 of the 11 (72.7%) T4 showed positive Fas-L expression ($p=0.288$). Thirteen of the 36 (36.1%) N0, 18 of the 30 (60%) N1, 7 of the 9 (77.8%) N2, and 4 of the 5 (80%) N3 showed positive Fas-L expression ($p<0.05$). Seven of the 8 (87.5%) patients with distant metastasis, and 35 of the 72 (48.6%) patients without distant metastasis showed positive Fas-L expression ($p=0.059$). These findings showed that the expression of Fas-L in NPC was associated with advanced disease stage and lymph node metastasis.

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Poster: 30

ALTERATIONS OF BIOLOGICAL PROPERTIES IN IMMORTALIZED NASOPHARYNGEAL EPITHELIAL CELLS BY THE EPSTEIN-BARR VIRUS ENCODED LATENT MEMBRANE PROTEIN 1

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The Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) is frequently expressed in NPC and is believed to play an important role in the pathogenesis of this disease. Previous in vitro studies have showed that LMP1 expression is oncogenic in certain rodent fibroblasts and alters growth properties in epithelial cells. However, the biological properties of LMP1 expression in non-malignant nasopharyngeal epithelial cells have not been examined because of the unavailability of such cell model.

Recently, we have immortalized a nasopharyngeal epithelial cell line (NP69) from primary and non-malignant nasopharyngeal epithelium using SV40T (Tsao et al. BBA, 2002, in press). The NP69 line harbors multiple genetic alterations but retains many differentiation properties of normal nasopharyngeal epithelial cells. The NP69 cells are non-tumorigenic in nude mice and exhibit anchorage-dependent growth property in vitro. Expression of LMP1 in NP69 cells confers many transformed properties including cell proliferation, anchorage independent growth, resistant to serum free induced apoptosis, enhanced cell migration, invasion properties etc.. These results support a role of LMP1 in promoting cell survival, migration and invasion in genetically altered nasopharyngeal epithelial cells.

NOTES:

Poster: 31

EBV-NPC IN INDONESIA : A CHALLENGE IN VACCINE DEVELOPMENT

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EBV is implicated in the development of NPC, which is the highest incidence carcinoma among men in the Yogyakarta province.

We genetically typed EBV from Indonesian NPC-isolates, showing type A (30.8%), type B (7.7%) and mixed type EBV strains (61.5%). PCR-sequence analysis was used to fine-map polymorphism on LMP-1 C-terminus and on LMP-2A at the known HLA-A2 ,-A11, and -A24 restricted CTL epitopes and revealed differences between local NPC-EBV and B95.8.

For LMP-1, codon insertion was found for GLY and ILE at position 314 and 334 respectively, insertion of 22 amino acid repeat sequence at position 371 and deletion of 5 amino acids at position 341 to 345.

For LMP-2A of 3 CTL epitopes showed 4 nucleotide changes namely G to C, A to G, T to C, TC to CG changing MET to VAL, CYS to SER and LEU to PRO at position 7, 8, 9 of the TYGPVFMCL epitope (HLA-A24). Other changes were found as G to C changing SER to THR at position 6 of SSCPLSKILL (HLA-A11) and G to C changing CYS to SER at position 1 of CLGGLLTMV (HLA-A2).

This polymorphism should be considered in future EBV-NPC vaccine development efforts specifically aiming for Indonesia.

NOTES:

Poster: 32

QUANTIFICATION OF EBV DNA COPY NUMBER IN SERA OF PATIENTS WITH NASOPHARYNGEAL CARCINOMA USING REAL-TIME QUANTITATIVE PCR

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Nasopharyngeal carcinoma (NPC) represents one of the major causes of cancer mortality in Malaysia. The unique characteristic that sets NPC apart from other head and neck cancers is its consistent association with Epstein-Barr virus (EBV). The recent discovery that cell-free EBV DNA was detectable in serum of patients with NPC prompted us to investigate the relationship between the viral DNA load (expressed in DNA copy number) with the development of NPC using real-time quantitative PCR (RTQ-PCR). The assay, based on TaqMan chemistry and performed on the Bio-Rad iCycler IQ System, could detect as low as 30 EBV DNA copies and has a dynamic range of 5 $\log_{10}$. Our preliminary investigation involving 15 NPC patients and 11 healthy controls revealed that the viral DNA load in the former was 10 to 269 times (mean=32) higher than the latter. These translate to a mean viral DNA load of 4301 copies/mL (median=2263 copies/mL) and 134 copies/mL (median=28 copies/mL) in NPC and the controls respectively. Interestingly, only an average of 15 DNA copies were detected in sera from two NPC patients who had undergone radiotherapy. We infer from these observations that high serum EBV load correlates positively to the tumour load and reduction in EBV DNA load after radiotherapy indicates the effectiveness of the treatment. Our study underscored both the sensitivity and precision of the quantitative EBV DNA assay, which warrants an evaluation on its prognostic implications.

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Poster: 33

EB VIRUS ENCODED LATENT MEMBRANE PROTEIN 1 MODULATES EPIDERMAL GROWTH FACTOR RECEPTOR PROMOTER ACTIVITY IN NASOPHARYNGEAL CARCINOMA CELL LINE

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EB virus encoded latent membrane protein 1 (LMP1) is expressed in most of the malignancies associated with EBV, including nasopharyngeal carcinoma (NPC). However, the question whether LMP1 regulates EGFR promoter activity remains to be answered. We revealed that in pTet-on LMP1 HNE2 cells (an NPC cell line), doxycycline (Dox) induced expression of LMP1 in a dose dependent manner. Dox-induced LMP1 in turn up-regulated EGFR expression and phosphorylation in a dose-dependent manner. Luciferase reporter assay showed that Dox-induced (0~0.6 μg/ml) LMP1 increased EGFR promoter activity up to 6.7 fold as compared with control, but high level of LMP1 (6 μg/ml Dox) reduced EGFR promoter activity 1.8 fold due most likely to the NFκB dependant manner in NPC cell. EGFR promoter activities were decreased significantly after the cells were transiently transfected with EGFR dominant negative mutant, LMP1 antisense expression plasmid, I κB α dominant negative, c-fos dominant negative (TAM67), JNK interaction protein (JIP), respectively. Collectively, we conclude that EB virus encoded LMP1 modulates EGFR promoter activity in a dose dependent manner as well as in a NFκB dependent manner. LMP1 modulates the activity of EGFR promoter may play a vital role in the development of nasopharyngeal carcinoma.

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Poster: 34

SPECIFIC ACTIVATION OF NF-κB p50 HOMODIMERS IN NASOPHARYNGEAL CARCINOMA

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LMP-1 CTAR1 retains the unique ability to activate three different NF-κB dimers in epithelial cells. To identify the forms of NF-κB activated in NPC, the LMP1+ C15 and LMP1- C17 nasopharyngeal carcinoma (NPC) tumors were analyzed. In both C15 and C17, only NF-κB p50 homodimers were detected in tumor nuclei by EMSA and ELISA, although Rel B/ p100, p50/p65, and p50/p50 dimers were detected in the cytosols of both C15 and C17. Western blot analysis of fractionated cells also detected additional NF-κB proteins in both tumor cytosols with increased p100 and RelB proteins in C15. These data indicate that NF-κB is activated in NPC even in the absence of the LMP-1 protein. Recent work has suggested that the IκB family member, bcl-3, can bind preferentially to NF-κB p50 and p52 providing a trans-activation function and that bcl-3 bound to p52 homodimers may play a role in the development of breast cancer. In both C15 and C17, bcl3/p50 complexes were identified in tumor nuclei by co-immunoprecipitation and Western blot analysis. The presence of nuclear p50 bound to bcl-3 in NPC suggests p50 homodimers may play a role in transcriptional activation.

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Poster: 35

IDENTIFICATION OF TWO CELLULAR GENES DIFFERENTIALLY EXPRESSED IN NASOPHARYNGEAL CARCINOMA BIOPSIES

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The molecular mechanisms leading to development of nasopharyngeal carcinoma (NPC) are not well understood. We used a combination of differential display and cDNA microarray analysis to identify genes differentially expressed in the NPC biopsies. Two cDNA clones, 3E5 and 4A5, were found to differ in expression between the biopsies and non-tumor nasopharyngeal tissues. Expression of 3E5, the osteoblast-specific factor-2 (OSF-2) gene, was detected at significantly higher levels in NPC biopsies than that in control tissues, a finding confirmed using real-time quantitative RT-PCR. On the other hand, expression of 4A5, whose sequences represent the 3'- untranslated region of the polymeric immunoglobulin receptor (pIgR) gene, was detected rarely in NPC specimens but frequently in non-tumor controls. The expression of pIgR in normal epithelial cells, but not in NPC tumor cells, was verified using RT-PCR and immunohistochemical staining. In addition, a correlation between expression of OSF-2 and its regulatory cytokine transforming growth factor-β was observed in non-tumor tissues but not in NPC biopsies, suggesting the tumors may have altered responses to cytokines.

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Poster: 36

THE MECHANISM AND DOWNSTREAM EFFECTS OF PROMYELOCYTIC LEUKEMIA BODY DISPERSION BY EPSTEIN-BARR VIRUS

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The immediate-early protein BZLF1 (Z) protein functions to transcriptionally activate the EBV early gene promoters. Z also manipulates its host cellular environment by binding to and/or interfering with the function of several cellular proteins. One such protein is the promyelocytic leukemia protein (PML), which normally resides the nuclear bodies. Z has acquired the ability to disperse nuclear bodies, creating a microspeckled pattern of PML in the nucleus.

To investigate the mechanism of PML dispersion, we have examined Z's effect upon interferon-induced expression of PML. We have also examined whether interferon treatment of cells could prevent Z-mediated dispersion of PML. Currently it is unknown why EBV disperses PML nuclear bodies, but it is presumed that PML dispersion plays a role in viral replication and survival[1]. Hence, I examined the effects of Z-mediated PML dispersion on known PML-related target proteins, such as MHC class I presentation[2] and p53 transcriptional targets[3]. PML/nuclear bodies appear to be involved with a number of important cellular processes including apoptosis, tumor suppression, and viral defense; therefore, the loss of these processes may have profound effects upon the cell.

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Poster: 37

TREATMENT OF PRODUCTIVE EBV INFECTION AND ORAL HAIRY LEUKOPLAKIA WITH VALACYCLOVIR

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Eighteen HIV-infected individuals with 19 cases of oral hairy leukoplakia and/or productive EBV replication were treated with valacyclovir (acyclovir prodrug with greater oral bioavailability) 1000 milligrams by mouth every 8 hours for 28 days. The clinical, histopathological, and molecular viral responses to therapy were assessed in biopsy specimens obtained before treatment, on the 28th day of treatment, and 28 days after discontinuing treatment. EBV replication was detected by RT-PCR of replicative EBV gene transcripts, immunohistochemistry of EBV EA-D or Z antigens, in situ hybridization for EBV DNA, or Southern blot hybridization of linear EBV genome termini. In 15 treated cases, hairy leukoplakia resolved and EBV replication was terminated (complete clinical and molecular viral response rate: 15/19 = 79%). In 4 of 12 resolved cases, hairy leukoplakia and/or EBV replication recurred after treatment (relapse rate: 4/12 = 33%). In 2 cases, hairy leukoplakia and EBV replication persisted (clinical failure rate: 2/19 = 10%), and in 2 other cases, hairy leukoplakia resolved grossly and histologically but EBV replication persisted in normal tongue epithelium (molecular viral failure rate: 2+2/19 = 21%). In summary: most hairy leukoplakia lesions respond to valacyclovir treatment, but some EBV strains may be naturally resistant to inhibition by acyclovir.

NOTES:

Poster: 38

MARKERS OF CLONALITY AND IDENTITY IN EBV-ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS OF IMMUNOCOMPROMISED HOSTS

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The clonality of EBV-associated lymphoproliferation may have important implications for diagnosis, prognosis, and therapeutics. Specimens of EBV–associated, B-cell lymphoproliferative disorders from immunocompromised hosts were analysed with three different molecular assays to determine the optimal approach for assessing clonality. Tumor cell clonality was determined by PCR-based detection of immunoglobulin gene JH region rearrangement. EBV clonality was determined by Southern blot hybridization to fused terminal genome fragments. EBV genotype identities were determined by strain-defining sequence variation in the LMP-1 gene, as detected by nested PCR amplification, cloning of the amplification products, and sequencing of multiple clones per specimen. The JH rearrangement and EBV termini results were concordant in 12 of 16 lymphoproliferation and control specimens (75%). For 3 of the 4 discordant results, the EBV termini assay gave the likely correct results. The number of EBV genotypes present in each specimen correlated with tumor clonality in only 7 of 13 cases (54%), but EBV genotyping established clear identity between different tumor specimens obtained from the same individual. These data indicate that EBV–associated lymphoproliferative tumor clonality is best assessed by applying the JH rearrangement and EBV termini assays together, and that EBV genotyping is more useful as a molecular epidemiologic tool.

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Poster: 39

SUCCESSFUL TREATMENT OF ADVANCED STAGE OF EBV-INFECTED NASAL T-CELL LYMPHOMA WITH UNRELATED ALLOGENIC BONE MARROW TRANSPLANTATION (UBMT).

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EBV-associated T/NK-cell LPD comprises chronic active EBV infection , Mosquito Allergy, Hydroa Vacciniforme, EBV-AHS , peripheral T-cell lymphoma, nasal/nasal type lymphoma and aggressive NK-cell leukemia. Its prognosis is very poor and fatal with conventional treatment. We report here a case of advanced EBV-associated nasal T-cell lymphoma who successfully treated with UBMT. A 9-year-old girl presented with nasal obstruction and a huge nasal mass on CT. The nasal mass biopsy revealed a T-cell lymphoma (CD2,3,4,7,TCRαβ(+),EBER(+),stage Ⅱ). During radiotherapy after induction chemotherapy, however, she developed fever, pancytopenia and liver disfunction. Laboratory data showed monoclonal proliferation of EBV-infected CD4+ T-cells in the PBL. Since high copies of EBV-DNA persisted in the PBL during intensive chemotherapy, we performed UBMT using a preconditioning regimen of TBI+VP-16+CY. Although aGVHD (Grade β) was observed, EBV-DNA disappeared in the PBL soon after UBMT. Thus, BMT is useful method to eliminate EBV-infected T-cells, and quantitation of EBV-DNA in the PBL is a useful marker for the treatment choice of patients with EBV-associated T/NK-cell LPD.

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Poster: 40

ANTIBODY RESPONSES TO EPSTEIN-BARR VIRUS ENCODED LATENT MEMBRANE PROTEIN -1 (LMP1) AND EXPRESSION OF LMP1 PROTEIN IN JUVENILE HODGKIN'S DISEASE

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The molecular characteristics of EBV serology was evaluated in detail in a group of juvenile HD patients (n=242). Mean age was 11.7 years (3.2-16.4) and 181 (75%) were EBV seropositive, with 19/181 (10.5%) showing evidence of recent infection. The EBV-status of the tumour was known in 129 cases. It was analysed whether antibodies to LMP1 were related to a specific EBV-serological pattern as detected by ELISA and immunoblot or to expression of LMP1 in the tumor cells. Antibodies to LMP1 were detected in 30% of the EBV seropositive HD patients, but no relation was found between the histopathological phenotype of HD and the presence of LMP1-specific antibodies. The presence of antibodies to LMP1 was not associated with a distinct anti-EBV antibody profile (ELISA), but a significantly higher percentage of patients with antibodies to LMP1 had antibodies to Zebra and VCA-p18 (immunoblot; $p<0.005$). No differences were found for anti-EAd antibodies. Significantly more patients with an EBV-positive tumor had detectable antibody responses to LMP1 ($p=0.006$), but the presence of antibodies to LMP1 did not reflect the expression of LMP1 in the tumor cells. In fact, patients with the strongest LMP1 antibody responses all had EBV negative tumors, suggesting immunological selection in vivo.

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Poster: 41

CHILDREN LIVING IN ENDEMIC MALARIA AREA HAVE INCREASED EBV LOAD

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The aim was to correlate the EBV genome load with severity of malaria in sera from children living in Ghana.

Plasma samples were collected from 73 Ghanian children; 32 had acute malaria infection and 41 were age-matched controls without ongoing malaria infection. Plasma from 20 Italian children served as controls.

EBV DNA was not detected in sera from Italian children. EBV DNA could be quantified in 29 (40%) of the 73 plasma samples from the Ghanian children. In the *p.falciparum* infected group 15/32 (47%) contained detectable EBV (median 1159 copies/mL) and in the non-infected control group 14/41 (34%, median 1170 copies/mL). The detection rate in the malaria-infected group was 1-7 times higher than in the non-infected group. The difference was not statistically significant.

In the cerebral malaria (CM) group 38,5% had detectable EBV DNA and in the uncomplicated malaria (UM) group more than 50% of the cases had detectable EBV DNA. The EBV DNA detection rate was lower in the CM group but the median copy number was 7 times higher than in the UM group. No significant correlation between malaria infection and or severity of disease and EBV load was detected.

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A COMPARATIVE ANAYLSIS OF LMP1 ASSOCIATED CYTOKINE EXPRESSION IN LYMPHOMA CELL LINES

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IL-10 modulates the immune response at certain levels, playing an important role in balancing humoral and cellular responses. It can function as a growth and differentiation factor for B cells. Expression of other cytokines, regulated by EBV, may play a role in the patho-physiology of lymphomas. Here we demonstrate that IL-6, IL-10 and TNF-α were specifically induced by LMP1 in certain lymphoma cell lines. Activation of NFκB by LMP1 plays a crucial role for TNF-α and in part for IL-6, but not for IL-10 expression. LMP1 derivatives of the CTAR/TES underline the differences in signaling associated with expression of TNF-α, IL-6 or IL-10. The overexpression of MEKK1, MEK1, PKA, AKT or MKK6 in comparison to LMP1 reveals additional differences between the analysed cytokines. Our data demonstrate that more than one signaling pathway is involved in this activation and suggests the necessity of a defined conformation of CTARs to activate IL-10.

Furthermore, we have characterized more than 10kbp of the 5' flanking region of the IL-10-gene identifying new DNA-sequence variations and their frequencies in different geographic regions. Their functional role in *in vitro* EBV immortalized B cells will be shown.

Supported by SFB502, DFG KU 954/4-1, DFG KU 954/5-1 & Wilhelm-Sander Stiftung.

NOTES:

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PREDIAGNOSIS EPSTEIN-BARR VIRUS SEROLOGIC PATTERNS IN EBV-POSITIVE AND EBV-NEGATIVE HODGKIN'S LYMPHOMA

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To examine the Epstein-Barr virus (EBV) serologic profile preceding diagnosis in relation to the molecular status of Hodgkin's lymphoma (HL) cases, we conducted a nested case-control study of 112 incident HL cases and matched controls from active-duty military personnel with archived serum in the US Department of Defense Serum Repository. Tissue blocks from cases were tested for EBV genome status. In conditional logistic regression analyses of 32 EBV-positive HL and matched controls, statistically significant risks were associated with elevated anti-EBV serum antibody titers for EA-D [OR=3.04 (95% CI=1.05-8.80)], EA-R [OR=3.93 (95% CI=1.14-13.53)], EBNA-2b [OR=5.05 (95% CI=1.25-20.42)] and the ratio of EBNA-1 to EBNA-2 <1 [OR=13.41 (95% CI=1.58-113.81)]. When the titers were mutually controlled in multivariate analyses, the only association that approached significance was with EBNA-1/EBNA-2, indicative of defective control of latent EBV infection. Analyses of 80 EBV-negative cases relative to their matched controls revealed a strikingly different pattern as null findings were found with all antibody titers. Moreover, a strong association was noted for EBNA-1/EBNA-2 in EBV-positive HL relative to EBV-negative HL. This contrasting antibody profile suggests differences in host response to the virus as well as differences in the natural history of disease based on EBV genome status.

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INVERSE ASSOCIATION BETWEEN NURSERY SCHOOL AND HODGKIN'S LYMPHOMA, INDEPENDENT OF EBV TUMOR STATUS

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Hodgkin's lymphoma (HL) features molecular evidence of Epstein-Barr virus (EBV) involvement in about one-third of tumors. HL, especially among young adults, is associated with delayed exposure to common infections during childhood. Pre-kindergarten attendance of nursery school or day care facilitates transmission of pathogens among young children, thereby priming the development of Th1 immunity. In a population-based case-control study of 573 HL patients and 664 controls in Massachusetts and Connecticut, a history of nursery school or day care attendance was inversely associated with HL risk: odds ratio (OR)=0.81, p=0.12, adjusting for age, sex, and state. Among persons who had attended for over one year, the protective effect was enhanced: OR=0.63, p=0.01. The OR did not change significantly with additional adjustment for other childhood social factors, and also remained consistent when stratified by age group. When EBV genome-positive cases (25% of all cases) were compared to controls, the OR for nursery school attendance was 0.87, p=0.62; for EBV genome-negative cases, OR=0.76, p=0.10. While the inverse association between nursery school attendance and HL suggests that early infection with common pathogens and development of Th1 immunity protect against HL, the consistency of the finding between EBV genome-negative and genome-positive cases implies that the biologic mechanism is independent of EBV status.

NOTES:

Poster: 45

EFFECTS OF EBV ON CELL GROWTH AND PHENOTYPE IN HODGKIN'S DISEASE-DERIVED CELL LINES

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Although EBV is associated with Hodgkin's disease (HD), little is known about the role of the virus in disease pathogenesis. In order to study the influence of EBV on the phenotype and growth of the malignant (HRS) cells of HD, we infected the EBV-negative, CD21-positive, HD cell line, KMH2, with Akata-derived EBV carrying a neo^r gene.

EBV-infected clones (as evidenced by nuclear expression of EBNA1 and EBERs) showed increased viability, enhanced proliferation and other phenotypic changes compared with their EBV-negative counterparts. The majority of infected cells displayed a restricted pattern of EBV gene expression being confined to Qp driven EBNA1, the EBERs and to transcription from the BamH1A region. Low-level transcription from Cp/Wp and of EBNA2 and LMP2, but not LMP1, was also detectable. By limiting dilution we have been also able to derive EBV-negative clones of L591 cells from the parental EBV-positive line. These cells proliferate less quickly and are less viable than their virus-positive counterparts.

Overall, these data suggest that EBV contribute to enhanced cell survival and to the growth of HRS cells, in some cases even in the absence of expression of the EBV oncogene, LMP1.

NOTES:

Poster: 46

EPSTEIN-BARR VIRUS POSITIVITY IS AN INDEPENDENT RISK FACTOR FOR FAILURE IN PEDIATRIC HODGKIN'S DISEASE

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Studies on the association of EBV with survival in adult Hodgkin's disease (HD) have shown significant differences in outcome. However, large population-based studies of prognostic factors for clinical outcome in childhood HD have not considered EBV. We have investigated whether there is an association between EBV-positivity and both survival and disease-free survival in a series of 175 pediatric HD patients diagnosed between 1957 and 2001.

Latent EBV infection was identified in 49% (85/175) of cases. EBV-positive status was associated with South Asian ethnicity ($P < 0.001$), males ($P=0.01$) children aged 0-9 years compared with those 10-14 years ($P=0.045$) and subtype ($P < 0.001$). EBV-positive cases were significantly more likely to derive from more deprived populations ($P=0.021$) or from homes where the main income earner had a manual as opposed to a non-manual occupation ($P=0.003$).

EBV-positivity significantly increased the hazard of failure (relapse or death), even after adjustment for other factors, including those shown to co-associate with EBV-positivity (HRs ranged from 2.00 to 2.57). Interestingly, this effect was more marked in the cases diagnosed between 1982 and 2001 in the period when treatment was likely to have been more effective (HRs from 2.44 to3.28). There was also an association between EBV-positivity and overall survival (HR=2.12, 95% CI 0.94 to 4.81).

In conclusion, our results show that children with EBV-positive HD are at least twice as likely to fail compared with their EBV-negative counterparts. This effect is independent of other factors known to influence risk of failure in pediatric HD.

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CELLULAR AND BIOLOGICAL STUDY OF HUMAN LYMPHOMAS INDUCED BY EB VIRUS

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Epstein-Barr virus is known as an etiologic agent of infectious mononucleosis and is closely associated with human NPC, BL and HD. However, animal experiment should be carried on to identify that EBV can induce tumors. It is very essential to establish the animal model in order to further explore the mechanism of EBV-induced tumors. Most important, we have to know more about the cellular and molecular mechanism of EBV-induced neoplasm. In the present work, we use CSA, an immunosuppressive drug to inhibit GVHR that may occur in the hu-PBL/SCID mice due to xenografts. The seral level of human sIL-2R are examined as to analyze the relationship among sIL-2R,GVHR and CSA. The seral value of human IgG are measured as to assess the association between IgG and development of lymphomas. Oncogenes/tumor suppressor genes such as p53,c-myc, bcl-2 and bax are also investigated in the EBV-induced tumors. Human peripheral blood lymphocytes (hu-PBLs) were isolated and were inoculated intraperitoneally into severe combined immuno- deficient(SCID) mice. The mice were divided into four groups according to the status of EBV infection and CSA application: the control group, the active infection group, the latent infection group and the CSA administration group. Our experiment showed that no mice in the CSA administration group and 15 mice of the other three groups died of GVHR. The medium life span was 17 days in the mice suffered from GVHR and the mortality rates were 55.56% (5/9), 30.43% (7/23), 42.86% (3/7) respectively. The difference was statistically significant between the CSA group and the other groups. It demonstrated that CSA could strikingly inhibit GVHR that might occur in hu-PBL/SCID mice. The sera levels of human sIL-2R were analyzed by ELISA in 7 mice of the CSA administration group and 8 mice of the active infection group at day 3,7,15,22,33 after hu-PBLs were inoculated. In the CSA administration group the levels of human sIL-2R were stable. However human sIL-2R levels increased gradually in the active infection group and reached highest at day 22.Significant difference existed between day 3 and day 15, day 3 and day 22($P<0.05$). There were 3 SCID mice in the active infection group died at day 12,14,16 respectively. There were also significantly difference between the two groups at day 15 and day 22. The results suggested that the increase of sIL-2R was associated with GVHR and CSA could reduce the release of sIL-2R. Of 38 survival SCID mice, 24 mice developed tumors in their body cavities . The tumor incidences were 0% (0/4), 68.75% (11/16), 50% (2/4), 78.57% (11/14) respectively in the aforementioned four groups. Immunohistochemical staining of the induced tumors revealed LCA positive, CD20 (human B cell marker) positive and CD3(T cell marker) negative. PCR showed that the tumors contained both EBV-DNA and Alu sequence of human genome. In situ hybridization exhibited resultant tumor cells had EBV encoded small RNA-1, i.e. EBER-1 and Alu sequence of human genome. There were EBV particles in nuclei of tumor cells observed by electron microscopy. The results confirmed that tumors were derived from human normal lymphocytes and induced by EBV.

The serum levels of human IgG were measured in 12 hu-PBL/SCID chimeras using single immunodiffusion assay. In 6 mice that developed visible tumors, the levels of human IgG could be found at day 15 after hu-PBL engraftment and increased as the day elapses. But IgG could not be detected in 3 mice only with microscopic tumors and in 3 mice without tumors. This result indicated……

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EBER-1 IN SITU HYBRIDIZATION, LMP1 IMMUNOHISTOCHEMICAL STAINING AND PCR FOR DETECTING EPSTEIN-BARR VIRUS FROM PARAFFIN-EMBEDDED HODGKIN'S LYMPHOMA (HL) TISSUES IN SOUTHERN CHINA

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To further explore the relationship between EBV and Hodgkin's lymphoma (HL), three methods were employed to detect EBV from 71 cases of paraffin–embedded tissue of HL in Southern China. All tissues were studied for immuno-histochemical staining of EBV latent membrane protein (LMP1), 39 cases of which were examined by *in situ* hybridization (ISH) for EBV-encoded RNA (EBER)-1 and PCR for EBV BamH1W fragment. The results indicate that Reed-Sternberg cells of 62.0% (44/71) cases were positive in LMP1 protein expression, including 22 of 32 (68.8%) with lymphocyte predominant, 16 of 27 (59.3%) with mixed cellularity, 3 of 7 (42.9%) with nodular sclerosis, and 3 of 5 (60.0%) with lymphocyte depletion subtype. And 66.7% (26/39) of them showed EBER-1 positive, almost matched with the results of LMP1 protein expression. Interestingly, 3 cases (in 39) of HL, negative in LMP1, showed positive in EBER-1, and 2 cases of 39 HL, EBER-1 negative, positive in LMP1 protein expression. However, the results of PCR, 43.6%(17/39) positive for HL, do not consistent with the results of LMP1 and EBER-1. Our results also indicate that EBER-1 positive rate in children (17/17) was much higher than that in adult patients (8/22).

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AN INVESTIGATION OF EPSTEIN-BARR VIRUS (EBV) IN CHINESE COLORECTAL TUMORS BY IN SITU HYBRIDIZATION (ISH), POLYMERASE CHAIN REACTION (PCR) AND IMMUNOHISTOCHEMICAL STAINING

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We investigated the presence of EBV in paraffin-embedded tissues from 130 Chinese patients with colorectal tumors, including 26 cases with adenomas (A), 23 cases of adenomas with dysplasia (AD), 22 cases of late adenomas with carcinoma (AC), 36 cases with colorectal cancer (CC), and 23 cases with HNPCC. Of 130 cases, we found EBV DNA (+) in 26 cases, LMP1 (+) in 20 cases, and EBER1/2(+) in 6 cases. LMP1 expression was found in 73.1%(19/26) of EBV DNA (+) cases. In addition, we found LMP1 (+) in 4 cases, EBER1/2(+) in 0 cases, and EBV DNA (+) in 5 cases in group A; 5, 1, and 6 cases in group AD; 5, 2, and 6 cases in group AC; And 6, 3, and 7 cases in CC group, respectively. None of HNPCC showed a positive result by ISH and IMC, but EBV (+) tumor infiltrating lymphocytes were found in 8.6% of patients in this group. Our results suggest that colorectal tumors in Chinese may be associated with EBV. HNPCC is not related to EBV. As a reservoir, EBV (+) lymphocytes can reach anywhere, providing a chance for epithelial cells to be exposed to EBV.

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HIGH LEVEL EBV IN PBMC OF PATIENTS WITH MYELODYSPLASTIC SYNDROME (MDS)

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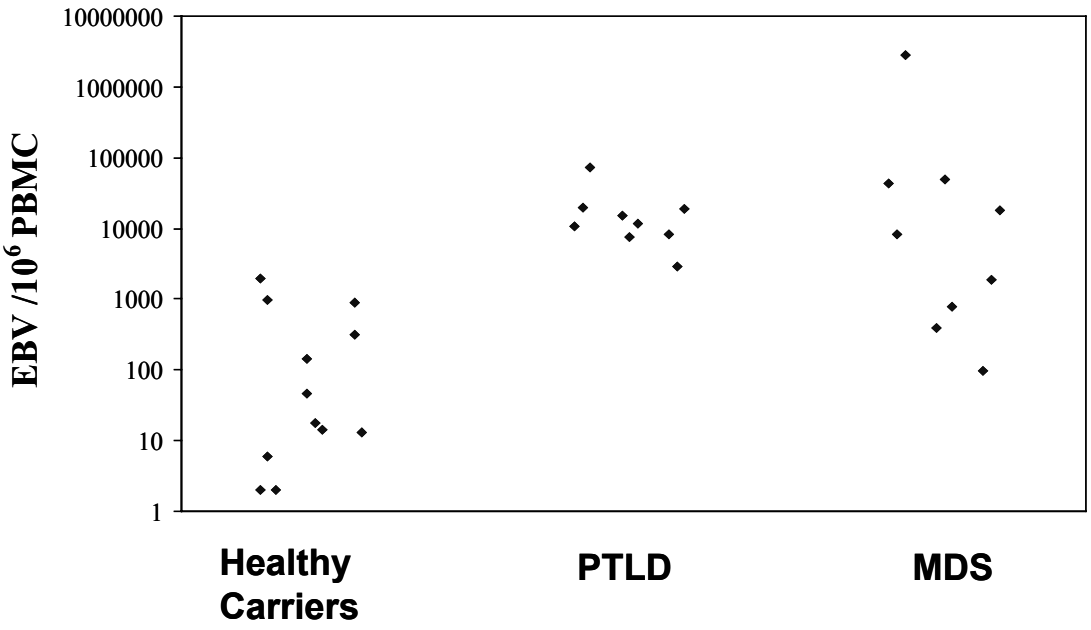
MDS is a clonal disorder of bone marrow stem cells that frequently evolves into acute myeloid leukemia. With the intent of monitoring drug effects on EBV load *in vivo*, we assayed patients at baseline entering a phase I study. In nine MDS patients, the median copy number of EBV genomes per million PBMCs was 8,133 (97-2,800,000). This is comparable to the EBV load in patients with active post-transplant lymphoma (PTLD) and is much higher than that in healthy controls. The pattern of viral gene expression in the PBMCs of patients with MDS was similar to that of healthy carriers and patients with active PTLD. Reverse-transcription PCR detected EBER1 but not EBNA2, EBNA3C, LMP1, BZLF1 or BLLF1 mRNA. Investigations of marrow specimens are underway to determine whether virus is present in malignant cells or only in lymphocytes.



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EBNA1 INDUCES DISTINCT CHANGES OF CELLULAR GENE EXPRESSIONS – IN 293 AND BJAB CELL LINES

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EBV is causally linked to several types of lymphomas and epithelial tumors including nasopharyngeal carcinoma, gastric adenocarcinoma, and possibly breast cancer. In its route of infection, EBV was shown to infect both B cells and epithelial cells in the oral cavity. The infection of B cells may result in the formation of lymphomas while the oral epithelial infection is associated with nasopharyngeal carcinoma. EBV nuclear antigen 1 (EBNA1) is expressed in all EBV associated tumors and is required for transformation. EBNA1 initiates the latent viral replication in dividing lymphoma cells, maintains the levels of viral genome copy number, and regulates transcription of other EBV oncogenes. Indeed, expression of EBNA1 in a transgenic mouse model resulted in the appearance of lymphoma clearly implicates EBNA1 in transformation. To further elucidate the role of EBNA1 in transformation of B cells and epithelial cells, we have examined the effect of EBNA1 on cellular gene expression by microarray analysis using the BJAB and 293 cell lines transfected with EBNA1. Analysis of the data from a 10.5K human gene array, revealed distinct profiles of cellular genes changes among BJAB and 293 cell lines. These results may suggest that EBNA1 affects tissue specific gene expression rather then specific genes.

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GENETIC AND BIOCHEMICAL CHARATERIZATION OF THE CO-ACTIVATION DOMAIN OF EBNA-3C

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The Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA-3C) is essential for EBV-mediated transformation of primary B lymphocytes, likely via regulation of virus and cell gene expression. We and others have observed that EBNA-3C co-operates with EBNA-2 to activate of the LMP-1 promoter, but inhibits the EBNA-Cp promoter under similar conditions. Genetic analysis of EBNA-3C revealed that amino acids (aa) 365 to 545, including most of the previously identified repression domain are necessary and sufficient for co-activation with wild-type EBNA-2. Of the proteins demonstrated to interact with EBNA-3C in a yeast two-hybrid screen, only the small ubiquitin-like proteins SUMO-1 and SUMO-3 mapped to this co-activation domain. EBNA-3C is also covalently modified by SUMO-1, SUMO-2, and SUMO-3 and aa 343-545 are required for this modification. We are in the process of determining which lysine residues are required for SUMO modification to test its importance for co-activation. Interaction between EBNA-3C and SUMO in yeast may reflect EBNA-3C association with SUMOlated proteins in vivo, and are now identifying cell proteins that interact with the coactivation domain in LCLs.

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THE ABILITY OF EPSTEIN-BARR VIRUS TO INFECT NAÏVE AND MEMORY B LYMPHOCYTES ISOLATED FROM BLOOD AND TONSIL

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B-cells were fractionated according to immunoglobulin class and EBV latency demonstrated by RT-PCR in all populations. Between 80-90% of the EBV genomes/10^5 $CD19^+$ cells are found in the IgA expressing fraction. This finding indicates either that: (i) the IgA-expressing cell is a major target for EBV infection or, (ii) the high viral copy number reflects the end stage of EBV maintenance in B-cell clones arising from initially infected IgM^+ naïve B cells that progress to isotype switching during the course of activation. EBV infection of B-cells requires CD21 and HLA cl II and we found their expression to be similar on blood and tonsil B-cells fractionated by virtue of carrying IgM, IgD, IgG or IgA.

Tonsil B-cells were infected and co-stained for EBNA5 and Ig isotype. The appearance of EBNA5 showed no preference for subsets defined by IgM, IgG, or IgA expression: as previously reported, IgD was actively down-regulated on EBV-infection. Furthermore, on fractionating tonsilar B cells into $CD38^{low}CD27^-$ "naïve" and $CD38^{low}IgD^-$ "memory" populations, no difference in EBV infectability was observed.

These results indicate that B cells present at the site of virus entry, or release, can be infected independently of the Ig isotype expression.

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Poster: 54

EBV-ENCODED EBNA-5 BINDS TO p14ARF AND PROLONGS THE SURVIVAL OF p14ARF EXPRESSING CELLS *IN VITRO*

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EBV carrying LCLs of normal origin express the full program of all nine growth transformation associated proteins. Their cellular p53 pathway is usually intact. This raises the question whether any of the virally encoded proteins impair the pathway functionally. Using the yeast two-hybrid system we have found that EBNA-5 interacts with the p14ARF protein, an upstream regulator of the p53 pathway. The interaction was confirmed *in vitro* by GST pull-down assay.

Over-expression of p14ARF induces growth arrest and ultimately cell death in cells that carry wild type p53 but lack endogenous p14ARF. In order to examine possible effect of EBNA-5 on p14ARF induced growth impairment changes we have compared the number of cells that survived two weeks selection with proper marker and expressed p14ARF in absence and presence of EBNA-5. At least two-fold increased number of p14ARF-positive cells survived in the presence of EBNA-5.

p14ARF localizes to the nucleoli and forms nuclear inclusions. EBNA-5 accumulates in the nuclear inclusions, where these two proteins showed high grade of co-localization. The same p14ARF-positive nuclear inclusions were targets for the PML-bodies, p53, Mdm2 and proteasomes. We hypothesize that such p14ARF-inclusions could serve as the sites for the protein processing.

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Poster: 55

REGULATION OF CDKI P27^{KIP1} IN EBV-TRANSFORMED LYMPHOCYTES

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Cyclin-dependent kinase inhibitor p27^{KIP1} plays a key role in controlling cell proliferation. The abundance of p27^{KIP1} is dramatically down regulated following EBV immortalisation of primary B-lymphocytes[1]. We demonstrate that this regulation is post-translational yet independent of ubiquitin-mediated proteolysis; the regulation is dependent on cleavage following amino acid residue D139 by a caspase-like activity[2,3]. The "p27^{KIP1} caspase" has been further characterised as being insensitive to broad spectrum caspase inhibitors and it is not associated with apoptosis.

We developed an *in vitro* fluorogenic assay to quantitate the p27^{KIP1} caspase activity using tetrapeptide derivatives and demonstrate that it is cell cycle dependent and its activity inversely correlates with the abundance of p27^{KIP1} within cells. This strongly suggests that the p27^{KIP1} caspase is a candidate to effect the regulation of p27^{KIP1} within these cells.

Furthermore, we have used a cell permeable tetrapeptide inhibitor to specifically inhibit p27^{KIP1} caspase within EBV-transformed cells and we demonstrate that this results in an increase in the abundance of p27^{KIP1} within the cells and a concomitant dramatic inhibition in cell proliferation. We propose that the p27^{KIP1} caspase plays a major role in regulating the cell cycle in EBV-transformed B-lymphocytes and the activity is undergoing further characterisation.

[1]Cannell et al., *Oncogene* **13**: 1413-1421, 1996

[2]Frost et al., *JGV* **82**: 3057-3066, 2001

[3]Frost et al., *Oncogene* **20**: 2737-2748, 2001

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Poster: 56

EPSTEIN-BARR NUCLEAR ANTIGEN-1 ACTIVATES TRANSCRIPTION FROM INTEGRATED TEMPLATES

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EBNA-1 supports replication and transcription of the EBV genome. The exact mechanism by which EBNA-1 activates transcription is unknown. Cytoplasmic microinjection experiments of *oriP*-based plasmids into EBNA-1 positive cells revealed a 20-fold increase of reporter gene activity compared to their nuclear injection (Langle-Rouault F, et al. J Virol, 72, 6181-5, 1998). This finding could mean that activation of transcription from transfected templates just reflects EBNA1's ability to move the transfected DNA's into the nucleus. This model is supported by Kang, et al., who found that EBNA-1 could not activate transcription from templates integrated within cellular DNA of a polyclonal B-cell line (Kang MS, et al. Proc Nat Acad Sci 98, 15233-8, 2001). We have tested the hypothesis that EBNA-1 can activate transcription from templates integrated into cellular DNA. We have established clones of BJAB cells with integrated copies of either tk-luciferase or FR-tk-luciferase. Expression of EBNA-1 had no effect on luciferase activity in cells carrying tk-luciferase but activity of FR-tk-luciferase was increased in a dose-dependent fashion (5-30 fold induction of luciferase activity). Different derivatives of EBNA-1 were examined and revealed that amino acids 40-89 are required to activate transcription from integrated templates. These experiments demonstrate that EBNA-1, in addition to transporting DNA's from the cytoplasm to the nucleus, can activate transcription from templates within the nucleus.

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Poster: 57

EPSTEIN-BARR VIRUS ASSOCIATED BURKITT LYMPHOMAGENESIS SELECTS FOR DOWNREGULATION OF EBNA2

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Epstein-Barr virus (EBV) is aetiologically linked to endemic Burkitt's lymphoma (BL) but its contribution to lymphomagenesis, vis-à-vis that of the chromosomal translocation leading to c-*myc* gene deregulation, remains unclear. The virus' growth transforming (Latency III) programme of gene expression is extinguished in tumour cells and reportedly only a single viral protein, the nuclear antigen EBNA1, is expressed via a programme called Latency I. It is not known if BL arises from a B cell subset in which EBV naturally adopts a Latency I infection or if a clone with limited antigen expression has been selected from an EBV-transformed Latency III progenitor pool. Here we identify early passage BL cell lines with a novel pattern of viral transcription in which the Latency III-associated EBNA promoter Wp is active but where differential splicing allows expression of the full range of EBNAs except EBNA2. Subsequent work with BL biopsy samples indicated that 3 of 15 endemic BLs examined displayed this "Wp-restricted" pattern of gene expression rather than the usual Latency I. This favours the view that, in at least some cases, BL is selected from a Latency III progenitor and the principal selection pressure is for downregulation of the c-Myc antagonist EBNA2.

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Poster: 58

PLASMA EPSTEIN-BARR VIRUS DNA CONCENTRATION IS CORRELATED TO SERUM EBV IGA/VCA ANTIBODY TITERS AND CLINICAL STAGE IN NASOPHARYNGEAL CARCINOMA

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Purpose: To quantitative analysis of plasma EBV-DNA level and its correlation with serum EBV IgA/VCA and IgA/EA antibody titers; to evaluate its application for clinical staging, monitoring recurrence and metastasis, and early diagnosis of nasopharyngeal carcinoma.

Patients and Methods: Patients of one hundred and twenty primary NPC, twenty-one NPC with distant metastasis, eight recurrent NPC, sixty remissive NPC after treatment, eight non-NPC tumor, and 47 normal controls were conducted to a study by using real-time polymerase chain reaction (PCR) assay for quantitative detection of plasma EBV-DNA.

Results: Plasma EBV-DNA was detectable in 96% (115/120) of primary NPC, 100% (21/21) of NPC with distant metastasis, 100% (8/8) of NPC with regional recurrent. The detectable proportion is significantly higher than that in control subjects (8%, 4/47), non-NPC tumor (12%, 1/8) and remissive NPC (12%, 7/60) ($p<0.001$). The plasma EBV-DNA level increase gradually in NPC with serum IgA/VCA antibody titers of ≤1:80, at 1:160 or 1:320, and to 1:640; however, decreased at titers of 1:1280 ($p=0.045$, Kruskal-Wallis Test). The disease TNM stage is also correlated to serum EBV IgA/VCA (Nonparametric correlations, $p<0.01$) and serum EBV IgA/EA (Nonparametric correlations, $p<0.05$) antibody titers. Advanced TNM stage (III+IV, median, 8530 copies/ml) and late T stage (T3+T4, median, 8530 copies/ml) NPC patients had significant higher plasma EBV-DNA concentration than those of in early TNM stage (I+II, median, 930 copies/ml, $P = 0.000$) and early T stage (T1+T2, median, 3700 copies, $p=0.006$) NPC. (Mann-Whitney rank-sum test). NPC patients with regional lymph nodes metastasis (N1+N2+N3) had higher plasma EBV-DNA concentration (median, 7670 copies/ml) than those without lymph node metastasis (N0, median 2025 copies/ml) ($p=0.023$, Mann-Whitney rank-sum test). The relapsed NPC patients had significant higher plasma EBV-DNA concentration (median, 1900 copies/ml) than that of clinical remissive NPC (median, 0 copies/ml) ($p=0.000$, Mann-Whitney rank-sum test). After completion of therapy, plasma EBV-DNA was undetectable in 15 of 31 patients; the plasma EBV-DNA concentration was sharply decreased from 13310 copies/ml (median, prior treatment) to 810 copies/ml ($p=0.001$, Wilcoxon Signed Ranks Test). In controls, two member who had high plasma EBV-DNA level were histologically diagnosed of NPC later, and in the group of clinical remissive NPC, 4 patients who had high plasma EBV-DNA concentration, were lately clinically confirmed regional recurrence of NPC tumor.

Conclusions: Our results suggest that the quantity of plasma EBV-DNA is a sensitive, valuable and reliable tumor marker for reinforcing clinical staging, monitoring of metastasis and recurrence, as well as improving prognostication of NPC.

This work is supported in part by the Chinese State Key Basic Research Project

(Project 973, Contract No. G1998051201).

Poster: 59

EBV-NPC IN INDONESIA : EPIDEMIOLOGY AND SCREENING

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EBV is implicated as causal factor in the development of NPC. Although the virus is considered to infect >90% of humans worldwide, limited documentation exists about EBV prevalence and its role in NPC in Indonesia. NPC ranks among the 4 most prevalent tumors in Indonesia.

An epidemiology study in Yogyakarta province showed that NPC is the highest incidence carcinoma among men.

During January 2001 to May 2002 we had collected 188 new NPC cases from Sardjito Hospital, who came from many different places in Java (99.5%) and outside Java (0.5%). Nearly all had Javanese ethnicity, only one being Chinese. Interestingly, about 29% came from Banyumas/ Kebumen, an area with special smoking habits using "klembak menyan". Histological type were undifferentiated Ca (72%), anaplastic Ca (7%), Squamous ca (9%) and others (12%) and most patients (90%) came at latest stage (stadium IV).

A simple synthetic peptide-based [VCA+EBNA] IgA-ELISA was developed for first-line screening and improving diagnosis of EBV-NPC, which showed 95% sensitivity and 90-95% specificity. Confirmation using immunoblot showed good results. Since immunoblot is laborious and expensive, we are developing specific EBV antigens for primary screening and confirmatory testing of this important and prevalent tumor in the Indonesian setting.

NOTES:

Poster: 60

ASSESSMENT OF THE IMMUNE RESPONSE TO EPSTEIN-BARR VIRUS IN HIGH-RISK SOLID ORGAN TRANSPLANT RECIPIENTS

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Epstein-Barr Virus (EBV) induced post-transplant lymphoproliferative disease (PTLD) arising in solid organ transplant patients is an increasingly important clinical problem. The early immune response in patients acquiring EBV from a transplanted organ, rather than through the conventional oral route, may predict the onset of PTLD. A recent clinical study recommended that "measurement of the EBV DNA load in peripheral blood at least once a week is essential for high risk patients for accurate monitoring of the dynamic balance between EBV-induced B cell proliferation and CTL-mediated clearance" (Stevens *et al.,* 2001. Blood 97(5):1165-1171). This demonstrated that EBV DNA loads can change rapidly, and can double within 2-3 days.

We are currently assessing ten EBV seronegative high-risk transplant patients on a weekly basis for the first three months following solid organ transplant, which may be the period where EBV seroconversion is most likely to occur, and then monthly for up to two years. Examining the EBV DNA load, generation of an EBV-specific T cell response, specific markers for maturation and differentiation of memory T cells, and the date of EBV seroconversion will provide an insight into the early immune response post-solid organ transplant, and may highlight risk factors for the onset of PTLD.

NOTES:

Poster: 61

PROTEIN PROFILING OF EBNA1 REVEALS A SPECIFIC INTERACTION WITH THE HERPESVIRUS-ASSOCIATED UBIQUITIN-SPECIFIC PROTEASE, USP7

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EBNA1 plays several roles in latent EBV infection, including viral DNA replication, segregation and transcriptional activation, and avoids immune detection due to the failure of EBNA1 to be processed by the proteasome. All of these functions likely involve specific interactions with cellular proteins. We have used an EBNA1 affinity column approach coupled with mass spectrometry to identify cellular proteins that bind EBNA1 and have eliminated those interactions that occur through nucleic acid and non-specific charge interactions. EBNA1 was found to specifically bind the nuclear ubiquitin-specific protease, USP7 (also called HAUSP), which was previously shown to interact with ICP0 of herpes simplex virus. The EBNA1-USP7 interaction was verified by co-immunoprecipitation and co-purification of the two proteins from human cells and the EBNA1 residues that bind USP7 were mapped to amino acids 395 to 452. The effect of deleting the USP7-binding region of EBNA1 on all EBNA1 functions is currently being assessed. The interaction with USP7 suggests that EBNA1 might avoid targetting to the proteasome due to the removal of conjugated ubiquitin from EBNA1. In keeping with this model, we have shown that purified EBNA1 can be efficiently ubiquitinated *in vitro* and that this conjugated ubiquitin can be removed by purified USP7.

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ROLE OF INTEGRINS AND FOCAL ADHESION KINASE IN CELL TRANSFORMATION INDUCED BY LATENT MEMBRANE PROTEIN 1 OF EPSTEIN-BARR VIRUS

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Epstein-Barr virus (EBV) is a human herpesvirus associated with a number of malignancies including Burkitt's lymphoma and nasopharyneal carcinoma. The EBV encoded latent membrane protein 1 (LMP1) is of particular interest as it resembles a classical oncogene in its ability to induce growth transformation. To understand the mechanisms involved in LMP1-induced cell transformation, we have established a tetracycline inducible LMP1 expression system in the human embryonic 293 cell line. The induction of LMP1 expression confers transformed phenotypes to these cells, which allows them to 1) pile up and finally form foci in confluent condition, 2) to grow in soft agar, and 3) to form tumors in nude mice. Among the molecules examined, we found the expression of LMP1 leads to elevated levels of several integrins and activation of focal adhesion kinase and mitogen-activated protein kinases. The mechanisms underlying these events and their relationship with cell transformation are currently under investigation.

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Poster: 63

CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES TO THE BHRF1 PROTEINS OF EPSTEIN-BARR VIRUS

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A monoclonal antibody designated 3E8 was produced against an Epstein-Barr virus early lytic cycle protein BHRF1 which sequence and functional homology to the antiapoptotic Bcl-2. The antibody recognizes a 17kDa protein in cell extracts from EBV-infected cell lines following lytic cycle activation and in EBV-negative cell line transfected with a plasmid expressed the BHRF1 gene. Epitope mapping revealed the antibody located on amino acid residues 28-33 over amino terminal of BHRF1, different from that of mAb 5B11. Using 3E8 to trace the kinetic expression of EBV BHRF1 in EBV-infection Akata cells and Akata EBV-infection NPC cell lines (NA), the western blotting results indicated that BHRF1 could be detected at 4hr and 6hr postactivation, respectively. In addition, the mAb could be applied in the detection of BHRF1 antigen by immunofluorescence, immunoprecipitation, immunoblot, immunohistochemistry and ELISA. The 3E8 mAb provides a powerful tool to elucidate the biological function of BHRF1

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Poster: 64

LMP1 AND LMP2 EXPRESSION IN B-CELLS *IN VIVO* EXPLORED

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In order to explore potential interactions between LMP1, LMP2 and EBNA-1 in B-cells *in vivo*, we have cross-bred EμLMP1, EμLMP2 and EμEBNA-1 transgenic mice (Wilson *et. al.,* 1990. *Cell* 61: 1315-1327; Wilson *et al.,* 1996 *EMBO J.* 15:3117-3126; Caldwell *et. al.,* 1998 *Immunity* 9:405-411). No cooperation in the latency to lymphoma onset was found, conversely, tumourigenesis was delayed in the tritransgenic mice compared to EμEBNA-1 mice. This could be due to the reduced population size of B-cells as a component of the EμLMP2 phenotype. An alternative hypothesis is that the roles of the transgenes in this system are counteractive, perhaps LMP2 promoting B-cell differentiation and thereby reducing the chances of oncogenesis.

EμLMP1 and EμLMP2 mice were able to mount humoural responses to antigen comparable to wild type mice, but show differences in their steady state levels of serum immunoglobulin isotypes. B-cell surface marker expression has been extensively characterized in these mice.

B-cells from immunized EμLMP2 mice have been used in adoptive transfer experiments to CD40 null recipients. Our preliminary data indicate that LMP2 is able to prolong the survival of memory B-cells without abrogating function.

NOTES:

Poster: 65

ADOPTIVE THERAPY OF EPSTEIN-BARR VIRUS INDUCED POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE.

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The treatment of Epstein-Barr virus (EBV)-associated lymphoproliferative disease (PTLD) in solid organ transplant patients poses a considerable challenge due to underlying immunosuppression which inhibits the virus-specific cytotoxic T cell (CTL) response in vivo. We have developed a novel protocol for activating autologous EBV-specific CTL lines from PTLD patients and show their potential use in immunotherapy against PTLD in solid organ transplant patients. Adoptive transfer of autologous EBV specific CTLs into a heart transplant patient with active subcutaneous PTLD was coincident with a very significant regression of the PTLD. Six adoptive transfers were delivered over a 10 month period during which real time quantitative PCR and ELISPOT analysis were used to assess longitudinal dynamics of EBV load and the EBV specific CTL frequencies respectively during the course of adoptive immunotherapy and for a two year follow up period. The opportunity to monitor a lytic antigen epitope, GLCTLVAML and latent antigen epitopes PYLFWLAAI and IEDPPFNSL in this patient indicated that the emergence of the latent responses was coincident with significant regression of PTLD. Interestingly, the activity of the lytic antigen epitope tended to correlate with the EBV viral load pattern.

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Poster: 66

PLASMA MEMBRANE-ASSOCIATED LMP1 DETECTED BY ANTIBODIES TO THE PUTATIVE LMP1 EXTRACELLULAR DOMAINS

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LMP1 is a membrane-associated oncogenic protein with six membrane spanning helices connected by three putative extracellular domains (loops 1-3). EBV-transformed cells in vitro and EBV-positive tumor cells in vivo show considerable heterogeneity in LMP1 expression and the precise subcellular localization of LMP1 remains undefined.

We designed structurally constraint synthetic peptides mimicking the putative loops of LMP1 and succeeded to create loop-specific polyclonal antibodies. These loop-specific antibodies were further purified by peptide-affinity chromatography and analysed for LMP1 reactivity. The resulting antibodies all reacted with purified recombinant LMP1 and with LMP1 expressed in EBV type-1 and type-2 transformed cell lines. Loop 1 and 3 reactive antibodies showed the best staining. Binding to cell surface-exposed LMP1 on viable cells EBV-positive cells revealed a patch-like pattern and was detected by indirect immunofluorescence and FACS analysis in about 3-15% of the cells depending on the cell line used, despite strong intracellular expression of LMP1 in >90% of these cells as revealed by different LMP1 monoclonal antibodies. Using antibody-dependent complement-mediated killing a maximal ^{51}Cr release of 8-15% was achieved, despite >85% release in control anti-HLA (ß2M) experiments.

These data suggest that only a fraction of LMP1 is inserted into the plasma membrane and can form a target for cytolytic antibodies.

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Poster: 67

HUMAN MONOCLONAL ANTIBODIES REACTIVE WITH LMP1 EXTRACELLULAR DOMAINS.

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LMP1 is a membrane-associated oncogenic protein with six membrane spanning helices connected by three putative extracellular domains (loops 1-3). In a recent study we used circular constraint peptides mimicking LMP1 loops 1-3 to produce mono-specific antisera that were capable of killing EBV-transformed B-cells in vitro (see separate abstract). Similar antibodies of human origin may provide a new therapeutic approach against EBV-driven malignancies.

Using a human antibody phage library and selection with biotinylated peptides, but also by using selection on viable LMP1 positive JY cells, we were able to isolate LMP1-specific loop1-3 reactive phages as defined by a positive response in loop1-3 peptide ELISA. Phages reactive with linear and constraint circular loop-peptides were cloned and functional Fab fragments were produced as soluble protein in E.coli and analysed for LMP1 binding using immunoblot and immunofluorescence assays. FACS analysis revealed binding in 3-12% of viable LMP1 positive cells depending on the cell line used, similar as found with polyclonal antisera. More Fab's reacted with LMP1-loops 1 and 3 compared to loop 2, correlating with the larger size of the putative extracellular domains 1 and 3. Genetic analysis showed that distinct clones were capable of binding to the individual LMP1 loops.

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Poster: 68

EXPRESSION PATTERNS OF LMP1 IN INDIVIDUAL EBV-CARRYING CELLS IN VITRO AND IN VIVO

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Monoclonal antibodies reactive with the N- or C-terminus of LMP1 were used to study the expression of LMP1 protein in EBV-carrying cell lines and in tumor biopsies. A surprising heterogeneous expression was seen at the single cell level in all EBV+ cell lines, with some cells being nearly negative and others showing strong cytoplasmic staining, mostly in a granular patch-like pattern. Sub-cloning of cell lines and selection for cells growing in clumps or as single cells did not bring any difference. Surprisingly, synchronising cell lines in either G1/S or in G2/M did not influence the heterogeneous distribution of LMP1 among individual cells and overall protein levels remained similar. However, in G2/M cells a significant increase of LMP1 mRNA was found. Only in B95-8 cells, but not in human LCL's, the N-terminus was cleaved as signal sequence. Induction of EBV lytic cycle by TPA+Butyrate resulted in a strong boost of LMP1 expression, in some cells preceding expression of ZEBRA. Finally, the chemicals used for synchronisation all induced EBV lytic cycle expression, complicating analysis of LMP1 expression upon cell cycle release. In vivo a similar heterogeneous expression of LMP1 is found which may be a fundamental characteristic of this viral oncogene.

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Poster: 69

ASSOCIATION OF EBV-ENCODED EBNA-5 WITH THE NUCLEAR PML-CONTAINING ONCOGENIC DOMAIN (POD) AND ITS INVOLVEMENT IN PROTEASOMAL PROTEIN DEGRADATION

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EBNA-5 is required for transformation and establishment of LCLs. We have previously shown that EBNA-5 is localized to PODs (PML oncogenic domains) in different cell lines. The aim of this project is to investigate the relationship of EBNA-5 to the PODs. Several recent findings indicate that the PODs themselves are involved in proteasomal protein degradation. To study the role of EBNA-5 targeting to the PODs and in proteasomeal protein degradation, we analyzed the changes in sub-cellular localization and in the total EBNA-5 levels upon proteasome inhibitor treatment. We found that a proteasome inhibitor MG132 induced the translocation of EBNA-5 to the nucleoli. A coordinated change in EBNA-5 and Hsp70 localization and effect on mutant p53 distribution upon MG132 treatment may reflect an involvement of EBNA-5 in the regulation of intracellular protein trafficking associated with the proteasome mediated degradation. The tight association of PML protein and PODs with proteasome-mediated protein degradation prompted us to analyze the intracellular distribution of proteasomes, different POD components and non-POD associated proteins in the presence of a proteasome inhibitor. Inhibition of the proteasomes in different cell lines resulted in a radical redistribution of the POD associated proteins PML, Sp100 and SUMO-1 and proteasomes into the nucleoli.

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Poster: 70

EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN-1 IS HIGHLY COLOCALIZED WITH CHROMATIN 'PREMATURELY CONDENSED' DURING INTERPHASE AND ITS NEWLY REPLICATED REGIONS IN PARTICULAR.

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Epstein-Barr virus nuclear antigen 1 (EBNA-1), which binds to both the EBV replication origin region, *oriP*, and metaphase chromosomes, is essential for the replication/retention and segregation/partition of *oriP*-containing plasmids. Here we have examined the chromosomal localization of GFP-EBNA-1 fusion protein by confocal microscopy combined with a 'premature chromosome condensation' (PCC) procedure. Our analyses show that GFP-EBNA-1 expressed in living cells lacking *oriP*-plasmids is associated with cellular chromatin that has been condensed rapidly by the PCC procedure into identifiable forms that are unique to each phase of interphase as well as metaphase chromosomes. Studies of cellular chromosomal DNAs labeled with BrdU or Cy3-dUTP indicate that GFP-EBNA-1 colocalizes highly with the labeled regions of prematurely condensed interphase chromatin in cells. These results suggest that EBNA-1 is associated not only with cellular metaphase chromosomes but also with condensing chromatin and/or chromosomes and probably with interphase chromatin, especially with its newly replicated regions.

NOTES:

Poster: 71

OXIDATIVE STRESS INVOLVED IN LMP1 MEDIATED TRANSFORMATION AND TUMORIGENESIS OF NIH 3T3 FIBROBLAST

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The involvement of oxidative stress in LMP1 induced transformation and tumorigenesis was investigated. Epstein-Barr virus oncoprotein latent infection membrane protein 1 (LMP1) is a constitutively aggregated pseudo-tumor necrosis factor receptor, and mediates thereof carcinogenetic potential through activation of transcription factor NF-κB and AP-1. In consistent with previous reports, the transfection of LMP1 significantly potentiated the proliferation, decreased the serum dependence of proliferation, increased the coloney formation efficiency, activated NF-κB and AP-1 in NIH 3T3 mice fibroblast. At the same time, transfection of LMP1 enhanced the level of lipid peroxide and induced oxidative DNA damage. The treatment of ascorbic acid restored the LMP1 induced malignant proliferation of NIH 3T3 without any observable effects on NIH 3T3. Tumorigenesis of LPM1 stable transfected NIH 3T3 was inhibited by ascorbic acid treatment regarding to the incidence of tumor production and the weight of individual tumor. These results strongly implicated that oxidative stress is involved in LMP1 mediated transformation and tumorigenesis.

NOTES:

Poster: 72

IDENTIFICATION OF PROTEIN KINASE CK2 AS A POTENT KINASE OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1

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The C-terminus of latent membrane protein 1 (LMP1) can be phosphorylated *in vivo*. However, the protein kinase responsible for LMP1 phosphorylation has not yet been identified. In this study, GST fusion proteins containing the C-terminus of LMP1 were generated and used as substrates in an *in-vitro* kinase assay to survey the kinases that phosphorylate LMP1. Among several purified protein kinases tested, only protein kinase CK2 (CK2) could specifically phosphorylate LMP1. Using the in-gel kinase assay in the absence and presence of a selective CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB), CK2 was determined to be the major kinase to phosphorylate LMP1 in lymphoma and epithelial cell lines. This is the first study to show that CK2 is a potent kinase to phosphorylate LMP1 *in vitro*.

NOTES:

Poster: 73

EBNA-1 SEQUENCE VARIATIONS REFLECT ACTIVE REPLICATION IN DISEASE STATUS OR QUIESCENT LATENCY IN LYMPHOCYTES

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EBV infects most of population worldwide; however only a limit percentage of individuals develop malignancy. In addition to host susceptibility, variation of virus strains has been studied. Since EBNA-1 functions as oriP binding protein and links the episomal form viral DNA to metaphase chromosomes, the variation of EBNA-1 was suggested to contribute to the different tissue tropism of EBV and the development of different EBV associated diseases. However other studies concluded EBNA-1 sequence variation simply reflects the geographic distribution of EBV. To clarify these possibilities, we collected DNA samples from patients with different EBV associated diseases and normal individuals for PCR and DNA sequencing. Results of this study indicated that (I) V-val EBNA-1 was detected in NPC and other EBV associated malignant diseases. (II) Recombinant forms of different types of N-terminal and C-terminal sequences were observed. (III) Amino acid changes other than original five subtypes were observed. (IV) Comparison of the paired tissue and serum samples from NPC patients reveals the possibility of quasispecies or dual infection. (V) The prototype p-ala strain can only be detected in PBL. The conclusion of this study indicated a possibility of EBNA-1 sequence derived from quiescent latency or active replication may be different.

NOTES:

Poster: 74

THE EBNA2 POLYPROLINE REGION IS DISPENSABLE FOR EBV-MEDIATED IMMORTALIZATION MAINTENANCE.

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The EBV latent protein EBNA2 is required for EBV-mediated B cell immortalization and is a transcriptional activator of viral and cellular genes. The protein contains a highly unusual motif in which 43 of 45 residues are prolines (polyproline region; PPR). Previously, it has been shown that recombinant viruses containing a deletion of the PPR were immortalization defective. In this study, we tested whether ΔPPR-EBNA2 proteins were sufficient to support EBV-mediated immortalization maintenance using a recently developed transcomplementation assay (J. Virol. 75:5899-912, 2001). Surprisingly, we found that a ΔPPR-EBNA2 was able to support B cell proliferation similar to wild-type EBNA2. However, ΔPPR-EBNA2 expressing LCLs have significantly reduced EBV genomic levels compared to LCLs expressing wild-type EBNA2. The ΔPPR-EBNA2 also stimulated the latent membrane protein promoters several-fold better than wild-type EBNA2 in transient cotransfection assays. The data are consistent with a model that lower EBNA2 target gene dosage may be selected for in ΔPPR-EBNA2-dependent cell lines to compensate for hyperactive stimulation of viral genes such as LMP-1, which is cytostatic for B cells when over-expressed. It is conceivable that the hyperactivity, rather than the loss of function, could be responsible for the inability of recombinant ΔPPR-EBNA2 EBVs to immortalize B cells.

NOTES:

Poster: 75

METHYLATION PROFILING AND MUTATION ANALYSIS OF THE ED-L1 AND ED-L1E PROMOTERS FOR LMP1 AND LMP2B IN VARIOUS CARCINOMAS AND LYMPHOMAS

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LMP1 is an oncoprotein only expressed in latency II and III and involved in EBV tumorigenesis. Various immune and gene therapy strategies towards EBV-associated tumors try to target this protein. In nasopharyngeal carcinoma (NPC), LMP1 is expressed in about half of the cases. We have investigated the methylation profile and mutations of the ED-L1 and ED-L1E promoters for LMP1/LMP2B in various tumors (NPC, Hodgkin's disease, posttransplant lymphoma, Burkitt's lymphoma and gastric carcinoma) and correlated them with the transcription level. The ED-L1 promoter (-353 to +114) was directly sequenced by PCR. The previously reported variation (GA to CC, -42 to -43) resulting in reduced LMP1 expression was found associated with Asian NPC. These NPC tumors also showed a special Asian-type spectrum of other variations in ED-L1. Both ED-L1 and ED-L1E promoters were found to be hypomethylated in latency III cell lines, but methylated in Rael. Hypermethylation of both promoters were seen in some NPC tumors and C666-1, but not in C15 and C18 tumors. Both promoters were occasionally methylated in some lymphomas. Hypermethylation correlated with transcription silencing, except for Burkitt's lymphoma. Upregulation of ED-L1 transcripts with 5-aza-2'-deoxycytidine was more profound in lymphoma cell line (Rael), while upregulation of ED-L1E and LMP2B transcripts was greater in carcinoma cell line (C666-1) though significant demethylation of the ED-L1E promoter was also observed in Rael, implying that ED-L1E might be more epithelial cell-specific. In NPC patients treated with 5-azacytidine *in vivo*, demethylation of both the ED-L1 and ED-L1E promoters was detected.

NOTES:

Poster: 76

FUNCTIONAL p53 CHIMERAS CONTAINING THE EPSTEIN-BARR VIRUS GLY-ALA REPEAT ARE PROTECTED FROM MDM2 AND HPV-E6 INDUCED PROTEOLYSIS

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Functional inactivation of the tumor suppressor protein p53 by accelerated ubiquitin-proteasome-dependent proteolysis is a common event in tumor progression. Proteasomal degradation is inhibited by the Gly-Ala repeat (GAr) of the Epstein-Barr virus nuclear antigen (EBNA)-1, which acts as a transferable element on a variety of proteasomal substrates. We demonstrate that p53 chimeras containing GAr domains of different length and position within the protein are protected from proteolysis induced by the ubiquitin ligases Mdm2 and E6-AP. The chimeras are efficiently ubiquitinated and interact with the S5a ubiquitin-binding subunit of the proteasome but retain the capacity to transactivate p53 target genes and induce cell cycle arrest and apoptosis. The chimeras have improved growth inhibitory activity in tumor cells with impaired endogenous p53 activity, thus providing an attractive new tool for gene replacement therapy of a wide variety of human malignancies.

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Poster: 77

ANALYSIS OF POLYTOPE ISCOM® EBV VACCINES FOR THE INDUCTION OF CTL

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The transforming potential of EBV proteins such as EBNA1 raises serious safety concerns regarding their use as vaccine antigens. In the present study, polytope technology has been utilised in the development of an EBV vaccine for the prevention of Infectious Mononucleosis (IM). We have designed an EBV polytope containing 26 CTL restricted epitopes (predominantly covering latent antigens) which is predicted to cover 94% of the United States population. The aim of this study was to demonstrate the induction of $CD8^+$ T cell responses to individual epitopes following the administration of an EBV polytope formulated vaccine. In addition, we evaluated if the order of epitopes within the polytope influenced the immune response. Two 26 epitope polytopes, differing by only the epitope order were linked to the C-terminus of EBV gp350 and expressed in *E. coli*. The proteins were purified and formulated as ISCOMs®. ELISPOT analysis of $CD8^+$ T cells from HLA-A2 mice vaccinated with the formulations demonstrated IFN-γ production in response to all 5 A2 epitopes incorporated in the polytope. There was no significant difference in the magnitude of $CD8^+$ T cell responses between the 2 polytopes, and thus epitope order did not appear to affect the ability of individual epitopes to induce $CD8^+$ T cell responses. These findings confirm that an EBV polytope ISCOM® invokes a strong $CD8^+$ T cell response to the individual epitopes within the polytope, and would therefore be suitable as a human vaccine candidate for prevention of IM.

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Poster: 78

LOCALIZATION PATTERNS IN MUTANTS OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 2

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Latently infected B cells are characterized by the expression of mRNA for one viral protein, Latent Membrane Protein 2a (LMP2a). The LMP2 gene encodes two messages, LMP2a and LMP2b differing only by a 119 amino acid N-terminal cytoplasmic domain in LMP2a. They share 12 putative transmembrane segments (TMs), short loops connecting the segments, and a 27 amino acid C-terminal cytoplasmic domain. Lack of anti-sera against LMP2b has impeded investigation concerning its localization and relationship with LMP2a. In this study, LMP2 proteins were tagged with EGFP or DsRed and epitope tags in TM-connecting loops. Tagged LMP2b was seen to co-localize with LMP2a; both molecules localized to a perinuclear region of transiently transfected B cells. C- and N-terminal truncation mutants have been produced to determine the minimal protein structures needed for retention to this perinuclear compartment. By immunofluorescent microscopy, a change in cellular distribution was seen with some truncated LMP2 proteins. Proteins containing epitope tags in extra-membrane loops were used to confirm the surface localization and orientation of truncation mutants. Interestingly, when truncated mutants were co-expressed with full-length proteins, an intracellular sequestration of the mutants was observed. These data suggest that LMP2 proteins specifically aggregate with each other as well as localize to a perinuclear region within B cells.

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Poster: 79

AP-2 TRANSCRIPTION FACTORS CO-OPERATE WITH EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 2 (EBNA2) IN ALLEVIATION OF THE HISTONE DEACETYLASE MEDIATED REPRESSION OF THE LMP1 PROMOTER

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We have previously identified a silencer element in the LMP1 promoter proximal region that binds to the Max-Mad-Sin3A transcription factors. The LRS region between -106 and -67 was shown to participate in the EBNA2-induced alleviation of the silencing effect. Inhibition of deacetylase activity with TSA and deletion analysis, showed that histone deacetylation is the main cause of the repression in this region. Electrophoretic mobility shift assays (EMSAs) and supershift experiments identified an Ikaros element at position -67 to -59 which bound Ikaros proteins, suggesting a repressive function of this site in addition to the identified E-box element. The EBNA2 responsive element was localised at position -107 to -95 in LRS, which contains an AP-2 consensus sequence. EMSAs and supershift experiments showed that AP-2 like factors bound to the AP-2 consensus sequence, and co-transfection of plasmids expressing AP-2 transcription factors activated reporter plasmids containing the LMP1 promoter via this element. The level of activation increased when AP-2 and EBNA2 were co-transfected as compared with when the AP-2 factors were expressed individually. Mutations of this site abolished this co-operative activation. We conclude that AP-2 like factors participate in EBNA2-induced alleviation of the repression caused by histone deacetylation in the LMP1 promoter proximal region.

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Poster: 80

HISTONE DEACETYLASE INHIBITOR TRICHOSTATIN A INDUCES A CELL CYCLE ARREST AND APOPTOSIS IN EBV-TRANSFORMED B-LYMPHOBLASTOIDS CELLS

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Effects of trichostatin A (TSA), a reversible histone deacetylase inhibitor, on proliferation of EBV-transformed B cells and BJAB lymphoma cells were studied. Growth of LCL's infected with either type 1 or type 2 EBV as well as BJAB cells were significantly inhibited in the presence of 100 nM or more TSA. The growth inhibition by TSA was due to the cell cycle arrest at G1 phase and apoptotic cell death as evidenced by flow cytometric analysis, nuclear morphological changes, DNA fragmentation, and cleavage of PARF. RNase protection analyses showed that in LCL's mRNA levels for cyclins D2, A, and B decreased but $p21^{WAF1}$ and cyclin D3 mRNAs did not change much 24 hours after treatment, whereas in BJAB cells $p21^{WAF1}$ mRNA significantly increased, cyclin D1 mRNA decreased and mRNA levels for cyclins D3, A, and B unchanged. mRNA levels of cdk 2 and other cdk inhibitors such as p16, p18, and p27 decreased or showed little changes by TSA treatment in both LCL and BJAB. Northern blotting indicated that in LCL p21 mRNA was also induced by TSA with a peak at 8 hr after TSA treatment but thereafter decreased rapidly down to the basal level at 24 hr. These results indicated that TSA caused the G1 arrest in LCL and BJAB in a different manner, mainly by decreasing expression of G1 cyclins but by up-regulating $p21^{WAF1}$, respectively. TSA had little effects on expression of EBNA-1, -2, LMP1, Bcl2, and A20. Taken together, these results suggest that TSA induces the G1 arrest and apoptosis in LCL without altering the EBV latency program.

NOTES:

Poster: 81

A CRITICAL ROLE FOR TRAF1 IN LMP1-MEDIATED JNK SIGNALLING

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The oncogenic Epstein-Barr virus (EBV)-encoded latent infection membrane protein 1 (LMP1) mimics a constitutive active tumour necrosis factor (TNF) family receptor in its ability to recruit TNF receptor-associated factors (TRAFs) and TNF receptor-associated death domain protein (TRADD) in a ligand-independent manner. As a result, LMP1 constitutively engages signalling pathways, such as the JNK and p38 mitogen-activated protein kinases (MAPK), the transcription factor NF-kB and the JAK/STAT cascade and these activities may explain many of its pleiotropic effects on cell phenotype, growth and transformation[1]. In this study we demonstrate the ability of the TRAF-binding domain of LMP1 to signal on the JNK/AP-1 axis in a cell-type dependent manner that critically involves TRAF1 and TRAF2. Thus, expression of this LMP1 domain in TRAF1-positive cells promotes robust JNK activation which is blocked by dominant-negative TRAF2. However, in cell lines lacking detectable TRAF1, JNK activation by the TRAF-binding domain of LMP1 requires reconstitution of TRAF1 expression. This critical role of TRAF1 in TRAF2-dependent JNK signalling is particular to LMP1, as the TRAF-binding region of CD40 signals on the JNK axis independently of TRAF1 status. These data further dissect the signalling components utilised by LMP1 and highlight the critical role of TRAF1 in the modulation of oncogenic signals engaged by this viral protein. [Supported by the Medical Research Council UK and Cancer Research UK]

[1]AG Eliopoulos and LS Young. *Sem. Cancer Biol*. 11:435, 2001.

NOTES:

Poster: 82

INHIBITION OF P38 MAPK PATHWAY DECREASES THE EXPRESSION OF LMP1 AFTER EBNA2 INDUCTION IN RESTING ER/EB2-5 CELLS

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We have previously shown that an ATF/CRE element at position –44 to –38 in the LMP1 regulatory sequence is essential for EBNA2 induced activation of the promoter. Two different heterodimeric transcription factors bind to this element, ATF-2/c-Jun and CREB-1/ATF-1.

We have now examined the phosphorylation levels of ATF-2 (Thr71), c-Jun (Ser73), and CREB-1 (Ser133) in resting ER/EB2-5-cells and after induction of EBNA2 with β-estradiol. Potential signaling pathways used for LMP1 expression was investigated by addition of Ser/Thr-kinase inhibitors to resting ER/EB2-5 cells followed by induction of EBNA2 with β-estradiol.

Our results showed that the phosphorylation level of ATF2 was low in resting ER/EB2-5 cells but increased 2h after induction with β-estradiol and remained high in proliferating ER/EB2-5 cells. The phosphorylation level of CREB-1 was similar in resting and proliferating ER/EB2-5 cells but increased temporarily after both withdrawal and addition of β-estradiol. Staurosporin, a broad range inhibitor of Ser/Thr kinases, and two specific inhibitors of the p38 MAPK pathway, SB203580 and SB202190, decreased the EBNA2 mediated activation of LMP1 substantially. Inhibitors of PKC (bisindolylmaleimide), PKA (H89), CaM Kinase (KN-93) and MEK1 (PD098059) did not considerably reduce the EBNA2 induced LMP1 expression.

NOTES:

Poster: 83

LMP1 ENHANCES CELL SURVIVAL OF EBV-TRANSFORMED LYMPHOBLASTOID CELLS INDEPENDENTLY OF NFκB SIGNALS

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We have used a dominant inhibitory mutant of LMP1 (dnLMP1), which inhibits signalling from the C-terminal cytosolic domain of wild-type LMP1, to examine the contribution of LMP1 to the maintenance of EBV-mediated transformation of B-cells. The dnLMP1 gene was cloned into a tetracycline regulated vector and stable transfectants established with IB4, an EBV-transformed lymphoblastoid cell line. Tetracycline efficiently repressed expression of dnLMP1, and removal of tetracycline induced high levels of expression. We have observed an inhibition of the co-immunoprecipitation of TRAFs with LMP1 from lysates of IB4 LCL expressing dnLMP1. However, no effect on NFκB DNA binding was observed, suggesting that LMP1 may be redundant for maintaining NFκB activation in EBV-transformed lymphoblastoid cells. A small (15-35%) but significant reduction was observed in expression of CD40 and CD54 which are known to be regulated by LMP1, but expression of Bcl-2 was unaffected. In contrast, expression of dnLMP1 had a dramatic effect on the response to apoptotic stimuli. Treatment with 20μM LY294002, an inhibitor of PI3-kinase, caused 50% cell death when dnLMP1 was induced compared to 10% death in cultures maintained in tetracycline. This striking effect of dnLMP1 suggests that LMP1 can enhance cell survival through mechanisms independent of NFκB and Bcl-2.

This work was funded by the Leukaemia Research Fund, London, and by the Medical Research Council.

NOTES:

Poster: 84

THE EPSTEIN-BARR VIRUS ONCOGENE PRODUCT, LATENT MEMBRANE PROTEIN 1, INDUCES THE DOWN-REGULATION OF E-CADHERIN GENE EXPRESSION VIA ACTIVATION OF DNA METHYLTRANSFERASES

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The latent membrane protein (LMP1) of Epstein-Barr virus (EBV) is expressed in EBV-associated nasopharyngeal carcinoma (NPC), which is notoriously metastatic. While it is established that LMP1 represses E-cadherin expression and enhances the invasive ability of carcinoma cells, the mechanism underlying this repression remains to be elucidated. In this study, we demonstrate that LMP1 induces the expression and activity of the methyltransferases 1, 3a and 3b, using real-time RT-PCR. This results in hypermethylation of the E-cadherin promoter and down-regulation of E-cadherin gene expression, as revealed by methylation PCR, real-time RT-PCR and Western blotting

data. The DNA methyltransferase inhibitor, 5'-Aza-2'dC, restores E-cadherin expression in LMP1-expressing cells and cells infected with a LMP1-recombinant adenovirus, which in turn blocks cell migration ability, as demonstrated by the Transwell cell migration assay. Our findings suggest that LMP1 down-regulates E-cadherin gene expression and induces cell migration activity by using cellular DNA methylation machinery.

NOTES:

Poster: 85

MUTATIONAL ANALYSIS OF BHRF-1

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BHRF-1 is an EBV early lytic cycle antigen that shares structural and functional homology with the cellular anti-apoptotic protein Bcl-2. We have isolated BHRF-1 homologues from simian γ-1 lymphocryptoviruses endemic to *Pan sp*. (chimpanzee) *Papio sp*. (baboon) (Williams *et al*., 2001) and *Gorilla sp*. In addition to conservation of the Bcl-2 family homology domains (BH1,2 and 3), extensive amino acid conservation with EBV BHRF-1 was found throughout the entire open reading frames (82% *H.pan* and 65% *H.papio*). In order to test the functional significance of domains of absolute conservation outside of the Bcl-2 family homology motifs, we are currently producing BHRF-1 deletion mutants which lack these regions of identity (amino acids 2-16; 25-35; 106-119; 128-143; 172-188). The functional effect following expression of the mutant BHRF-1s in a lymphoid cell background *in vitro* in response to apoptotic stimuli will be assayed. The mutants' ability to delay the terminal differentiation and modulate the proliferation of epithelial cell lines *in vitro* will also be investigated.

NOTES:

Poster: 86

A NOVEL DOMINANT NEGATIVE MUTANT OF THE EBV LATENT MEMBRANE PROTEIN 1 (LMP1) AFFECTS TRAF- AND TRADD-DEPENDENT TRANSDUCTION PATHWAYS

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LMP1 has been described as a major oncogene of EBV. This integral membrane protein is functionally related to the tumor necrosis factor (TNFa) receptor family. The main signal transduction pathways triggered by this protein are NFkB and JNK/AP1. The two LMP1 domains responsible of these activations act through recruitment of adaptors as Traf2 (TNF-receptor associated factor 2) and TRADD (TNF-receptor associated death domain) and are localized in the cytoplasmic C-terminal region (CT).

In order to study the role of LMP1 in cellular transformation, we have generated a dominant-negative form of this protein composed of the sole C-terminal region fused to Green Fluorescent Protein (GFP). Using transfection experiments in HEK293 cells, we show that this protein termed GFP-CT is not only unable to activate appropriate signaling pathways, but also inhibits signaling from wild type LMP1. By contrast with other classical LMP1 mutants, GFP-CT is unable to form an inactive complex with wild type LMP1 at the membrane. GFP-CT rather depletes membrane wild type LMP1 complexes from signaling outputs by trapping Traf2 and TRADD in the cytoplasm, as shown by confocal microscopy and co-immunoprecipitation techniques. Our preliminary data indicate that this property could confer to GFP-CT a dominant-negative function towards other members of TNF-receptor family using TRAF2 and/or TRADD as adaptors.

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MONOCLONAL ANTIBODIES AGAINST MUTATED LMP1-PROTEIN

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The latent membrane protein 1 (LMP1) of Epstein-Barr virus shows a number of mutations and deletions in the C terminal region and it has been proposed that these changes affects its signalling properties. Currently available antibodies are unable to distinguish between the wild-type and mutated LMP1s. In an attempt to solve this problem we created a pATH-fusion protein from the Exon 3 region of "CAO"-LMP1 featuring a 33-bp deletion and produced a number of mAbs. 3 clones were initially identified that were able to distinguish between the CAO-deleted LMP1 and the prototype B95-8 in formalin-fixed, normal biopsy tissue as well as in Western blotting and immunofluorescence. When biopsy samples of Hodgkin Disease, PTLD and Nasal T-NK lymphoma were studied and the LMP1 DNA was sequenced, we found that the antibodies were reliably able to differentiate between the CAO LMP1 and the wild-type B95-8, however 1 case of Mediterranean LMP1 (Sandvej et al., 1997, Blood 90:323) were also stained with the antibodies. Double labelling with FITC-labelled CS1-4 antibodies with the Cy-3-labelled mix of the CAO-antibodies showed complete co-localization of the signals. Whilst no antibodies specific to the epitope created by the CAO-deletion were generated, the new clones allow a reliable determination of LMP1 type in archival tissue sections and protein extracts.

Supported by a joint grant from the Deutscher Akademischer Austauschdienst (DAAD)(to F.G.) and the Hong Kong research council (to J.N.).

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Poster: 88

LMP1 INDUCES ACTIN STRESS FIBRE FORMATION AND INFLUENCES THE ADHESIVE AND MIGRATORY PROPERTIES OF EPITHELIAL CELLS

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The small Rho GTPases are key modulators of actin filiament organisation. In this study we show that LMP1 induces actin stress fibre formation in Swiss 3T3 fibroblasts following microinjection of LMP1 expression vectors. This induction is mediated through the small Rho GTPases, as co-microinjection of LMP1 with dominant negative forms of Rho, Rac and CDC42 inhibited LMP1-mediated actin stress fibre formation to varying degrees. Microinjection of expression vectors encoding mutant LMP1 proteins, identified CTAR1 as the domain of LMP1 responsible for actin stress fibre formation. We also show that LMP1 affects both the adhesive and migratory properties of epithelial cells, as Boyden Chamber analysis and wound-healing experiments indicated accelerated rates of migration and invasion in SCC12F cells and MDCK cells stably expressing LMP1. These data are in accordance with findings that NPC tumours that express LMP1 possess increased metastatic potential.

NOTES:

Poster: 89

FUNDAMENTAL DIFFERENCES BETWEEN EBV-INFECTED AND CD40 LIGAND/IL-4 TREATED HUMAN B CELLS

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Human B-lymphocytes isolated from peripheral blood were activated and induced to proliferate by either EBV or CD40 Ligand (CD40L) plus IL-4. Although both populations proliferated as B-blasts following both viral infection and the more physiological stimulation, significant differences were revealed. The EBV infected cells initially showed a biphasic pattern of DNA synthesis that was sensitive to TGF-β but the CD40L/IL4 driven cells showed no biphasic response and were refractory to TGF-β. EBV infection resulted in continuously proliferating lymphoblastoid cell lines, whereas CD40L/IL4 stimulated B-lymphocytes had a finite proliferative lifespan. These cells did not die but appeared to exit from the cell cycle. This occurred concomitantly with an increase in the cdki $p21^{WAF1}$ and Cyclin D2. This increase did not occur after infection with EBV**.** Both populations secreted immunoglobulins, but whereas EBV infected cells retained B-lymphocyte surface marker expression, cells treated with CD40L/IL4 down-regulated all B-lymphocyte surface markers analysed, with the exception of CD38. These CD38-expressing cells show an increase in the levels of cytoplasmic Ig and underwent morphological changes consistent with plasma cell differentiation. The B cells stimulated with CD40L/IL4 secreted much higher levels of IL-6 than EBV-infected cells and this may contribute to the differential response.

NOTES:

Poster: 90

INHIBITION OF EPSTEIN BARR VIRUS LMP2A SIGNALING

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Epstein-Barr virus (EBV) is a human herpesvirus that establishes a lifelong latent infection in the majority of the human population. The virus resides in a latent state in B lymphocytes and is associated with a variety of cancers. In normal individuals, EBV latent infection poses no health risk, but upon immunosuppression, following organ transplantation or HIV infection, malignancies and lymphoproliferative diseases can result. Latent membrane protein 2A (LMP2A) is a virally encoded protein that is expressed in EBV latent infection and in most of the EBV-associated cancers. Previous studies have indicated that LMP2A expression alters the activity of the Src family protein tyrosine kinases (PTK), the Syk PTK, the Btk PTK, and phosphatidylinositol 3-kinase. In this study, inhibitors of each of these kinases were tested using an *in vitro* system dependent on LMP2A expression for B cell colony formation. Of the inhibitors tested, only piceatannol, a Syk PTK inhibitor, demonstrated a specific effect on LMP2A expressing cells and not control cells. These studies provide a basis for targeting LMP2A function in EBV latency and may allow for the identification of novel therapeutics for the treatment or eradication of EBV latent infections and associated cancers.

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Poster: 91

INCREASED HISTONE ACETYLATION IS REQUIRED FOR TRANSCRIPTIONAL ACTIVATION OF THE EPSTEIN-BARR VIRUS LMP1 PROMOTER; DIFFERENCES IN LMP1 PROMOTER ACTIVATION BETWEEN RESTING AND PROLIFERATING B LYMPHOCYTES

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The EBV nuclear antigen 2 (EBNA2) is a transcriptional activator of the Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) gene. In this study we show that EBNA2 and the histone deacetylase inhibitor Trichostatin A (TSA) respectively increase the level of acetylation of histone H3 and H4 at the LMP1 promoter in the oestrogen-regulated EBNA2 cell line ER/EB2-5. However, only EBNA2 was able to activate transcription from the LMP1 ED-L1 promoter in resting B-cells. This was in contrast to the situation in the proliferating EBV positive but EBNA2 deficient B-cell line P3HR1 where inhibition of histone deacetylation by TSA induced activation of the LMP1 promoter. Mutation of an ATF/CRE element in the proximal part of the LMP1 regulatory sequence severely impaired the responsiveness for TSA induced activation. These results show that increased histone acetylation is required for transcriptional activation of the Epstein-Barr virus LMP1 promoter and that an intact ATF/CRE element is important for this process but also that there are important differences in LMP1 promoter activation between resting and proliferating B lymphocytes.

NOTES:

Poster: 92

ASSESSMENT OF EPSTEIN-BARR VIRUS DNA LOAD IN WHOLE BLOOD WITH AUTOMATED EXTRACTION COUPLED TO REAL-TIME PCR DURING EBV-ASSOCIATED DISEASES

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The methods to assess EBV DNA load in blood often require a prior blood separation. Here, we compared the EBV DNA load monitoring in unfractionated whole blood (WB) after an automated extraction (MagNAPure® extractor) followed by a real-time quantitative PCR (LightCycler®) to a strategy coupling manual DNA extraction and real time PCR in peripheral blood mononuclear cells (PBMCs) and plasma (P) after blood separation. EBV DNA load was assessed in 100 patients with either EBV-related diseases (n=20) or HIV-infection without EBV associated disease (n= 66) and healthy immunocompetent EBV carriers (n=14). 68/100 samples were positive in both WB (17 to 271,000 copies/µg DNA) and PBMC (12 to 124,000 copies/µg DNA) with a good correlation for high as well as low copy numbers ($r= 0.93$; $p<0.0001$). Only 7 plasma samples were positive for EBV (250 to 135,700 copies/ml). The highest levels of EBV DNA in WB, PBMC or plasma were observed during EBV-associated post transplantation lymphoproliferative diseases and in 4 serious EBV primary infections. Automated nucleic acid extraction from WB followed by real time quantitative PCR appear as a useful method for EBV DNA measurement. Value of EBV DNA monitoring during serious EBV-primary infection will also be discussed.

NOTES:

Poster: 93

A SYNERGY BETWEEN TWO REGIONS OF EBV LATENT MEMBRANE PROTEIN 1 (LMP-1) IS REQUIRED FOR THE INDUCTION OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1 (STAT-1)

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Epstein-Barr virus (EBV) latent membrane protein 1 (LMP-1) is required for EBV transformation of primary B cells in vitro. Signal transducers and activators of transcription (STATs) play a pivotal role in the initiation and maintenance of certain cancers. STAT proteins, especially Stat1/3/5, are persistently tyrosine phosphorylated or activated in many cancers. We have discovered that type III latency cells, in which LMP-1 is present, expressed high levels of four STATs (STAT1/2/3/5A) and LMP-1 was responsible for the induction of at least three STATs (STAT1/2/3). The most abundant STAT member induced was apparently STAT-1. In addition, C-terminal activator region 1 (CTAR-1) and CTAR-2 of LMP-1 synergistically induced the expression of STAT-1. The synergy was only present when CTAR-1 and CTAR-2 were in the same molecule in *cis*, but was not present when the two were in different molecules in *trans*. Most of the induced STAT-1 was not phosphorylated at the tyrosine residue 701, the critical one activated by cytokines. Finally, the induced STAT-1 was functional because it could be activated by IFN, and an IFN-inducible gene was upregulated. The high expression of STATs in type III latency may be a part of the viral programming that leads to cellular transformation.

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Poster: 94

THE EBV LATENT MEMBRANE PROTEIN 1 INDUCES IFN-γ INDUCIBLE PROTEIN-10 IN LYMPHOMA CELLS

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EBV is detectable in a number of malignancies including Burkitt lymphoma (BL), Hodgkin lymphoma (HL) and Nasopharyngeal carcinomas (NPC). EBV infection is associated with activation of cellular signaling pathways leading to phenotypic changes of the infected cells. Growth and survival of EBV-infected cells is regulated by secretion of immunomodulatory cytokines and chemokines. Here we demonstrate that the transforming latent membrane protein 1 (LMP1) of EBV is necessary and sufficient for IFN-γ-inducible protein-10 (IP-10) induction in lymphoma cells of different origin. Deletion or mutation of the LMP1 C-terminal activation region (CTAR) 1 abolishes IP-10 protein expression, indicating that this domain is involved in IP-10 induction. CTAR2 deficient mutants with amino acid substitutions disturbing the CTAR2 show a better IP-10 induction capacity than the variants with a deletion directly behind the CTAR1. Signaling pathways involved in IP-10 gene regulation by EBV will be discussed. Our results further supports our previous hypothesis, that a defined structure of C-terminus is required for activation of some cellular pathways activated by LMP1.

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C-MYC OVEREXPRESSION ACTIVATES ALTERNATIVE PATHWAYS FOR INTRACELLULAR PROTEOLYSIS IN LYMPHOMA CELLS

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Burkitt's lymphoma (BL) is a highly malignant B-cell tumor characterised by chromosomal translocations that constitutively activate the *c-myc* oncogene. Here we show that BL cells are resistant to apoptosis and do not accumulate ubiquitin-conjugates in response to otherwise toxic doses of inhibitors of the proteasome. Deubiquitinating enzymes and the cytosolic subtilisin-like protease tripeptidylpeptidase II (TPPII) are upregulated in BLs and were rapidly induced by overexpression of *c-myc* in normal B cells carrying estrogen-driven recombinant Epstein-Barr virus (EBV). Apoptosis was induced by inhibition of TPPII, suggesting that the activity of this protease may be required for BL cells survival. We thus demonstrate, for the first time, a regulatory link between *c-myc* activation and changes in proteolysis that may affect malignant transformation.

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Poster: 96

INHIBITION OF GENE EXPRESSION BY LMP1 IS MEDIATED THROUGH INDUCTION OF PHOSPHORYLATION OF EIF2-α.

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LMP inhibits gene expression and cell proliferation when expressed at high physiological levels. Neither the mechanism nor the biological significance of this inhibition is understood. Given that LMP1 inhibits protein synthesis, we investigated whether phosphorylation of EIF2-α is induced when LMP1 is expressed at high physiological levels. In BJAB cells, the induction of LMP1 or its derivative lacking carboxy-cytoplasmic domain increased phosphorylation of EIF2-α by more than 10-fold. In clones of EBV-positive cells before and after immortalization, cells that expressed higher levels of LMP1 had higher levels of EIF2-α phosphorylated. The levels of LMP1 per cell in these clones varied by more than 100-fold while the levels of phosphorylated EIF2-α varied by 7.5-fold from the 5% of the cells with the lowest level of LMP1 to 5% with the highest levels of LMP1. Cells expressing high levels of LMP1 incorporated less BrdU than those expressing low levels, indicating that high levels of LMP1 did inhibit cell proliferation. These findings indicated that LMP1 in clones of exponentially growing cells limited protein synthesis and proliferation in those cells expressing it at high levels by inducing phosphorylation of EIF2-α.

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Poster: 97

EPIGENETIC INACTIVATION OF THE TUMOR SUPPRESSOR GENE *ARF* IN NASOPHARYNGEAL CARCINOMA (NPC) AND HODGKIN'S DISEASE (HD)

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Epigenetic inactivation of tumor suppressor genes by promoter hypermethylation is frequently involved in tumorigenesis. *ARF* is a tumor suppressor gene involved in p53 signaling pathway. Its product p14 targets MDM2 to interfere with p53 degradation. An inverse correlation between the expression of p14 and p53 has been reported in cell lines. We studied the epigenetic inactivation of this gene in EBV-associated tumors NPC and HD where p53 accumulation is common but with few mutations. Hypermethylation of the *ARF* promoter was detected in 8/18 (44%) of NPC and 22/37 (59.5%) of HD cases by methylation-specific PCR, but only in 2/10 Burkitt's lymphoma and not in any normal samples. This hypermethylation was confirmed by bisulfite genomic sequencing. Hypermethylation correlated with the down-regulation of *ARF*. 5-azacytidine treatment demethylated the *ARF* promoter and restored its expression in cell lines. Moreover, no mutation in the coding exons of p14 was detected in any tumor. Thus epigenetic inactivation of the *ARF* gene is common and the sole abnormality of this gene in HD and NPC, suggesting that it might be involved in their oncogenesis.

NOTES:

Poster: 98

REGULATION OF GAMMAHERPESVIRUS LATENCY BY NF-κB

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Gammaherpesviruses encode a conserved immediate-early transactivator Rta which activates viral lytic genes, leading to reactivation. Rta can auto-stimulate its own expression. We hypothesised that mechanisms exist to inhibit the function of Rta in lymphocytes, where gammaherpesviruses establish and maintain latency. We found that the cellular transcription factor NF-κB, which is highly active in lymphocytes, plays a key role in this process. In epithelial cells which are permissive for virus replication and where endogenous NF-κB activity is low, overexpression of NF-κB(p65 subunit) inhibits the Rta-mediated activation of KSHV, EBV and MHV-68 lytic promoters. In addition, overexpression of p65 inhibits the replication of MHV-68 in these cells. The inhibitory activity of p65 in these assays maps to its C terminal activation domain. In lymphocytes, which are non-permissive for virus replication, the inhibition of NF-κB results in reactivation of both latent KSHV and EBV. Therefore, we propose that Rta functions as a molecular switch and NF-κB sets the threshold level for the switch that regulates the balance between latency and lytic replication of these gammaherpesviruses. The study also suggests NF-κB as a potential drug target for the disruption of viral latency, a strategy to destroy infected tumour cells in the presence of acyclovir.

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Poster: 99

EFFECT OF TRANSFORMING GROWTH FACTOR-β1(TGF-β1) ON THE CELL GROWTH IN EBV-INFECTED EPITHELIAL CELL LINES: THE LATENT MEMBRANE PROTRIN-1 (LMP-1) BLOCKS TGF-β1-MEDIATED MAPK/p21 SIGNALING

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Transforming growth factor (TGF)-β1 plays important roles in regulating cell growth. We found that gastric tissue-derived EBV-infected epithelial cell lines GT38 and GT39 had resistance to TGF-β1-mediated growth inhibition compare to a TGF-β1-susceptible gastric carcinoma cell line HSC -39, although TGF-β1 induced EBV reactivation in GT38 and GT39 cells. The both cell lines produced the TGF-β1 which was sufficient for inhibiting cell growth of HSC -39 cells. These results suggested abnormalities on TGF-β1-mediated signaling pathways for growth inhibition but not for EBV reactivation in GT38 and GT39 cells. We analyzed the abnormalities on TGF-β/MAPK/p21 pathway in the cells. TGF-β1 activated MAPK (ERK 1/2) and p21 in HSC-39 but not in GT38 cells. GT38 cells had constitutively a higher level of ERK 1/2 phosphorylation and p21 expression than those of HSC-39 cells. U0126, a specific inhibitor of MEK, suppressed TGF-β1-mediated ERK 1/2 phosphorylation and p21 expression in HSC -39 cells and the constitutive phosphorylation of ERK 1/2 in GT38 cells. LMP1 induced constitutive phosphorylation of ERK 1/2 in the LMP1 transfected HSC-39 cells, and the cells became resistant to TGF-β1-mediated growth inhibition and did not any longer respond to the TGF-β1-mediated phosphorylation of ERK 1/2 and the induction of p21. These results are consistent with a conclusion that the growth inhibition by TGF -β1 is mediated via TGF-β/MAPK/p21 pathway and the resistance to TGF-β1 on GT38 cells is due to blocking of the pathway by the constitutive phosphorylation of MAPK induced by LMP1.

NOTES:

Poster: 100

HUMAN 2B4 (CD244) MEDIATES SIGNAL FOR CYTOKINE PRODUCTION AND VIRUS REPLICATION IN EBV-POSITIVE NATURAL KILLER CELLS

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Activation of human 2B4 (h2B4, CD244) molecule induces an association of SAP with cytoplasmic region following the augmentation of cytotoxicity in natural killer (NK) cells. The loss of signal transduction with SAP gene mutation has been reported in NK cells from patients with X-linked lymphoproliferative disease. Cytokine productions and replication of EBV by h2B4 stimulation was examined in newly established EBV-positive three NK cell lines (NK-TS, NK-YM, NK-TE) and NK-like cell line, YTN10. Three NK cell lines expressed h2B4, CD2, CD56, CD94, perforin, granzyme B and TIA-1 and retained cytotoxicity against NK-sensitive tumor cell. They expressed EBV-related mRNA (EBER1, LMP-1, -2A, BZLF-1 and vIL-10) and showed no mutation in SAP gene. Cross-linking of h2B4 by immortalized C1.7 antibody did not induced proliferation and CD69 expression, but augmentated TNF-α, IFN-γ, soluble Fas ligand, and granzyme B releases in different manner in each cell lines. Augmentation of phosphorylation of 2B4 was detected by pervanadate treatment . Cell free EBV-DNA was detected in the culture supernatant from all cell lines and an increase of cell free EBV by h2B4 stimulation was found in NK-YM. These results indicated that cell activation through 2B4 antigen mediates the pathogenesis of EBV-positive NK lymphoproliferative diseases.

NOTES:

Poster: 101

SIGNALING ROLE OF EPSTEIN-BARR VIRUS ENCODED LATENT MEMBRANE PROTEIN 1 IN NASOPHARYNGEAL CARCINOMA AND THE MECHANISMS INVOLVED

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Our previous data indicated that EBV-LMP1 activated NF-κB signaling pathway in nasopharyngeal carcinoma (NPC) cells. P^{65} and P^{50} are the major components responsible for this NF-κB activity. To further elucidate the mechanism of LMP1, a tetracycline-regulated LMP1-expressing NPC cell line, Tet-on-LMP1-HNE2 is established. Results show that IκBα rather than IκBβ is phosphorylated and degraded after inducible expression of LMP1. The IκBα can completely inhibit both translocation and transactivation of NF-κB induced by LMP1.

Using this cell line, we give clear evidence that LMP1 can increase activities of not only NF-κB but also AP-1. Transferring JNK-interacting protein (JIP), c-jun negative mutant (TAM67) can break AP-1 signaling pathway. These abnormal activities are related with the malignant phenotye in NPC. Further data indicate that cross-talk between NF-κB and AP-1 contributes to the carcinogenesis of NPC.

Cyclin D1 is a target protein in these activated signaling pathways. We show here cyclin D1 induction appears to be mediated by both NF-κB and AP-1. LMP1 deletion mutants lacking either C-terminal activation regions 1(CTAR1) or CTAR2 have a decreased ability to induced cyclin D1 expression. The deletion of both active regions completely abolishes the induction of cyclinD1 expression. This novel finding may thus represents a mechanism of action by which LMP1 activate signaling pathway involved cell cycle abnormally.

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Poster: 102

EPSTEIN-BARR VIRUS BZLF 1 GENE IS ACTIVATED BY TRANSFORMING GROWTH FACTOR-β THROUGH COOPERATIVITY OF SMADS AND C-JUN / C-FOS PROTEINS

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The expression of BZLF1 gene is initiated from promoter Zp, which is normally suppressed in EBV-transformed B cells. The BZLF1 gene can be induced for expression by activating agents, such as transforming growth factor-β (TGF-β) and 12-*o*-tetradecanoylphorbol-13-acetate (TPA). The TPA responsive element located in the Zp is the AP-1 motif. The TGF-β responsive element, however, has not been determined. Here, we demonstrated that the Smad4 Binding Element (SBE) site, GTCTG, from -233 to -229, was located in the regulatory region of the Zp relative to the BZLF1 transcription initiation site and physically associated with Smad4. This association is important for the TGF-β induction of Zp. We also showed here that from the result of co-transfection experiments, both the AP-1 motif and SBE site appeared to be required for the TGF-β-induced activation of Zp. This effect was mediated through the cooperation of Smad3/Smad4 and c-Jun/c-Fos that formed a complex. Those data showed that BZLF1 gene was activated by TGF-β through cooperativity of smads and c-Jun/c-Fos proteins.

NOTES:

Poster: 103

EVOLUTION OF EBV-SPECIFIC CD8 T CELL RESPONSES FROM PRIMARY TO MEMORY PHASE

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EBV allows one to follow a virus-induced human CD8+ T cell response from primary infection, as seen in infectious mononucleosis (IM) patients, through to a persistent carrier state. Using HLA class I/peptide tetramers to identify responder cells in blood, we find (i) primary and memory populations can be different with respect to both their range of detectable reactivities and the ratio of one reactivity to another, (ii) responses to lytic and latent cycle epitopes often show different kinetics, with lytic responses reaching high peaks in acute IM then falling, whereas latent responses can be relatively delayed and are less dramatically culled, and (iii) lytic and latent memory populations tend to be distinct in their expression of differentiation markers such as the CD45 isoforms and migration markers such as CCR7, distinctions which seem to hold irrespective of the absolute size of each epitope-specific response. We are currently studying whether the above kinetic differences reflect available levels of lytic versus latent antigen load in vivo, and to what extent the representation of epitope-specific reactivities in peripheral blood mirrors that existing in lymphoid tissues where the majority of EBV-infected target cells are likely to be situated.

NOTES:

Poster: 104

LACK OF EBV-SPECIFIC EFFECTOR CELL DIFFERENTIATION IN HIV+ INDIVIDUALS DEVELOPING AIDS-NHL IS ASSOCIATED WITH FUNCTIONAL LOSS OF EBV-SPECIFIC CD8+ T CELLS, WHICH CAN BE RESTORED AFTER HAART

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In HIV-infected patients, loss of control over Epstein-Barr virus (EBV) may cause AIDS-related Non Hodgkin's Lymphomas (AIDS-NHL). We previously showed that in AIDS-NHL patients EBV-specific CD8+ T cells did not disappear but lost their capability to produce IFNγ which was paralleled by an increase in EBV load. To investigate this loss of viral control further, we studied the phenotype of EBV-specific T cells using CD45RO and CD27 expression. Furthermore, we studied the effect of highly active anti-retroviral therapy (HAART) on EBV-specific CD8+ T cells and EBV load.

EBV-specific T cells in AIDS-NHL patients had high expression of CD27, whereas the expression of CD27 decreased in the course of HIV-infection in subjects without lymphoma. HAART did not lead to significant changes in EBV-specific tetramer+ T cells. Interestingly, there was an increase in the ratio IFNγ-producing T cells/ total number of EBV-specific T cells in the majority of individuals. Despite this relative functional improvement of EBV-specific T cells, no significant changes were observed in EBV load. Furthermore, no change in CD27-expression on EBV-specific T cells was observed after HAART.

In conclusion, functional loss of EBV-specific T cells is associated with a block in effector differentiation. HAART improves the antigen responsiveness of EBV-specific T cells without affecting the phenotype and not leading to a decrease in EBV load.

NOTES:

Poster: 105

AN INACTIVE LMP1 MUTANT ENABLES LMP1-SPECIFIC CTL GENERATION FOR THE ADOPTIVE IMMUNOTHERAPY OF EBV-POSITIVE HODGKIN'S DISEASE

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The Epstein-Barr virus (EBV) encoded LMP1 protein is expressed in EBV^+ Hodgkin's disease (HD) and can be targeted by cytotoxic T-lymphocytes (CTL). However, the LMP1-specific CTL frequency is low and its cytotoxic effects have impeded LMP1-specific CTL expansion. We reasoned that expression of inactive, non-toxic LMP1 (ΔLMP1) in dendritic cells (DC) would enable the activation and expansion of LMP1-specific CTL. Recombinant LMP1 or ΔLMP1 adenoviral vectors (Ad) were tested in their ability to transduce DC. LMP1 expression was toxic within 48 hours where as high levels of ΔLMP1 expression was achieved with minimal toxicity. ΔLMP1 expressing DC were used to generate LMP1-specific CTL from three healthy EBV^+ donors. Polyclonal LMP1-specific CTL were detected by IFN-γ ELISPOT assays using the HLA-A2 restricted LMP1 peptide, YLQQNWWTL (YLQ). Prior to stimulation the frequency of YLQ-specific CTL was low (<0.001%). After stimulations the frequency increased to 0.6-4%, showing an at least 600-4,000x fold expansion. In addition, the LMP1-specific CTL lysed autologous targets expressing LMP1. In contrast the frequency of YLQ-specific CTL in EBV-specific CTL generated by lymphoblastoid cell lines (LCL) was low (<0.01%) and no LMP1-specific cytotoxic activity was observed. Thus ΔLMP1 expression in DC is non-toxic and enables the generation of LMP1-specific CTL.

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Poster: 106

QUANTITATION OF EBV TRANSCRIPTS IN CLINICAL MATERIAL DETERMINED BY REAL TIME RT-PCR ASSAYS

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Epstein-Barr virus can adopt one of several programs of virus latency which are characterised by distinct patterns of viral protein expression and latent promoter usage. Identification of the latent transcripts by RT-PCR serves as a useful diagnostic marker for EBV-associated diseases including infectious mononucleosis (IM), Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). However most studies of EBV latency employ conventional end-point RT-PCR analyses and these may not accurately reflect the true levels of viral transcripts because the data are obtained when the PCR products have reached a plateau. To overcome this problem we have exploited the Taqman-based detection system which monitors the accumulation of PCR products in real time thereby allowing quantitation during the exponential phase of the PCR reaction. Here we describe a series of novel quantitative assays which measure several EBV latent and lytic transcripts. Reconstruction experiments using cell lines indicate that these assays are quantitative over a 5-log range, can detect transcripts from as little as one EBV-infected cell and correlate strongly with levels of expression of the corresponding proteins. Transcription data will also be presented from our studies on BL and NPC biopsies and on tonsillar material isolated from IM patients.

NOTES:

Poster: 107

ADOPTIVE IMMUNOTHERAPY FOR POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE: LESSONS FROM THE HUMANISED SCID MOUSE MODEL

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EBV-specific immune control is primarily mediated by virus-specific cytotoxic T lymphocytes (CTL). In immunosuppressed organ transplant recipients, CTL immunity is reduced which may lead to uncontrolled proliferation of EBV+ve B lymphocytes. This can result in post-transplant lymphoproliferative disease (PTLD). PTLD arises in up to 10% of organ graft recipients, is aggressive and difficult to treat with up to 70% fatality. Our laboratory collaborates with organ transplant centres in UK to study the effectiveness of *ex vivo* expanded polyclonal CTL against PTLD.

We have used the humanised scid mouse to investigate CTL control of EBV+ve B lymphocytes with a view to refining current adoptive immunotherapeutic regimes for PTLD. In the scid mouse, sc inoculated B lymphoblastoid cell lines (BLCL) regularly give rise to PTLD-like tumours which can be directly measured and thus monitored for the effectiveness of novel therapeutic strategies. Using this model, we have demonstrated tumour regression following administration of autologous EBV-specific polyclonal CTL. We have extended these studies to include allogeneic polyclonal CTL as well as virus peptide-stimulated monospecific CTL and peptide antigen-specific CTL clones in order to examine our hypothesis that these cells are superior to autologous polyclonal CTL in destroying PTLD tumour cells. We will present results of these *in vivo* studies and discuss the implications for future immunotherapeutic approaches to the disease.

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Poster: 108

ADOPTIVE IMMUNOTHERAPY TO A PATIENT WITH CHRONIC ACTIVE EBV INFECTION BY *EX VIVO* EXPANDED T LYMPHOCYTES

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Chronic active EBV infection (CAEBV) is characterised by chronic or recurrent, infectious mononucleosis-like symptoms persisting over a long period of time and by an unusual pattern of anti-EBV antibodies. CAEBV is a high mortality, high morbidity disease with life threatening complications, but treatment of CAEBV has not been established. Successful treatment by hematopoietic stem cell transplantation has been reported, but it constitutes a considerable risk. We performed adoptive immunotherapy by ex vivo expanded T lymphocytes to a 13-year-old patient with CAEBV. Autologus periperal blood lymphocytes were activated and expanded with an immobilized anti-CD3 antibody and IL2. After infusion of the ex vivo expanded T lymphocytes (10^6 cells/kg), EBVspecific CD8+ T-cells was transiently increased and viral load was decreased. Along with the decrease of the viral load, the patient showed improvement of liver function and fever. The treatment continued for more than 2 years, but no adverse effects were observed. Adoptive immunotherapy by ex vivo expanded T lymphocytes may be safe and useful treatment for CAEBV.

NOTES:

Poster: 109

THE B-SUBUNIT OF E. COLI HEAT-LABILE ENTEROTOXIN ENHANCES THE IMMUNOGENICITY OF EBV LATENT MEMBRANE PROTEINS

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In some Epstein-Barr virus (EBV)-associated malignancies, viral antigen expression is limited to EBNA1 and latent membrane proteins (LMP) 1 and 2, which elicit poor cytotoxic T-cell (CTL) responses. We describe a novel method of enhancing the immunogenicity of LMP1 and 2 using a non-toxic, recombinant B-subunit of *E. coli* heat-labile enterotoxin (EtxB). EtxB undergoes rapid aggregation and internalisation following binding to its ganglioside receptor GM1 found within glycosphingolipid-rich (GSL) rafts on mammalian cell membranes. Both LMP1 and 2 are also found within these rafts. Colocalisation of EtxB and LMP1 on the cell surfaces of EBV-positive lymphoblastiod cell lines (LCLs) was confirmed using confocal microscopy. Both EtxB and LMP1 underwent capping and internalisation following binding of EtxB to GM1. There was a substantial increase in HLA class I-mediated killing of EtxB-treated LCL targets by CD8+ CTL lines specific for known LMP1 and 2 epitopes. These effects were proteasome-dependent and limited to raft-associated viral antigens. Enhanced CTL responses were found against both TAP-dependent and TAP-independent LMP2 epitopes. Using mutant forms of EtxB, we concluded that GM1 binding by EtxB but not its signaling properties was essential. These findings demonstrate potential therapeutic applications for EtxB in EBV-positive nasopharyngeal carcinoma and Hodgkin's disease.

NOTES:

Poster: 110

IMPROVED INFECTIVITY OF FIBER-MUTANT ADENOVIRUS VECTOR AGAINST EPSTEIN-BARR VIRUS-POSITIVE LYMPHOID CELLS

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Adenovirus type 5 (Ad5), belonging to adenovirus subgroup C, is commonly used as a template of an efficient vector (usually E1/E3-deleted) for gene transduction to cells. However, Ad5 has the limited infection tropism, especially for lymphoid cells. We have newly constructed a fiber-mutant of Ad5, in which the fiber knob gene was replaced with that of Ad35, belonging to subgroup B. This fiber-mutant vector expressing EGFP (SV40 promoter-driven, Ad5/35f-EGFP) was examined for infectivity against various Epstein-Barr virus (EBV)-positive tumor cells, in comparison with Ad5-EGFP.

Among 21 lymphoid cell lines tested that included Burkitt lymphoma (BL) cell lines, EBV-immortalized B-cells and EBV-positive T-cell lines, 16 cell lines were susceptible to both Adv vectors. The efficiency of gene transduction assessed by FACS analysis, however, was 20-50% higher in Ad5/35f-EGFP than Ad5-EGFP. In addition, 3 BL cell lines were found to be infectable only by Ad5/35f-EGFP. All EBV-converted cells of epithelial origin examined in this study showed high infectability to both Adv's. These results suggest that our fiber-mutant of Adv can be a useful vector for EBV-specific gene therapy against the virus-associated lymphoid malignancy.

NOTES:

Poster: 111

A CENTRAL ROLE FOR EBNA-1 IN LUPUS AUTOIMMUNITY.

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A powerful association of EBV infection with Systemic Lupus Erythematosus (SLE) is well known (odds ratio>40) (*J Clin Invest* 100:3019,1997; *Arthritis Rheum* 42:1782,1999; 44:1122,2001). In addition, a possible molecular mimicry pathway may begin with EBNA-1 in anti-Sm positive SLE (*NY Acad Sci* 815:124,1997; *Immunol Rev* 164:185,1998). We suspected that the molecular immunopathogenesis of SLE involves EBNA-1. All 36 (100%) of the SLE patients tested had detectable anti-EBNA-1 antibody, which was present in fewer, 25 of the 36 (69%), matched normal controls (odds ratio>20; $p<0.001$). Studies of EBNA-1 expressed fragments showed that SLE patients and controls produced antibodies that tend to bind different EBNA-1 fragments. This result was confirmed by studies of all possible overlapping octapeptides of EBNA-1 (accession NP_039875), showing that anti-EBNA-1 in normal EBV infected individuals tends to bind only the Gly-Ala repeat of EBNA-1, while the SLE patients bind many different epitopes of EBNA-1 ($p<0.001$). Meanwhile, SLE patients and matched normal controls have qualitatively indistinguishable fine specificity antibody responses against a control antigen, CMV Immediate Early Antigen. These results support the hypothesis that the control of the humoral immune response against EBNA-1 generates risk for SLE and is responsible for the association of EBV with SLE.

NOTES:

Poster: 112

BZLF-1 IS COMMONLY RECOGNIZED BY $CD4^+$ AND $CD8^+$ T CELLS DURING ACUTE INFECTIOUS MONONUCLEOSIS

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EBV-specific $CD4^+$ T cell effector responses were evaluated at presentation with acute infectious mononucleosis (AIM) and sequentially through convalescence. A GST fusion-tagged EBV lytic protein, BZLF-1, was used in *in vitro* stimulation assays for measuring cytokine production by antigen-specific $CD4^+$ T cells by flow cytometry. IFN-γ-producing, BZLF-1-specific $CD4^+$ T cells were detected in the peripheral blood of all individuals (n=8) presenting with AIM (range: 0.07% to 0.96%; mean: 0.34% of all $CD4^+CD45RO^+$ T cells). TNF-α production was also detected at lower frequencies in 7 of these individuals tested (range: 0.05% to 0.62%; mean: 0.17% of all $CD4^+CD45RO^+$ T cells). Frequencies of BZLF-1-specific $CD4^+$ T cells declined over time following AIM and were undetectable by 1 year post infection. Overlapping peptides of BZLF-1 were used for concurrent analysis of $CD4^+$ and $CD8^+$ T cell responses during AIM. We found that regions of the BZLF-1 protein targeted by $CD4^+$ and $CD8^+$ T cells within an individual were different. Fine mapping of HLA class II-restricted BZLF-1 epitopes is currently in progress. Our studies suggest that BZLF-1 is well recognized by $CD4^+$ and $CD8^+$ T cells over the course of acute EBV infection.

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PRESENTATION OF A HYDROPHOBIC CD8+ T CELL EPITOPE FROM EBV LMP2 IS TAP-INDEPENDENT BUT REQUIRES THE IMMUNOPROTEASOME FOR ITS PROCESSING

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Recent work showed that a subset of CD8+ T cell epitopes within the EBV latent membrane protein LMP2, though requiring the standard multi-subunit proteasome for their generation, were presented in cell lines (such as T2) lacking the peptide transporter TAP. We proposed that this unique phenotype of cytosolic generation combined with TAP-independence was a specific property of epitopes with inherently high hydrophobicity. Here, we identify another highly hydrophobic LMP2 epitope, the HLA-A*0201-resticted peptide FLYALALLL (designated FLY), which unexpectedly was not presented in T2 cells. However TAP-transfected T2 cells also failed to present FLY, implying that the epitope could not be generated in T2, perhaps because these cells also lack two subunits specific to the immunoproteasome, a specialized form of the proteasome normally only expressed in professional antigen presenting cells. Subsequent work indeed showed that non-lymphoid cells such as fibroblasts were unable to present FLY from endogenously expressed LMP2 unless first treated with interferon gamma to induce the immunoproteasome. Then using interferon-induced fibroblasts from a TAP2-deficient patient we found that, as originally predicted, FLY could be presented in the absence of TAP. This identifies FLY as a rare example of a TAP-independent but immunoproteasome-dependent epitope .

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Poster: 114

EBV GENE ACTIVITY AND CHANGES OF HLA ANTIGENS IN THE DEVELOPMENT OF NPC

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EBV gene activity is detected indirectly by mARN through RT- PCR and nested PCR with 2 EBNA1 primers 293 bp PRC product HLA antigens are detected by Terrasaki cytotoxicity (1964 for class I) and by Van Rood (1976 for class II).

Obtained results show that:

- The GMT of IgA/VCA is high or very high (85 -147), of IgA/EBNA is high (70-389 IU/I) in the onset or relapse of disease.
- The EBV gene activity is detected in 100% of investigated cases with UCNT (47/47 cases) and (7/9 cases), 77.7% with non keratinizing carcinoma and no gene and gene activity are not seen in keratinizing carcinoma.
- The changes of HLA antigens are concentrated in class I with significant differences on A11 (0.36/ 0.10), A2 (0.51/ 0.23) and B17 (0.58/ 0.26). The linkage disequilibrium is detected with A2 - B17 and A11 - B17 (Δr: 1.44, $p < 0.01$ and $\Delta r = 1.59$, $p < 0.01$ respectively, or with the frequency of 14.6% and 14.55% comparing with the normal people 3.3% and 2.7%, respectively ($p < 0.05$).

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Poster: 115

DEFECTIVE *DE NOVO* METHYLATION OF EBV AND CELLULAR GENES IN ICF SYNDROME CELLS

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ICF syndrome (immunodeficiency, centromere instability, and facial anomalies) is an autosomal recessive disease resulting from mutations in the DNA methyltransferase 3B (*DNMT3B*) gene. ICF patients exhibit numerous chromosomal abnormalities including abnormal decondensation, pairing, separation and breakage. Global levels of DNA methylation in ICF cells are only slightly reduced, however certain repetitive sequences and genes on the inactive X chromosome of female ICF patients are significantly hypomethylated. Here we analyzed the molecular defect of *de novo* methylation in ICF cells in greater details, by making use of EBV based system and three members of the unique cancer-testis (C-T) gene family (two on X chromosome, one autosomal gene). Results with the EBV-based system indicate that all new *de novo* methylation is defective in ICF cells. Some *de novo* methylation capacity, however, is retained in these cells indicating that the mutations in *DNMT3B* do not lead to complete loss of its function or other DNMTs cooperate with DNMT3B. Analysis of the C-T genes revealed that loss of methylation from cellular genes is highly selective and that genes on the inactive X chromosome in female ICF cells may be particularly sensitive to mutations in *DNMT3B*. Aberrant hypomethylation at a number of loci examined correlated with altered gene expression levels. Lastly, no consistent change in the protein levels of the DNA methyltransferases was noted when normal and ICF cell lines were compared.

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Poster: 116

CD4$^+$ T CELLS INDUCE PLASMACYTOID DIFFERENTIATION OF EPSTEIN-BARR VIRUS-TRANSFORMED LYMPHOBLASTOID CELLS

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CD4$^+$ helper T cells play a central role in normal B cell activation, proliferation, and differentiation to antibody-secreting plasma cells. In this report, we show that CD4$^+$ T cells can also influence the differentiation state of Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (LCL). Coculture of LCL with EBV-specific CD4$^+$ T cells resulted in a shift to a plasmacytoid phenotype, characterized by elevated CD38 expression and decreased capacity for proliferation. In comparison with control LCL, the cocultured LCL were markedly less susceptible to lysis by EBV-specific CD8$^+$ cytotoxic T lymphocytes (CTL). In contrast, CD4$^+$ T cell-induced plasmacytoid differentiation of LCL did not diminish sensitivity of LCL to lysis by CD8$^+$ CTL specific for an exogenously loaded peptide antigen or lysis by alloreactive CD8$^+$ CTL, suggesting that differentiation is not associated with intrinsic resistance to CD8$^+$ T cell cytotoxicity and that evasion of lysis is confined to EBV-specific CTL responses. Transwell cocultures, in which direct LCL-CD4$^+$ T cell contact was prevented, indicated a major role for CD4$^+$ T cell cytokines in differentiation of LCL. Finally, CD4$^+$ T cell-induced plasmacytoid differentiation of LCL and concomitant insensitivity of LCL to lysis by EBV-specific CD8$^+$ CTL was associated with reduced expression of viral latent genes.

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Poster: 117

THE CHARACTERIZATION OF EPSTEIN-BARR VIRUS-INFECTED B CELLS IN THE PERIPHERAL BLOOD OF PEDIATRIC SOLID ORGAN TRANSPLANT RECIPIENTS

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The majority of immunocompromised pediatric solid organ transplant recipients develop a persistent Epstein-Barr viral load several orders of magnitude greater than normal immunocompetent individuals in their peripheral blood. These organ recipients may be persistent high load carriers (>200 genome copies/10^5 lymphocytes for two months) or persistent low load carriers (<200 genome copies/10^5 lymphocytes for two months). As shown by fluorescent in situ hybridization, low load carriers carry their viral load predominately in low copy number cells (1-2 genomes/nucleus), while high load carriers have a dual population of both high copy cells (>10 genomes/nucleus) and low copy cells. Both high and low load patients carry their viral load in the IgD-negative population of B cells. We have characterized the Ig isotype of the high and low copy infected cells in the persistent load carriers by FISH and immunofluorescence. Surprisingly, in most high load carriers, the high copy number cells had no detectable surface Ig, while the low copy cells are predominately IgM+. The phenotype of high copy cells with respect to expression of surface Ig and other B cell markers suggests that these cells have undergone a different developmental program than the low copy virus-infected B cells.

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Poster: 118

STEALTH-ADAPTED VIRUSES AS POTENTIAL VECTORS FOR CELLULAR ONCOGENES

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Stealth-adaptation is a mechanism that allows cytopathic viruses to evade immune elimination through the deletion of genes coding the major antigens targeted by the cellular immune system. A prototype stealth-adapted virus, repeatedly cultured from a patient with chronic fatigue syndrome (CFS) was cloned and partially sequenced. It has a fragmented, genetically unstable, genome. It has retained numerous viral sequences that can be aligned to various regions of the genome of human cytomegalovirus. Where the comparison can be made, the sequences match much more closely to those of African green monkey simian cytomegalovirus (SCMV) indicating an unequivocal origin from SCMV. The SCMV-derived stealth-adapted virus has seemingly acquired cellular sequences from infected cells, including a set of three divergent genes that potentially code for proteins related to the putative oncogenic CXC chemokine known as melanoma growth stimulatory activity (MGSA/Gro-alpha). Interestingly, two of the three MGSA-related genes lack a major intron present in genomic DNA, suggesting that cellular RNA was assimilated into viral DNA, presumably through reverse transcription. The capacity of stealth-adapted viruses to acquire potentially oncogenic sequences led to studies of stealth-adapted viruses in cancer patients. Evidence will be presented of stealth virus infection in many of the cancer patients tested.

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Poster: 119

HIGH INCIDENCE OF IMMEDIATE EPSTEIN-BARR VIRUS (EBV) REACTIVATIONS AFTER KIDNEY TRANSPLANTATION FOLLOWED BY ALLOGRAFT REJECTION EPISODES

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Immunosuppression *in vivo* substantially increases the incidence of EBV reactivations; however, their onset and frequency after initiating immunosuppressive therapy are widely unknown. EB viremia together with increases of EB viral load have been shown to reflect asymptomatic EBV reactivations.

We investigated 25 patients, who underwent allogeneic kidney transplantation, before and every 2–3 days posttransplant up to 12 weeks after beginning immunosuppressive triple therapy. Four patients also received monoclonal interleukin-2 receptor antibodies, one patient anti-thymocyte globulin for induction. Patients with a stable viral load and without viremia throughout the study were regarded not to have reactivated EBV infection. EBV reactivations were seen in 12 patients 0–6 days posttransplant (median 2.5 days). Two patients showed serologic and molecular evidence of primary EBV infection (2/4 days posttransplant). Rejection episodes were diagnosed clinically and histologically in 13 patients, of whom 11 had EBV reactivations/primary infection 2–45 days prior to rejection diagnosis (median 6 days; $p<0.01$). Two patients with reactivation and one with primary infection did not show a clinical course suggestive of rejection. Our data suggest that EBV reactivations upon immunosuppression are immediate and frequent events, which – in transplanted patients – are significantly associated with early rejection episodes and graft failure.

NOTES:

Poster: 120

THE EPSTEIN-BARR VIRUS U LEADER EXON CONTAINS AN INTERNAL RIBOSOME ENTRY SITE

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Eukaryotic translation can be initiated either by a cap-dependent mechanism or by internal ribosome entry, a process by which ribosomes are directly recruited to structured regions of the mRNA (1). Here we report the findings of an internal ribosome entry site (IRES) in the 5´untranslated region (5´UTR) of the Epstein-Barr virus nuclear antigen 1 (EBNA1) gene. EBNA1 is the only nuclear protein expressed in all states of EBV latency and in the virus lytic cycle, and is required for the maintenance of the EBV episome (2). Using cDNA reporter constructs in transfection assays we found that sequences contained in the 5´UTR of the EBNA1 mRNA increased the protein expression in different Burkitt lymphoma cell lines. The U leader exon, located within the 5´UTR, common to all known EBNA1 transcripts and also included in the EBNA3, 4 and 6 mRNAs, was demonstrated by bicistronic expression analysis to contain an IRES. RNase protection analysis showed that the increase in translation of the second cistron did not result from an increase of mRNA expression, demonstrating that translational regulation of EBNA1 can be mediated through cap-independent mechanism.

1. S. Vagner, et al. 2001. *Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep* **2:**893-8.
2. A. Rickinson, et al. 2001. *Epstein-Barr Virus,* p. 2575-2627. *Fields Virology,* 4 ed.

NOTES:

Poster: 121

SCREENING OF HHV-8 SEROPREVALENCE IN REPEATEDLY BLOOD TRANSFUSED PATIENTS

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Routes of transmission of HHV-8 are not fully understood thus far and a link between blood transfusion and HHV-8 infection has not been conclusively demonstrated as yet. In the present study we investigated whether subjects that live in areas with high prevalence of HHV-8 infection and that are affected by diseases that require repeated blood transfusion are at risk of being infected by HHV-8.

The survey was carried out in Sardinia, a geographic area where a seroprevalence of 25-30% among healthy individuals has been previously reported. The screening was performed on individuals affected by thalassemia, an hereditary disease with a high prevalence in the Mediterranean basin. Patients affected by thalassemia undergo blood transfusions too frequently (every 15-20 days) throughout their life.

We also analyzed patients affected by thalassemia living in Rome, an area with a lower seroprevalence to HHV-8 in the general population (10%) when compared to Sardinia, but still high in comparison to the seroprevalence reported in Northern Europe and North America.

Results show that both in Sardinia and in Rome repeatedly transfused patients have a three-fold increase in the rate of HHV-8 seroprevalence, thus suggesting that transfusion from blood donors living in areas at high HHV-8 prevalence could be regarded as a risk factor for HHV-8 infection.

NOTES:

Poster: 122

FUNCTIONAL INTERACTION OF NUCLEAR FACTOR Y (NF-Y) AND SP1 IS REQUIRED FOR ORIP-EBNA1-INDEPENDENT AND -DEPENDENT ACTIVATION OF THE EPSTEIN-BARR VIRUS C PROMOTER

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We have previously shown that the NF-Y transcription factor interacts with a CCAAT box in the -71/-63 Cp region of the Epstein-Barr virus genome (1). To further evaluate the functional consequences of this interaction, we have conducted transient transfection assays in latency I and III phenotype B lymphoid cell lines, and in the Schneider's *Drosophila* cell line 2 (SL2). A dominant negative analogue of the DNA-binding subunit (NF-YA) was used in order to elucidate the importance of the NF-Y interaction in the B lymphoid cell lines. Furthermore, the concerted effects of NF-Y, Sp1 and EBNA1 on Cp activity were studied in cotransfection experiments in the SL2-cells, as these cells do not express the above mentioned proteins endogenously (2,3).

We demonstrate that i) a dominant negative analogue of NF-Y abolishes o*riP*-independent as well as –dependent Cp-activity, ii) a functional interaction of NF-Y and Sp1 is necessary for the activation of the Cp, iii) the EBNA1-*oriP* induced transactivation of Cp requires concomitant expression of NF-Y and Sp1, but additional factors seem necessary for optimal activation.

1. T. Nilsson, 2001. *J Virol.* **75**: 5796-5811
2. A. Courey, 1988. *Cell* **55**: 887-98
3. M. Magana, 2000. *J Biol Chem* **275**: 4726-33

NOTES:

Poster: 123

REDUCED SEROPREVALENCE TO THE NEW WORLD LYMPHOCRYPTOVIRUS: INHERENT BIOLOGIC DIFFERENCE OR ARTIFACT OF CAPTIVITY?

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We have recently described that the natural host range for EBV-related lymphocryptoviruses is not restricted to humans and Old World primates. Natural LCV infection in New World primates has been demonstrated by virus isolation from lymphomatous and healthy marmosets and by peripheral blood PCR amplification from marmosets and squirrel monkeys. The complete genome sequence identifies significant differences in the gene repertoire compared to EBV. The impact of these genetic differences on the biology of marmoset LCV infection is unknown. In order to assess the prevalence of marmoset LCV infection, we developed a serologic assay for antibodies to the viral capsid antigen that is immunodominant in humans and Old World primates. 268 marmosets from 3 independent colonies were screened. Seroprevalence rates were 42%, 36% and 60% in these three colonies. This is in sharp contrast to the high seroprevalence rates in adult humans and Old World primates, eg >80% seropositivity at 12 months of age in captive rhesus macaque and baboon colonies. How much of this epidemiologic variance from Old World and human LCV is due to an inherent difference in marmoset LCV genetics and biology versus consequences of housing and breeding marmosets in captivity remains to be determined.

NOTES:

Poster: 124

A NEW, NON-INVASIVE, TECHNIQUE FOR STUDYING ORAL EPITHELIAL EBV INFECTION

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EBV productively replicates in oral hairy leukoplakia and in normal tongue epithelial cells of HIV-infected individuals. Previous studies of oral EBV infection have utilized surgically excised tissues. We tested a newly-developed oral epithelial cell collection brush as a non-invasive alternative to biopsy. Paired, same-site, samples of lateral tongue epithelium were obtained first by brush and then by biopsy, from HIV-infected research subjects. The brush collected both squamous and basal epithelial cells, which were pelleted, fixed, and paraffin embedded for histologic sectioning. Brush cell pellet sections proved equivalent to histologic sections of paired biopsy tissues for the diagnosis or exclusion of hairy leukoplakia by H&E staining, immunohistochemical staining for EBV EA-D, and in situ hybridization for EBV DNA. Nucleic acids were extracted from both brush cells and biopsy tissues for amplification of EBV and cellular sequences by PCR and RT-PCR. The amplification results were concordant in 85% of 240 paired brush and biopsy specimens. Among the discordant results, amplification from brush specimens was more sensitive than from paired biopsy specimens in 73% of cases. In summary: this new, non-invasive, brush technique safely and painlessly obtains oral epithelial cells equivalent or superior to biopsy for the study of oral epithelial EBV infection.

NOTES:

Poster: 125

NEW EBV VIRAL SETPOINT AFTER HIV SEROCONVERSION

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High EBV load in HIV-infected individuals doesn't correlate with lower CD4 counts or progression to AIDS. Factors as lower CTL function and increased immune activation may lead to a renewed EBV viral setpoint early after HIV infection. To investigate this, we measured EBV load in 20 homosexual men participating in the Amsterdam Cohort Studies on AIDS at 1-3 years before and 1 year after HIV seroconversion. Furthermore, in 7 individuals we measured numbers and function of EBV-specific CTL using HLA-peptide tetramers and ELISPOT for IFNγ.

EBV load increased significantly after HIV seroconversion (from 1797 to 18702 copies / 10^6 PBMC; p=0,001, Wilcoxon Signed Ranks Test). Numbers of EBV-specific $CD8^+$ T-cells increased, though not significantly, in 6/7 individuals (0,387 to 0,705 % of lymphocytes), while IFNγ-production remained stable (0,095 to 0,090 % of lymphocytes). The increase in EBV-specific $CD8^+$ T-cells was completely accounted for by an increase in T-cells against lytic epitope RAK, while latent epitope specific T-cells remained stable.

In conlusion, it seems that a new EBV viral setpoint is reached already early after HIV seroconversion. The increased immune response towards a lytic EBV epitope supports the idea of viral reactivation in the setting of HIV.

NOTES:

Poster: 126

ROLE OF EBV-SPECIFIC CD4$^+$ T LYMPHOCYTES IN AIDS-RELATED NON-HODGKIN LYMPHOMA

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Loss of control of EBV infection in HIV-infected individuals can lead to B-cell malignancies.

To investigate the role of EBV-specific CD4$^+$ T cell help, we measured CD4$^+$ T cell responses in both HIV$^-$ and HIV$^+$ EBV carriers, using ELISpot and FACS analysis to enumerate cytokine producing T cells after *ex vivo* antigenic stimulation.

Stimulation with a range of EBV-infected cell-lysates, to get an idea of the global anti-EBV response, gave inconsistent results, due to poor antigen presentation. In about half of both HIV$^-$ and HIV$^+$ individuals, peptides derived from EBV latent proteins EBNA1 and EBNA3C induced low frequencies of IFNγ producing cells (0.015 to 0.045% of CD4$^+$ T cells). In addition, we studied responses to peptides from lytic proteins BZLF1 and BHRF1. No responses to BZLF1 were found in 6/6 HIV$^-$ individuals. BHRF1 induced IFNγ production in the same range as the latent proteins in 1/9 HIV$^-$ and 3/11 HIV$^+$ individuals. In order to get an idea of the responses to whole proteins, we are currently setting up an expression system to produce both latent and lytic antigens.

Until now our data suggest no difference in EBV-specific CD4+ T cell response between HIV$^-$ and recently infected HIV$^+$ individuals.

NOTES:

Poster: 127

PROGNOSTIC FACTORS FOR CHRONIC ACTIVE EPSTEIN-BARR VIRUS INFECTION

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Chronic active Epstein-Barr virus infection (CAEBV) is a high mortality and high morbidity disease. Recently, successful treatment by hematopoietic stem cell transplantation has been reported. Since transplantation constitutes a considerable risk, its indication should be restricted to patients with poor prognosis. The purpose of this study was to clarify the prognostic factors of the disease. A national survey of CAEBV was performed in Japan and 82 cases that met the criteria of CAEBV were enrolled. Out of them, 47 were alive and 35 had already died. Clinical and laboratory data were analyzed and compared between live and dead cases. On multivariate analysis, onset age and thrombocytopenia were associated with death. Probability of survival at 5 years was 0.45 for older patients (onset age $\geq$ 8years) and 0.94 for younger patients ($P < 0.001$), and was 0.53 for patients with thrombocytopenia (platelet $< 15x10^4/\mu l$ at diagnosis) and 0.76 for patients without it ($P = 0.01$). Furthermore, patients with T cell infection had shorter survival than those with natural killer cell infection. CAEBV patients with late onset, thrombocytopenia, and T cell infection had significantly shorter survival. These patients might be treated with aggressive therapies such as transplantation.

NOTES:

Poster: 128

EPSTEIN-BARR VIRUS INFECTION OF NON-PRIMATE CELL LINES

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EBV is supposed to infect only humans and a few species of New World monkeys. EBV-GFP/NeoR is a recombinant EBV bearing a green fluorescence protein (GFP) gene and the neomicin resistant (NeoR) gene at the BXLF1 site. Using this EBV, susceptibility of EBV infection was examined in 9 kinds of non-human mammalian cells, including MDCK (dog), BHK (hamster), 9L, cKDH, c-SST-2, Rat-1 (rat), NIH3T3 (mouse), VERO, and COS-7 (African green monkey). Two days after EBV-GFP/NeoR infection, cells were incubated in the G418 selection media. G418 resistant, EBV-infected clones were obtained from five cell lines (MDCK, 9L , cKDH, c-SST-2, COS-7). NIH3T3 and Rat-1 cells transiently expressed GFP, but could not grow stable infection of EBV. The frequency of appearance of the EBV-infected cells was 3 to 14 per 10^6 cells, which was more than 100 times lower than the frequency in human CD21 expressing MDCK cells.

We have previously reported that when human CD21 gene was introduced into nonprimate mammalian cells and the initial barrier for attachment was overcome artificially, EBV could establish a stable infection in nonprimate cells (J.Virol.74, 10745). In this study, we showed that EBV could infect non-human cells without artificial expression of human CD21. Further study will be required to clarify whether animals can be the reservoir of EBV.

NOTES:

Poster: 129

A RAPID AND RELIABLE ENZYME IMMUNOASSAY (EIA) PCR-BASED SCREENING METHOD TO IDENTIFY EPSTEIN-BARR VIRUS (EBV)-CARRYING GASTRIC ADENOCARCINOMAS

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EBV is associated with a substantial number of gastric adenocarcinomas, as confirmed by EBER1/2-RNA in situ hybridization (RISH). We developed a rapid and sensitive PCR-EIA based pre-screening method for the detection of EBV in gastric adenocarcinomas to reduce the amount of laborious EBER1/2-RISH assays to be performed. The method was evaluated by testing paraffin-embedded tumors (n=246), using both BamHI W PCR-EIA and EBER1/2-RISH, in combination with appropriate DNA and RNA quality controls.

Seventy-four percent of the samples had good DNA quality as shown by β-globin PCR, after proteinase K and boiling pre-treatment, while after DNA purification this was increased to 90%. After PCR-EIA, 32% of all cases were EBV-DNA positive, whereas 10% of these gastric cancers contained EBV-transcripts in the neoplastic cells by EBER1/2-RISH. Interestingly, only samples with high Optical Density (OD) (405/630) in PCR-EIA, equivalent to the maximum OD reading of the assay as determined by the positive control, contained EBV transcription positive tumor cells. In contrast, the weak positive samples, were EBER1/2 RISH negative and represented the presence of latently infected lymphocytes. In conclusion, high OD values in PCR-EIA are very valuable to pre-screen gastric carcinomas for the presence of EBV.

NOTES:

Poster: 130

LIGHTCYCLER REAL-TIME PCR FOR MONITORING EPSTEIN-BARR VIRUS DNA LOAD IN UNFRACTIONATED WHOLE BLOOD: A COMPARISON WITH QUANTITATIVE COMPETITIVE PCR

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LightCycler(LC)-based real-time PCR was developed for monitoring Epstein-Barr virus (EBV) DNA load in unfractionated whole blood and compared with quantitative competitive EBV PCR (Q-PCR) and TaqMan EBV-PCR.

The LC assay was highly sensitive and reproducible for quantifying plasmid DNA, either in presence or absence of healthy donor blood. Amplifying plasmid DNA in blood from different donors slightly increased variation of quantification, indicating sample influence on quantification. TaqMan PCR showed somewhat inferior results. In follow-up samples of transplant recipients good correlation was observed between EBV DNA load dynamics by LC and Q-PCR, although correlation between absolute values of EBV DNA loads was weak and occasional samples were false negative in LC. In 253 cross-sectional blood samples from Burkitt's Lymphoma, Infectious Mononucleosis and HIV-infected patients weak but significant correlation between both methods was found (r^2=0.37, P<0.001).

Our results indicate that clinical specimen background may influence absolute values of EBV DNA load as measured in LC and TaqMan. LightCycler PCR is very suited for monitoring of longitudinal EBV DNA load dynamics and comparable to Q-PCR. To avoid false negativity or underestimation of viral load, internal calibration of real-time amplification assays is recommended. This would also improve EBV load assay standardization and interinstitute comparisons.

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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR MEASURING THE INTERACTION BETWEEN EBV LATENT MEMBRANE PROTEIN 1 AND TNFR-ASSOCIATED FACTOR 2 AND DEATH DOMAIN PROTEIN

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The pleiotropic and profound effects of latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) on diverse biological processes seem to be due to its comparable properties to a constitutively active tumour necrosis factor receptor (TNFR) superfamily member. Molecules such as TNFR-associated factors (TRAFs) and TNFR-associated death domain protein (TRADD) interact directly with the C-terminus activating region 1 and 2 (CTAR1 and CTAR2) in the C-terminal domain of LMP1, respectively. These interactions are essential for LMP1-mediated activation of the transcription factor, NF-κB. To facilitate the measurement of these interactions in vitro, such as by ELISA, we have expressed and purified the recombinant TRAF2, TRADD and the C-terminal cytoplasmic portion of LMP1. Meanwhile, we have also generated two anti-LMP1 peptide antibodies and demonstrated their utility in ELISA for LMP1. Using these materials, interaction between LMP1 and TRAF2 (or TRADD) can be detected by ELISA, although the interaction signal is relatively weak. This procedure involves coating of the TRAF2 (or TRADD) protein on microtiter plates, blocking with non-fat milk, binding the C-terminal cytoplasmic portion of LMP1 to coated TRAF2 (or TRADD), and detection of the bound LMP1 using the anti-LMP1 peptide antibodies and secondary antibody conjugated with alkaline phosphatase, followed by reaction with p-nitrophenyl phosphate. We also tried to use the biotin-labelled peptide corresponding to the CTAR2 region of LMP1 (designated as biotin-LMP1C-16) as the binding partner to the precoated TRADD, and detect the bound peptide by streptavidin-conjugated alkaline phosphatase. The ELISA signal detected by this way was much stronger than that using the C-terminal cytoplasmic portion of LMP1 as the binding partner to TRADD. Irrelevant proteins such as ovalbumin and bovine serum albumin did not bind biotin-LMP1C-16 significantly, and the interaction signal between TRADD and biotin-LMP1C-16 could be blocked by LMP1C-16 peptide. These results indicate a relative good specificity of this ELISA for the measurement of interaction between LMP1 and TRADD. The ELISA may be helpful to screen compounds that can block the LMP1-mediated signal transduction through the interference of the interactions between LMP1 and its associated molecules.

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SEXUAL HISTORY AND EPSTEIN-BARR VIRUS INFECTION

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A cohort comprising 2000 students has been recruited to investigate the pathogenesis and current epidemiology of infectious mononucleosis in this susceptible group. New students starting degree courses lasting 4 or more years, at Edinburgh University, were asked to complete an anonymous, confidential questionnaire about their lifestyle prior to arrival at university, and to donate a blood sample which was tested for IgG to Epstein Barr Virus capsid protein. Analysis of the first 1006 students showed that 25% were seronegative. The prevalence of EBV seropositivity was significantly greater in female (79.2%), than male students (67.4%) ($p<0.001$), and in those who had ever been sexually active (82.7%), than in those who had not (63.7%) ($p<0.001$). Amongst EBV seropositives 110 reported prior infectious mononucleosis, of whom 89 (80.9%) had been sexually active before university. In contrast, only 39.5% of seronegatives, and 61.1% of asymptomatic seroconverters, reported sexual activity prior to university. Increasing numbers of sexual partners were a highly significant risk factor for EBV seropositivity (p trend = 0.001). The findings suggest that EBV may be transmitted during sexual intercourse or closely associated behaviours.

This study is funded by The Medical Research Council of the United Kingdom.

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THE EPSTEIN-BARR VIRUS GLY-ALA REPEAT AND THE CELLULAR GLN REPEAT INHIBIT PROTEASOMAL DEGRADATION BY DISTINCT MECHANISMS

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The Gly-Ala repeat (GAr) of the Epstein-Barr virus nuclear antigen 1 (EBNA1) blocks proteasomal degradation. The stabilizing repeat prolongs the half-life of EBNA1 and abrogates the generation of antigenic epitopes. No cellular equivalents have been found of this viral repeat. However, a possible candidate is the expanded poly-Gln repeat (polyQr) involved in neurodegenerative disorders. These viral and cellular domains share a number of striking characteristics: 1) both are simple repetitive sequences, 2) they interfere with proteasomal degradation, 3) they have a length dependent effect, and 4) they are transferable elements. An attractive hypothesis is that the proteasome cannot efficiently process repetitive sequences. We investigated whether the viral GAr and the cellular polyQr display similar inhibitory mechanisms using green fluorescent protein (GFP)-based proteasome substrates. This revealed that the polyQr interferes with proteasomal degradation through the formation of insoluble aggregates. On the contrary, GFP substrates carrying the GAr did not form aggregates but were nevertheless efficiently protected from proteasomal degradation. These data indicate that the EBNA1 GAr and the cellular polyQr both interfere with proteasomal degradation but through different mechanisms.

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ISOLATION AND CHARACTERISATION OF BALF1, A NOVEL BCL-2 HOMOLOGUE, FROM EPSTEIN-BARR VIRUS AND PRIMATE VIRUS ANALOGUES

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Epstein-Barr virus (EBV) encodes two viral Bcl-2 homologues (v-bcl-2s) – BHRF1 and BALF1. Members of the Bcl-2 family either oppose or promote apoptosis and possess at least one of four Bcl-2-homology (BH) domains. BHRF1 has been well characterised, in contrast, the function and expression status of BALF1 is less well defined. BALF1 is unusual amongst other v-bcl-2s, in that it possesses a conserved BH4 domain.

In a comparative approach, homologues of BALF1 were isolated from EBV-like viruses of the chimpanzee, baboon and gorilla. The BALF1 ORF was found to be highly conserved - except at the amino-terminus. Interestingly, the primate virus BALF1 homologues lack the amino-terminal BH4 domain, as they initiate at a site equivalent to the second methionine of EBV BALF1. Additionally, all four viruses possess a Kozak sequence at this methionine, raising the possibility that translation of EBV BALF1 may also occur from this point. However, we found the BALF1 amino-terminal domain to be absolutely conserved in several different EBV isolates, suggesting full-length BALF1 may function during EBV infection.

In order to investigate the role of the two forms of BALF1 in apoptosis, stable B-cell transfectants, expressing full-length and amino-terminal truncated EBV BALF1, are currently being established.

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CELL GROWTH RETARDATION AND P27^{KIP1} PROTEIN STABILIZATION IN RESPONSE TO EPSTEIN-BARR VIRUS DNASE

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DNA damage usually induces cell cycle arrest accompanying the alteration of cell cycle regulators. The cellular response to DNase-induced DNA damage is less characterized. Epstein-Barr virus (EBV) DNase possesses both endonuclease and exonuclease activities and utilizes both double-stranded DNA and single-stranded DNA as substrates. This enzyme is localized in the nucleus, and likely digests cellular DNA. In order to investigate the effects of EBV DNase, it was expressed in human cells under the control of inducible lactose operator. This inducible system allowed us to express EBV DNase in a dose-dependent manner. The expression of EBV DNase resulted in DNA-strand breaks, detected by comet assay. The induction of DNase retarded cell growth in Raji and HEp2 cells. p27^{Kip1}, a cyclin dependent kinase inhibitor, was found to be stabilized in response to EBV DNase expression in various cell lines. The response of p27^{Kip1} to DNase was in a p53-independent manner. H89 and 3-aminobenzamide blocked the EBV DNase-induced signals and reduced the response of p27^{Kip1} to EBV DNase. These results indicate that expression of EBV DNase leads to cell growth retardation and p27^{Kip1} protein stabilization.

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THE EBNA- 3 GENE FAMILY PROTEINS DISRUPT THE G2M CHECKPOINT

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The Epstein Barr Nuclear Antigens (EBNA), EBNAs -3, -4 and -6 (the EBNA-3 gene family proteins), have previously been shown to act as transcriptional regulators, however, this study identifies an additional function for these proteins, disruption of the G2/M checkpoint. Following treatment of lymphoblastoid cell-lines (LCLs) with a G2/M initiating drug azelaic bishydroxamine (ABHA), the cells did not show a G2/M checkpoint response as measured by DNA cell cycle analysis, rather they continued through the cell cycle. DNA cell cycle analysis demonstrated that EBNAs -3, -4 and -6 are capable of disrupting the G2/M checkpoint response induced by ABHA, whereas EBNAs -2, and -5 were not. EBNA-3 gene family protein expression also disrupted the G2/M checkpoint initiated in response to the genotoxin etoposide and the S phase inhibitor hydroxyurea. Immunoblotting for the G2/M regulators cyclin B1 and the phosphorylated form of cdc2 confirmed these findings. Co-immunoprecipitations experiments performed in DG75 cells expressing the individual EBNA proteins and in lymphoblastoid cell lines demonstrated that EBNA3 was capable of interacting with chk2, a kinase implicated in the G2/M DNA damage and replication checkpoint responses. A possible mechanism for the G2/M checkpoint disruption observed in LCLS and cell lines expressing the EBNA-3 gene family proteins could be via interaction and inactivation of chk2. The function of EBNAs -3, -4 and -6 proteins now appears to be far more complex than anticipated and suggest a role for these proteins in cell cycle progression.

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IMMUNE RESPONSE TO EPSTEIN-BARR VIRUS DISRUPTS SELF-TOLERANCE BY MOLECULAR MIMICRY AND BYSTANDER ACTIVATION

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The memory response to Epstein-Barr virus (EBV) in HLA-B8+ virus carriers is dominated by CD8+ cytotoxic T lymphocytes (CTLs) that respond to the RAKFKQLL peptide from the immediate early BZLF1 protein. Here we show that these antiviral CTLs recognise peptide mimics from a cohort of self proteins and common environmental bacteria. The crossreactive CTLs are activated and abundant in the peripheral blood during acute primary infection, and are present in the synovial fluid of affected joints in chronic rheumatoid arthritis. Importantly, self-reactive CTLs can be induced *in vitro* by stimulation of peripheral blood lymphocytes with the cognate viral peptide, and with the self peptide in the presence of the costimulatory CD40 ligand or an immunogenic bystander viral peptide. Moreover, CD4+ T cells are dispensable in the induction of this autoreactivity. These findings reveal a novel functional network linking persistent virus, self, and bacteria in the disruption of self-tolerance by molecular mimicry and bystander activation, two processes strongly incriminated in the pathogenesis of autoimmune disease.

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REGULATION OF SECRETORY LEUKOCYTE PROTEASE INHIBITOR BY EPSTEIN-BARR VIRUS-ENCODED IMMEDIATE EARLY RANSACTIVATOR BZLF1

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Epstein-Barr virus (EBV) acts as an opportunist in immunocompromised patients to produce hairy leukoplakia (HLP), the only pathologic manifestation of permissive EBV infection. This HIV-associated oral lesion is characterized by marked hyperproliferation and minimal inflammatory infiltration. Salivary levels of secretory leukocyte protease inhibitor (SLPI), an epithelial cell-derived serine protease inhibitor and anti-inflammatory protein, were significantly higher in HIV-infected patients with HLP compared to infected patients without HLP ($p<0.01$, unpaired t-test). In this study, SLPI was detected in HLP and in HIV-negative tongue biopsies by RT-PCR and immunohistochemical methods. SLPI gene expression occurs at the transcriptional level, with the promoter region of the gene containing four AP-1 sites. Reporter assays were performed using SLPI promoter constructs that contained either the wildtype promoter or single-site mutations in each of the AP-1/BZLF1-responsive sites. In epithelial cells expressing BZLF1 immunoblot and ELISA analyses detected enhanced SLPI production and secretion. A 10-fold increase in SLPI promoter activity was detected in cells expressing wildtype BZLF1 compared to mutant BZLF1($p<0.05$). This regulatory mechanism likely contributes to the modulation of the immune response to EBV in oral infections. The induction of SLPI by EBV may modulate the immune response to EBV in oral infection.

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DETECTION OF KSHV IN THE OROPHARNYX OF IMMUNOCOMPETENT AND IMMUNOSUPPRESSED INDIVIDUALS

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Although the high frequency of Kaposi's sarcoma in HIV-infected homosexual men suggests that the causative agent, Kaposi's Sarcoma associated Herpesvirus (KSHV), is sexually transmitted, recent studies suggest that oral transmission also occurs. The objective of this investigation was to determine the prevalence of KSHV in HIV-negative, KS-negative heterosexual individuals. We examined throat washings and blood samples from HIV-positive and HIV-negative individuals and detected KSHV in the oral cavity in healthy and immune-suppressed individuals. Furthermore, KSHV DNA was detected more readily in saliva than in sera of the HIV-negative, KS-negative individuals. Viral strain analysis by of KSHV K1 detected a variety of strains previously detected in KS lesions. Immunofluorescence for Latency Associated Nuclear Antigen detected consistent nuclear expression of the protein in oral epithelial specimens. These data suggest that KSHV infection is common in the oropharynx of healthy individuals and may be transmitted by saliva. Like most herpesviruses, KSHV is a successful opportunistic organism prevalent in the human population.

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TRANSCRIPTIONAL ACTIVATION OF THE *BFL-1* GENE BY THE EBV LATENT MEMBRANE PROTEIN 1

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The Epstein Barr virus (EBV) Latent Membrane Protein 1 (LMP1) exhibits transforming and anti-apoptotic properties in B lymphocytes. We have previously reported that expression of LMP1 as the sole EBV latent protein leads to an upregulation of the mRNA levels of the anti-apoptotic gene *bfl-1* in EBV-negative Burkitt's lymphoma (BL) cell lines and that this effect is mediated in part by an increase in *trans*-activation of the *bfl-1* promoter (D'Souza *et al*., J. Virol, 2000, 74: 6652-6658). We now report that LMP1-mediated transactivation of the *bfl-1* promoter is at least partly NF-κB-dependent, since expression of dominant negative IκBα inhibited this effect of LMP1 in B lymphocytic cell lines. Studies using LMP1 molecules mutated in the functional domains have shown that both CTAR1 and CTAR2 are required for this effect, with CTAR2 contributing to a greater extent. Furthermore, co-expression of a dominant negative TRAF2 molecule or over-expression of A20 also inhibited the upregulation of *bfl-1* promoter activity by LMP1 indicating the requirement of TRAF2 recruitment by LMP1 in upregulating *trans*-activation of *bfl-1*. Analysis of the 5' region of the *bfl-1* gene by promoter-reporter assays has revealed that a 129 bp sequence just upstream of the transcription start site is essential for this LMP1-mediated effect. This sequence contains binding sites for candidate transcription factors including NF-κB and AP-1 and the requirement of these sites for increased transcription of the *bfl-1* gene by LMP1 is being investigated.

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